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UNIVERSITÉ D'OTTAWA
UNIVERSITY OF OTTAWA

In memory of my father

Michael Hrycko

1927-1985

"Tant que tu ne cesses de monter, il y a toujours des marches,
il y en a toujours qui se dessinent, plus haut que tes pas."

F. Kafka "Fürsprecher" Die Erzählungen,
S. Fischer Verlag, p.330

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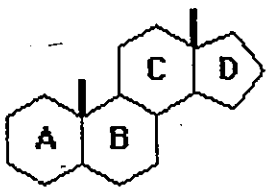
GLOSSARY

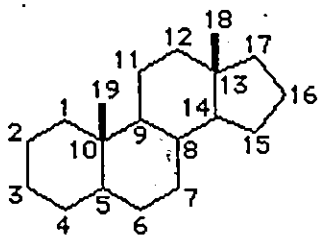
A. Systematic[1] and Trivial[2] Names of Steroids.

In this thesis, systematic names[1] (according to the IUPAC-IUB 1967 Revised Tentative Rules for Steroid Nomenclature) will be used. Examples of systematic and trivial names are given below.

Estrone	3-Hydroxy-1,3,5(10)-estratrien-17-one
Progesterone	4-Pregnene-3,20-dione
Testosterone	17β-Hydroxyandrost-4-en-3-one
Androstenedione	Androst-4-ene-3,17-dione

B. Numbering of the Steroid Skeleton.

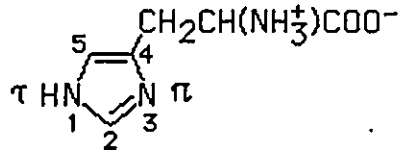




α below the plane ⋯⋯
β above the plane ——
ξ unknown stereochemistry ∿∿

C. Numbering of the Imidazole Ring[3].

In this thesis, the nitrogen atoms of the imidazole ring of histidine are denoted by *pros* ("near", π) and *tele* ("far", τ) to show their position relative to the side chain, according to the IUPAC-IUB Nomenclature and Symbolism for Amino Acids and Peptides.



D. Abbreviations and Symbols.

Ala	L-Alanine
Asp	L-Aspartic acid
br	Broad
CD	Circular dichroism
Ci	Curie (2.22×10^{12} dpm)
CM	Carboxymethyl
Cys	L-Cysteine
d	Doublet
δ	Chemical shift (in ppm)
dpm	Disintegrations per minute
DTT	Dithiothreitol
ε	Extinction coefficient
γ_{max}	Wave number (cm^{-1})
g	Acceleration due to gravity
Glu	L-Glutamic acid
Gly	Glycine
[^{3}H]	When follows a number, refers to the tritiated molecule
h	Hour
His	L-Histidine
HSD	Hydroxysteroid dehydrogenase
IR	Infrared
Ile	L-Isoleucine
λ_{max}	Wavelength at maximum intensity of absorption
Leu	L-Leucine
Met	L-Methionine
μ	Micro (10^{-6})
m	Milli (10^{-3}) or multiplet in ^{1}H NMR
mA	Milliampere
MCPBA	*m*-chloroperoxybenzoic acid
min	Minute
M	Molar
mol	Mole
mp	Melting point
MS	Mass spectrum
n	nano

NADP, NADPH	Nicotinamide-adenine dinucleotide phosphate (oxidized and reduced forms).
nm.	nanometer
NMR	Nuclear magnetic resonance
Phe	L-Phenylalanine
PPO	2,5-Diphenyloxazole
q	Quartet
R_f	Displacement of compound/Displacement of solvent front
s	Singlet
Ser	L-Serine
sh	Shoulder
sp. radioactivity	Specific radioactivity
THF	Tetrahydrofuran
Thr	L-Threonine
tlc	Thin layer chromatography
tr	Triplet
TRIS	2-Amino-2-hydroxymethylpropane-1,3-diol
Tyr	L-Tyrosine
UV	Ultraviolet
Val	L-Valine
V	Volt
v/v	By volume
W/v	Weight in volume

ABSTRACT

Three forms of a soluble 17β-hydroxysteroid dehydrogenase (17β-HSD) with activity toward both androgen and estrogen substrates have been purified from female rabbit liver. The enzyme forms also exhibit 3α-hydroxy-steroid dehydrogenase activity toward androgens of the 5β-androstane series. The binding sites of the enzyme forms I and III and the orientation of the steroid in the binding site for 17β- and 3α- enzyme activity were investigated using the affinity labeling technique. Substrate analogues having the alkylating group at C-3, C-6, C-11, C-17 or C-19 of the steroid have been synthesized. These analogues were found to inhibit the 17β-enzyme activity of both forms of the hydroxysteroid dehydrogenase. The radioactive iodoacetoxy substrate analogues synthesized were found to label primarily cysteine residues. A comparison of the radioactive peptide maps obtained from the alkylated 17β-HSD with ^{14}C-iodoacetoxy steroid derivatives indicates that the binding sites are different for the 17β-HSD forms I and III. The similarity of the radioactive peptide maps obtained upon alkylation of the 17β-HSD with 19-iodo[2'-^{12}C]acetoxyandrost-4-ene-3,17-dione **33** and 19-iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37** suggests that a single active site would be responsible for the C-3 and C-17 activities of the 17β-HSD enzyme.

Attempts to prepare 4,19-dihydroxyandrost-4-ene-3,17-dione **63**, a precursor to a bifunctional affinity label, are described. One method examined to obtain this compound required the synthesis of 19-hydroxy-4β,5-epoxy-5β-

androstane-3,17-dione **62**. It was found that alkaline hydrogen peroxide treatment of 19-hydroxyandrost-4-ene-3,17-dione **4** could give the desired 4β,5β-epoxide or a hydroperoxidic steroid depending on the conditions used for the reaction. The structure and chemistry of the hydroperoxidic steroid were investigated. Baeyer-Villiger reactions of 19-hydroxy steroids were studied and found to involve the participation of the 19-hydroxyl group.

INTRODUCTION

A. Affinity Labeling Reagents.

In order to establish the relationship between the structure and biological function of an enzyme, detailed knowledge of the active site is necessary. Affinity labeling is one of the techniques which has been used to obtain information about the topography of the active sites of enzymes. Affinity labeling is the term for a procedure in which covalent bonds are generated between the complexing molecules, e.g. the affinity label or active site directed reagent and the protein. Three distinct classes of affinity labeling reagents have been designed:

1) classical affinity labeling reagents [4,5,6];
2) mechanism based irreversible inhibitors [7,8];
3) photoaffinity labeling reagents [9,10,11].

The classical reagents are substrate analogues containing chemically reactive functional groups. The specific binding of these inhibitors to the active site regions of the enzyme can lead to the formation of a covalent bond provided a suitable amino acid residue is properly positioned for a reaction to occur. The mechanism based inhibitors (k_{cat} or suicide inhibitors) are substrates which are chemically unreactive but which are converted by the enzyme to a highly reactive molecule. This reactive product may react with an active site amino acid thus irreversibly inhibiting the enzyme. The photoaffinity reagents, like the suicide inhibitors, possess a masked group but in this case activation is *via* light absorption. Suicide inhibitors offer the advantage of being more stable than classical reagents prior to enzymic activation. Also because of their specificity they can be used to examine an enzyme in a crude system. The major difficulties encountered in photochemical labeling studies are low incorporation of the label and non specific labeling.

Several criteria have been established to define affinity label [12]:

1) the affinity label should be a substrate for the enzyme,
2) the stoichiometry should be one molecule of label bound for each active site of the enzyme and
3) the rate of inactivation in the presence of excess labeling reagent should decrease upon addition of real substrate.

An additional term, catalytic competence, describes the ability of the affinity labeled enzyme to catalyze its normal reaction on its covalently bound ligand. For example, Engel and co-workers[13], in studies on the 17β-hydroxy-steroid dehydrogenase of human placenta, demonstrated that the affinity label 3-bromoacetoxy-1,3,5(10)-estratrien-17-one satisfies the catalytic competence criterion since this compound, which was covalently bound to the enzyme, was converted to 1,3,5(10)-estratriene-3,17β-diol. This criterion will be satisfied only when the affinity label, which contains a functional group capable of undergoing reaction, reacts with a group which is not essential for catalysis and when the covalently bound affinity label is able to achieve the orientation required for enzymic catalysis.

B. Affinity Labeling of Hydroxysteroid Dehydrogenases.

Affinity labeling has been used extensively to study enzymes involved in steroid metabolism. Steroid substrates are amenable to such studies because they are rigid molecules possessing a variety of carbon atoms and functional groups able to undergo addition and substitution reactions[14]. Several sites on the steroid skeleton may be converted to affinity labeling groups and eventually provide clues about the topography of the active site of enzymes. The particular group of steroid metabolising enzymes which has been examined in this manner are the

hydroxysteroid dehydrogenases. The designation hydroxysteroid dehydrogenase (ketosteroid oxido-reductase) has been widely adopted for the group of enzymes which catalyze the interconversion of hydroxyl and carbonyl functions on the steroid skeleton and side chain. These reactions are represented by the following equation:

$$\text{Hydroxysteroid} + \underset{(NAD^+)}{NADP^+} \longrightarrow \text{Ketosteroid} + \underset{(NADH)}{NADPH} + H^+$$

1. 17β-Estradiol Dehydrogenase.

The human placental 17β-estradiol dehydrogenase (E.C.1.1.1.62) has been extensively studied by affinity labeling. This enzyme uses either NAD or NADP as cofactor and is highly selective for estrogen substrates[15]. Crastes de Paulet and co-workers [16,17,18] synthesized haloacetate and haloacetamide derivatives of 3-hydroxy-1,3,5(10)-estratrien-17-one and 1,3,5(10)-estratriene-3, 17β-diol with the alkylating group at nine different positions of the steroid nucleus: C-1, C-2, C-3, C-4, C-6, C-7, C-11, C-16 and C-17. Among all the derivatives, the best affinity label was 3-iodoacetoxy-1,3,5(10)-estratrien-17-one[19]. When the placental enzyme was inactivated with 3-iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one in the presence of cofactor and subjected to chymotryptic digestion to generate peptides, a single peptide was radiolabeled and the label was recovered as N^{τ}-carboxymethyl-L-histidine[20]. However, when the estradiol dehydrogenase was completely inactivated in the absence of coenzyme three amino acid residues were labeled, two cysteine and one histidine residues. The histidine residue was located in the same peptide isolated in the presence of the cofactor

while the two cysteine residues were located on different peptides, designated as C_3* and C_5* by the authors. These investigators concluded that the two cysteine peptides were part of the hydrophobic coenzyme binding site.

When 3-chloroacetyl pyridine adenine dinucleotide, an NAD coenzyme analogue directing the alkylating group towards the catalytic site of dehydrogenases [21], was used as an affinity label, it was found to alkylate mainly the C_3* cysteine residue along with C_1* and C_5* residues. On the other hand, only the cysteine C_5* residue was labeled when 3-chloroacetyl pyridine adenine dinucleotide phosphate, an NADP analogue, inactivated the enzyme [22]. Since this label had a better affinity for estradiol dehydrogenase than the previous NAD analogue it was concluded that C_5* should be located nearer the cofactor site than C_3*. It was also concluded[16] that the histidine residue was located in the vicinity of the A-ring of the bound steroid since the labeling of this histidine residue decreased when the alkylating group was shifted towards the B- or C-rings.

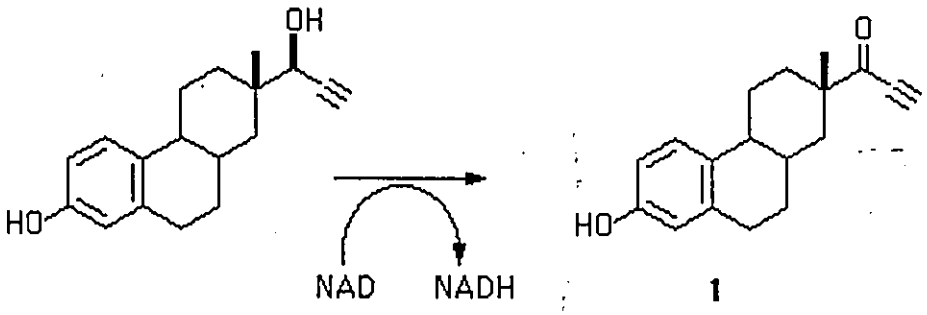
Chin and Warren [23], using 16α-bromoacetoxy-3-methoxy-1,3,5(10)-estratrien-17β-ol as an affinity label for the 17β-estradiol dehydrogenase of human placenta, identified a histidine residue in the catalytic region of the active site. This residue was thought to be different from the histidine residue located near position C-3 of the steroid, previously demonstrated by Crastes de Paulet and co-workers[19,20], since no modified cysteine residues were found in the presence or absence of cofactor. Moreover, the fact that N^{τ}-carboxymethyl-L-histidine predominates early in the inactivation and N^{π},N^{τ}-dicarboxymethyl-L-histidine dominates later in the inactivation indicates, according to the authors, that a single

histidine residue is being doubly alkylated. Warren's group [24] also synthesized 4-bromoacetamido-3-methoxy-1,3,5(10)-estratrien-17-one and found it to modify lysine and cysteine residues of the placental 17β-estradiol dehydrogenase. In the presence of cofactor, they found that no cysteine residues were modified. Similarly, 2-bromoacetamido-3-methoxy-1,3,5(10)-estratrien-17-one was synthesized and found to carboxymethylate cysteine (65%), histidine (25%), and lysine (8%)[25]. The presence of 1,3,5(10)-estratriene-3,17β-diol was found to inhibit the alkylation of cysteine and histidine residues suggesting that they may be located in the region of the active site close to the steroid A-ring.

Warren and co-workers [26,27] suggested, on the basis of Dreiding models, that if 12β-bromoacetoxy-4-estrene-3,17-dione alkylates the same histidine residue previously identified using the 16α-bromoacetoxy derivative [23], this residue would be located at the site of the catalytic event. However, the results indicated that a different N^{τ}-carboxymethylated histidine residue was obtained with these two analogues. These workers concluded that the histidine residue obtained with the 12β-bromoacetoxy derivative is not located in the region of the steroid where the catalytic event occurs, most likely because the angular 18-methyl group would restrict access of the 12β-bromoacetoxy group to the β-face, D-ring region of the steroid. Alkylation of a histidine group in the A,B-rings region would then be favoured. In fact, it was found that the same carboxymethylated histidine residue is labeled both by 3-bromo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one and 12β-bromo[2'-^{14}C]acetoxy-4-estrene-3,17-dione[28]. As mentioned, Warren and co-workers[26] demonstrated that the 16β-bromoacetoxy derivative and the 12β-bromoacetoxy derivative label different histidine residues to form

N^{π}-carboxymethyl-L-histidine. However, the data obtained did not exclude the possibility of also labeling a single histidine at different positions in the imidazole ring by these two steroid derivatives. Recently a tryptic peptide of 17β-estradiol dehydrogenase containing a histidine residue modified by 16β-bromo[2'-^{14}C]-acetoxy-3-methoxy-1,3,5(10)-estratrien-17β-ol and by 12β-bromo[2'-^{14}C]-acetoxy-4-estrene-3,17-dione was sequenced[29]. Thus it was demonstrated that there are at least two different histidine residues present at the active site, one located near the A-ring and the other near the D-ring of the steroid. Interestingly, the sequenced peptide contains two arginine residues. Inano and Tamaoki[30] employed 3-hydroxy-1,3,5(10)-estratriene-16,17-dione as an affinity label and presented evidence for two catalytically active arginine residues.

The placental enzyme has also been studied with mechanism based inactivators [31,32,33]. Auchus and Covey[34] recently reported that a lysine residue was modified when the enzyme was inactivated by the enzyme-generated 3-hydroxy-14,15-secoestra-1,3,5(10)-trien-15-yn-17-one **1** .



The mechanism is assumed to be a Michael addition of the N^{6}-amino group of a lysine residue (nucleophile) to the acetylenic ketone (potent electrophile). This result contrasts with the histidine and cysteine residues

modified by the haloacetoxy (or acetamido) steroidal affinity labels previously described. Perhaps the higher pH (9.2 compared to pH 6.3 [28,29] or 7.2[18] in previous studies) deprotonates the lysine amino acid rendering it sufficiently nucleophilic to compete with other nucleophilic residues in the active site.

2. Bifunctional Enzyme Activity.

In 1981, Strickler, Tobias and Covey[35] suggested that the purified 17β-estradiol dehydrogenase from human placenta also possessed 20α-hydroxysteroid dehydrogenase activity. Such enzymes possessing activities for two different positions on the steroid molecule are often designated as bifunctional enzymes. Affinity alkylation studies were undertaken in order to find if the estrogen and 4-pregnene-3,20-dione oxido-reductase activities were occurring at a single active site. Affinity radioalkylation studies using 16α-bromo[2'-^{3}H]acetoxy-4-pregnene-3,20-dione indicated a stoichiometry of two mol of steroid bound per mol of inactivated enzyme dimer and indicated also that a N^{π},N^{τ}-dicarboxymethyl-L-histidine residue was formed. Also, the fact that both the 17β-estradiol dehydrogenase and 20α-hydroxysteroid dehydrogenase activities were identically inactivated suggested that only one site was responsible for both activities. Moreover, the affinity labeling nucleotide analogue 5'-[*p*-(fluorosulfonyl)-benzoyl]adenosine, which binds at the cofactor site as a competitive inhibitor of NADH, was found to decrease both 17β- and 20α- activities[36]. Further evidence that both 17β- and 20α- activities were present in the same protein were revealed by Thomas and Strickler[37] using 6β-bromoacetoxy-4-pregnene-3,20-dione. Affinity radioalkylation of the placental enzyme with 6β-[2'-^{14}C]bromoacetoxy-4-

pregnene-3,20-dione, indicated that cysteine (56%), histidine (22%), and lysine (8%) residues were carboxymethylated. These results support those reported for 2-bromo[2'-^{14}C]acetamido-3-methoxy-1,3,5(10)-estratrien-17-one radioalkylation of the 17β-estradiol dehydrogenase[25].

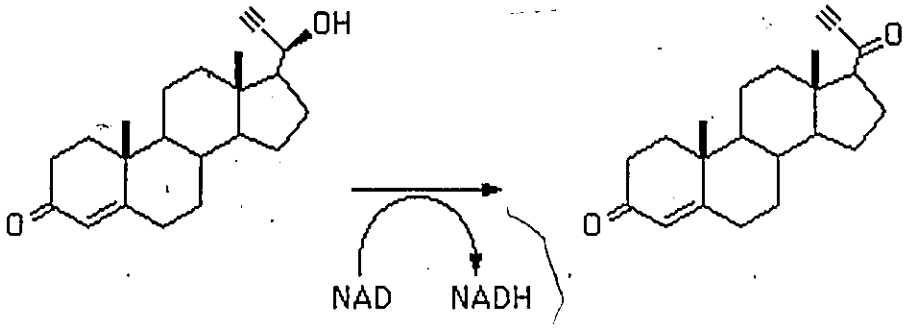
Strickler's group[38] found that 3-bromoacetoxy-1,3,5(10)-estratrien-17-one caused a loss of both the 17β- and 20α-enzyme activities of the human placental enzyme. Both activities were recovered when the pH of the inactivated enzyme solution was raised to 9.3. Based on their computer graphic model of estrogen and progestin substrate binding, Strickler and co-workers predicted that 11α-bromoacetoxy-4-pregnene-3,20-dione could label amino acid residues in the region of the enzyme active site which is also accessible to 3-bromoacetoxy-1,3,5(10)-estratrien-17-one. Therefore the 17β,20α-hydroxysteroid dehydrogenase was inactivated with 3-bromoacetoxy-1,3,5(10)-estratrien-17-one, reactivated and affinity radioalkylated with 11α-bromo[2'-^{14}C]acetoxy-4-pregnene-3,20-dione to determine if a common active site amino acid was modified by both steroid derivatives. They concluded that both derivatives alkylate a common histidine residue in the enzyme active site since an observed 5-fold decrease in the production of radiolabeled N^{τ}-carboxymethyl-L-histidine in the reactivated enzyme is found relative to control enzyme, i.e. enzyme which was treated similarly with 3-hydroxy-1,3,5(10)-estratrien-17-one and 11α-hydroxy-4-pregnene-3,20-dione. The initial alkylation by 3-bromoacetoxy-1,3,5(10)-estratrien-17-one prior to reactivation labels the histidine residue which then cannot be radiolabeled by the 4-pregnene-3,20-dione derivative.

Bifunctional enzyme activity at the same active site has also been reported for the 3α,20β-hydroxysteroid dehydrogenase from *Streptomyces hydrogenans* (E.C.1.1.1.53) [39,40,41] and for the 3β,20α-hydroxysteroid oxido-reductase from fetal blood [42]. Affinity labeling has been used to examine this bifunctional activity in both of these enzymes.

In studies on the 3α,20β-hydroxysteroid dehydrogenase affinity alkylation was initially accomplished with 21-iodoacetoxy-17α-hydroxy-4-pregnene-3,11,20-trione[43] and the results indicated that a histidine residue was modified. In a more extensive study 2α-, 6β-, 11α-, and 16α-bromoacetoxy-4-pregnene-3,20-dione [44,45,46] and 21-bromoacetamido-4-pregnene-3,20-dione[47] were synthesized and these compounds were used to affinity alkylate the 3α,20β-hydroxysteroid dehydrogenase. The following topography of the active site could be deduced from these experiments: two different methionyl residues were located near positions C-2 and C-11 of the A- and C-rings[44], a cysteine residue was located near position C-6 of the B-ring [45,48] and a histidine residue near positions C-16 and C-21 of the D-ring[46,47].

It should also be mentioned that bifunctional affinity labels have been synthesized and used to study hydroxysteroid dehydrogenases. 2,4-Bis(bromo-methyl)-3-methoxy-1,3,5(10)-estratrien-17-one was found to inactivate the 17β-estradiol dehydrogenase[49] enzyme of human placenta and 6β,21-bis(bromoacetoxy)-11β, 17-dihydroxy-4-pregnene-3,20-dione inactivates 3α,20β-hydroxysteroid dehydrogenase[50]. Also, with this 3α,20β-hydroxysteroid dehydrogenase, a mechanism based inactivation was performed with an enzyme-

generated ethoxyacetylenic ketone shown below[51].



In this thesis, affinity labeling studies of two forms of the 17β-hydroxysteroid dehydrogenase isolated from female rabbit liver will be described. Previous studies in this laboratory[52] have shown that multiple forms of this enzyme are present in female rabbit liver and each of these forms exhibits bifunctional enzyme activity. The enzymes catalyze the oxidation of both 17β- and 3α-hydroxysteroids. The 3α-dehydrogenase activity is selective for steroids of the 5β-androstane series. The 17β-dehydrogenase activity is highly selective for Δ^4-unsaturated steroids. The purpose of this study is to compare the steroid binding features of the two enzyme forms and to examine the possibility that the 3α- and 17β-hydroxysteroid dehydrogenase share a common active site.

The second part of this thesis deals with the introduction of a C-4 hydroxyl group in C-19 substituted steroids. The structure and chemistry of a hydroperoxidic steroid isolated from an epoxidation reaction of a C-19 substituted steroid will be presented along with the results of Baeyer-Villiger rearrangements with C-19 substituted steroids.

PART I

AFFINITY LABELING OF 17β-HYDROXYSTEROID DEHYDROGENASE.

RESULTS AND DISCUSSION

A. Isolation and Purification of 17β-Hydroxysteroid Dehydrogenase.

The 17β-HSD enzymes were isolated from the liver of female New Zealand white rabbits essentially by the procedure previously developed in our laboratory[52,53]. This procedure is summarized in Figure 1. The principal modification from the published procedure is the elimination of the initial Sephadex G-25 chromatographic step and the utilisation of histidine-HCl buffer instead of imidazole-HCl buffer for chromatofocusing. The enzyme profiles obtained by affinity chromatography and chromatofocusing are shown in Figures 2 and 3, respectively. Three 17β-HSD, designated enzyme forms I, II and III (Figure 3), were separated by chromatofocusing. Subsequent studies were undertaken with forms I and III since purification of these enzyme forms was most readily effected. The purity of the enzyme forms I and III was monitored by polyacrylamide-gel electrophoresis at pH 8.3[54] and by isoelectric focusing polyacrylamide-gel electrophoresis using a modification of the method of Hayes and Wellner[55]. Isoelectric focusing polyacrylamide-gel electrophoresis of both enzyme forms I and III is shown in Figure 4.

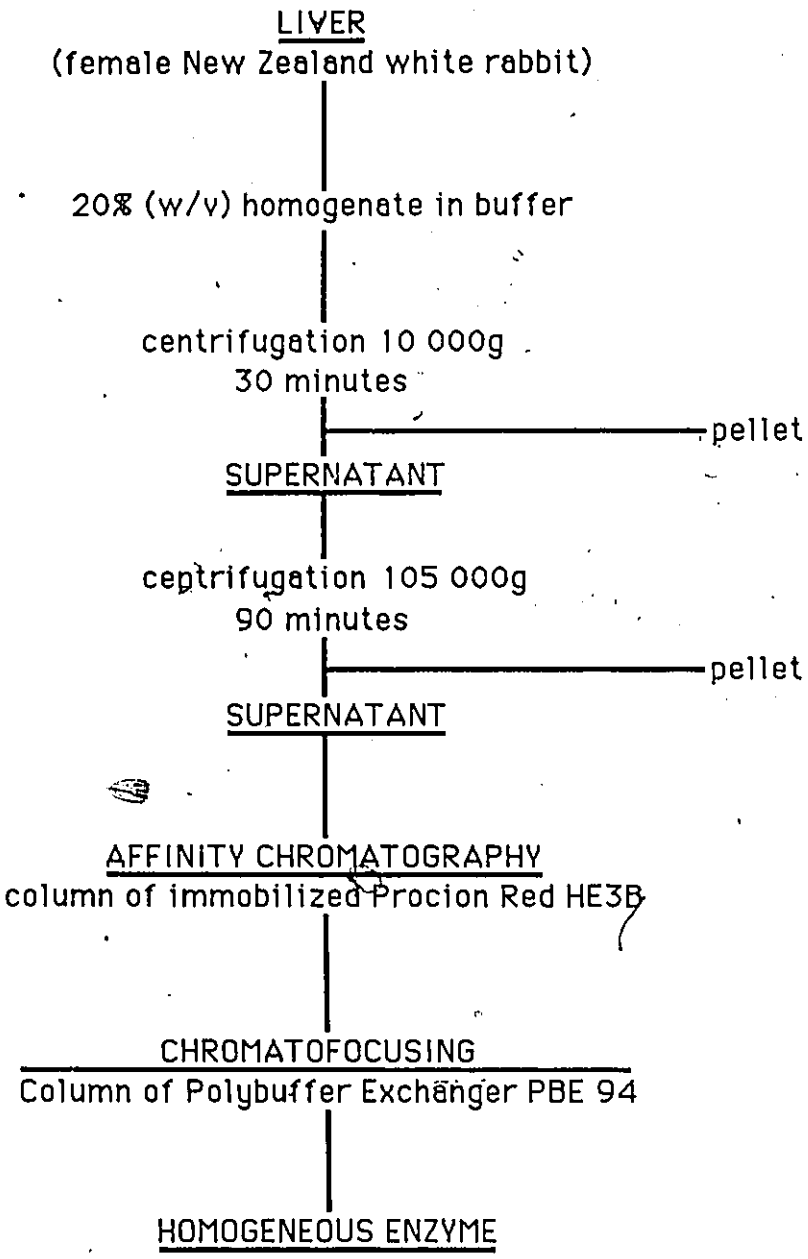


Figure 1. Scheme for the Purification of the Soluble 17β-Hydroxysteroid Dehydrogenase from Rabbit Liver.

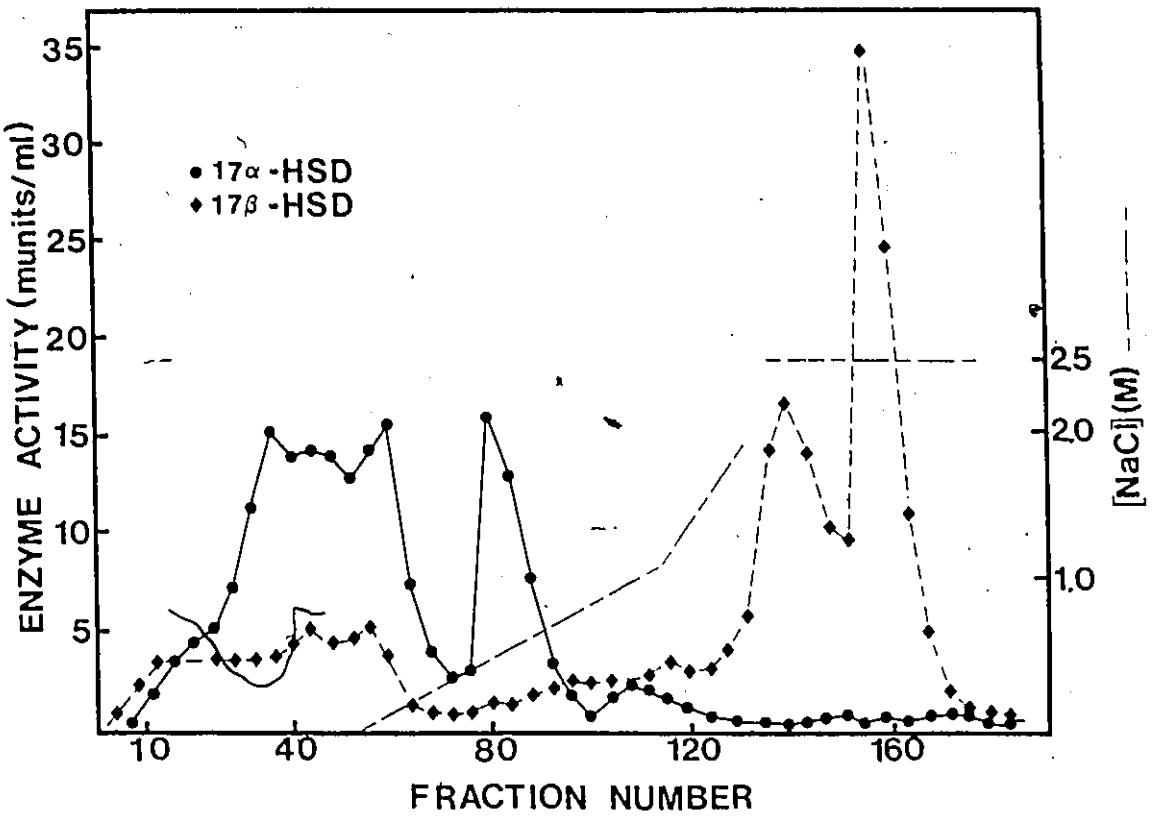


Figure 2. Affinity Chromatography of the Soluble 17β-Hydroxysteroid Dehydrogenase of Female Rabbit Liver.

The concentrated supernatant was applied at a flow rate of 18 ml/h on a column of agarose immobilized Procion Red HE3B (1.5 cm x 40 cm) previously equilibrated with 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT. The column was eluted first with 150 ml of 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT containing 1 mM EDTA and 5 mM $MgCl_2$. Then followed a 600 ml linear gradient of NaCl from 0 M to 1 M in 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT containing 1 mM EDTA and 5 mM $MgCl_2$. The elution was continued with a 200 ml linear gradient of NaCl from 1M to 2 M in 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT containing 5 mM $MgCl_2$. Finally the column was eluted with 400 ml of 2.5 M NaCl in 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT containing 10 mM $MgCl_2$. Aliquots (1 µl) from the fractions collected were assayed for 17α- (•) and 17β-HSD activities (♦) with 17α-hydroxyandrost-4-en-3-one and 17β-hydroxyandrost-4-en-3-one **5** as substrates, respectively. The enzyme activity and the concentration of NaCl are plotted along the ordinate and the fraction number is along the abscissa.

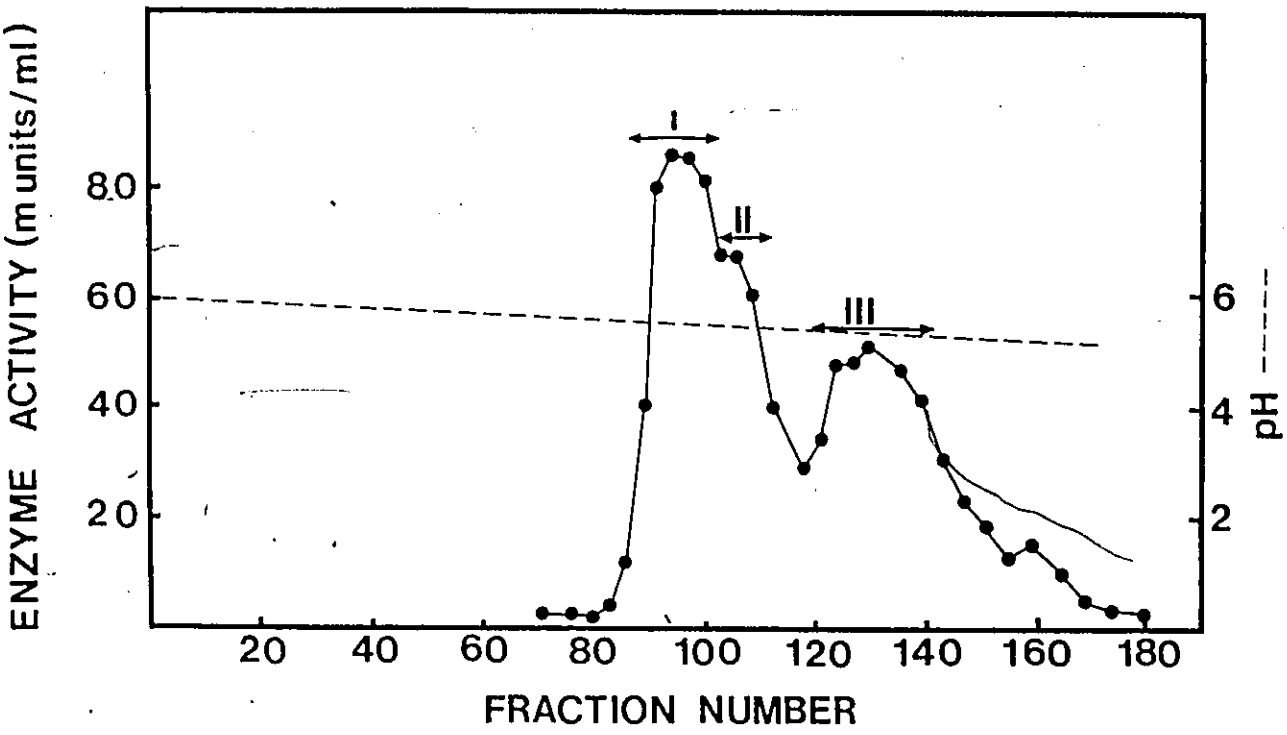


Figure 3. Chromatofocusing of the Soluble 17β-Hydroxysteroid Dehydrogenase from Female Rabbit Liver.

The desalted and concentrated 17β-HSD activity fraction from the affinity chromatography, in 0.025 M histidine buffer, pH 6.0, 0.5 mM DTT was applied on a column of PBE 94 Polybuffer Exchanger previously equilibrated with 0.025 M histidine, pH 6.0, 0.5 mM DTT. The column was eluted with a solution of Polybuffer 74 (diluted 10 times with deaerated distilled water) and fractions (1 ml) were collected. Aliquots (1 µl) from the collected fractions were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The enzyme activity and the pH of the column are plotted along the ordinate and the fraction number is along the abscissa.

I III

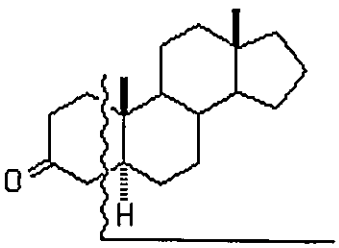
Figure 4. Isoelectric Focusing Polyacrylamide-Gel Electrophoresis of 17β-HSD Forms I and III.

Isoelectric focusing and staining were carried out as described in the Experimental section B.16. The enzyme fraction (25 to 30 μg) was applied to the gel and isoelectric focusing was carried out initially at 400 V for 30 min and then at 500 V for 4 h.

B. Analysis of Steroids by Mass Spectrometry.

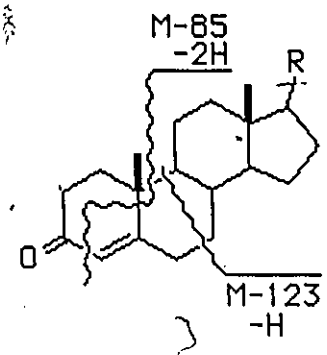
In the present study mass spectrometry was used to identify the steroid products obtained after enzyme incubations and to confirm the structures of the various steroid products synthesized. The main fragmentation patterns of steroids are well documented and reviewed[56-59].

The spectrum of 5α-androstan-3-one **2** has a doublet of intense peaks at m/z 202 and 203[60]. Labeling experiments have shown that these ions originate from the cleavage of the C_1-C_{10} and C_4-C_5 bonds and from the loss of one and two hydrogen atoms respectively from the charged fragment. It is also known that for saturated 3-ketones the patterns found from the mass spectrum analysis of one representative of a given series may not be applied to other compounds of that type[57].

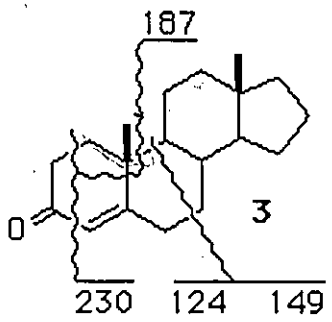


2

In contrast to their saturated counterparts, α,β-unsaturated 3-keto steroids exhibit characteristic mass spectral fragmentations of a general type which are usually independent of ring substituents and are consistent from one class of steroids to another[61]. The bond cleavages giving rise to the major diagnostic ions seen in the mass spectra of Δ^4-3-keto steroids are illustrated below.

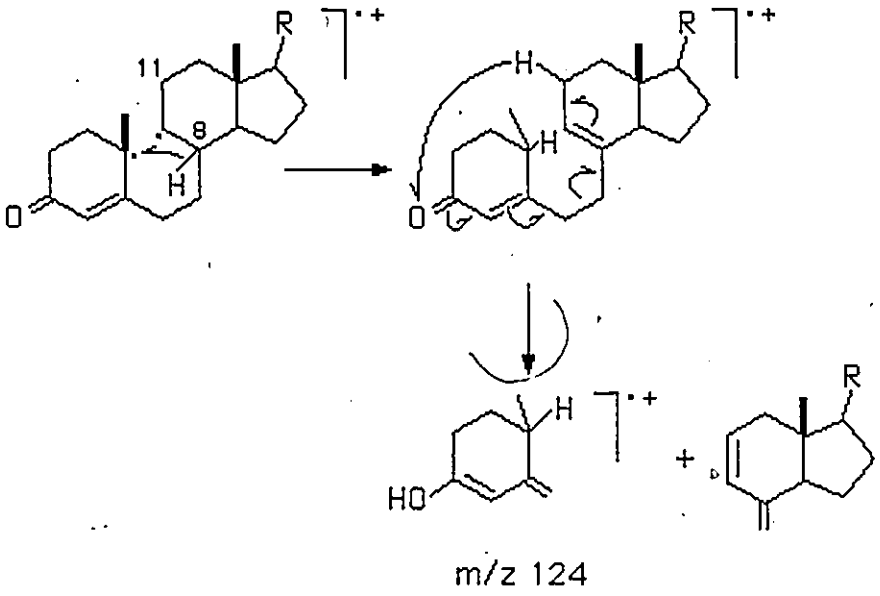


For example, the prominent peaks in the high-mass region of androst-4-en-3-one **3** are m/z 230 (M-42, loss of ketene from the A-ring), m/z 215 (M-57, loss of ketene plus a methyl radical), m/z 187 (M-85, loss of C-1, -2, -3, -10 and -19), m/z 149 (M-123) and m/z 124[61,62].



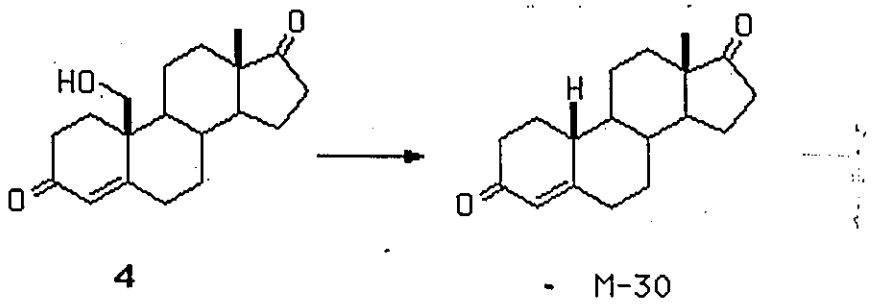
Hydrogen migrations play an important role in the ring cleavages of Δ^4-3-keto steroids and Djerassi and co-workers[61,62] unraveled these complex processes by means of deuterium labeling. The m/z ion is formed from the A-ring plus C- 6 and C-19 and two additional hydrogen atoms. C-8 and C-11 are the major sources of the two itinerant hydrogen atoms[62]. Metastable defocusing indicated that the molecular ion was the sole parent of the m/z 124 ion[61]. The fragmentation can be visualized as occurring by the process shown below.

This mechanism could also be written with the hydrogen atom migration from C-11 preceding that from C-8. Unfortunately an attempt to differentiate between these two processes was not successful[61].



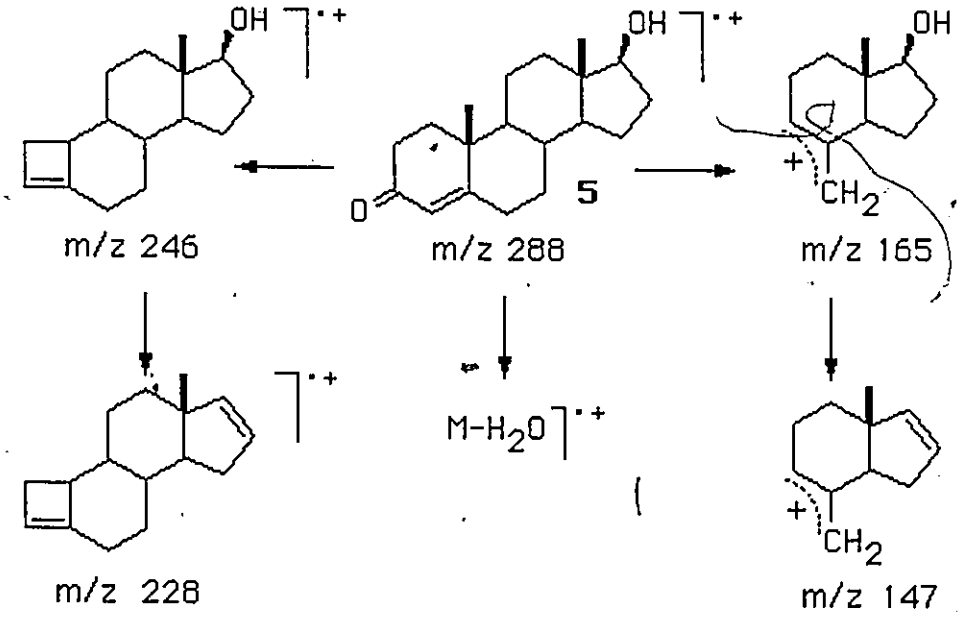
The M-123 ion, resulting from the fragmentaion of the C_6-C_7 and C_9-C_{10} bonds with the charge being retained by the hydrocarbon portion (C- and and D-rings), is also characteristic of Δ^4-3-ketones. The molecular ion is the unique progenitor and one hydrogen atom is transferred away from the charged fragment[61]. This hydrogen atom transfer is not very specific; significant contributions are made by C-8, C-9, C-14 and C-15. The M-85 ion is also characteristic of Δ^4-3-ketones and originate from the M-42 ion (loss of ketene) and not from the molecular ion as shown by metastable defocusing[61].

An interesting feature is revealed in the fragmentation of 19-hydroxyandrost-4-ene-3,17-dione **4**. The base peak in the mass spectrum of **4** is due to elimination of the angular CH_2OH group as formaldehyde[63].



4 - M-30

The mass spectrum of 17β-hydroxyandrost-4-en-3-one **5** has been studied[64,65,66] and the principal fragments are summarized below[67].



17-Keto steroids were studied extensively by Djerassi and co-workers[68] and loss of a methyl group and water are observed among other fragments.

C. Verification of Steroids as Substrates for 17β-HSD.

Before using various steroid derivatives as affinity labels, it was necessary to establish that the parent steroids (the underivatized steroids) were substrates for the enzymes. The steroids of interest for our active site studies were incubated with 17β-HSD enzyme forms I and III, in 10 mM Tris-HCl, pH 8.0, with NADP or with 0.1M Tris-maleate, pH 7.6 if NADPH was the cofactor utilized. The product and remaining starting material were isolated, separated by thin layer chromatography and analyzed by mass spectrometry. Table I summarizes the steroids which were incubated and the products obtained. Previous studies in our laboratory had demonstrated that forms I and III of the rabbit liver 17β-HSD catalyzed the C-17 oxido-reduction of 3-keto-Δ^4-androgens and estrogens and the C-3 oxido-reduction of 5β-androstanes. The present studies confirm this specificity (Table I). The results in Table I also show that the presence of hydroxyl groups at C-3 (3-hydroxy-1,3,5(10)-estratrien-17-one, **16**), at C-4 (4-hydroxy-androst-4-ene-3,17-dione, **6**), at C-6 (6β-hydroxyandrost-4-ene-3,17- dione, **10**), or at C-11 (11β-hydroxyandrost-4-ene-3,17-dione, **8**) does not preclude the steroid as a substrate for the 17β-HSD enzyme forms I and III and indicates that these substrate analogues could be suitable affinity labels. For 17β-hydroxy-androst-4-en-3-one **5** , 19-hydroxyandrost-4-ene-3,17-dione **4** and 3α-hydroxy-5β-androstan-17-one **20** the incubation of the corresponding tritiated compound indicates that **5, 4** and **20** are substrates for both forms I and III of the 17β-HSD.

TABLE I

Substrate Specificity of 17ß-Hydroxysteroid Dehydrogenase for Enzyme Forms I and III (with NADP or NADPH as Cofactor).

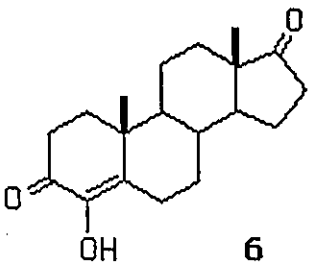
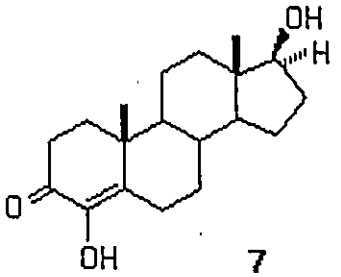
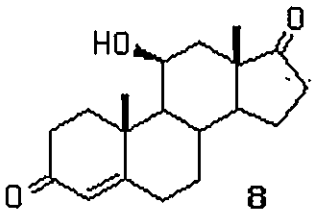
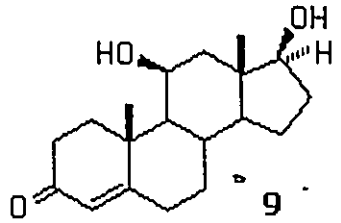
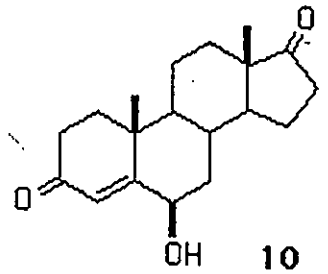
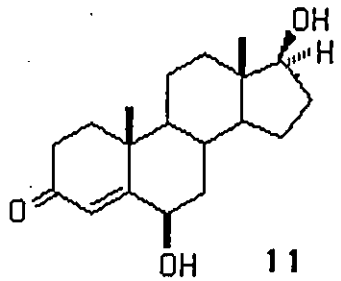
Substrate	Product
 6	 7
$C_{19}H_{26}O_3$ 302(M^+), 287(M-15,CH_3), 284 (M-18, H_2O), 260(M-42,C_2H_2O), 217(M-85, C_5H_9O), 163(M-140),-1H).	$C_{19}H_{28}O_3$ 304(M^+), 289(M-15,CH_3), 286(M-18,H_2O).
 8	 9
$C_{19}H_{26}O_3$ 302(M^+), 287(M-15,CH_3), 284(M-18,H_2O), 260(M-42,C_2H_2O), 217(M-85,C_5H_9O), 179(M-123).	$C_{19}H_{28}O_3$ 304(M^+), 289(M-15,CH_3), 286(M-18,H_2O), 262(M-42,C_2H_2O), 219(M-85,C_5H_9O), 180(M-123,-1H).
 10	 11
$C_{19}H_{26}O_3$ 302(M^+), 287(M-15,CH_3), 284(M-18,H_2O), 260(M-42,C_2H_2O), 217(M-85,C_5H_9O),163(M-141).	$C_{19}H_{28}O_3$ 304(M^+), 289(M-15,CH_3), 286(M-18,H_2O), 262(M-42,C_2H_2O), 219(M-85,C_5H_9O), 165(M-141).

TABLE I(continued)

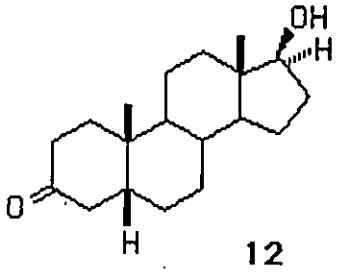
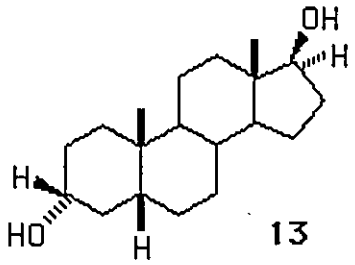
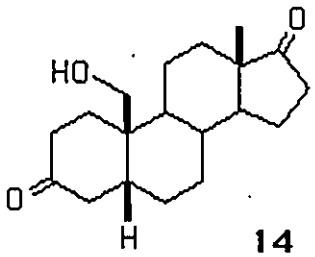
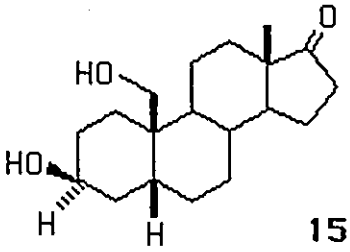
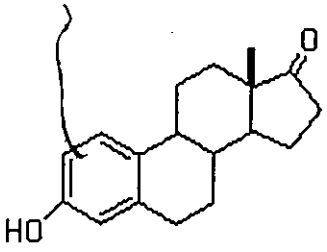
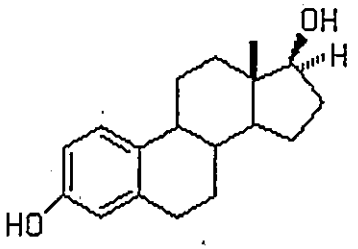
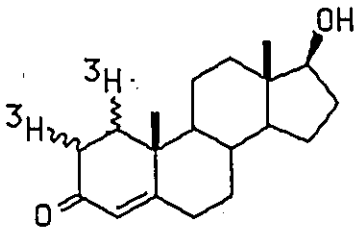
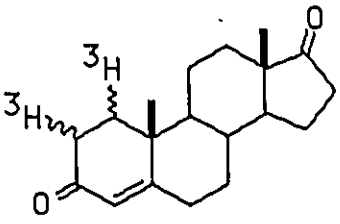
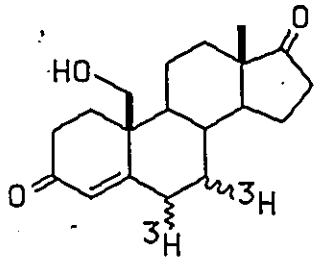
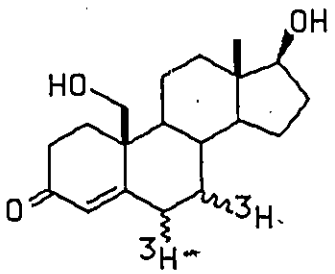
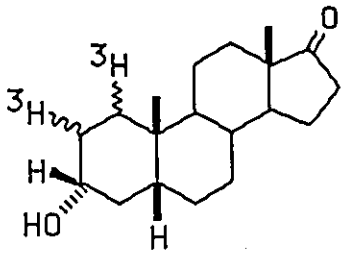
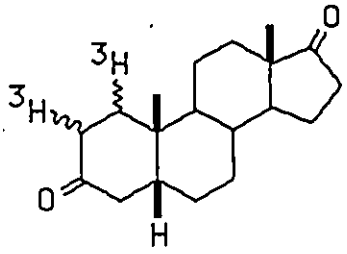
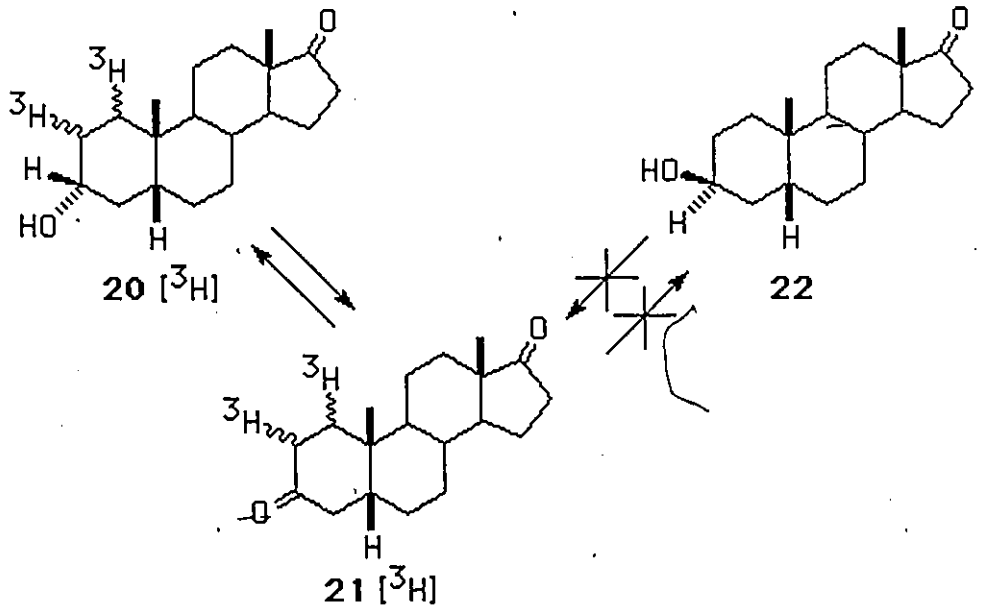
Substrate	Product
 12	 13
$C_{19}H_{30}O_2$ 290(M^+), 275(M-15,CH_3), 272 (M-18, H_2O).	$C_{19}H_{32}O_2$ 292(M^+), 277(M-15,CH_3) 274(M-18,H_2O), 256(M-36,M-2(H_2O)).
 14	 15
$C_{19}H_{28}O_3$ 304(M^+), 289(M-15,CH_3), 286(M-18,H_2O), 274(M-30).	$C_{19}H_{30}O_3$ 306(M^+), 288(M-18,H_2O), 276(M-30).
 16	 17
$C_{18}H_{22}O_2$ 270(M^+), 255(M-15,CH_3).	$C_{18}H_{24}O_2$ 272(M^+), 257(M-15,CH_3).

TABLE I(continued)

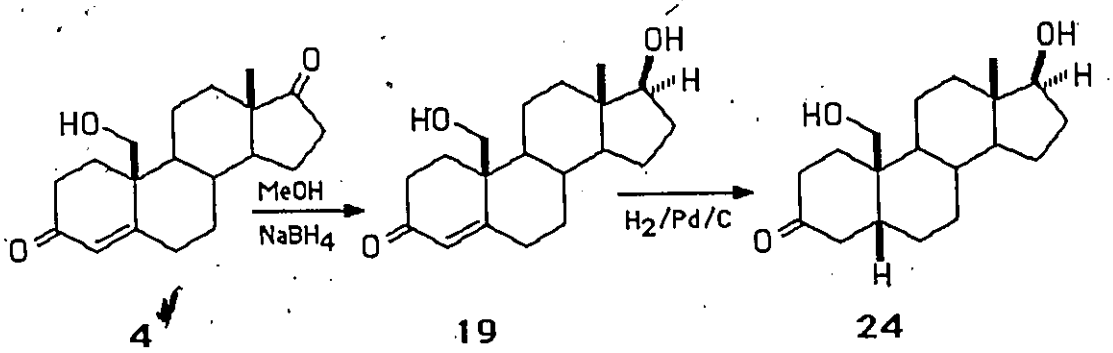
Substrate	Product
	
5 [^{3}H]	18 [^{3}H]
	
4 [^{3}H]	19 [^{3}H]
	
20 [^{3}H]	21 [^{3}H]

Previous studies in our laboratory had indicated that the activity of enzyme forms I and III towards 5β-steroids was specific for 3α-hydroxysteroids[52]. This was again confirmed for both enzyme forms by incubating 3α-hydroxy-[1,2-^{3}H]5β-androstan-3-one **20** [^{3}H] in the presence of NADP and observing that it was converted to [1,2-^{3}H]5β-androstane-3,17-dione **21** [^{3}H]. Similarly the incubation of both enzyme forms with [1,2-^{3}H]5β-androstane-3,17-dione **21** [^{3}H] gave only the 3α-hydroxy derivative **20** [^{3}H]. It was also observed that upon incubation with 3β-hydroxy-5β-androstan-3-one **22**, both enzyme forms I and III failed to give 5β-androstane-3,17-dione **21** thus confirming the specificity of the dehydrogenase for 3α-alcohols.



Based on this result, it was expected that the incubation of 19-hydroxy-5β-androstane-3,17-dione **14**, in the presence of NADPH, would give

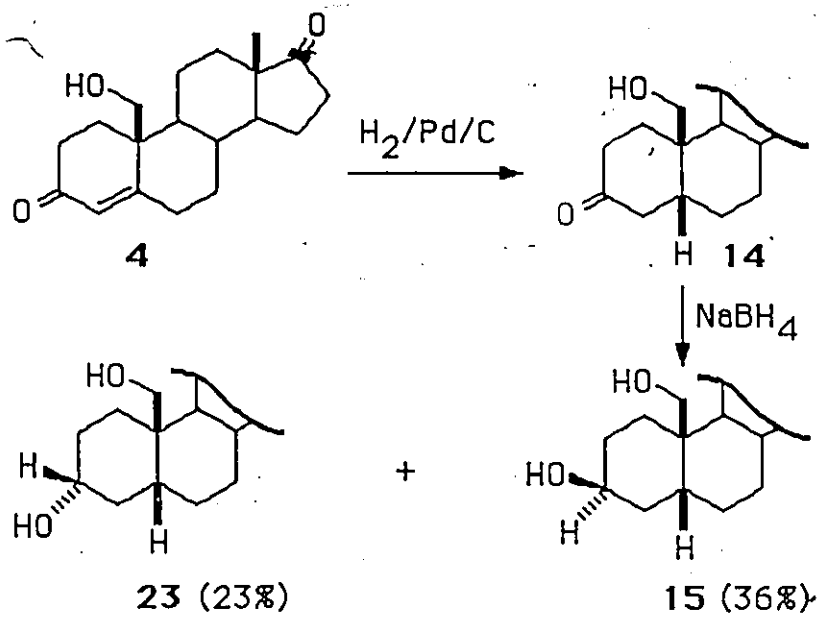
3α,19-dihydroxy-5β-androstan-17-one **23**. The mass spectrum of the product allowed us to be sure that we were not isolating 3α,17β,19-trihydroxy-5β-androstane because this compound would have a mass of 308 and the isolated product had a mass of 306. However, the mass spectrum did not permit us to distinguish between the product of reduction at the 3- or the 17- position. We therefore synthesized the known 17β,19-dihydroxy-5β-androstan-3-one **24**[69] by first reducing the 17-ketone **4** with sodium borohydride to give **19**. Hydrogenation of the double bond was done in 95% ethanol with 10% palladium on charcoal to afford 65% of **24**.



4 **19** **24**

Sodium borohydride reduction of 19-hydroxy-5β-androstane-3,17-dione **14**, previously obtained from hydrogenation of the unsaturated compound **4**, gave a mixture of two products. The major product (36%) was determined to be the 3β-compound **15** and the minor product (23%) was assigned the 3α-configuration **23**. This assignment is based on the half-band width (W/2) of the proton signal at C-3[70]. The broader signal at δ 3.61 (W/2 of *ca* .16.7 Hz) is attributed to the axial-3β hydrogen atom because of two large diaxial and two smaller diequatorial vicinal couplings. With the 3β-alcohol, the equatorial

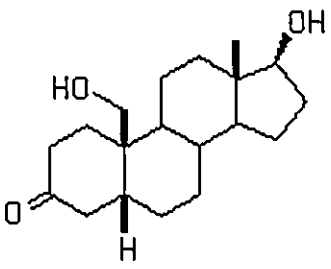
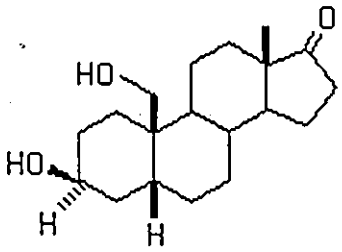
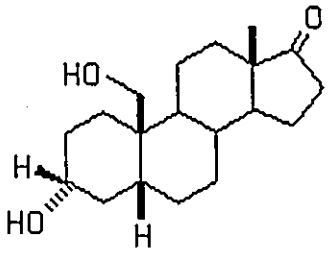
3α-hydrogen atom is submitted only to smaller diequatorial and equatorial-axial vicinal couplings thus giving a half-band width (W/2) of *ca.* 7.6 Hz.



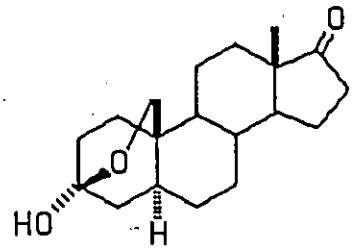
Thin layer chromatography of the enzymatic product isolated after incubation of **14** and the synthesized standards **24, 15** and **23** was carried out. The R_f of the enzymatic product corresponded to the R_f of 3β,19-dihydroxy-5β-androstane-17-one **15** as summarized in Table II. The enzymatic product isolated from the incubation of 19-hydroxy-5β-androstane-3,17-dione **14** was also analyzed by proton NMR. The half-band width at δ 3.61 is 8 Hz thus confirming that the product generated by both forms of the 17β-HSD is 3β,19-dihydroxy-5β-androstan-17-one **15**. It was also observed that upon incubation with 3α- or 3β,19-dihydroxy-5β-androstan-17-one, in the presence of NADP, both forms of the 17β-HSD gave 19-hydroxy-5β-androstane-3,17-dione **14**. It is then concluded that the specificity of both enzyme forms I and III toward 3-hydroxysteroids is altered when a hydroxymethyl group rather than a methyl group is present at C-19.

TABLE II

R_f Values (in methylene chloride : acetone, 6 :4) for some Hydroxysteroids.

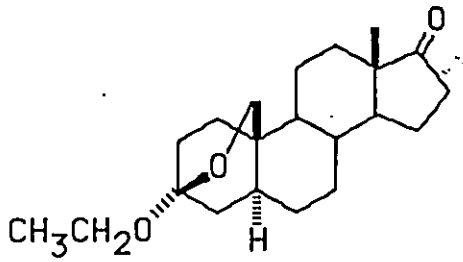
STEROID	R_f
 **24**	0.50
 **15**	0.40
 **23**	0.29
Product isolated from the enzymatic reaction	0.40

The hydrogenation of 19-hydroxyandrost- 4-ene-3,17-dione **4** is accompanied by the formation of a second product whose structure was initially assigned as **25** by Edwards and co-workers[69].



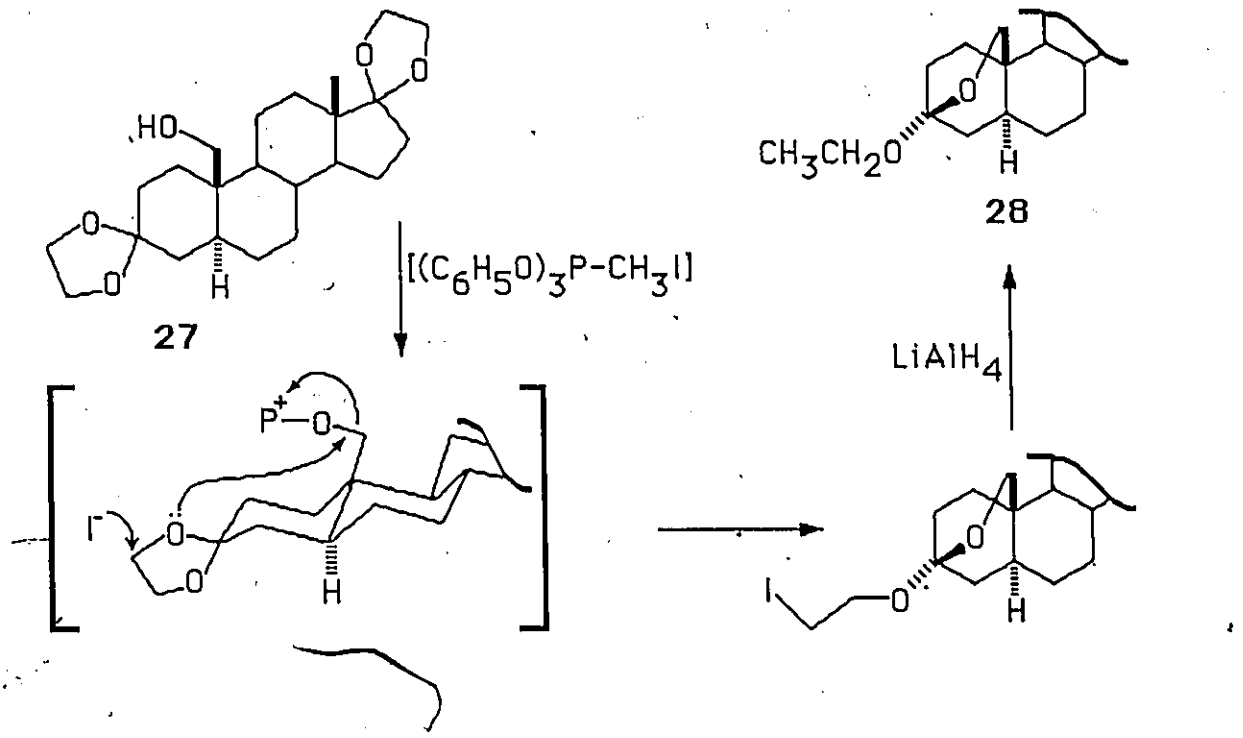
25

The minor product was isolated in 6% yield in our case (*cf.* 10.5%[69]) and had the same melting point as the one reported for **25**. However, the high resolution mass spectrum indicated a molecular weight corresponding to the formula $C_{21}H_{32}O_3$. This represents the addition of C_2H_4 to **25**. The proton NMR indicated that an ethyl group was present with a triplet at δ 1.15 integrating for 3 protons, and a quartet at δ 3.61 integrating for 2 protons. Therefore the reported structure[69] of **25** should be modified to **26**. In agreement also with our structure were the elemental analysis and the ^{13}C spectrum. A Dreiding molecular model of **26** clearly indicates that the 5α-stereochemistry is much more favorable than a 5β- junction.



26

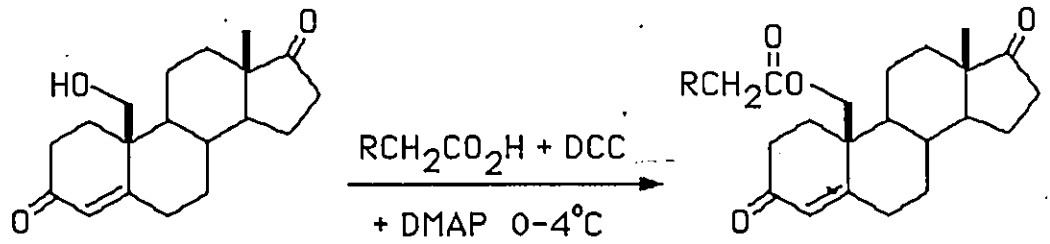
The synthesis of 3α-ethoxy-3β,19-oxido-5α-androstan-17-one **26** had previously been achieved by treatment of **27** with triphenyl phosphite-methyl iodide followed by reduction with lithium aluminum hydride[71].



The same authors reported that hydrolysis of **28** gave 19-hydroxy-5α-androstane-3,17-dione which, on treatment with ethylene glycol and *p*-toluene-sulfonic acid gave **27**. Furthermore, it was found that 19-hydroxy-5β-androstane-3,17-bis(ethylene dioxide) did not react when treated with triphenyl phosphite-methyl iodide. According to the authors[71], the reason why the reaction did not proceed in the A/B cis series was not clear (steric factors or lack of anchimeric assistance of the C-3 ketal moiety).

D. Synthesis of Haloacetoxy Steroids.

The synthesis of the acetate and haloacetate derivatives of the steroids established as substrates for forms I and III of the 17β-HSD (Table II) was carried out. The synthesis with each steroid follows the general equation illustrated with 19-hydroxyandrost-4-ene-3,17-dione 4.



4 29

Either iodoacetic acid or bromoacetic acid was used as acylating agent; dicyclohexylcarbodiimide (DCC) served as a coupling agent and 4-dimethyl-aminopyridine (DMAP) was found to greatly enhance the reaction rate. In initial studies iodo[2-^{3}H]acetic acid was used to synthesize radiolabeled **32**. However, a loss of radioactivity was observed upon acid hydrolysis of the alkylated enzyme because of tritium exchange. The more stable iodo[2-^{14}C]acetic acid was subsequently used in the synthesis of the radiolabeled steroids. It is very interesting to note that tritiated haloacetoxy steroids, for example, 6β-bromo-[2'-^{3}H]acetoxy-4-pregnene-3,20-dione[45] and 11α-bromo[2'-^{3}H]acetoxy-4-pregnene-3,20-dione[45] were synthesized and reported to label histidine, methionine or cysteine residues in the active site of 20β-HSD. Also 21-bromo[2'-^{3}H]acetylamino-4-pregnene-3,20-dione[47], 16α-bromo[2'-^{3}H]acetoxy-3-methoxy-1,3,5(10)-estratrien-17β-ol[23]

and 16α-bromo[2'-^{3}H]acetoxy-4-pregnene-3,20-dione[35] were found to label histidine residues. These results are surprising since it was found by Chin and Wold[72] that the rate of exchange of the methylene hydrogen atoms of S-[^{3}H]carboxymethyl-L-cysteine was rapid (6 h) in 6N HCl at 110°C. After 20 h in 6N HCl at 110°C, N^{6}-[^{3}H]carboxymethyl-L-lysine did not lose any tritium but N^{π}- and N^{τ}-[^{3}H]-carboxymethyl-L-histidine had lost 70% of their tritium.

All the steroid derivatives synthesized are summarized in Table III. Attempts to synthesize the iodoacetoxy derivative of 4-hydroxyandrost-4-ene-3,17-dione **6** were unsuccessful, precluding the utilization of this compound as an affinity label. 11β-Hydroxyandrost-4-ene-3,17-dione **8** reacts very slowly to form the iodoacetoxy derivative. This is explained by the fact that the 11β-hydroxyl group is sterically hindered because of severe 1,3-diaxial interactions with the two angular methyl groups, C-18 and C-19. We did not succeed in making the ^{14}C-labeled 11β-iodoacetoxy derivative **43** in quantities required for labeling the 17β-HSD.

The yields of the non radioactive derivatives were generally high but they were not optimized. In contrast, the yields of the radiolabeled derivatives were poor mainly because of losses during the purification and because the reactions had to be carried out on such a small scale. The non radioactive derivatives were identified by the usual spectroscopic methods (mass spectrometry, ^{1}H NMR and IR). Some compounds have already been reported: **31**[69], **36**[69], **39**[73], **42**[74], **45**[75], **46**[76], **50**[77], **52**[19], **53**[78], **54**[19]. The radiolabeled derivatives were identified by mass spectrometry and their radiochemical purity was verified by tlc.

TABLE III

Steroid Derivatives Synthesized.

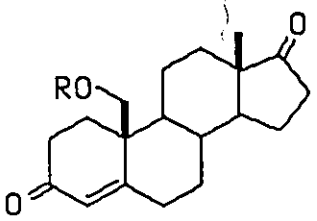
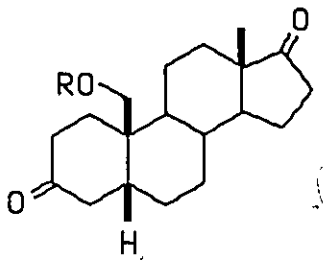
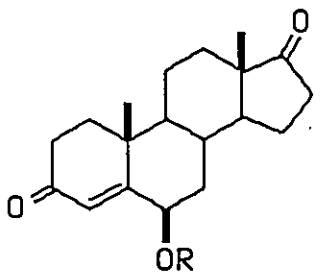
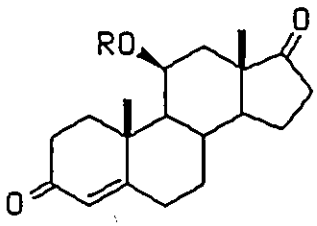
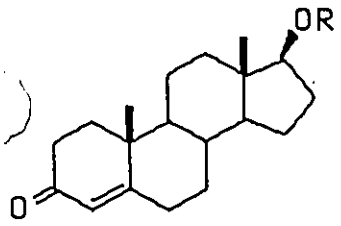
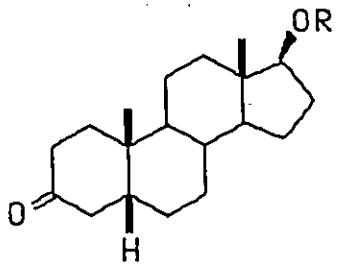
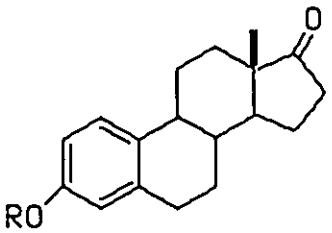
Steroid	R
	**29** R= $\overset{O}{\overset{\|}{C}}CH_2I$ **30** R= $\overset{O}{\overset{\|}{C}}CH_2Br$ **31** R= $\overset{O}{\overset{\|}{C}}CH_3$ **32** R= $\overset{O}{\overset{\|}{C}}C^3H_2I$ **33** R= $\overset{O}{\overset{\|}{C}}{}^{14}CH_2I$
	**34** R= $\overset{O}{\overset{\|}{C}}CH_2I$ **35** R= $\overset{O}{\overset{\|}{C}}CH_2Br$ **36** R= $\overset{O}{\overset{\|}{C}}CH_3$ **37** R= $\overset{O}{\overset{\|}{C}}{}^{14}CH_2I$
	**38** R= $\overset{O}{\overset{\|}{C}}CH_2I$ **39** R= $\overset{O}{\overset{\|}{C}}CH_3$ **40** R= $\overset{O}{\overset{\|}{C}}{}^{14}CH_2I$
	**41** R= $\overset{O}{\overset{\|}{C}}CH_2I$ **42** R= $\overset{O}{\overset{\|}{C}}CH_3$ **43** R= $\overset{O}{\overset{\|}{C}}{}^{14}CH_2I$

TABLE III (continued)

Steroid	R
	**44** R= $-C(=O)CH_2I$ **45** R= $-C(=O)CH_2Br$ **46** R= $-C(=O)CH_3$ **47** R= $-C(=O)^{14}CH_2I$
	**48** R= $-C(=O)CH_2I$ **49** R= $-C(=O)CH_2Br$ **50** R= $-C(=O)CH_3$ **51** R= $-C(=O)^{14}CH_2I$
	**52** R= $-C(=O)CH_2I$ **53** R= $-C(=O)CH_3$ **54** R= $-C(=O)^{14}CH_2I$

E. Stability of Iodoacetoxy Steroids at pH 7.0.

Haloacetoxy steroids must be stable under the conditions used to inactivate the enzyme. Hydrolysis of the haloacetoxy group must be avoided otherwise non specific labeling of amino acids by liberated haloacetic acid may occur. Most haloacetoxy steroids used as affinity labels are reported to resist hydrolysis at 25°C, over a wide pH range and for a relatively long period of time. For example, 6β- and 11α-bromoacetoxy-4-pregnene-3,20-dione are reported to be stable for 24 h at 25°C in 0.05 M potassium phosphate buffer, pH 7.0[45]. Similarly, 21-bromoacetamido-4-pregnene-3,20-dione is reported to be stable over a period of 72 h at 25°C within a pH range between 3 to 10.5[47]. In contrast to the above bromoacetoxy steroids, 3-iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54** was previously reported to be completely hydrolyzed in approximately 30 min in 0.07 M phosphate buffer, 20% glycerol, pH 7.2[19]. We also observed a complete hydrolysis of compound **54** after 30 min at 25°C in 0.2 M Tris-maleate, pH 7.0, 10% glycerol. However, we found that in the absence of glycerol, the rate of hydrolysis decreased and about 40% of 3-iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54** was still present after 30 min. The stability profile of 3-iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54** is shown in Figure 5. Similarly, Figures 6 to 10 represent the stability profiles of the other iodoacetoxy steroids synthesized. Most derivatives are stable for 3 h at 25°C, at pH 7.0. In addition to 3-iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54**, the less stable derivatives are 17β-iodo[2'-^{14}C]acetoxy-5β-androstan-3-one **51** and 17β-iodo[2'-^{14}C]acetoxyandrost-4-en-3-one **47**.

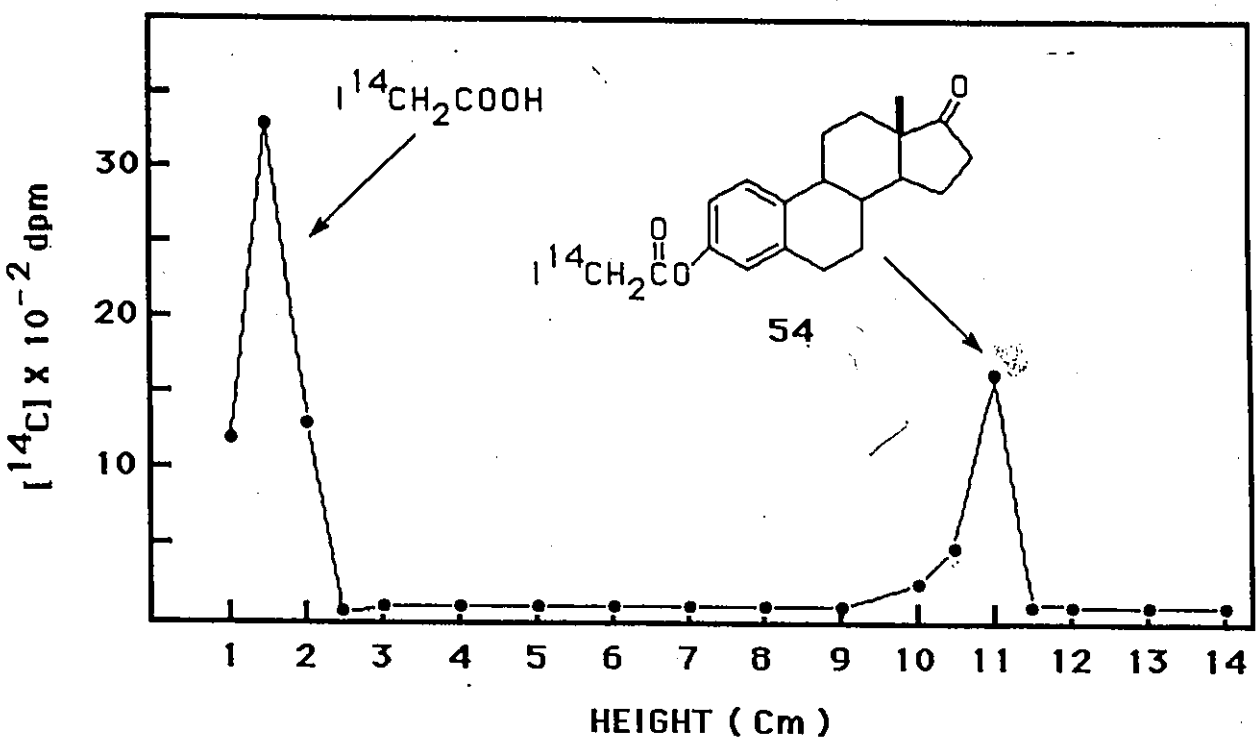


Figure 5. Radioactive Profile of 3-Iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54** after 30 min at 25°C in 20 mM Tris-maleate Buffer pH 7.0.

The incubation contained the following: 3-iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54** (7×10^{-8} mol in 10 µl of 95% ethanol) and 20 mM Tris-maleate buffer, pH 7.0 (2 ml). The incubation was terminated after 30 min at 25°C by extracting with chloroform (2 ml). The chloroform fraction was dried, applied on a tlc plate and eluted in chloroform : ethyl acetate, 9 : 1. After the elution, the tlc plate was divided in 0.5 or 1.0 cm sections and the corresponding silica gel was scraped and the radioactivity measured. The height of the tlc, the origin being located at 1 cm, is plotted along the abscissa and the radioactivity is along the ordinate.

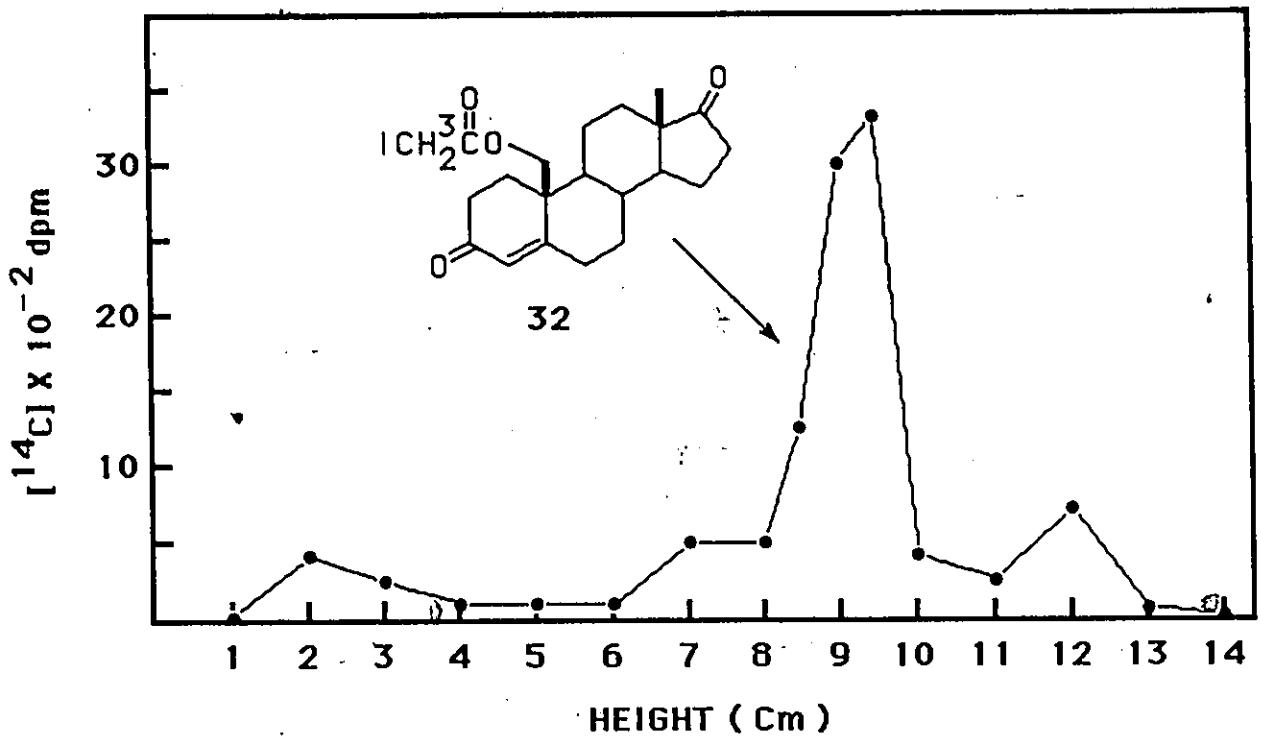


Figure 6. Radioactive Profile of 19-Iodo[2'-$^3H_2$]acetoxyandrost-4-ene-3,17-dione **32** after 3 h at 25°C in 20 mM Tris-maleate Buffer, pH 7.0.

The incubation contained the following: 19-iodo[2'-$^3H_2$]acetoxyandrost-4-ene-3, 17-dione **32** (5 nmol in 20 µl of 95% ethanol) and 20 mM Tris-maleate buffer, pH 7.0 (1 ml). The incubation was terminated after 3 h at 25°C by extracting with ethyl acetate (2 ml). The ethyl acetate fraction was dried, applied on a tlc plate and eluted in methylene chloride : ethyl acetate, 90 : 10. After the elution, the tlc plate was divided in 0.5 or 1.0 cm sections and the corresponding silica gel was scraped and the radioactivity measured. The height of the tlc, the origin being located at 1 cm, is plotted along the abscissa and the radioactivity is along the ordinate.

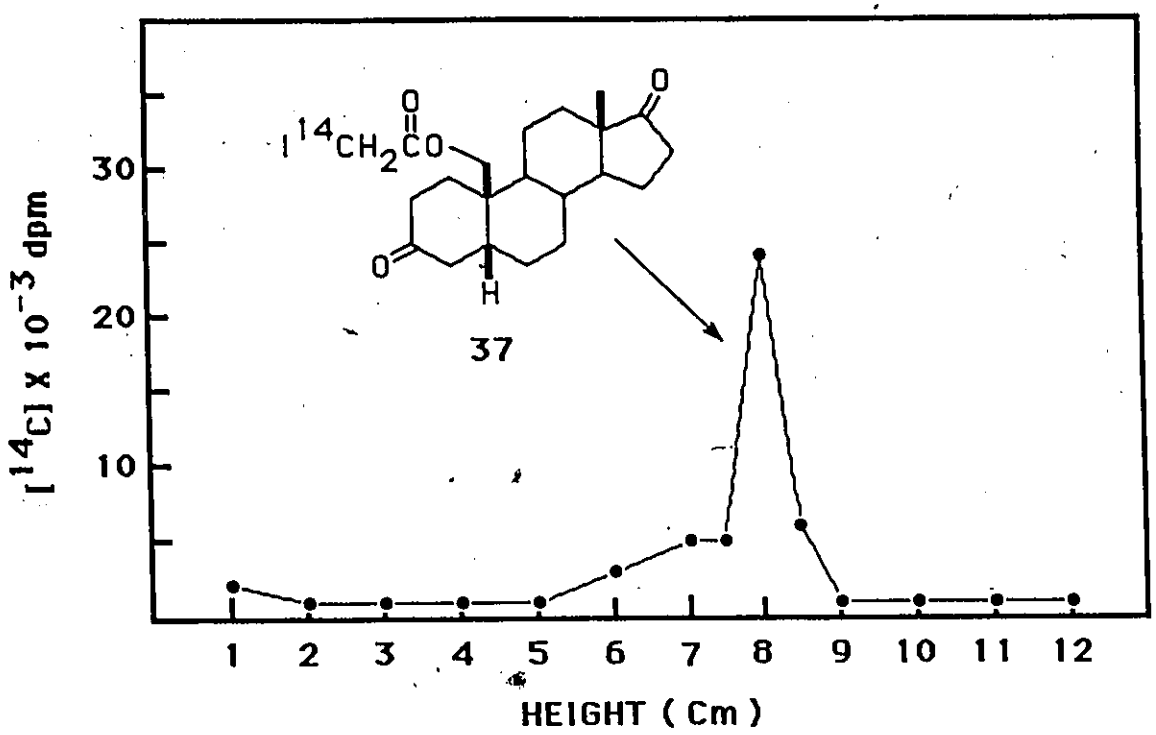


Figure 7. Radioactive Profile of 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37** after 3 h at 25°C in 20 mM Phosphate Buffer, pH 7.0.

The incubation contained the following: 19-iodo[2'-^{14}C]acetoxy-5β-androstane-3, 17-dione **37** (5 nmol in 10 µl of 95% ethanol) and 20 mM phosphate buffer, pH 7.0 (1 ml). The incubation was terminated after 3 h at 25°C by extracting with ethyl acetate (2 ml). The ethyl acetate fraction was dried, applied on a tlc plate and eluted in methylene chloride : ethyl acetate, 90 : 10. After the elution, the tlc plate was divided in 0.5 or 1.0 cm sections and the corresponding silica gel was scraped and the radioactivity measured. The height of the tlc, the origin being located at 1 cm, is plotted along the abscissa and the radioactivity is along the ordinate.

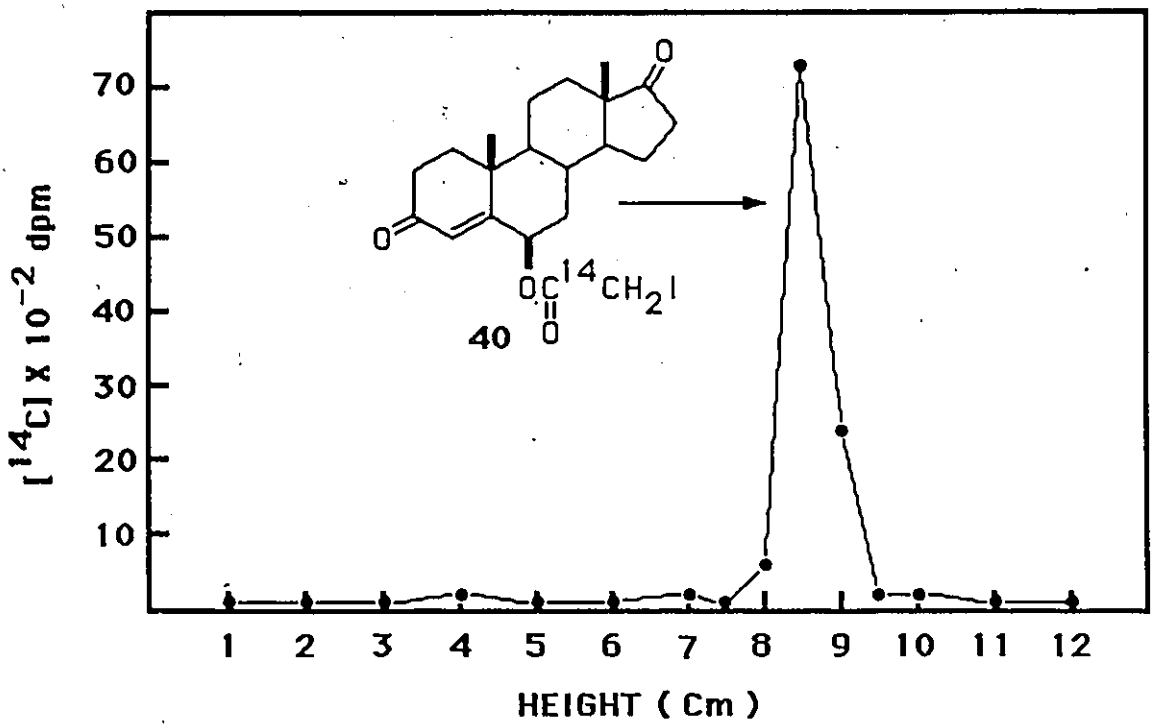


Figure 8. Radioactive Profile of 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **40** after 3 h at 25°C in 20 mM Phosphate Buffer, pH 7.0.

The incubation contained the following: 6β-iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **40** (5 nmol in 10 µl of 95% ethanol) and 20 mM phosphate buffer, pH 7.0 (1 ml). The incubation was terminated after 3 h at 25°C by extracting with ethyl acetate (2 ml). The ethyl acetate fraction was dried, applied on a tlc plate and eluted in methylene chloride : ethyl acetate, 65 : 35. After the elution, the tlc plate was divided in 0.5 or 1.0 cm sections and the corresponding silica gel was scraped and the radioactivity measured. The height of the tlc, the origin being located at 1 cm, is plotted along the abscissa and the radioactivity is along the ordinate

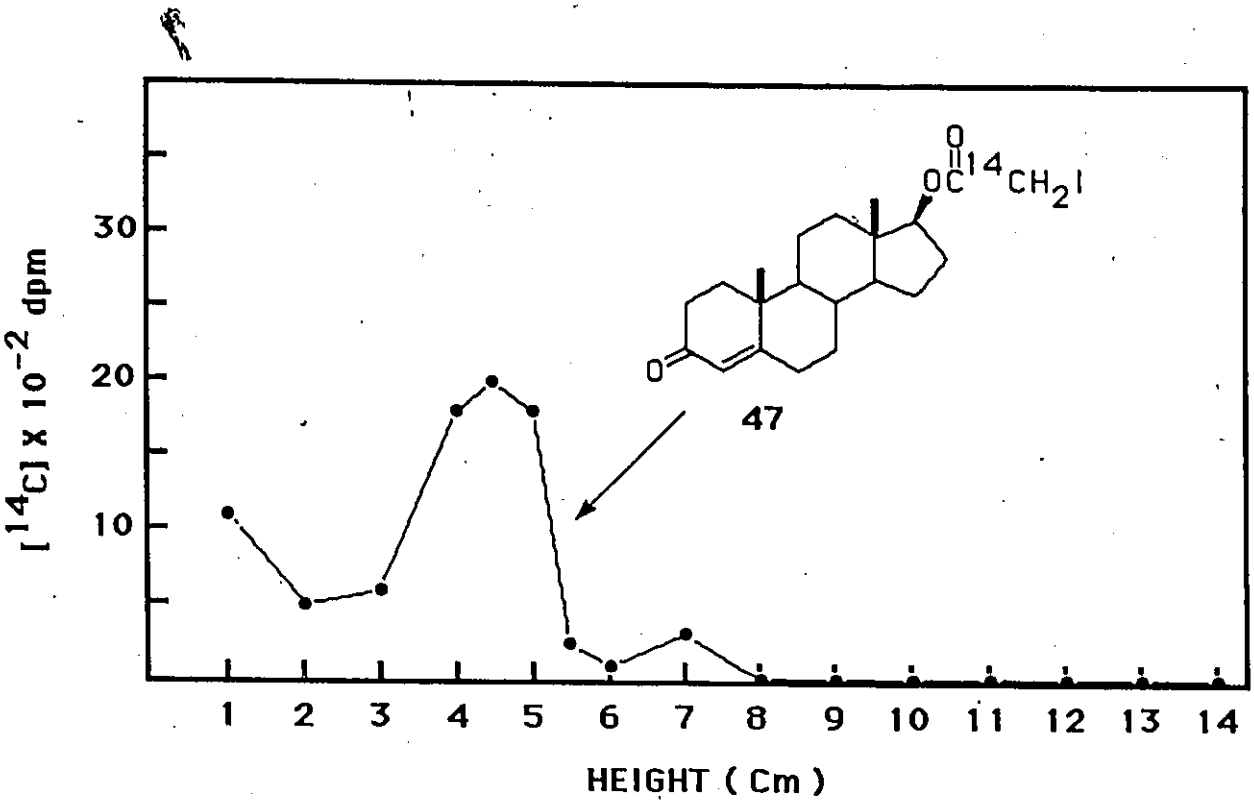


Figure 9. Radioactive Profile of 17β-Iodo[2'-^{14}C]acetoxyandrost-4-en-3-one 47 after 3 h at 25°C in 20 mM Phosphate Buffer, pH 7.0.

The incubation contained the following: 17β-iodo[2'-^{14}C]acetoxyandrost-4-en-3-one 47 (5 nmol in 10 µl of 95% ethanol) and 20 mM phosphate buffer, pH 7.0 (1 ml). The incubation was terminated after 3 h at 25°C by extracting with ethyl acetate (2 ml). The ethyl acetate fraction was dried, applied on a tlc plate and eluted in methylene chloride : ethyl acetate, 90 : 10. After the elution, the tlc plate was divided in 0.5 or 1.0 cm sections and the corresponding silica gel was scraped and the radioactivity measured. The height of the tlc, the origin being located at 1 cm, is plotted along the abscissa and the radioactivity is along the ordinate.

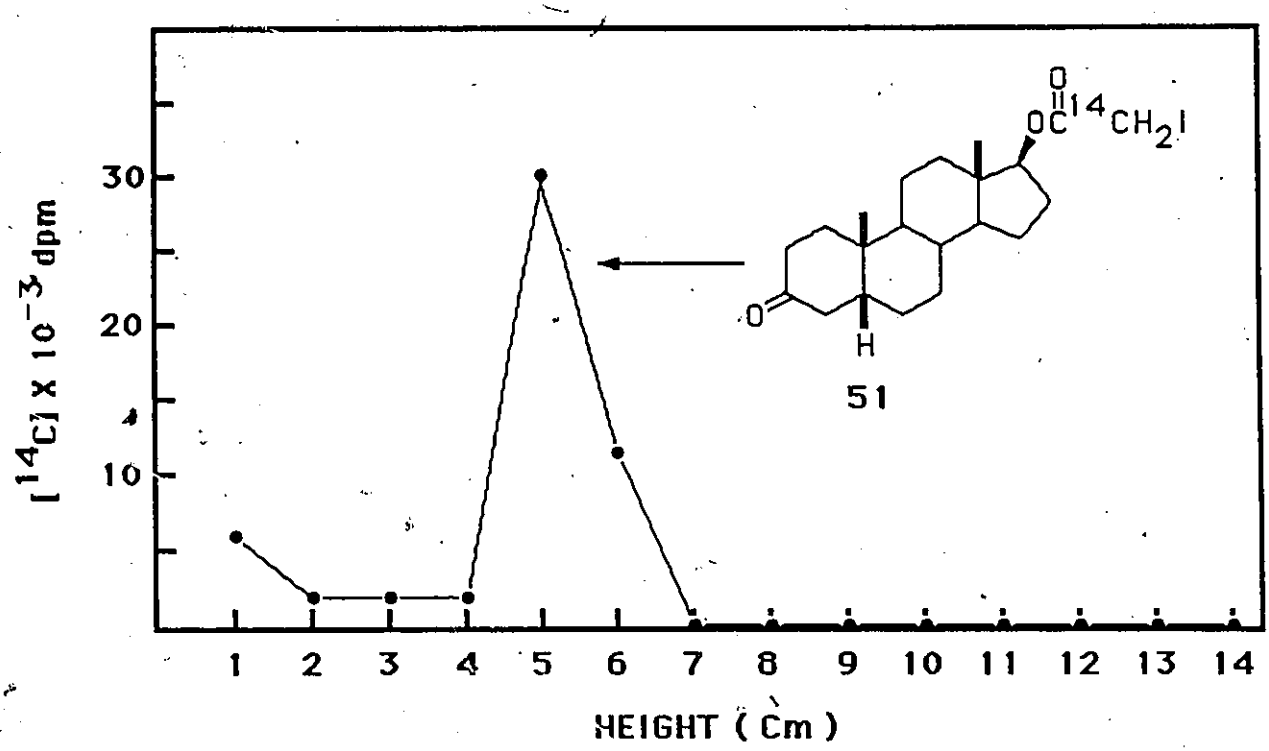


Figure 10. Radioactive Profile of 17β-Iodo[2'-^{14}C]acetoxy-5β-androstan-3-one **51** after 3 h at 25°C in 20 mM Phosphate Buffer, pH 7.0.

The incubation contained the following: 17β-iodo[2'-^{14}C]acetoxy-5β-androstan-3-one **51** (5 nmol in 10 µl of 95% ethanol) and 20 mM phosphate buffer, pH 7.0 (1 ml). The incubation was terminated after 3 h at 25°C by extracting with ethyl acetate (2 ml). The ethyl acetate fraction was dried, applied on a tlc plate and eluted in methylene chloride : ethyl acetate, 90 : 10. After the elution, the tlc plate was divided in 0.5 or 1.0 cm sections and the corresponding silica gel was scraped and the radioactivity measured. The height of the tlc, the origin being located at 1 cm, is plotted along the abscissa and the radioactivity is along the ordinate.

F. Stability of 17β-HSD at pH 7.0.

The stability of the 17β-HSD enzyme forms I and III was studied in 20 mM phosphate buffer, pH 7.0 at 25°C. In the absence of DTT, removed by centrifugal gel filtration[79] on Sephadex G-25 medium column, there is a rapid loss of activity of both enzyme forms I and III as illustrated in Figures 11 and 12. The addition of iodoacetic acid does not affect the enzyme activity whereas the presence of NADP or NADPH prevents the loss of activity with both enzyme forms I and III. We found that changing the incubation temperature from 25°C to 0°C prevented the loss of enzyme activity (Figure 13). However, at 0°C 19-iodo-acetoxyandrost-4-ene-3,17-dione **29** or 17β-iodoacetoxyandrost-4-en- 3-one **44** did not inactivate the enzyme over a period of time. It was therefore decided to carry out the inactivations at 25°C even though the enzyme is less stable at that temperature.

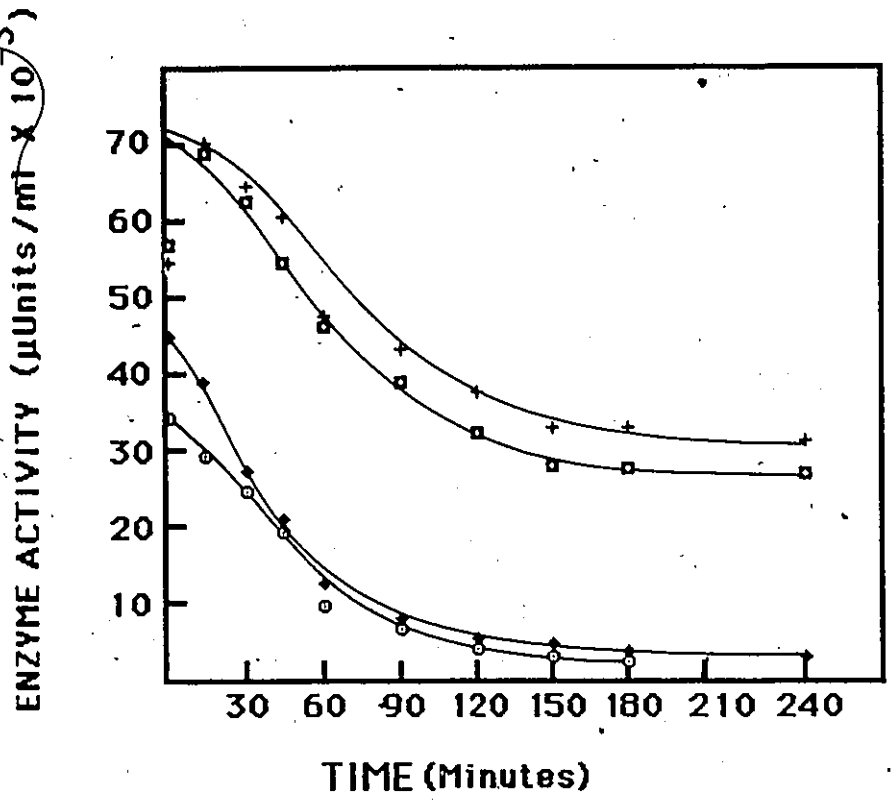


Figure 11. Stability of 17β-HSD-I at 25°C, after Sephadex G-25 Filtration. Effect of NADP, NADPH and Iodoacetic Acid on the Enzyme Activity.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 μl) and EGME (3 μl) was incubated alone (•), in the presence of iodoacetic acid (15 nmol) (⊙), NADP (15 nmol) (+) and NADPH (15 nmol) (⊡). At various time intervals, duplicate aliquots (1 to 3 μl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The enzyme activity is plotted along the ordinate and the time interval is along the abscissa.

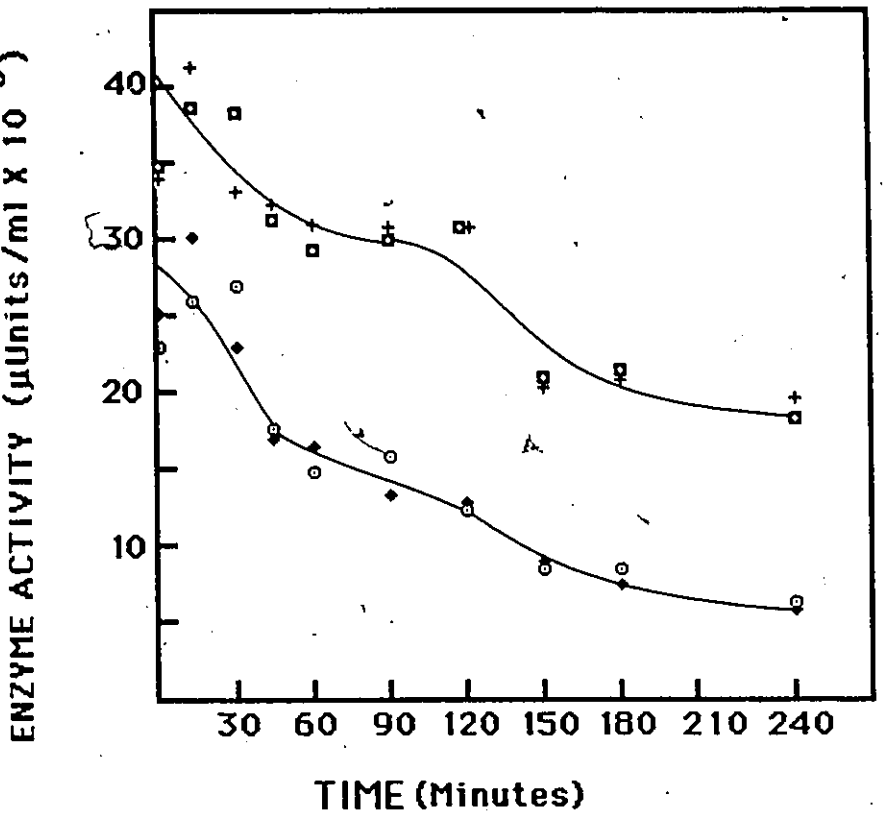


Figure 12. Stability of 17β-HSD-III at 25°C, after Sephadex G-25 Filtration. Effect of NADP, NADPH and Iodoacetic Acid on the Enzyme Activity.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 μl) and EGME (3 μl) was incubated alone (◆), in the presence of iodoacetic acid (15 nmol) (⊖), NADP (15 nmol) (+) and NADPH (15 nmol) (□). At various time intervals, duplicate aliquots (1 to 3 μl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The enzyme activity is plotted along the ordinate and the time interval is along the abscissa.

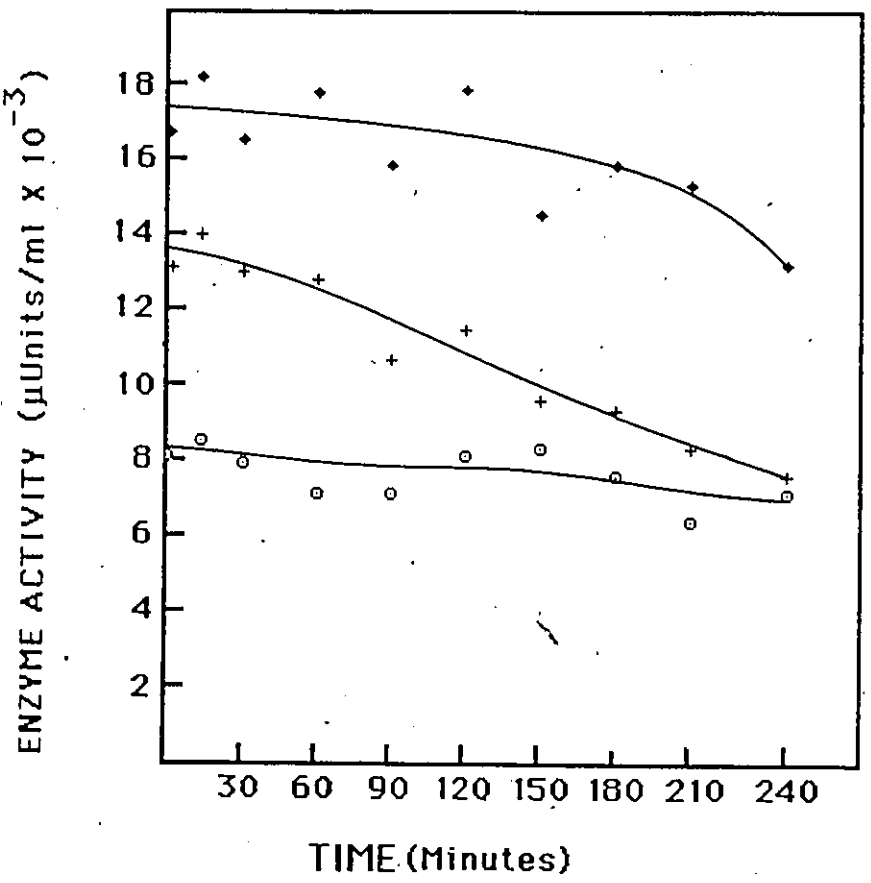


Figure 13. Stability of 17β-HSD-III at 0°C, in 20 mM Phosphate Buffer, pH 7.0. Effect of 19-Iodoacetoxyandrost-4-ene-3,17-dione **29** (+) and of 17β-Iodoacetoxyandrost-4-en-3-one **44** (⊙).

Enzyme (1 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 μl) and EGME (3 μl) was incubated alone (•), in the presence of 19-iodoacetoxyandrost-4-ene-3,17-dione (45 nmol) (+) or 17β-iodoacetoxy-androst-4-en-3-one (45 nmol) (⊙). At various time intervals, duplicate aliquots (2 to 6 μl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The enzyme activity is plotted along the ordinate and the time interval is along the abscissa.

G. Inactivation of 17β-HSD at pH 7.0 by Iodoacetoxy Steroids.

Initial studies were undertaken in order to determine the appropriate concentrations of iodoacetoxysteroids for inactivation of the 17β-HSD. We found that an iodoacetoxy steroid to enzyme ratio of 5 : 1 resulted in an effective inhibition of enzyme activity. As an example, inactivation of enzyme form I by a 5- and 40-fold excess of 6β-iodoacetoxyandrost-4-ene-3,17-dione **38** is shown in Figure 14. The inactivation of 17β-HSD enzyme forms I and III by the various iodoacetoxy steroids synthesized is illustrated in Figures 15 to 33. In 20 mM phosphate buffer, pH 7.0 and at 25°C both enzyme forms are inactivated by most of the iodoacetoxy steroids tested. However, it was observed that when 17β-HSD enzyme form I is inactivated with 17β-iodoacetoxyandrost-4-en-3-one **44** (Figure 17), the inactivation profile is different from the profile obtained with enzyme III and with other iodoacetoxy steroids. When the latter experiment was repeated the profile shown in Figure 17 was again obtained. These results could be due to the presence of the affinity label at the steroid position undergoing reaction and suggests that placement of the affinity label at this position should be avoided. Acetoxy steroids were utilized as controls in order to take into account the loss of enzyme activity exhibited when the enzyme alone is submitted to the same conditions. Therefore the inactivations are expressed as percentage of control.

The effects of added steroid substrate and cofactor (NADP) were also studied. If the affinity label (the iodoacetoxy steroid) sits in the enzyme active site the addition of substrate, in the presence of affinity label, should decrease the rate of inactivation since the substrate will also compete for the active site. The same reasoning applies if the cofactor is added along with the affinity label to the incubation. If the iodoacetoxy steroid sits in the cofactor binding site, it will

compete for this site with added cofactor. Therefore the rate of inactivation should decrease. Since we found that the cofactor protects the enzyme against inactivation in the absence of the iodoacetoxy steroids(Figures 11 and 12) it was necessary to incorporate the cofactor with the acetoxy steroid as control. Generally, the substrates prevent slightly or not at all the inactivation by iodoacetoxy steroids whereas prevention of inactivation by the cofactor is much more extensive. The data suggests that the affinity label may be occupying the cofactor site, or part of it, due to the hydrophobic character of this region of the enzyme active site. Such an example of the binding of a steroid in the hydrophobic region normally occupied by the adenine portion of the pyridine nucleotide has been reported for the 17β-HSD of human placenta[12,16]. In one case however, the cofactor did not prevent the inactivation. Figure 24 illustrates that the inactivation of 17β-HSD enzyme form I by 17β-iodoacetoxy-5β-androstan-3-one **48** is not prevented by the cofactor NADP.

H. Stoichiometry of ^{14}C-Carboxymethyl Incorporation.

The stoichiometry criterion implies that one molecule of label should be bound for each active site of the enzyme. For example, affinity alkylation of 3α,20β-HSD from *Streptomyces hydrogenans* with 17-[2'-^{3}H]bromoacetoxy-4-pregnen-3-one requires one mol of steroid to completely inactivate one mol of enzyme[39]. Because this experiment requires a large amount of enzyme, we were not able to determine the stoichiometry for both enzyme forms I and III with all the iodoacetoxy steroids. In fact, the stoichiometry was successfully determined only for 17β-HSD-III with the 19-iodoacetoxy derivative and it was found to be 0.71 mmol of steroid/mmol of enzyme after 30 min of inactivation (30% of enzyme

inactivated) and 1.1 mmol of steroid/mmol of enzyme after 120 min of inactivation (85% of enzyme inactivated). The experimental details are summarized in the Experimental section B.22.

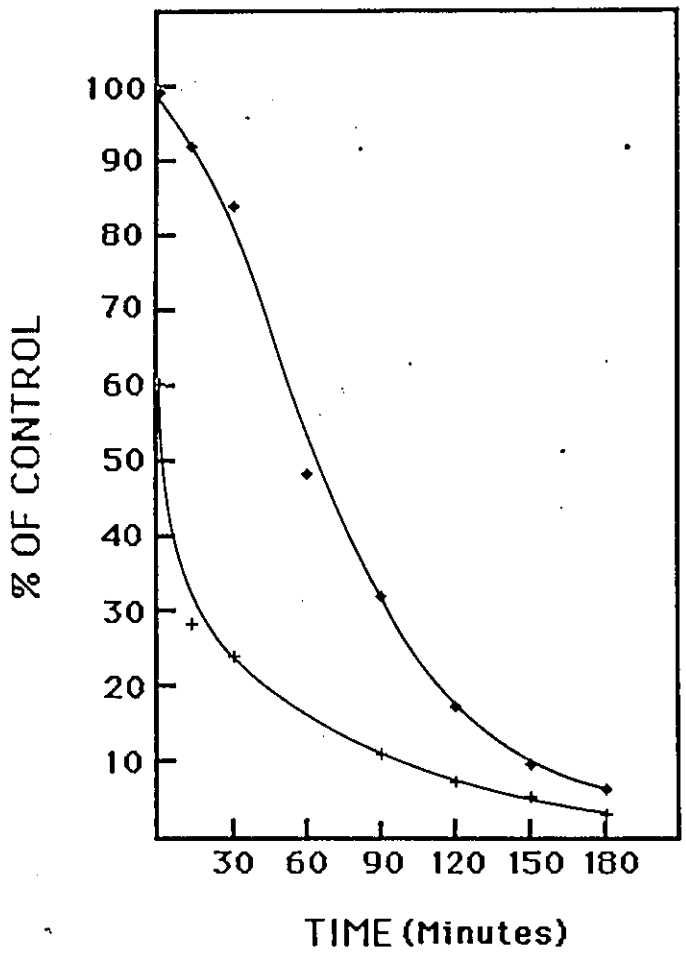


Figure 14. Inactivation of 17β-HSD-I by 5-Fold (•) and 40-Fold (+) of 6β-Iodo-acetoxyandrost-4-ene-3,17-dione **38** at 25°C.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 6β-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol (•) or 30 nmol (+) in 3 µl of EGME) Identical incubations containing 6β-acetoxyandrost-4-ene-3,17-dione **39** (3.75 nmol or 30 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control is plotted along the ordinate and the time of incubation is along the abscissa.

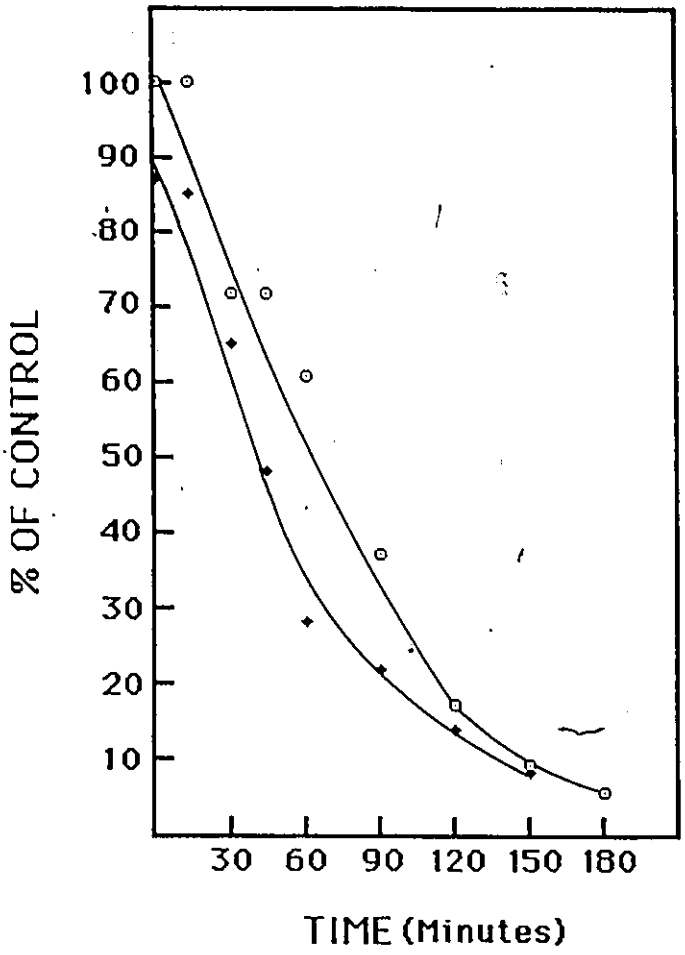


Figure 15.- Inactivation of 17β-HSD-I by 19-Iodoacetoxyandrost-4-ene-3,17-dione **29** at 25°C in the Presence (⊙) and Absence (•) of 17β-Hydroxy-androst-4-en-3-one **5**.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 19-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol in 1 µl of EGME) and with EGME (2 µl) or with 17β-hydroxyandrost-4-en-3-one (3.75 nmol in 2 µl of EGME). Identical incubations (with and without 17β-hydroxyandrost-4-en-3-one) containing 19-acetoxyandrost-4-ene-3,17-dione **31** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one as substrate. The percentage of control with (⊙) and without (•) 17β-hydroxyandrost-4-en-3-one is plotted along the ordinate and the time of incubation is along the abscissa.

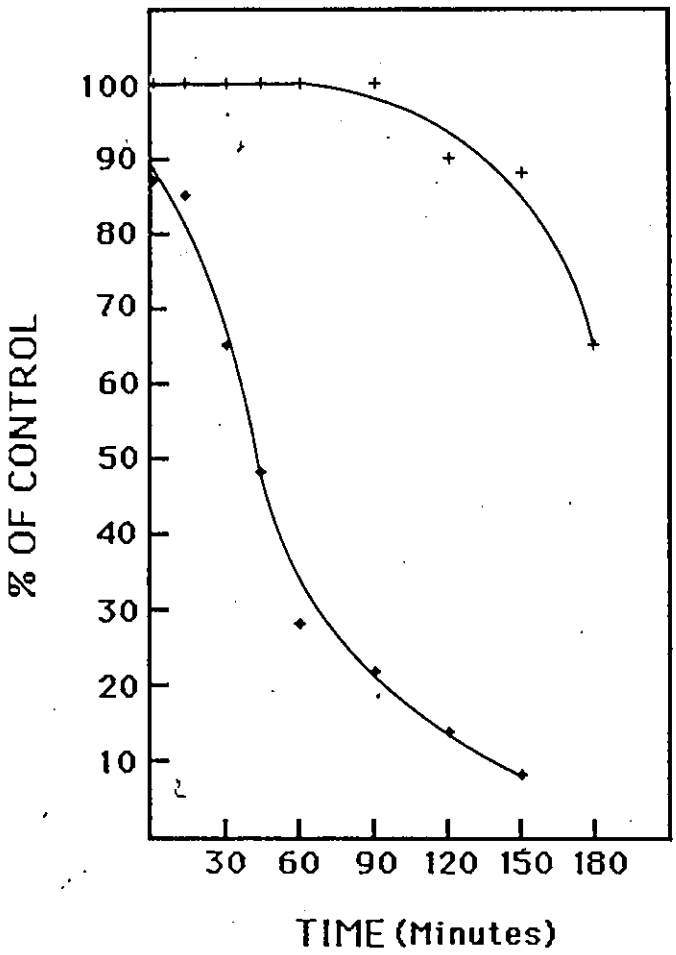


Figure 16. Inactivation of 17β-HSD-I by 19-Iodoacetoxyandrost-4-ene-3,17-dione **29** at 25°C in the Presence (+) and Absence (•) of NADP.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 19-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol in 3 µl of EGME) alone or in addition with NADP (1.5 nmol). Identical incubations (with and without NADP) containing 19-acetoxyandrost-4-ene-3,17-dione **31** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 25 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (+) and without (•) NADP is plotted along the ordinate and the time of incubation is along the abscissa.

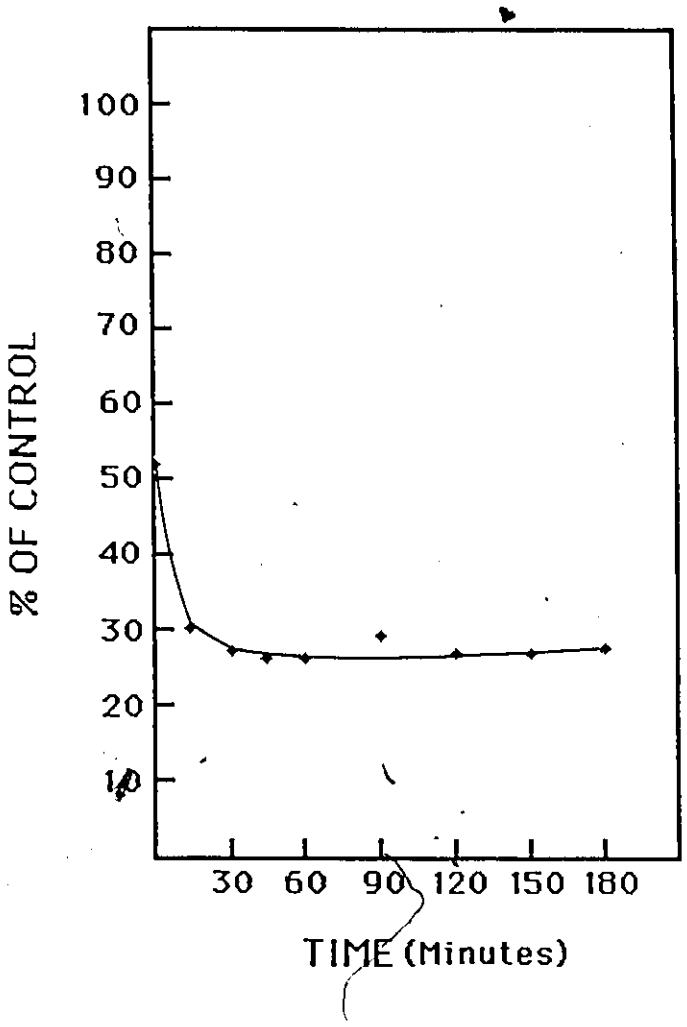


Figure 17. Inactivation of 17β-HSD-I by 17β-Iodoacetoxyandrost-4-en-3-one 44 at 25°C .

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 17β-iodoacetoxy-androst-4-en-3-one (3.75 nmol in 3 µl of EGME). Identical incubations containing 17β-acetoxyandrost-4-en-3-one **46** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 8 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control is plotted along the ordinate and the time of incubation is along the abscissa.

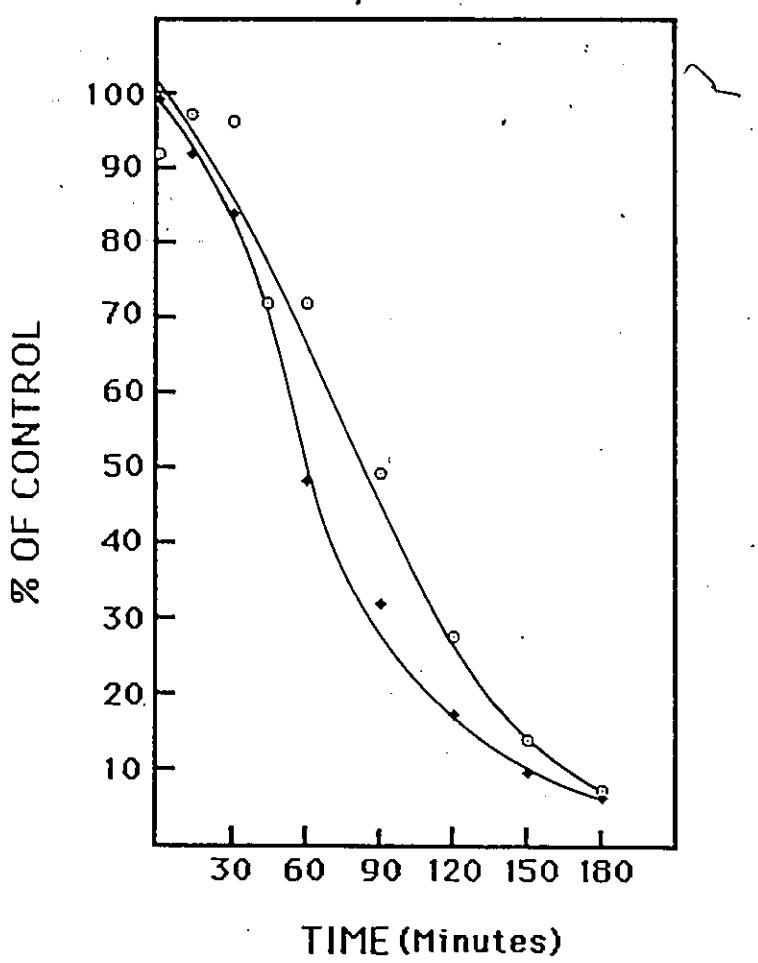


Figure 18. Inactivation of 17β-HSD-I by 6β-Iodoacetoxyandrost-4-ene-3,17-dione **38** at 25°C in the Presence (⊙) and Absence (•) of 17β-Hydroxy-androst-4- en-3-one **5**.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 6β-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol in 1 µl of EGME) and with EGME (2 µl) or with 17β-hydroxyandrost-4-en-3-one (3.75 nmol in 2 µl of EGME). Identical incubations (with and without 17β-hydroxyandrost-4-en-3-one) containing 6β-acetoxyandrost-4-ene-3,17-dione **39** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one as substrate. The percentage of control with (⊙) and without (•) 17β-hydroxyandrost-4-en-3-one is plotted along the ordinate and the time of incubation is along the abscissa.

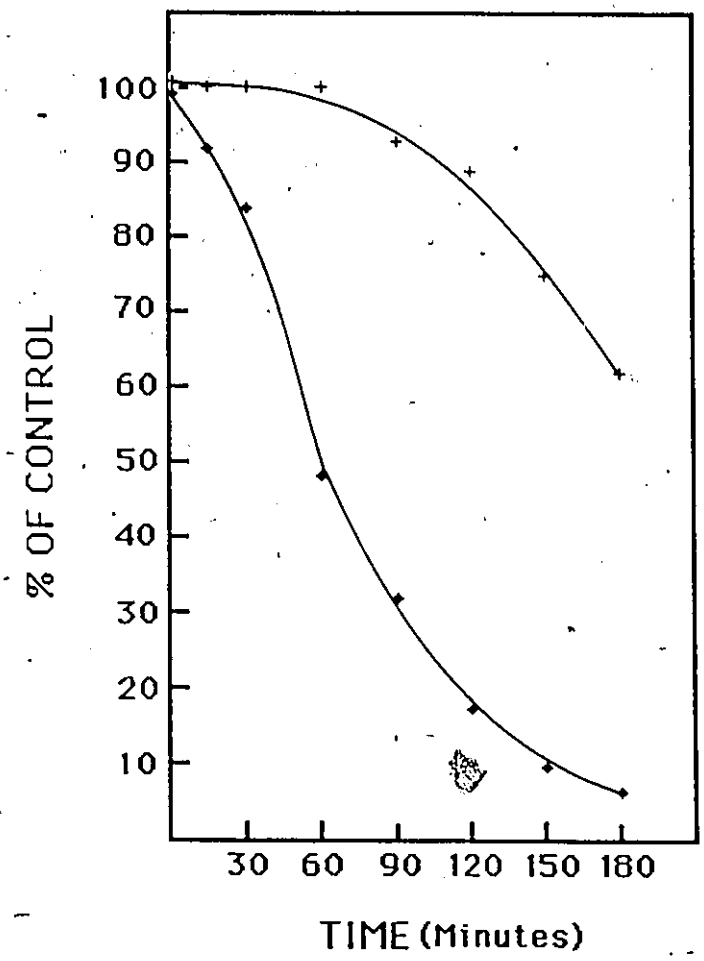


Figure 19. Inactivation of 17β-HSD-I by 6β-Iodoacetoxyandrost-4-ene-3,17-dione **38** at 25°C in the Presence (+) and Absence (•) of NADP.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 6β-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol in 3 µl of EGME) alone or in addition with NADP (1.5 nmol). Identical incubations (with and without NADP) containing 6β-acetoxyandrost-4-ene-3,17-dione **39** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (+) and without (•) NADP is plotted along the ordinate and the time of incubation is along the abscissa.

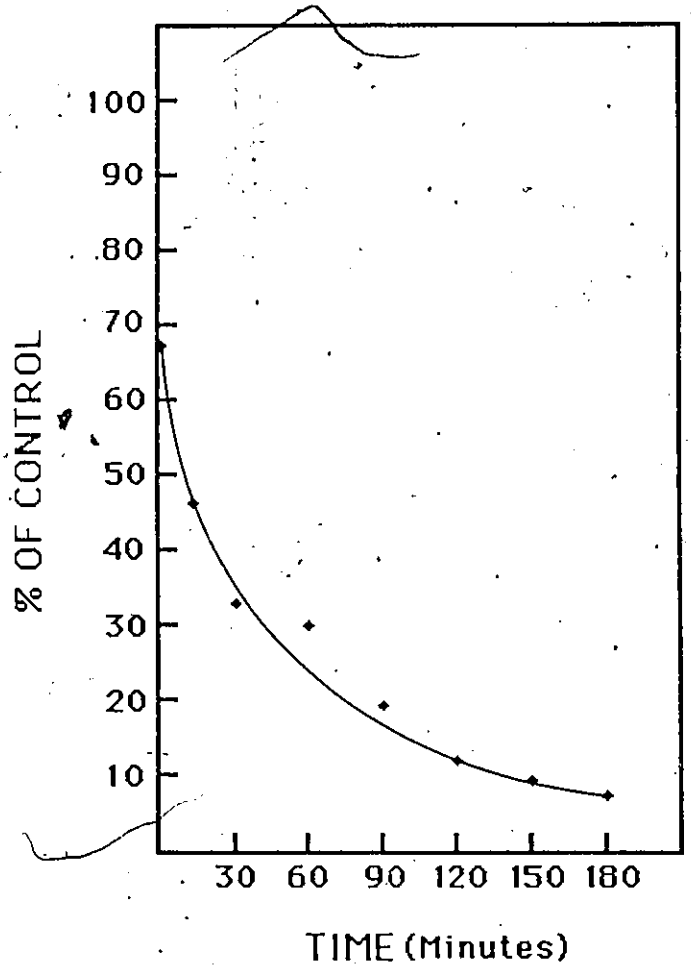


Figure 20. Inactivation of 17β-HSD-I by 11β-Iodoacetoxyandrost-4-ene-3,17-dione **41** at 25°C.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 11β-iodoacetoxy-androst-4-ene-3,17-dione (30 nmol in 3 µl of EGME). Identical incubations containing 11β-acetoxyandrost-4-ene-3,17-dione **42** (30 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 25 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control is plotted along the ordinate and the time of incubation is along the abscissa.

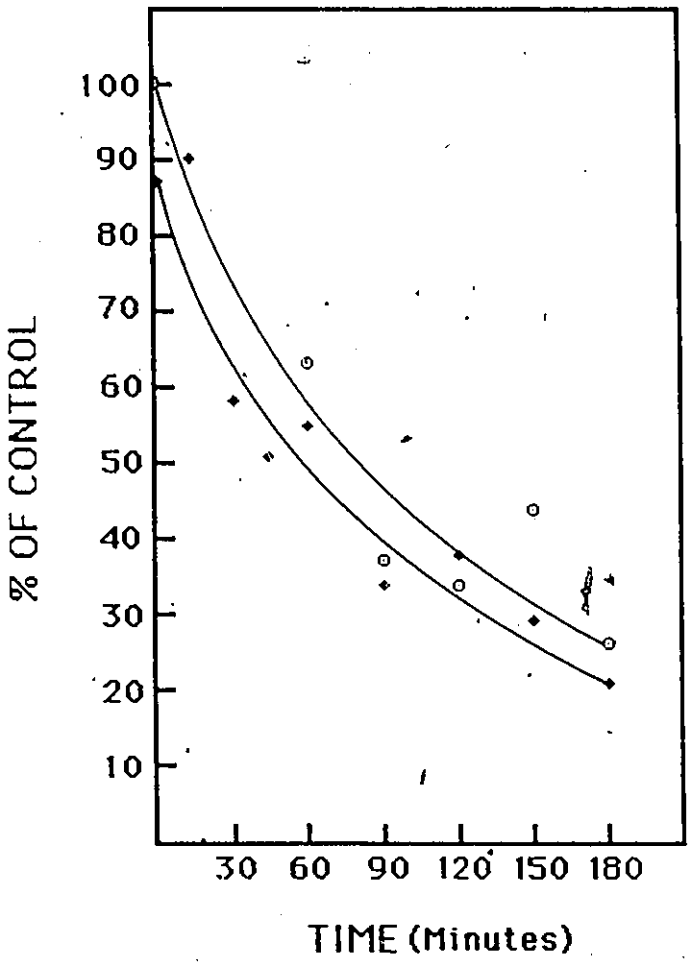


Figure 21. Inactivation of 17β-HSD-I by 3-Iodoacetoxy-1,3,5(10)-estratrien-17-one **52** at 25°C in the Presence (⊙) and Absence (•) of 17β-Hydroxy-androst-4- en-3-one **5**.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 3-iodoacetoxy-1,3,5(10)-estratrien-17-one (3.75 nmol in 1 µl of EGME) and with EGME (2 µl) or with 17β-hydroxyandrost-4-en-3-one **5** (3.75 nmol in 2 µl of EGME). Identical incubations (with and without 17β-hydroxyandrost-4-en-3-one) containing 3-iodoacetoxy-1,3,5(10)-estratrien-17-one **53** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 15 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one as substrate. The percentage of control with (⊙) and without (•) 17β-hydroxyandrost-4-en-3-one is plotted along the ordinate and the time of incubation is along the abscissa.

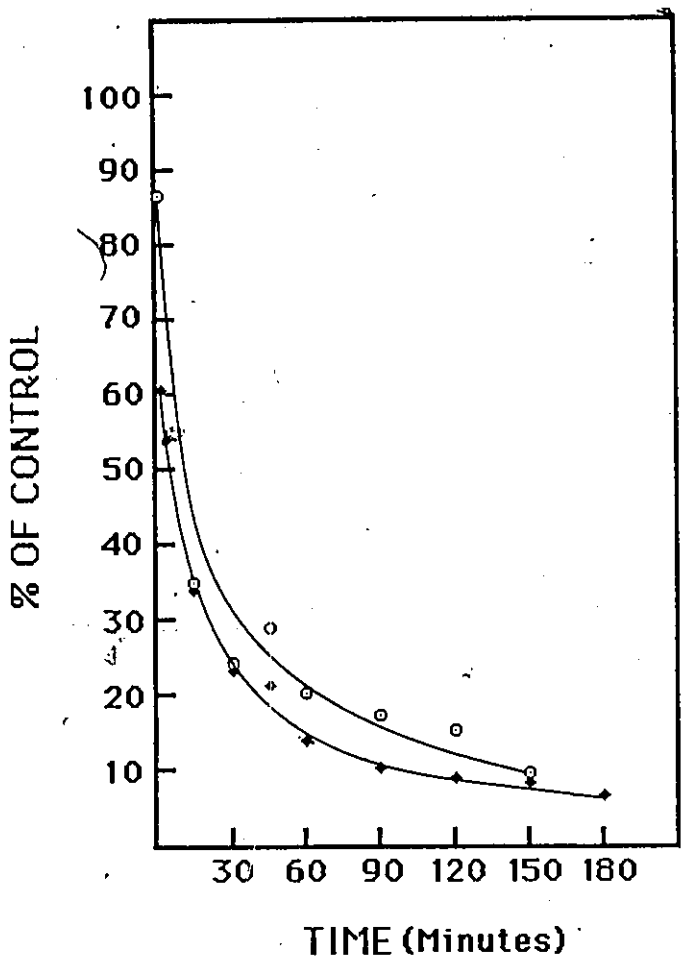


Figure 22. Inactivation of 17β-HSD-I by 19-Iodoacetoxy-5β-androstane-3,17-dione **34** at 25°C in the Presence (⊙) and Absence (•) of 3α-Hydroxy-5β-androstan-17-one **20**.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl), was incubated at 25°C with 19-iodoacetoxy-5β-androstane-3,17-dione (3.75 nmol in 1 µl of EGME) and with EGME (2 µl) or with 3α-hydroxy-5β-androstan-17-one (3.75 nmol in 2 µl of EGME). Identical incubations (with and without 3α-hydroxy-5β-androstan-17-one) containing 19-acetoxy-5β-androstane-3,17-dione **36** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 25 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (⊙) and without (•) 3α-hydroxy-5β-androstan-17-one is plotted along the ordinate and the time of incubation is along the abscissa.

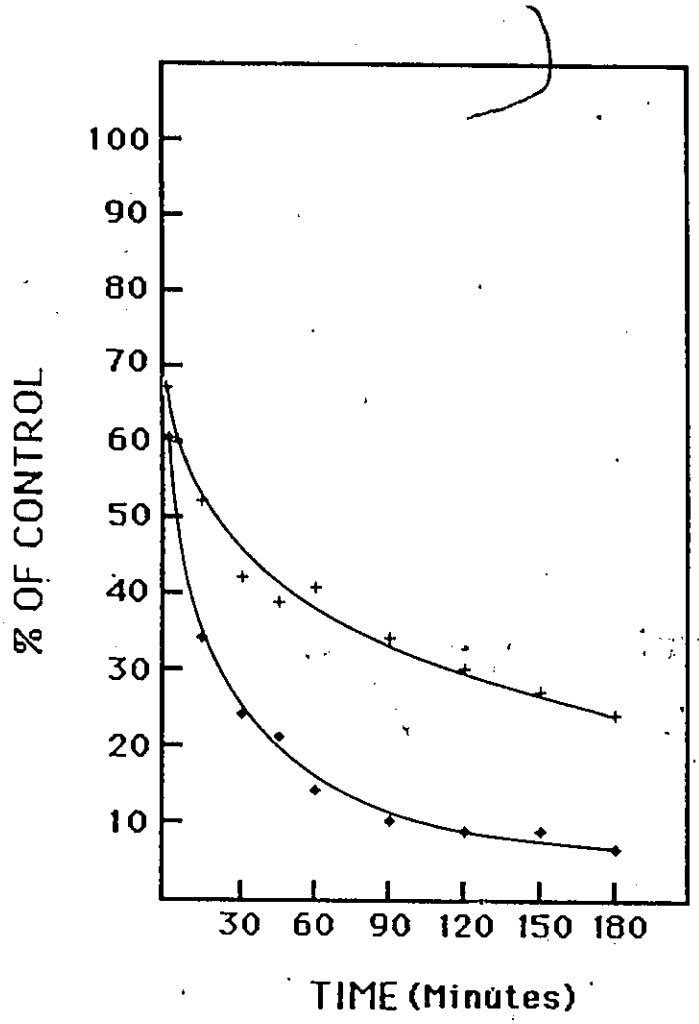


Figure 23. Inactivation of 17β-HSD-I by 19-Iodoacetoxy-5β-androstane-3,17-dione **34** at 25°C in the Presence (+) and Absence (•) of NADP.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 19-iodoacetoxy-5β-androstane-3,17-dione (3.75 nmol in 3 µl of EGME) alone or in addition with NADP (1.5 nmol). Identical incubations (with and without NADP) containing **19-acetoxy-5β-androstane-3,17-dione 36 (3.75 nmol in 3 µl of EGME) were** performed as control. At various time intervals, duplicate aliquots (1 to 4 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (+) and without (•) NADP is plotted along the ordinate and the time of incubation is along the abscissa.

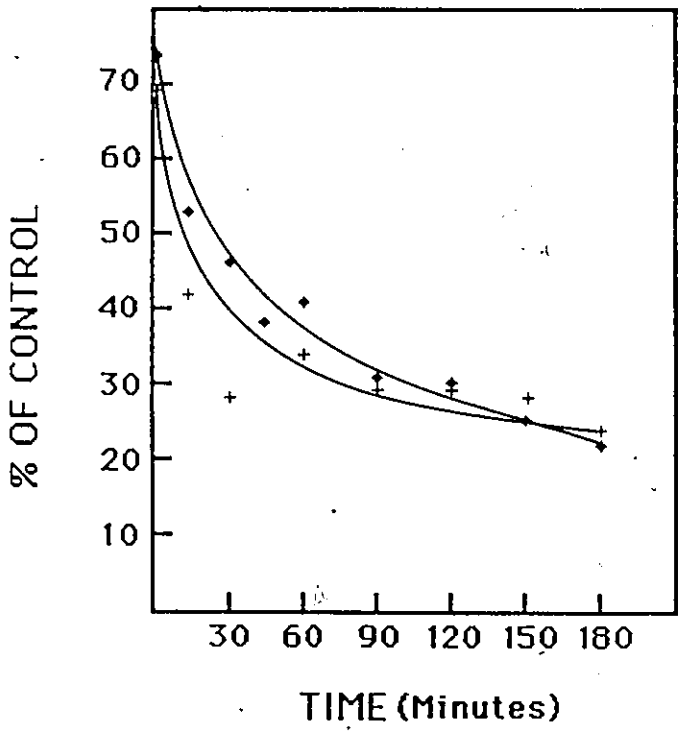


Figure 24. Inactivation of 17β-HSD-I by 17β-Iodoacetoxy-5β-androstan-3-one 48 at 25°C in the Presence (+) and Absence (•) of NADP.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 17β-iodoacetoxy-5β-androstan-3- one (3.75 nmol in 3 µl of EGME) alone or in addition with NADP (1.5 nmol). Identical incubations (with and without NADP) containing 17β-acetoxy-5β-androstan- 3-one **50** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 3 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (+) and without (•) NADP is plotted along the ordinate and the time of incubation is along the abscissa. The values are the mean of duplicate experiments.

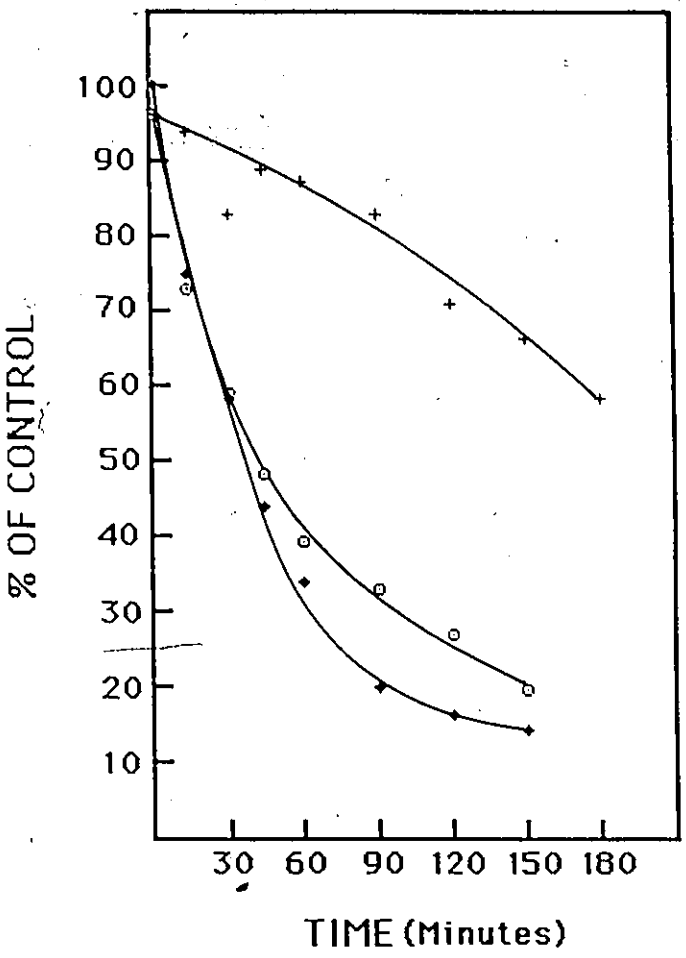


Figure 25. Inactivation of 17β-HSD-III by 19-Iodoacetoxyandrost-4-ene-3,17-dione **29** at 25°C alone (•) and in the Presence of 17β-Hydroxy-androst-4-en-3- one **5** (⊙) or NADP (+).

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 19-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol in 1 µl of EGME) and with EGME (2 µl). Similar incubations were performed containing in addition 17β-hydroxyandrost-4-en-3-one (3.75 nmol) or NADP (1.5 nmol). Identical incubations (with and without 17β-hydroxyandrost-4- en-3-one or NADP) containing 19-acetoxy-androst-4-ene-3,17-dione **31** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4- en-3-one as substrate. The percentage of control alone (•) or with 17β-hydroxyandrost-4-en-3-one (⊙) or with NADP (+) is plotted along the ordinate and the time of incubation is along the abscissa.

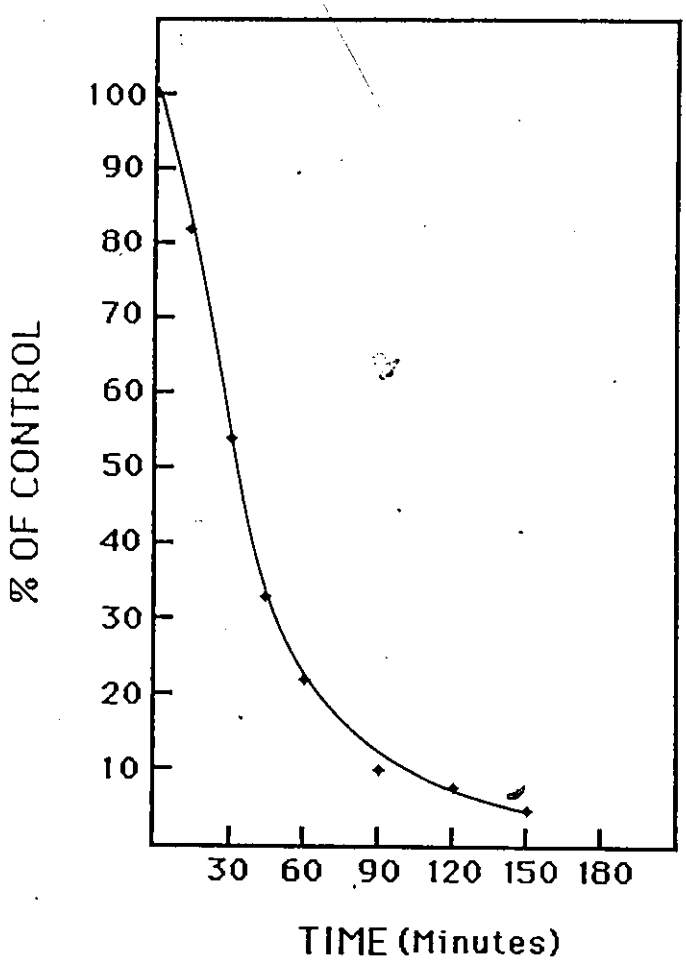


Figure 26. Inactivation of 17β-HSD-III by 17β-Iodoacetoxyandrost-4-en-3-one 44 at 25°C.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 17β-iodoacetoxy-androst-4-en-3-one (3.75 nmol in 3 µl of EGME). Identical incubations containing 17β-acetoxyandrost-4-en-3-one **46** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 15 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control is plotted along the ordinate and the time of incubation is along the abscissa.

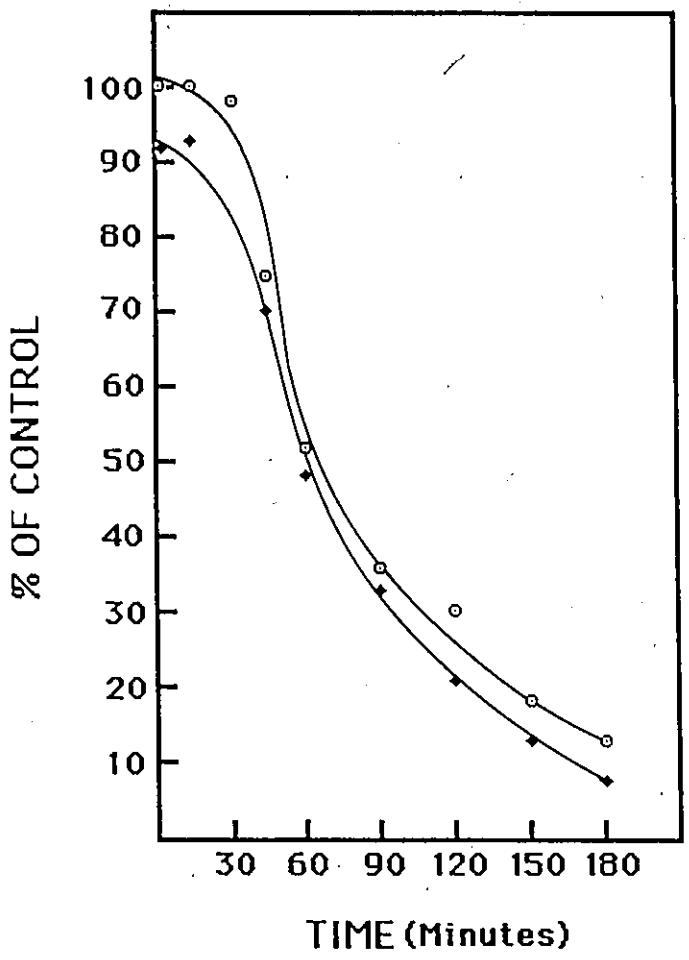


Figure 27. Inactivation of 17β-HSD-III by 6β-Iodoacetoxyandrost-4-ene-3,17-dione **38** at 25°C in the Presence (⊙) and Absence (⬥) of 17β-Hydroxy-androst-4-en-3-one **5**.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 6β-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol in 1 µl of EGME) and with EGME (2 µl) or with 17β-hydroxyandrost-4-en-3-one (3.75 nmol in 2 µl of EGME). Identical incubations (with and without 17β-hydroxyandrost-4-en-3-one) containing 6β-acetoxyandrost-4-ene-3,17-dione **39** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one as substrate. The percentage of control with (⊙) and without (⬥) 17β-hydroxy-androst-4-en-3-one is plotted along the ordinate and the time of incubation is along the abscissa.

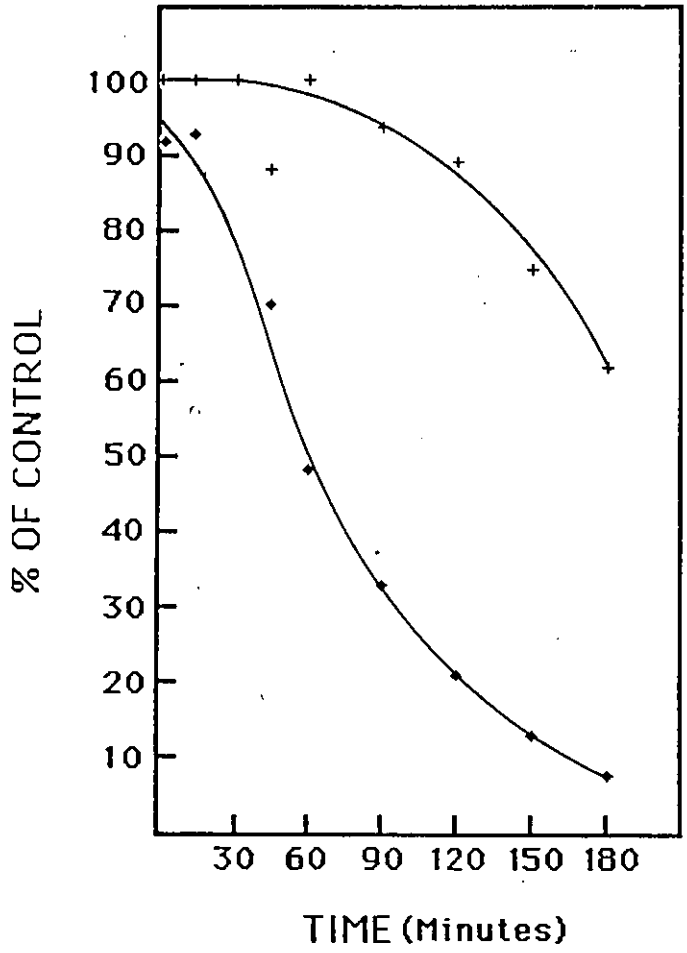


Figure 28. Inactivation of 17β-HSD-III by 6β-Iodoacetoxyandrost-4-ene-3,17-dione **38** at 25°C in the Presence (+) and Absence (•) of NADP.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 6β-iodoacetoxy-androst-4-ene-3,17-dione (3.75 nmol in 3 µl of EGME) alone or in addition with NADP (1.5 nmol). Identical incubations (with and without NADP) containing 6β-acetoxyandrost-4-ene-3,17-dione **39** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (+) and without (•) NADP is plotted along the ordinate and the time of incubation is along the abscissa.

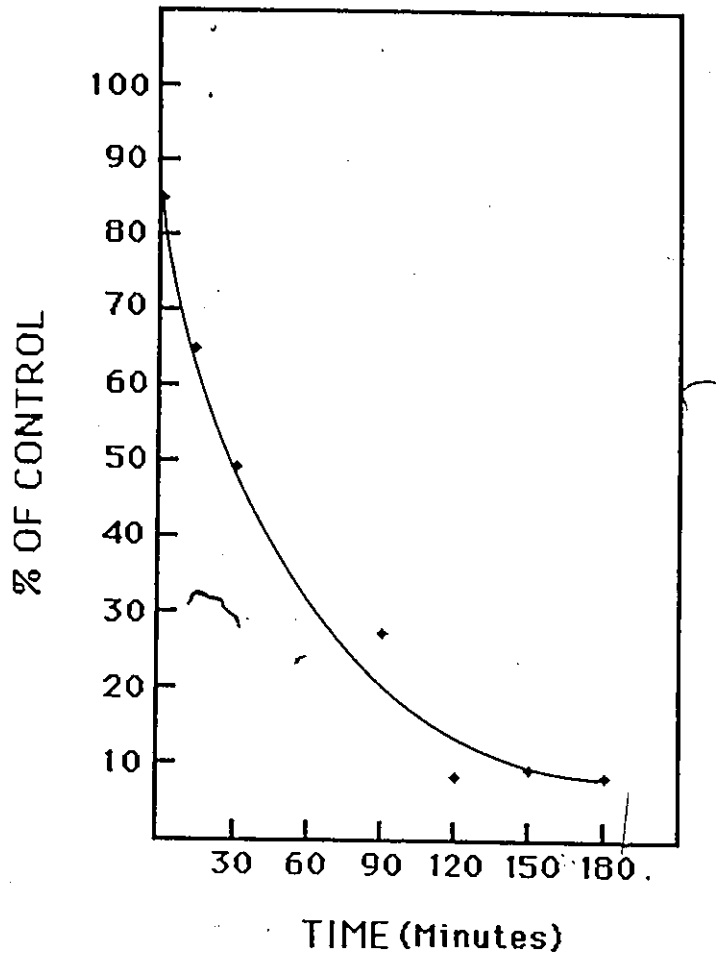


Figure 29. Inactivation of 17β-HSD-III by 11β-Iodoacetoxyandrost-4-ene-3,17-dione **41** at 25°C.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 11β-iodoacetoxy-androst-4-ene-3,17-dione (30 nmol in 3 µl of EGME). Identical incubations containing 11β-acetoxyandrost-4-ene-3,17-dione **40** (30 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (2 to 16 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control is plotted along the ordinate and the time of incubation is along the abscissa.

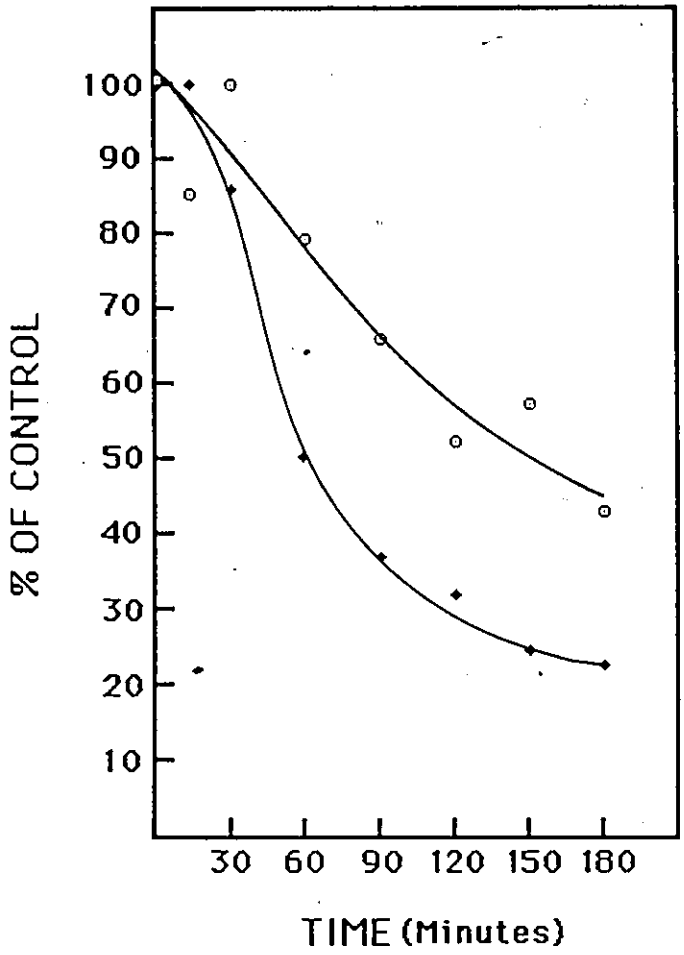


Figure 30. Inactivation of 17β-HSD-III by 3-Iodoacetoxy-1,3,5(10)-estratrien-17-one **52** at 25°C in the Presence (⊙) and Absence (•) of 17β-Hydroxy-androst-4- en-3-one **5**.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 3-iodoacetoxy-1,3, 5(10)-estratrien-17-one (3.75 nmol in 1 µl of EGME) and with EGME (2 µl) or with 17β-hydroxyandrost-4-en-3-one (15 nmol in 2 µl of EGME). Identical incubations (with and without 17β-hydroxyandrost-4-en-3-one) containing 3-iodoacetoxy-1,3,5(10)-estratrien-17-one **53** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 20 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one as substrate. The percentage of control with (⊙) and without (•) 17β-hydroxy-androst-4- en-3-one is plotted along the ordinate and the time of incubation is along the abscissa.

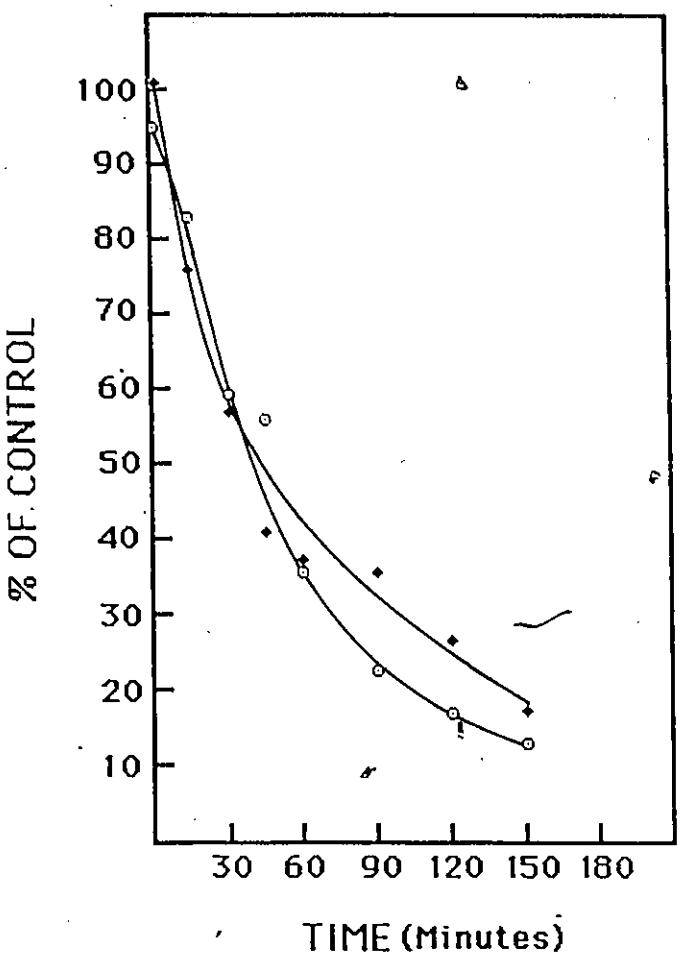


Figure 31. Inactivation of 17β-HSD-III by 19-Iodoacetoxy-5β-androstane-3,17-dione **34** at 25°C in the Presence (⊙) and Absence (•) of 3α-Hydroxy-5β-androstan-17-one **20**.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 19-iodoacetoxy-5β-androstane-3,17-dione (3.75 nmol in 1 µl of EGME) and with EGME (2 µl) or with 3α-hydroxy-5β-androstan-17-one (3.75 nmol in 2 µl of EGME). Identical incubations (with and without 3α-hydroxy-5β-androstan-17-one) containing 19-acetoxy-5β-androstane-3,17-dione **36** (3.75 nmol in 1 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 30 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (⊙) and without (•) 3α-hydroxy-5β-androstan-17-one is plotted along the ordinate and the time of incubation is along the abscissa.

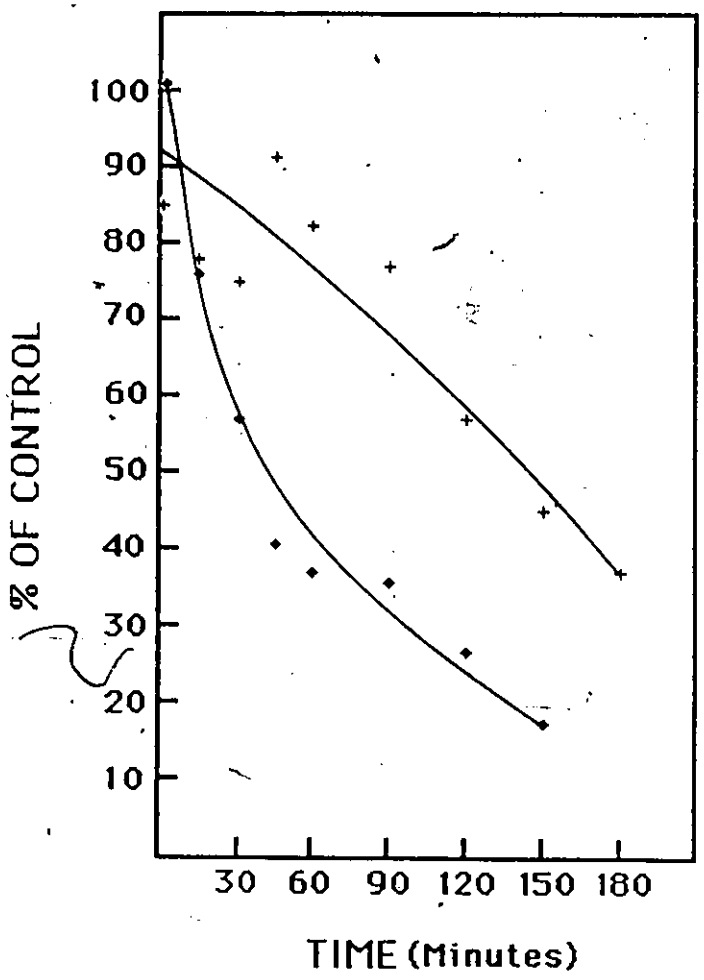


Figure 32. Inactivation of 17β-HSD-III by 19-Iodoacetoxy-5β-androstane-3,17-dione **34** at 25°C in the Presence (+) and Absence (•) of NADP.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 19-iodoacetoxy-5β-androstane-3,17-dione (3.75 nmol in 3 µl of EGME) alone or in addition with NADP (1.5 nmol). Identical incubations (with and without NADP) containing **19-acetoxy-5β-androstane-3,17-dione 36** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 3 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (+) and without (•) NADP is plotted along the ordinate and the time of incubation is along the abscissa.

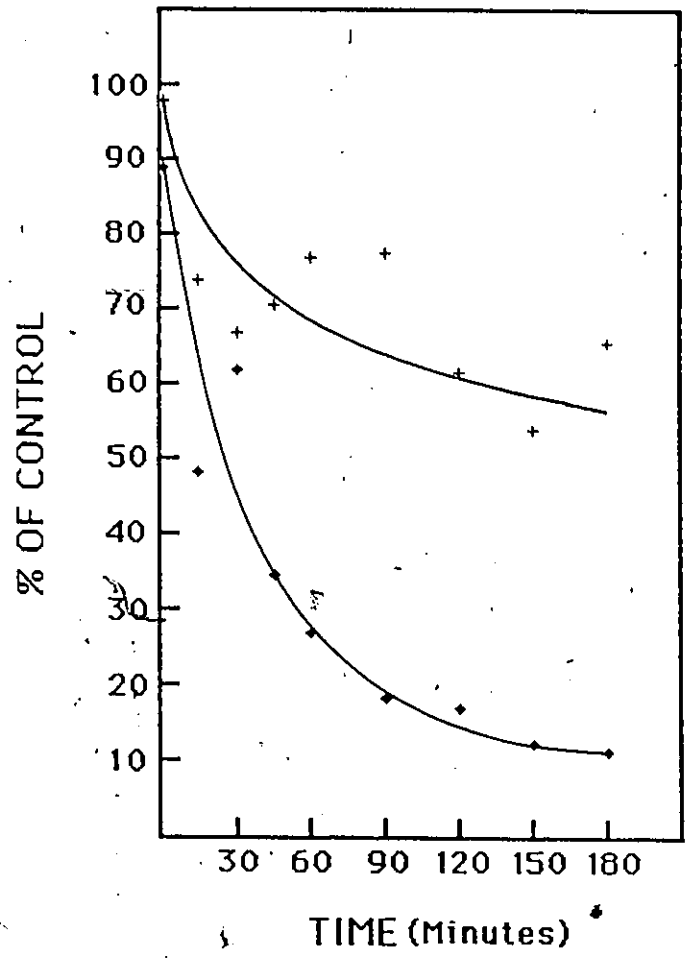


Figure 33. Inactivation of 17β-HSD-III by 17β-Iodoacetoxy-5β-androstan-3-one **48** at 25°C in the Presence (+) and Absence (•) of NADP.

Enzyme (0.75 nmol), after Sephadex G-25 filtration, dissolved in 20 mM phosphate buffer, pH 7.0 (127 µl) was incubated at 25°C with 17β-iodoacetoxy-5β-androstan-3-one (3.75 nmol in 3 µl of EGME) alone or in addition with NADP (1.5 nmol). Identical incubations (with and without NADP) containing 17β-acetoxy-5β-androstan-3-one **50** (3.75 nmol in 3 µl of EGME) were performed as control. At various time intervals, duplicate aliquots (1 to 3 µl) were assayed for 17β-HSD activity using 17β-hydroxyandrost-4-en-3-one **5** as substrate. The percentage of control with (+) and without (•) NADP is plotted along the ordinate and the time of incubation is along the abscissa.

I. Amino Acid Analysis After Inactivation of 17β-HSD with Iodo[2'-^{14}C]acetoxy Steroids.

Amino acid residues modified by iodo[^{14}C]acetoxy steroids were identified by comparing the high pressure liquid chromatography (HPLC) fractions containing radioactivity to the retention times of standard carboxymethylated amino acids. Standard carboxymethylated amino acids were first synthesized and their retention times were determined as summarized in Table IV. Amino acid analysis was then performed on the 6 N HCl hydrolysis products of the 17β-HSD inactivated with iodo[2'-^{14}C]acetoxy steroids. The elution patterns from amino acid analysis of hydrolysis products from inactivation of 17β-HSD are illustrated in Figures 34, 35 and 36 for 6β-iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **40** with enzyme form I and for 19-iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37** with enzyme forms I and III, respectively. In all three cases, the major peak of radioactivity coincides with the retention time of S-carboxymethyl-L-cysteine. Similarly, the radioactive profile of inactivated 17β-HSD enzyme form III with 19-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33** is shown in Figure 37. It should be noted in Figure 37 that only one radioactive peak corresponding to S-carboxymethyl-L-cysteine is found, after 1 or 3 h, when the 17β-HSD enzyme form III has been inactivated with the 19-iodoacetoxy derivative **33**. The difference between 1 or 3 h inactivation is in the amount of radioactivity incorporated. To confirm that the radioactivity was really due to S-carboxymethyl-L-cysteine, we carried out a performic acid oxidation. It was reported by Takahashi[80] that the product of performic oxidation of S-carboxymethyl-L-cysteine is S-carboxymethyl-L-cysteine sulfone, which has a shorter retention time than S-carboxymethyl-L-cysteine. As illustrated in Figure 37, the radioactivity is

shifted to a shorter retention time upon performic acid oxidation, thus confirming that the modified amino acid is S-[^{14}C]carboxymethyl-L-cysteine.

The radioactive profiles from amino acid analysis of hydrolysis products obtained after inactivation (3 h) of 17β-HSD enzyme forms I and III are summarized in Tables V and VI, respectively. In most cases, the majority of the radioactivity coincides with S-carboxymethyl-L-cysteine. But in some cases, N^{π},N^{τ}-dicarboxymethyl-L-histidine is found to account for 25% of the radioactivity when, for example, 17β-HSD-I is inactivated with 17β-iodo[2'-^{14}C]-acetoxyandrost-4-en-3-one **47**. One possible explanation for the formation of N^{π},N^{τ}-dicarboxymethyl-L-histidine is that a single alkylation of the active site histidine predisposes it to a second alkylation of the imidazole ring[35,47]. We see that for both enzyme forms I and III, the unsaturated iodoacetoxy steroids **33**, **40** and **47** give mainly S-carboxymethyl-L-cysteine although with the derivative **47** we notice a more complex profile. The complexity of the profile of carboxymethylated amino acids increases with the saturated iodoacetoxy derivatives **37** and **51**. There are minor differences between the enzyme forms I and III when we compare their radioactive profiles on inactivation by the unsaturated derivatives **47**, **40** and the saturated derivative **51**. The difference is in the proportion of labeled amino acids for the derivatives **47** and **51**. For the derivative **40**, the difference is in the labeling of two other amino acids, besides cysteine for enzyme form III, whereas for the enzyme form I only cysteine is found labeled.Two radioactive peaks, with retention times of 6.50 and 19.00 min, did not correspond to any of our standards. It is possible that the unknown derivative with a retention time of 6.50 min could be glycolic acid since the unknown is eluted at a position similar to that reported for glycolic acid by Sweet and Samant[39].

Glycolic acid was found to be the major radioactive peak (80%) when 17[2'-^{3}H]-bromoacetoxy-4-pregnen-3-one inactivated 3α,20β-HSD from *Streptomyces hydrogenans* thus showing that this bromoacetoxy derivative reacted with an aspartic or glutamic acid residue at the active site of the enzyme[39]. To confirm if the unknown residue at 6.50 min is glycolic acid we would need to add authentic tritiated glycolic acid to the [^{14}C]hydrolysate before amino acid analysis and determine whether the [^{3}H] and [^{14}C] peaks co-elute since glycolic acid does not react with ninhydrin.

As we previously mentioned in the introduction, histidine and cysteine are the most frequent amino acids found to react with haloacetoxy steroids. Histidine, cysteine and lysine residues were identified in the active site of the 17β-estradiol dehydrogenase from human placenta[16,23]. Similarly, histidine[46,47], cysteine[45,48] and methionine[44] residues were found in the active site of 3α,20β-HSD from *Streptomyces hydrogenans*.

TABLE IV

RETENTION TIME OF STANDARD AND CARBOXYMETHYLATED AMINO ACIDS.

AMINO ACID	RETENTION TIME (minutes)
L-Histidine	81.23
N^{π}, N^{τ}-Dicarboxymethyl-L-Histidine	4.64
N^{π}-Carboxymethyl-L-Histidine	22.89
N^{τ}-Carboxymethyl-L-Histidine	34.39
L-Cysteine	34.79
S-Carboxymethyl-L-Cysteine	9.70
L-Lysine	75.81
N^{6}-Carboxymethyl-L-Lysine	30.48
L-Methionine	41.94
Carboxymethyl-L-methionine	18.53

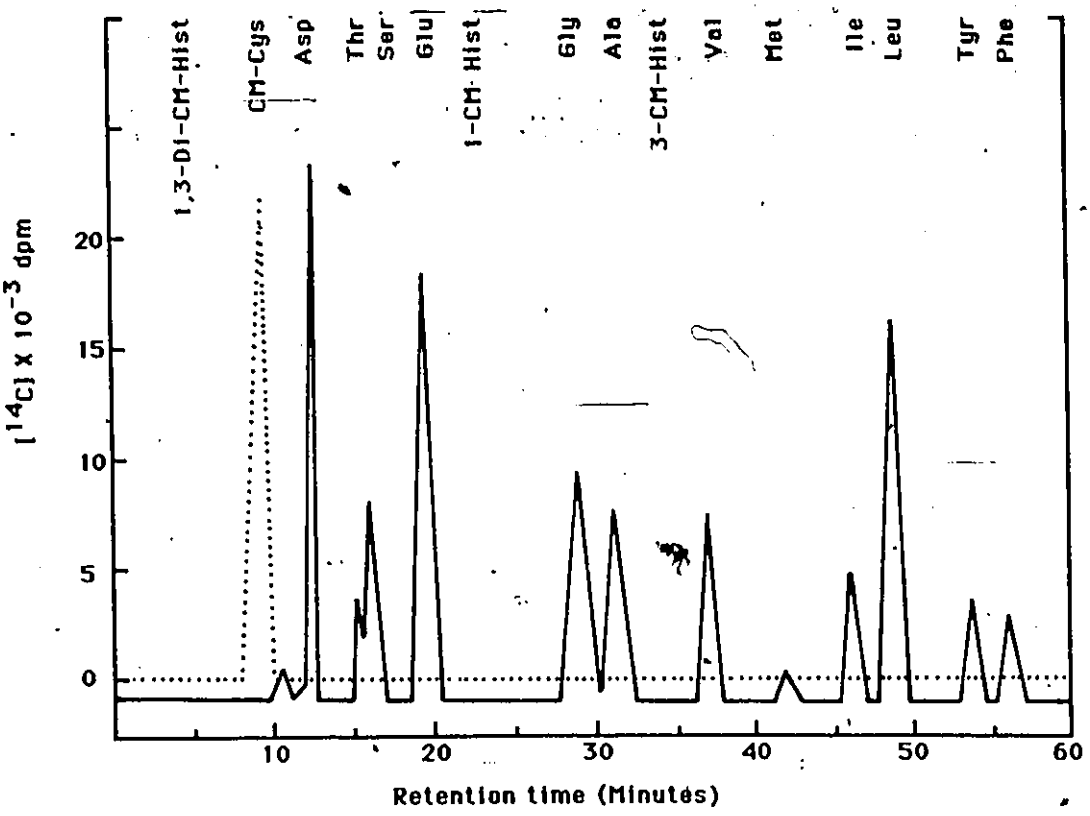


Figure 34. Elution Pattern from Amino Acid Analysis of Hydrolysis Products from Inactivation of 17β-HSD-I with 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **40**.

Enzyme (10 nmol) after Sephadex G-25 filtration, was inactivated with 6β-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione (50 nmol) for 3 h at 25°C and hydrolyzed as described in the Experimental section 20. Standard carboxymethylated histidine and cysteine were added to the sample mixture prior to amino acid analysis. The retention time is plotted along the abscissa. The ninhydrin absorption is along the ordinate (full line) as well as the radioactivity (dotted line). The ninhydrin absorption peaks for the standard carboxymethylated amino acids are not drawn but indicated by letters.

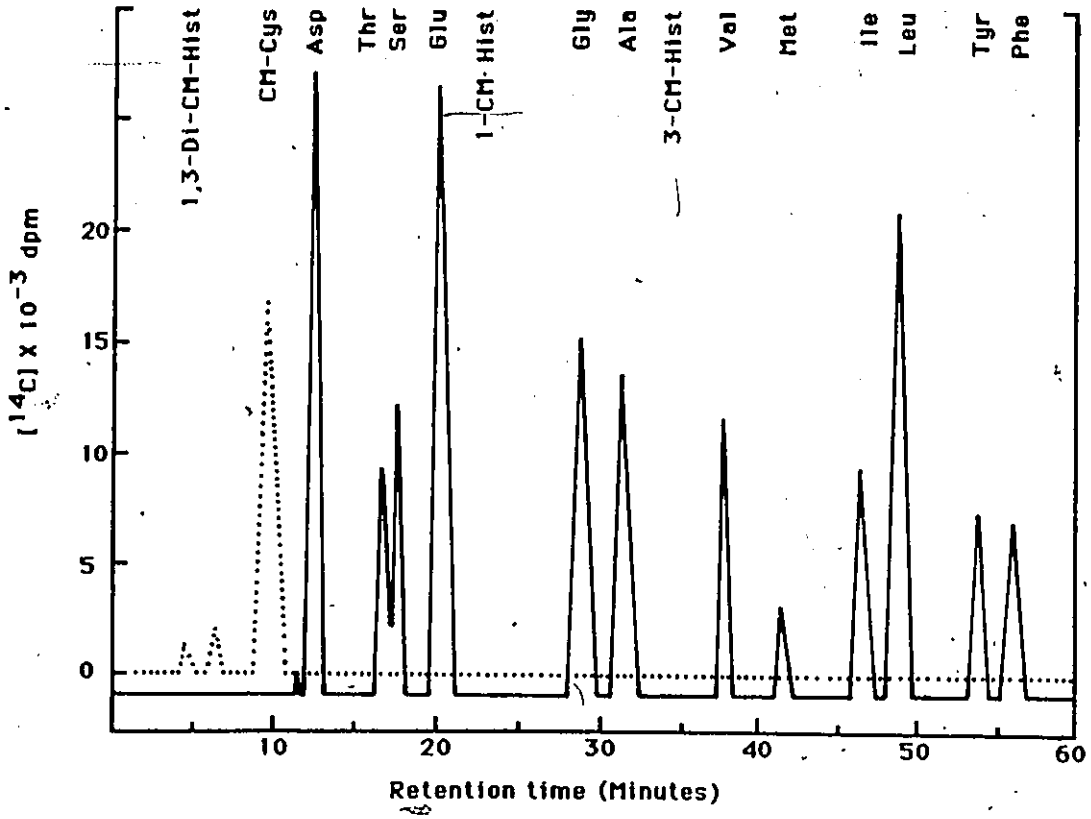


Figure 35. Elution Pattern from Amino Acid Analysis of Hydrolysis Products from Inactivation of 17β-HSD-I with 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione 37.

Enzyme (10 nmol) after Sephadex G-25 filtration, was inactivated with 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione (50 nmol) for 3 h at 25°C and hydrolyzed as described in the Experimental section 20. Standard carboxymethylated histidine and cysteine were added to the sample mixture prior to amino acid analysis. The retention time is plotted along the abscissa. The ninhydrin absorption is along the ordinate (full line) as well as the radioactivity (dotted line). The ninhydrin absorption peaks for the standard carboxymethylated amino acids are not drawn but indicated by letters.

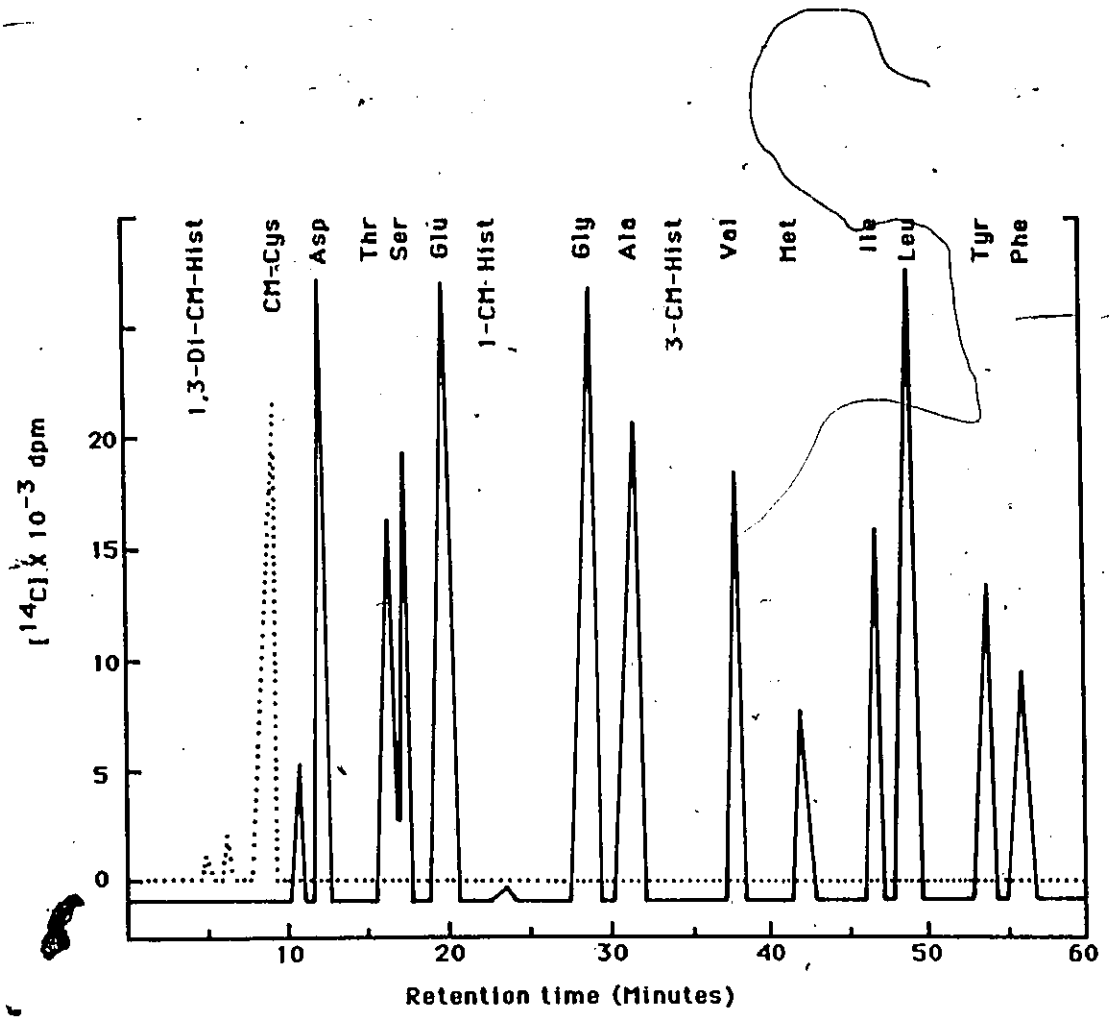
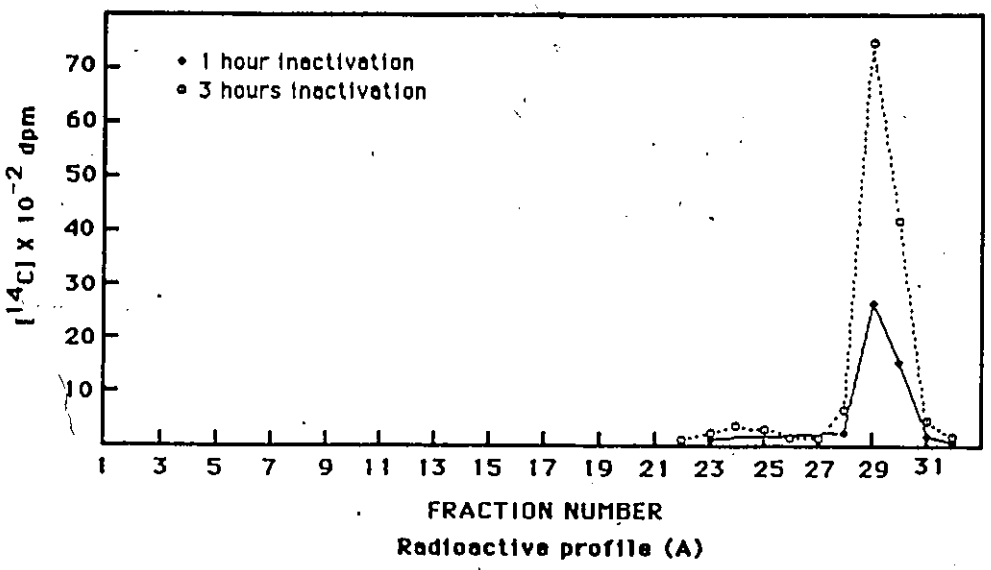


Figure 36. Elution Pattern from Amino Acid Analysis of Hydrolysis Products from Inactivation of 17β-HSD-III with 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione 37.

Enzyme (10 nmol) after Sephadex G-25 filtration, was inactivated with 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione (50 nmol) for 3 h at 25°C and hydrolyzed as described in the Experimental section 20. Standard carboxymethylated histidine and cysteine were added to the sample mixture prior to amino acid analysis. The retention time is plotted along the abscissa. The ninhydrin absorption is along the ordinate (full line) as well as the radioactivity (dotted line). The ninhydrin absorption peaks for the standard carboxymethylated amino acids are not drawn but indicated by letters.



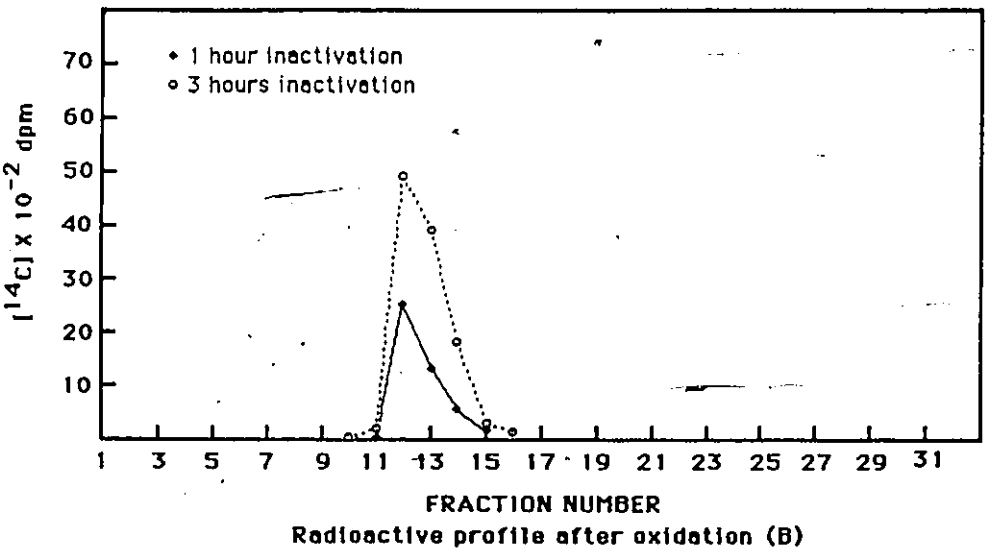


Figure 37. Radioactive Profile Before and After Oxidation from Amino Acid Analysis of Hydrolysis Products from 1 and 3 h Inactivation of 17β-HSD-III with 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33**.

Enzyme (10 nmol) after Sephadex G-25 filtration, was inactivated with 19-Iodo-[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione (50 nmol) for 1 and 3 h at 25°C, hydrolyzed and oxidized as described in the Experimental sections B.20 and B.21. The radioactivity is plotted along the ordinate and the fraction number is along the abscissa.

TABLE V

Radioactive Profile from Amino Acid Analysis of Hydrolysis Products from 3 h Inactivation of 17β-HSD-I with Various Iodoacetoxy Steroids.

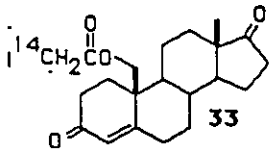
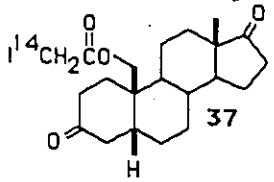
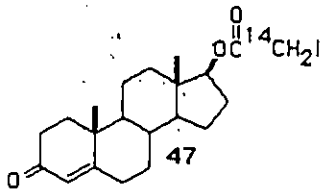
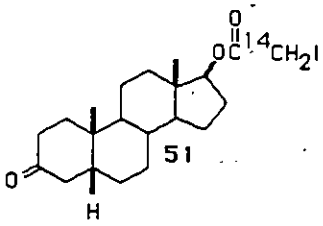
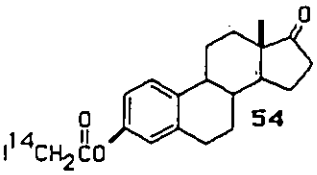
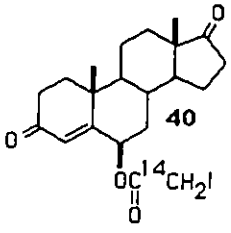
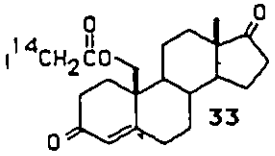
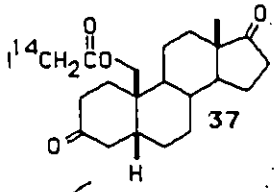
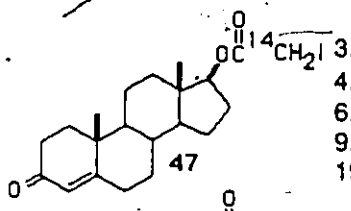
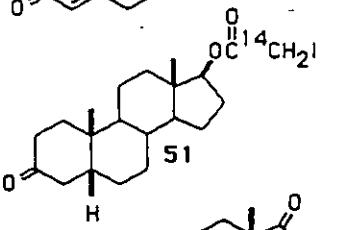
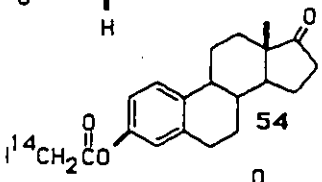
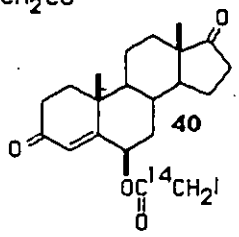
Steroids	Retention time (minutes)	%Radio-activity	Carboxymethy-lated amino acid	%Reco-very	%Radio-activity after oxi-dation
 33	3.50	0		60	100
	9.70	60	CM-Cys		0
 37	3.50	0		93	~90
	4.64	6	N^{π}, N^{τ}-Di-CM-His		
	6.50	8			
	9.70	71	CM-Cys		0
	19.00	4			
	34.39	4	N^{τ}-CM-His		
 47	3.50	0		86	~77
	4.64	25	N^{π}, N^{τ}-Di-CM-His		
	6.50	16			~6
	9.70	45	CM-Cys		0
 51	3.50	0		91	~90
	4.64	18	N^{π}, N^{τ}-Di-CM-His		
	6.50	21			~5
	9.70	44	CM-Cys		
	19.00	8			
 54	3.50	0		74	
	9.70	74	CM-Cys		
 40	3.50	0		100	~90
	9.70	98	CM-Cys		0

TABLE VI

Radioactive Profile from Amino Acid Analysis of Hydrolysis Products from 3 h Inactivation of 17β-HSD-III with Various Iodoacetoxy Steroids.

Steroids	Retention time (minutes)	%Radio-activity	Carboxymethy-lated amino acid	%Reco-very	%Radio-activity after oxi-dation
 33	3.50	0		100	~99
	6.50	1			
	9.70	99	CM-Cys		
 37	3.50	0		96	~90
	4.64	2	N^{π}, N^{τ}-Di-CM-His		
	6.50	4			
	9.70	87	CM-Cys		0
	19.00	2			
	22.89	1	N^{π}-CM-His		
 47	3.50	0		82	~80
	4.64	10	N^{π}, N^{τ}-Di-CM-His		
	6.50	17			17
	9.70	50	CM-Cys		0
	19.00	5			
 51	3.50	0		95	~50
	4.64	7	N^{π}, N^{τ}-Di-CM-His		
	6.50	7			6
	9.70	77	CM-Cys		0
	19.00	2			
	34.39	2	N^{τ}-CM-His		
 54	3.50	0		99	
	9.70	99	CM-Cys		
 40	3.50	0		99	~93
	4.64	10	N^{π}, N^{τ}-Di-CM-His		
	6.50	10			7
	9.70	79	CM-Cys		0

J. Radioactive Peptide Maps After Inactivation of 17β-HSD with Iodo[2'-^{14}C]acetoxy Steroids.

Since cysteine is the major amino acid modified by the different iodoacetoxy steroids, the following question needed to be answered: are these cysteines located at the same or at different positions in the enzyme active site?

Preliminary work was done to determine the number of cysteine residues present in each form of the 17β-HSD. However, we were not able to determine the number of cysteine groups using the electrophoretic method of Stan-Lotter and Bragg[81]. This procedure is based on Creighton's method[82] and involves the reaction of cysteine-containing denatured and reduced polypeptides with iodoacetic acid or iodoacetamide thus introducing acid carboxymethyl groups or neutral iodoacetamide groups, respectively. Then by using various ratios of iodoacetamide : iodoacetic acid, a spectrum of polypeptide molecules is generated having 0 to n acidic carboxymethyl groups, where n is the number of cysteine residues per molecule of polypeptide. These species are separated by gel electrophoresis based on charge and the number of cysteine groups is determined by counting the bands corresponding to the n+1 species present. It was hoped that having found the number of cysteine groups by this method, we could first inactivate the enzyme with the [^{14}C]-iodoacetoxy steroid, then apply Stan-Lotter and Bragg's method to determine how many radioactive cysteine groups have been modified. It was possible to establish conditions which prevented the formation of carbamates since we got one major protein band of inactivated 17β-HSD-I with 19-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33**, after overnight urea denaturation (Figure 38). When the urea was not treated with the mixed-Bed resin, multiple bands were found when urea isoelectric focusing gel was performed with

pure 17β-HSD enzyme. However, we were unable to obtain only one band when the enzyme was treated with iodoacetic acid or iodoacetamide.

The purpose of peptide mapping was to get more information about the location of the labeled cysteine residues and to compare the active sites of enzyme forms I and III. Peptides were generated by treating the inactivated enzyme with chymotrypsin and the peptides were separated by electrophoresis and chromatography on cellulose sheets. These maps show only the radioactive peptides. The maps obtained for both enzyme forms I and III are shown in Figures 39 to 52. Similar maps are obtained after 1 or 3 h inactivation, as illustrated by comparing Figures 39-40, 41-42 and 49-50. However, comparison of the peptide maps for enzyme form I with those for enzyme form III indicates differences in the active site regions of these enzymes. The similarity of the peptide maps of enzyme form I inactivated with 6β-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **40** (Figure 43) and 19-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33** (Figure 40) suggests that both of these analogues inactivate the same or neighbouring cysteines. Examination of Drieding molecular models for both compounds **33** and **40** clearly indicates that the 19-iodoacetoxy group is free to rotate and may reach a position which is also accessible to the 6β-iodoacetoxy group. With enzyme form III, this similarity is less apparent (Figures 47 and 50) but the same dominant spot is present in both radioactive peptide maps.

We previously found that inactivation by substrate analogues is prevented to a greater extent by NADP than by steroid substrate. This suggests that these analogues may be reacting with a cysteine residue present in the cofactor binding site. To verify our hypothesis, 17β-HSD enzymes were inactivated with

6β-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **40** and 19-iodo[2'-^{14}C]acetoxy-androst-4-ene-3,17-dione **33** in the presence of NADP (Figures 53 to 56). However, there is almost no difference in the radioactive peptide maps (Figures 40 and 53, 47 and 55) for both enzyme forms when they are inactivated, in the presence or absence of NADP, with the 19-iodoacetoxy steroid **33**. Similarly, when both enzyme forms are inactivated in the presence or absence of NADP with the 6β-iodoacetoxy steroid **40** (Figures 43 and 54, 50 and 56), only very minor differences can be detected. From this result, we may postulate that the cysteine residue is possibly located nearer the cofactor binding site than the substrate binding site. These results indicate that no alternative binding site is accessible to the affinity label thus it is competing with the NADP cofactor for the same site containing the reactive cysteine.

Initially, 19-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33** and 19-iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37** were designed to react with amino acid residues located in the site of the 17β-HSD and 3α-HSD, respectively. The objective was to find out if one or two sites were responsible for both 3- and 17-HSD activities. Sweet and Samant[39] proposed two models for steroid binding at the active site of 3α,20β-HSD. The first model represents a single steroid binding cavity with two cofactor (NADH) sites, one located near C-3 thus delivering the 3β-hydride and the other located near C-20 thus delivering the 20α-hydride. In this model the steroid substrate is restricted to a single enzyme-substrate complex. The second model represents two different enzyme-substrate complexes: there is only one site for the cofactor (NADH) delivering the 3β- or 20α-hydride. The two enzyme-substrate complexes are related through 180^{o} rotation of the steroid at the active site. The radioactive peptide maps obtained upon inactivation of 17β-HSD-I

with both compounds **33** and **37** (Figures 40 and 42) are similar thus suggesting that both analogues may share the same active site. This similarity is less apparent for 17β-HSD-III (Figures 47 and 48), one peptide (indicated by an arrow) is present when the 17β-HSD-III is inactivated with the unsaturated analogue **33** and is absent when the enzyme is inactivated with the saturated analogue **37**. The peptide maps obtained when 17β-HSD-I is inactivated with both 17β-iodo[2'-^{14}C]-acetoxy-5β-androstan-3-one **51** and 17β-iodo[2'-^{14}C]acetoxyandrost-4-en-3-one **47** (Figures 45 and 44) are similar. With 17β-HSD-III, however, the same conclusion cannot be made, the peptide maps being different and more difficult to compare (Figures 52 and 51).

K. Summary.

In summary, the purpose of this investigation was to obtain information on the topography of the 17β-HSD enzyme forms I and III isolated from female rabbit liver utilizing the affinity labeling technique. Besides the comparison of the active site of both enzyme forms I and III, the possibility of one or two active sites responsible for both 3α- and 17β-hydroxysteroid dehydrogenase activities was also raised.

We found that all substrate analogues synthesized inactivated both forms of the 17β-HSD enzyme reacting primarily with one or more cysteine residues present in the enzymes. The fact that inactivation by the substrate analogues is prevented to a greater extent by NADP than by steroid substrate suggests that the cysteine residue is nearer the cofactor binding site than the substrate binding site.

Substrate analogues with iodoacetoxy groups located at C-6 or C-19 likely label the same or neighbouring cysteine residues as indicated by the similarity of the peptide maps obtained when both C-6 and C-19 derivatives reacted with 17β-HSD enzyme forms I and III. The similarity of the peptide maps obtained following inactivation with 19-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33** and 19-iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37** suggest that a single active site would be responsible for the C-3 and C-17 activities of both enzyme forms of the 17β-HSD.

It was previously emphasized by Murdock and co-workers[29], with 17β-HSD from human placenta, that the majority of residues modified by steroid derivatives were cysteine residues. Since Crastes de Paulet and co-workers[15] have shown that most of these cysteine residues are nonessential for catalytic activity or are located in the cofactor binding region of the enzyme, recent studies[29] were directed toward evaluation of the involvement of the minor histidine residues present in the active site also labeled with affinity reagents but to a lesser extent. In order to get a more complete understanding of the 17β-HSD active site of both enzyme forms I and III studies should be oriented toward the isolation, sequencing and comparison of the radioactive peptides obtained by the various iodoacetoxy steroids.

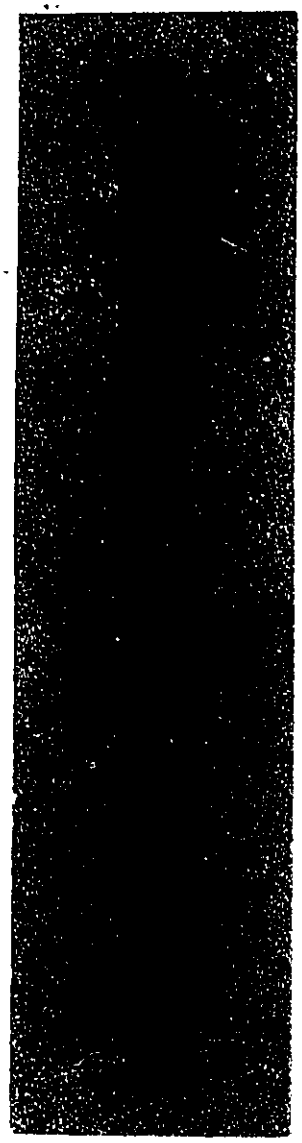


Figure 38. Urea Isoelectric Polyacrylamide-Gel Electrophoresis of Inactivated 17β-HSD-I with 19-Iodo[2'-^{14}C]acetoxy-androst-4-ene-3,17-dione **33**.

Enzyme (2 nmol) was inactivated with 19-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione for 3 h at 25°C. The inactivated enzyme was treated with 8 M urea overnight. The solution was concentrated with a Centricon apparatus and applied on an urea polyacrylamide gel (described in the Experimental section B.17). Isoelectric focusing was carried out at 4°C for 30 min at 450 V followed by 4 h at 500 V.

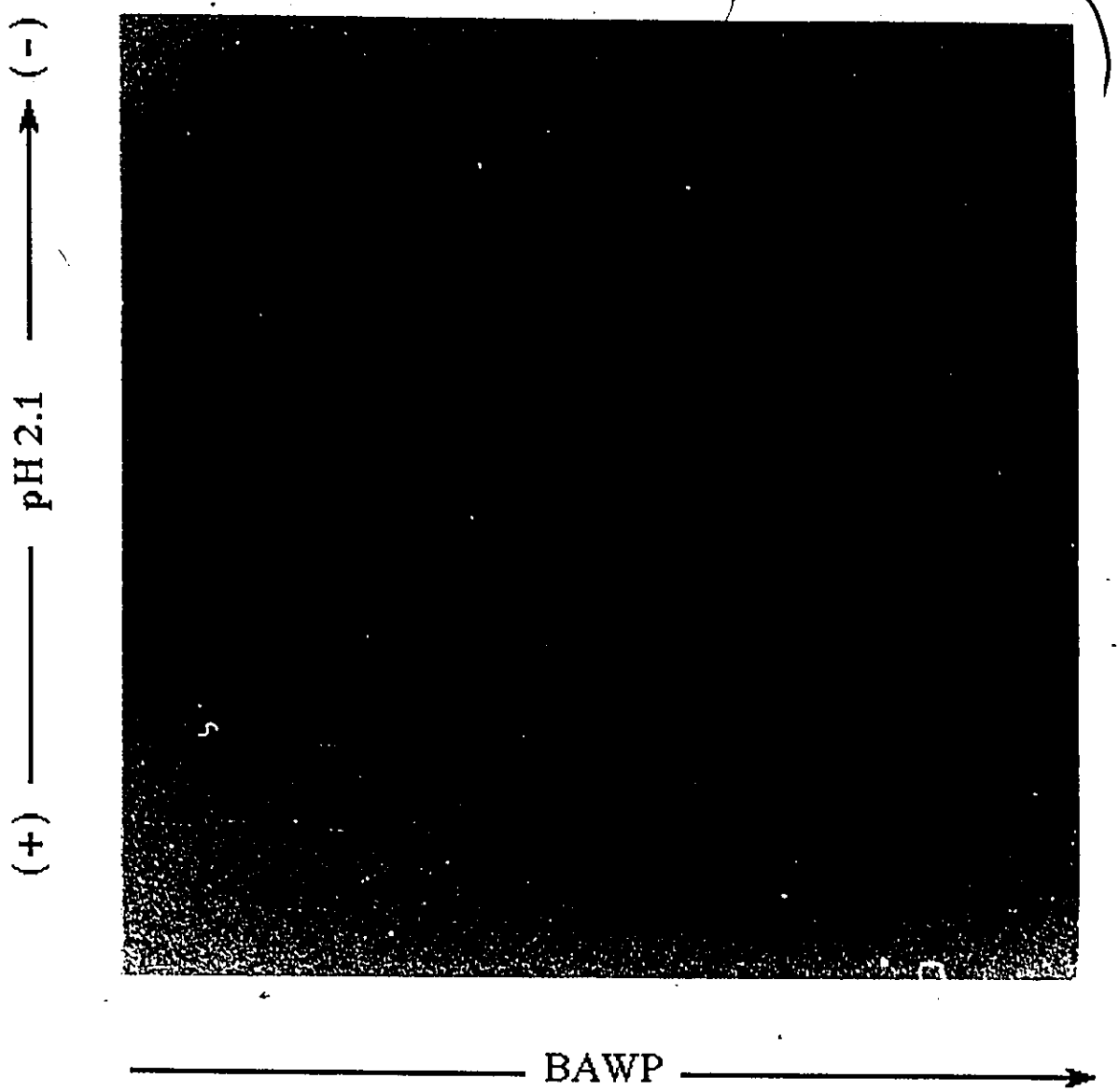


Figure 39. Peptide Map of the Chymotrypsin Digest of 1 h Inactivation of 17β-HSD-I by 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **33**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

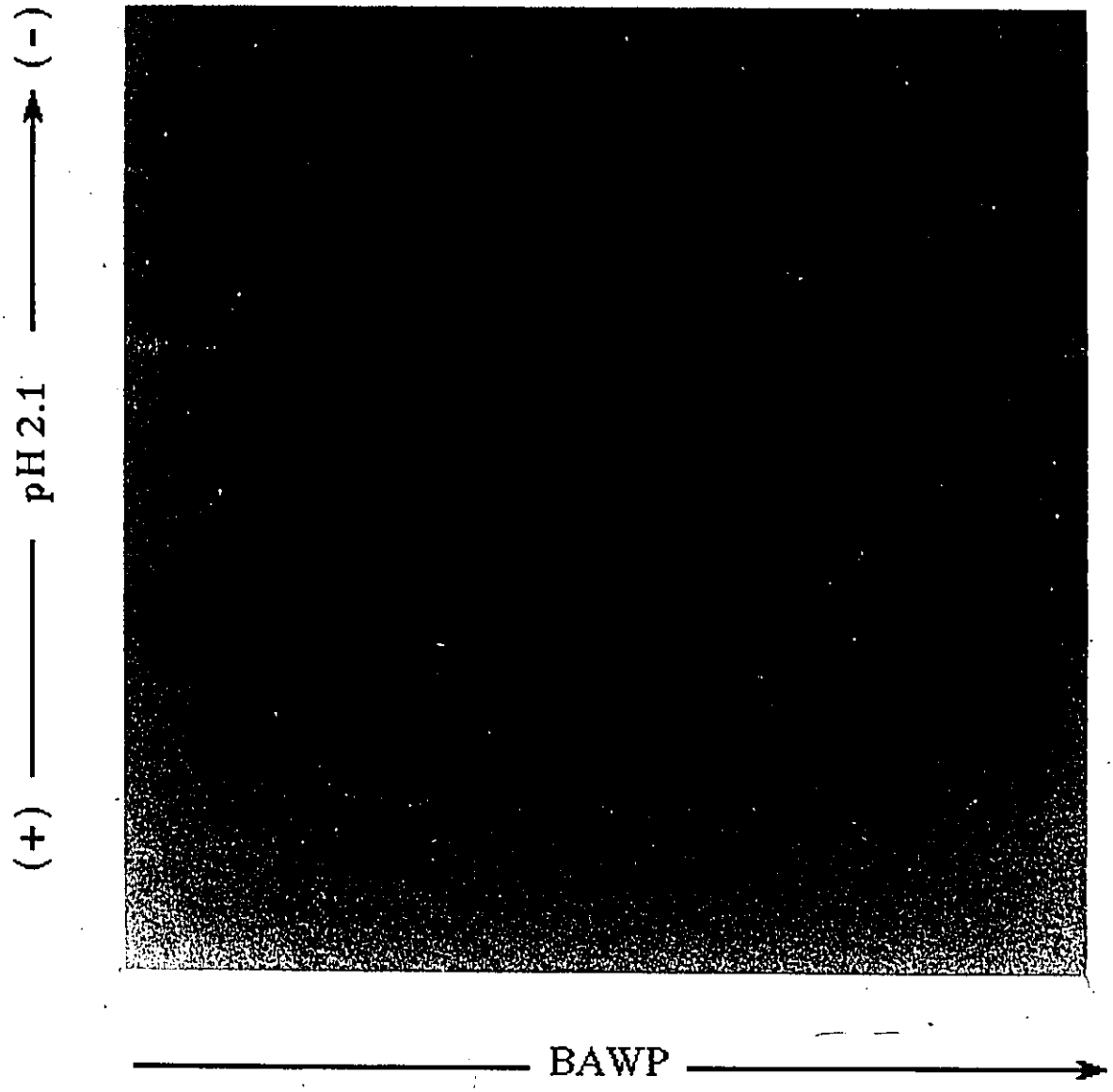


Figure 40. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-I by 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione 33.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

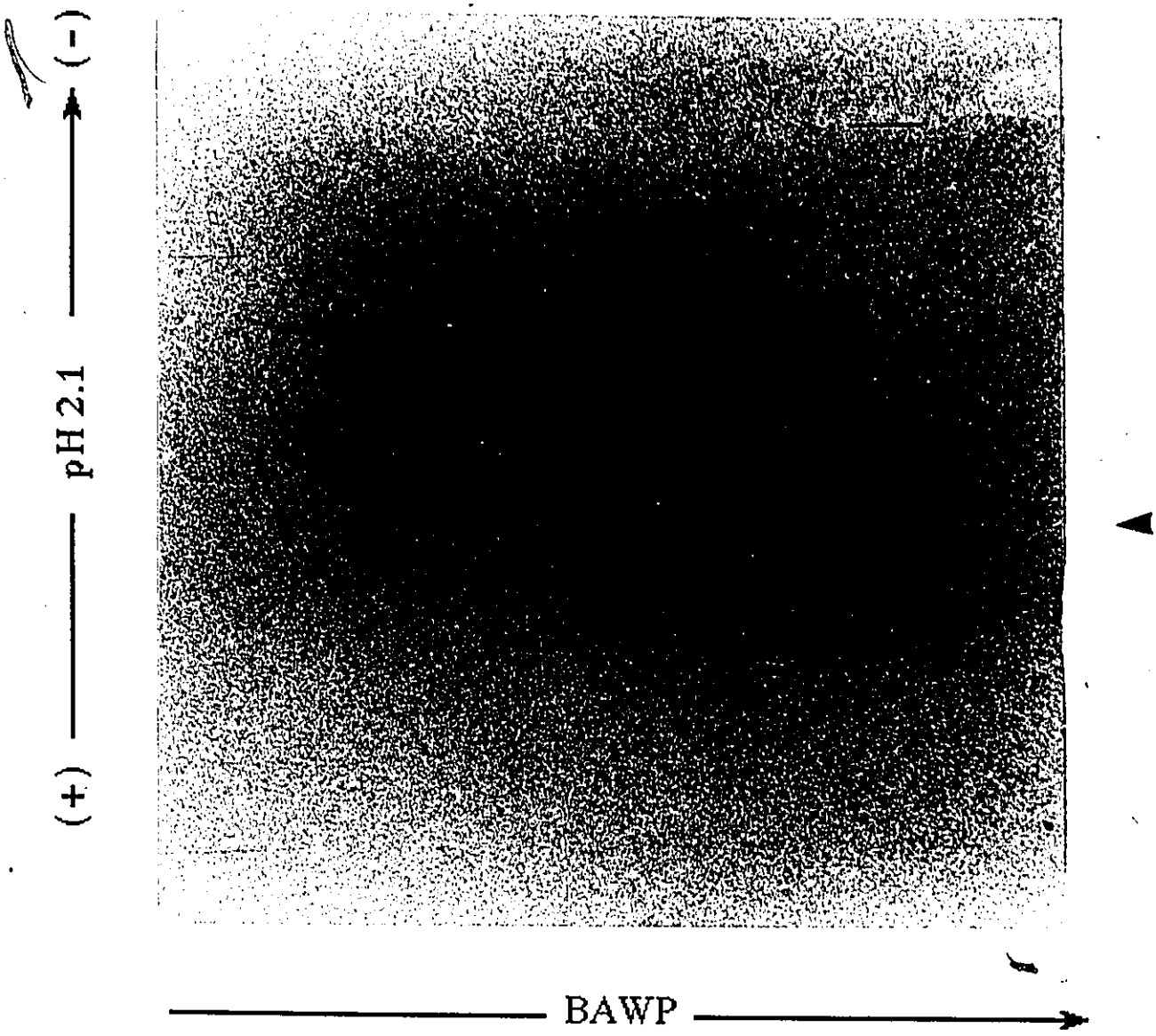


Figure 41. Peptide Map of the Chymotrypsin Digest of 1 h Inactivation of 17β-HSD-I by 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3, 17-dione **37**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

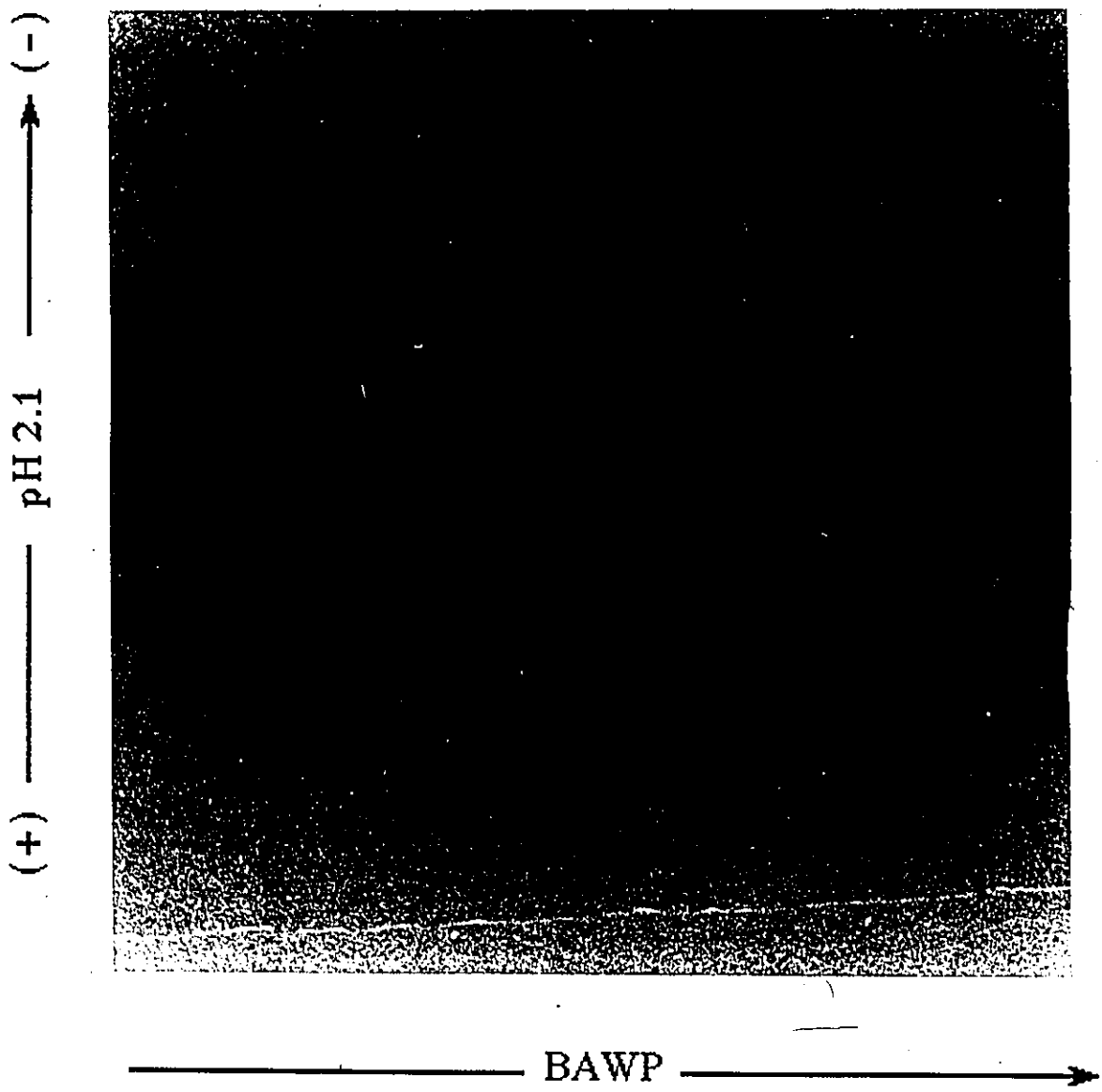


Figure 42. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-I by 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3, 17-dione **37**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

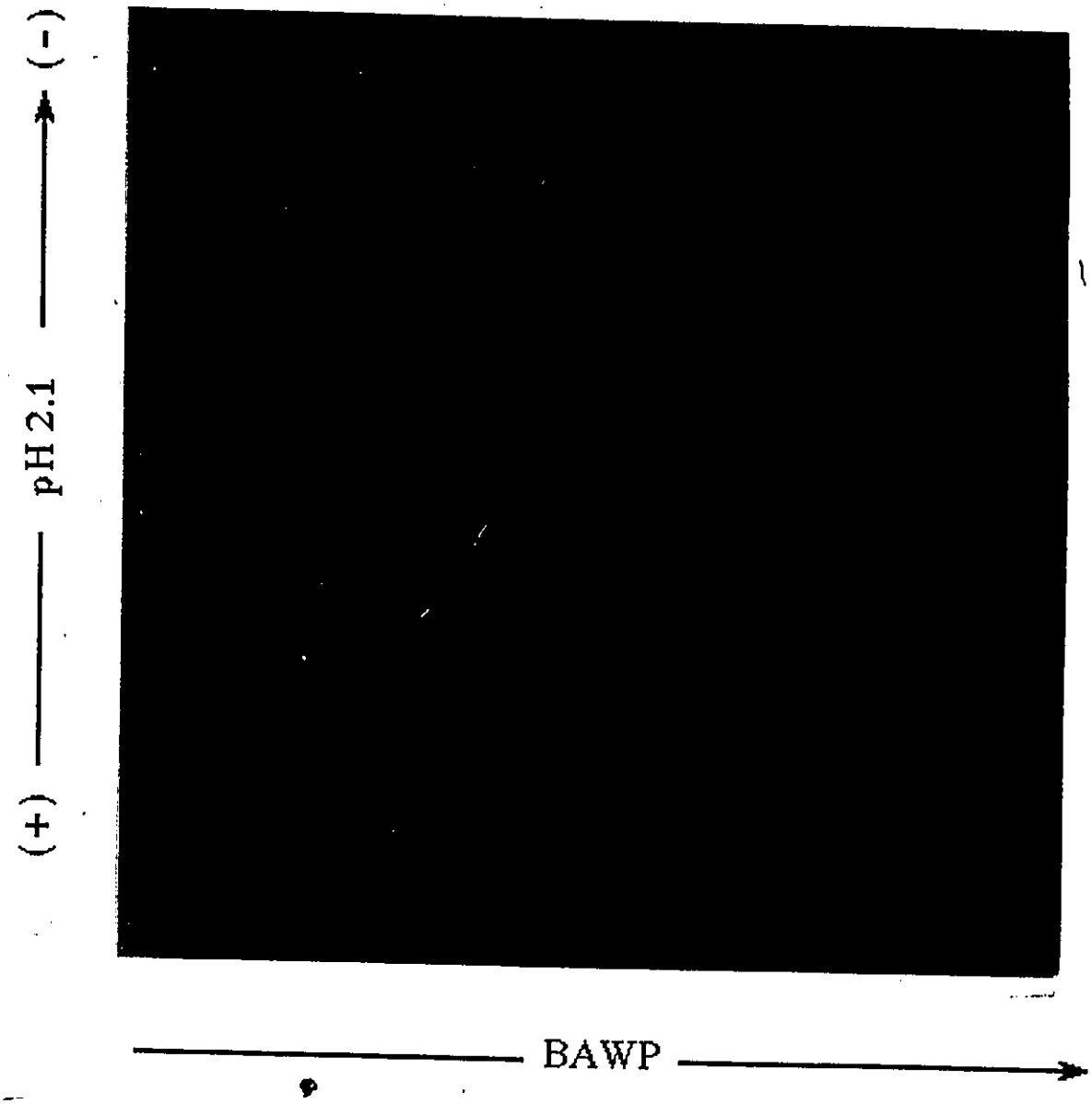


Figure 43. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-I by 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **40**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 ~~in the first~~ dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

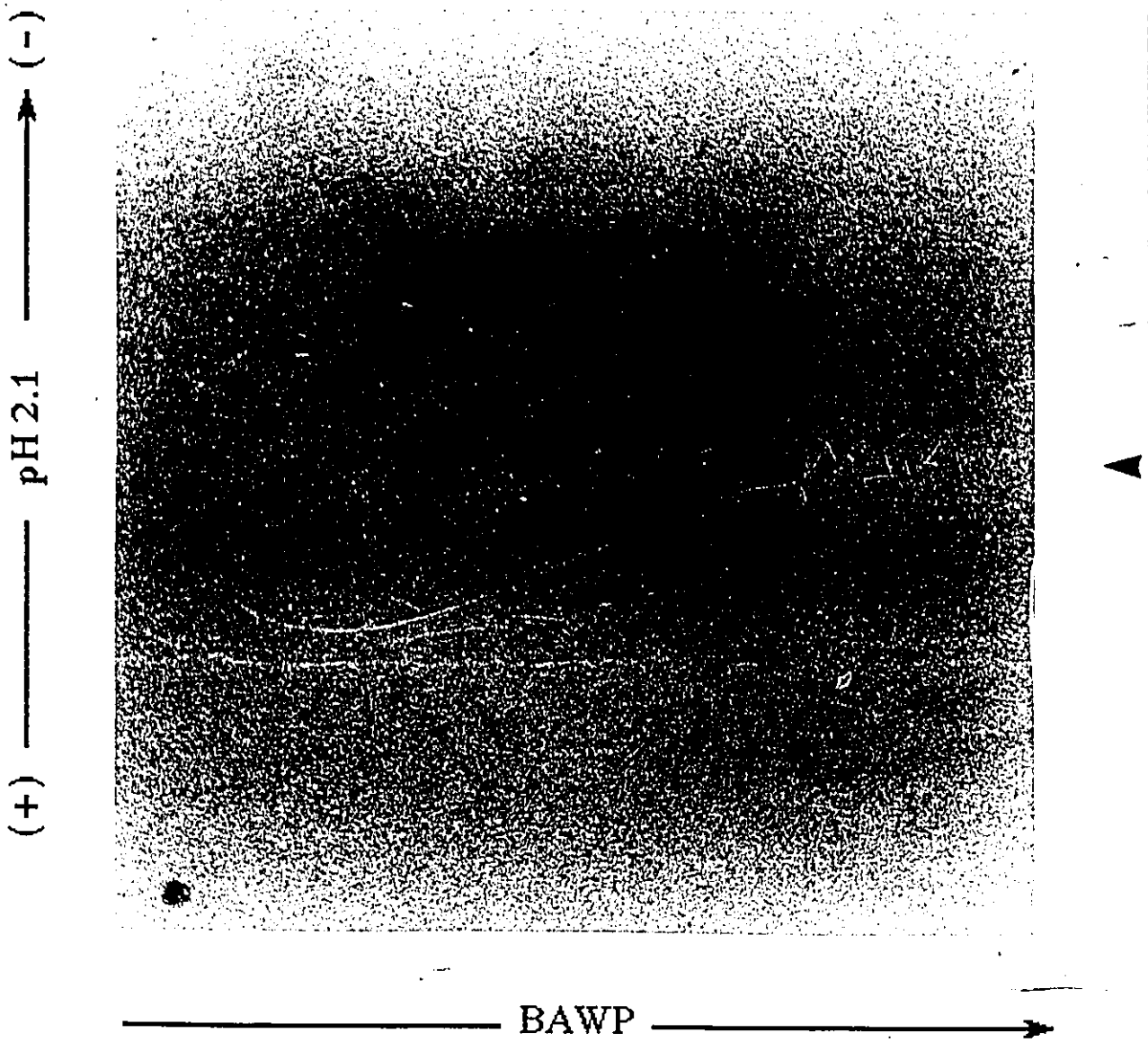


Figure 44. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-I by 17β-Iodo[2'-^{14}C]acetoxyandrost-4-en-3-one 47.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

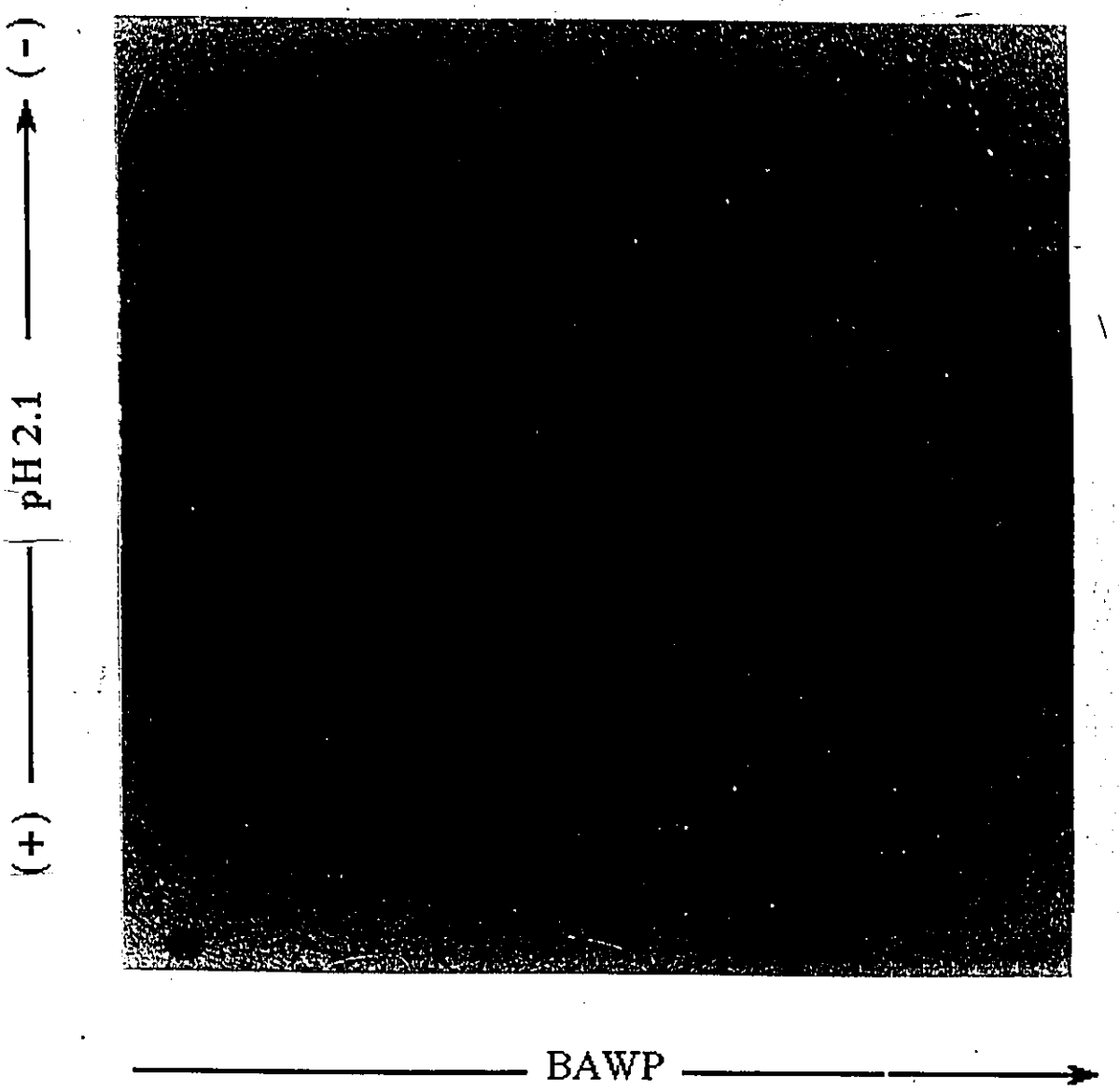


Figure 45. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-I by 17β-Iodo[2'-^{14}C]acetoxy-5β-androstan-3-one **51**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

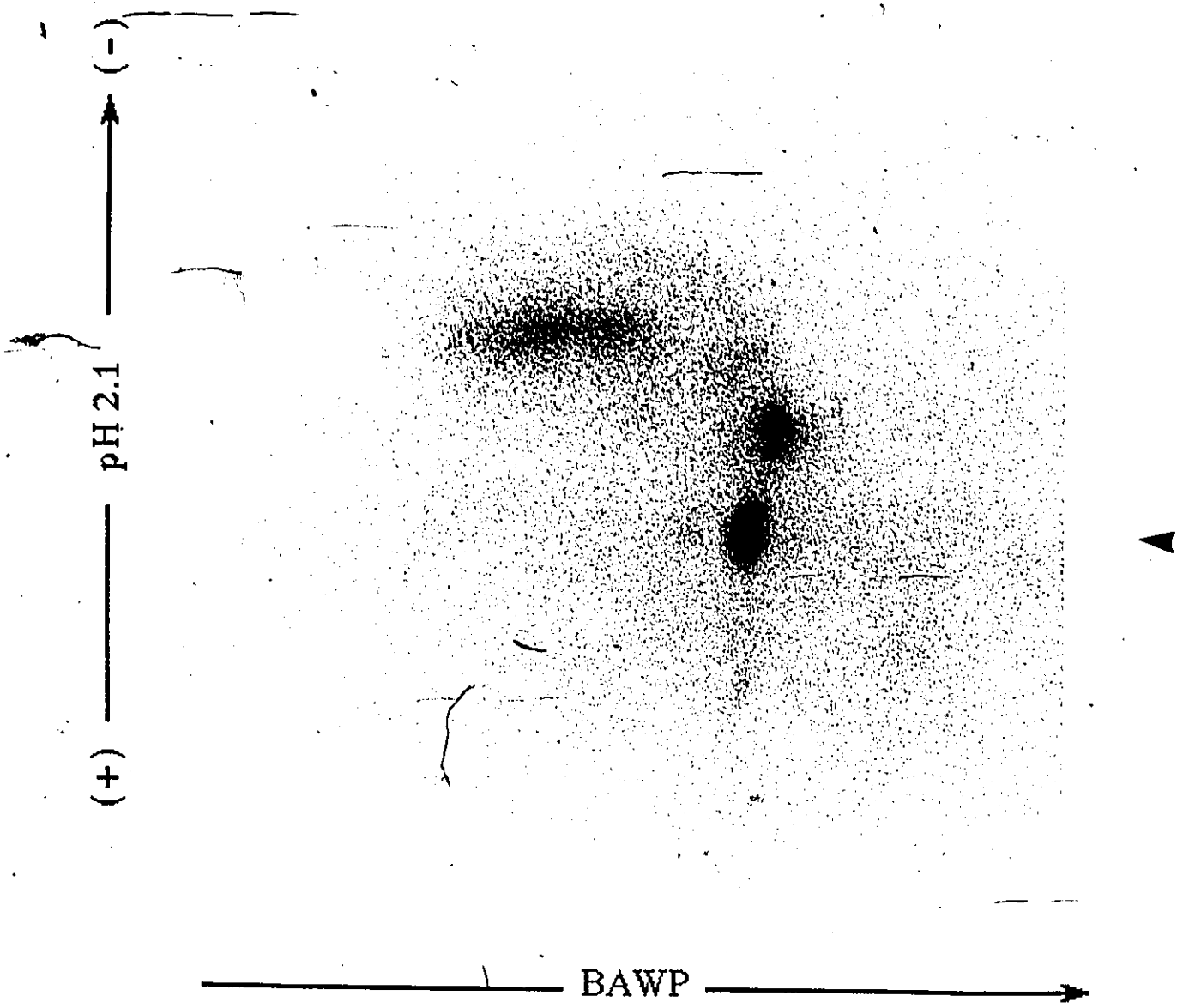


Figure 46. Peptide Map of the Chymotrypsin Digest of 1 h Inactivation of 17β-HSD-III by 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **33**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

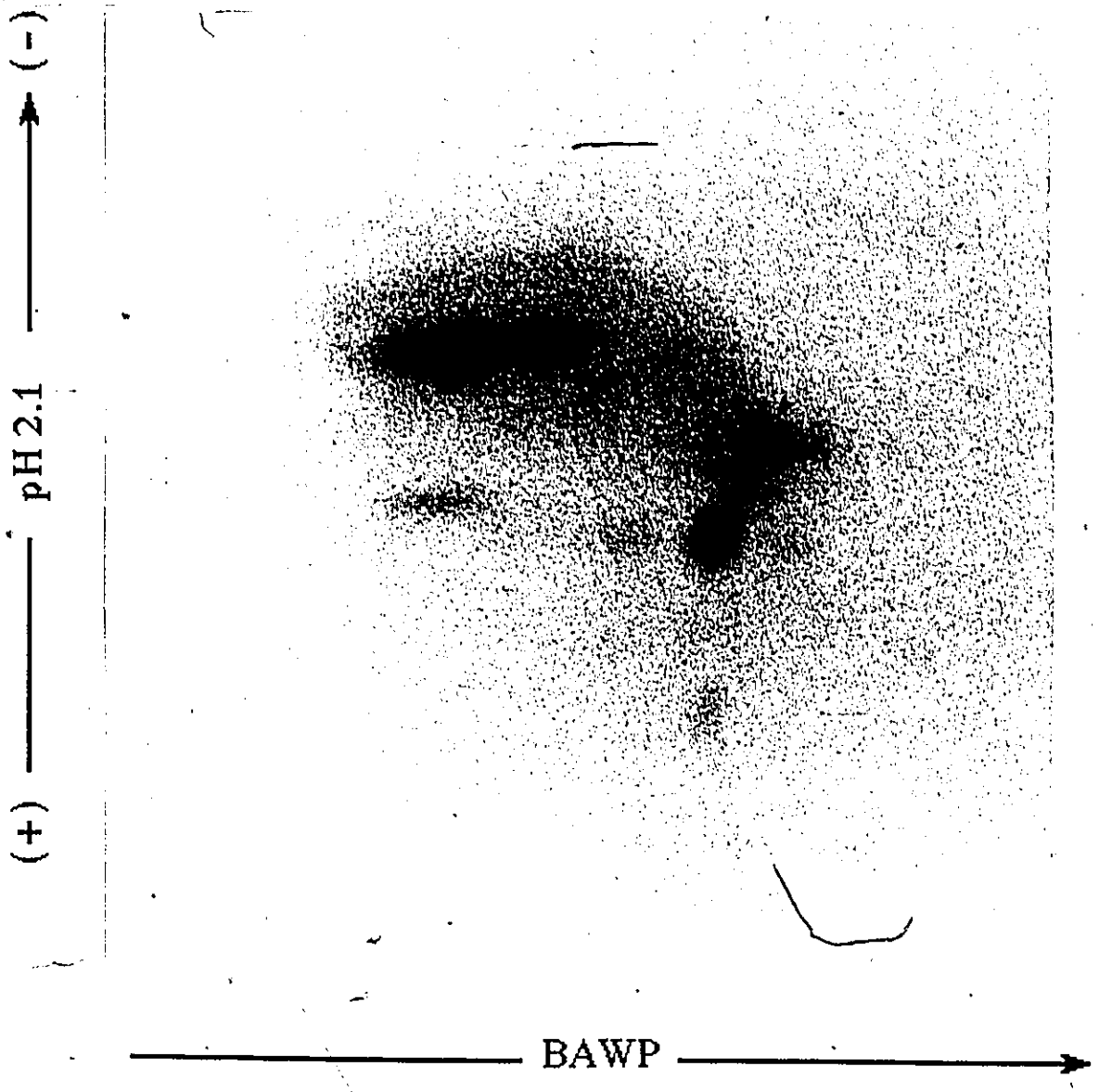


Figure 47. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-III by 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **33**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

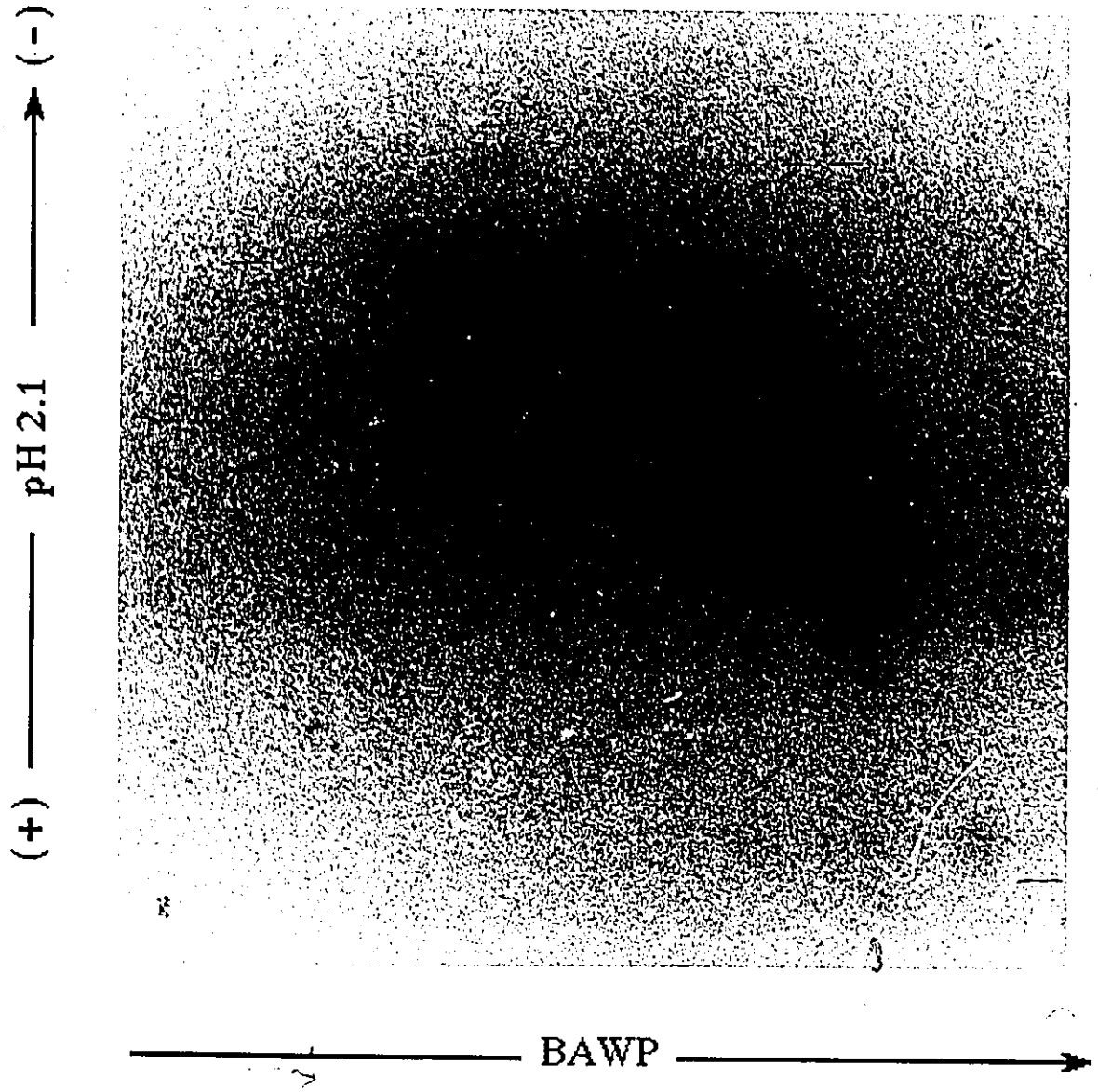


Figure 48. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-III by 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

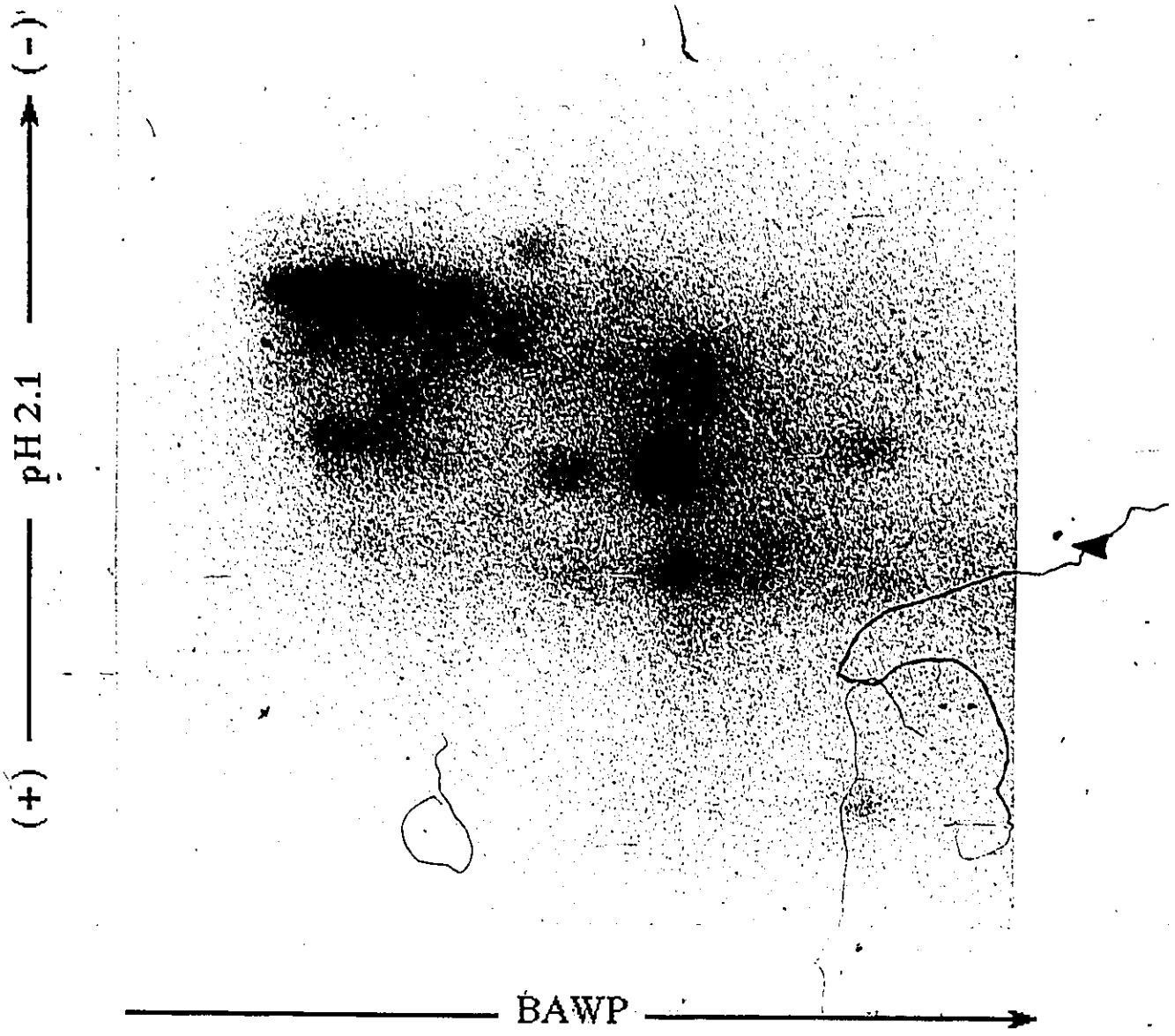


Figure 49. Peptide Map of the Chymotrypsin Digest of 1 h Inactivation of 17β-HSD-III by 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **40**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

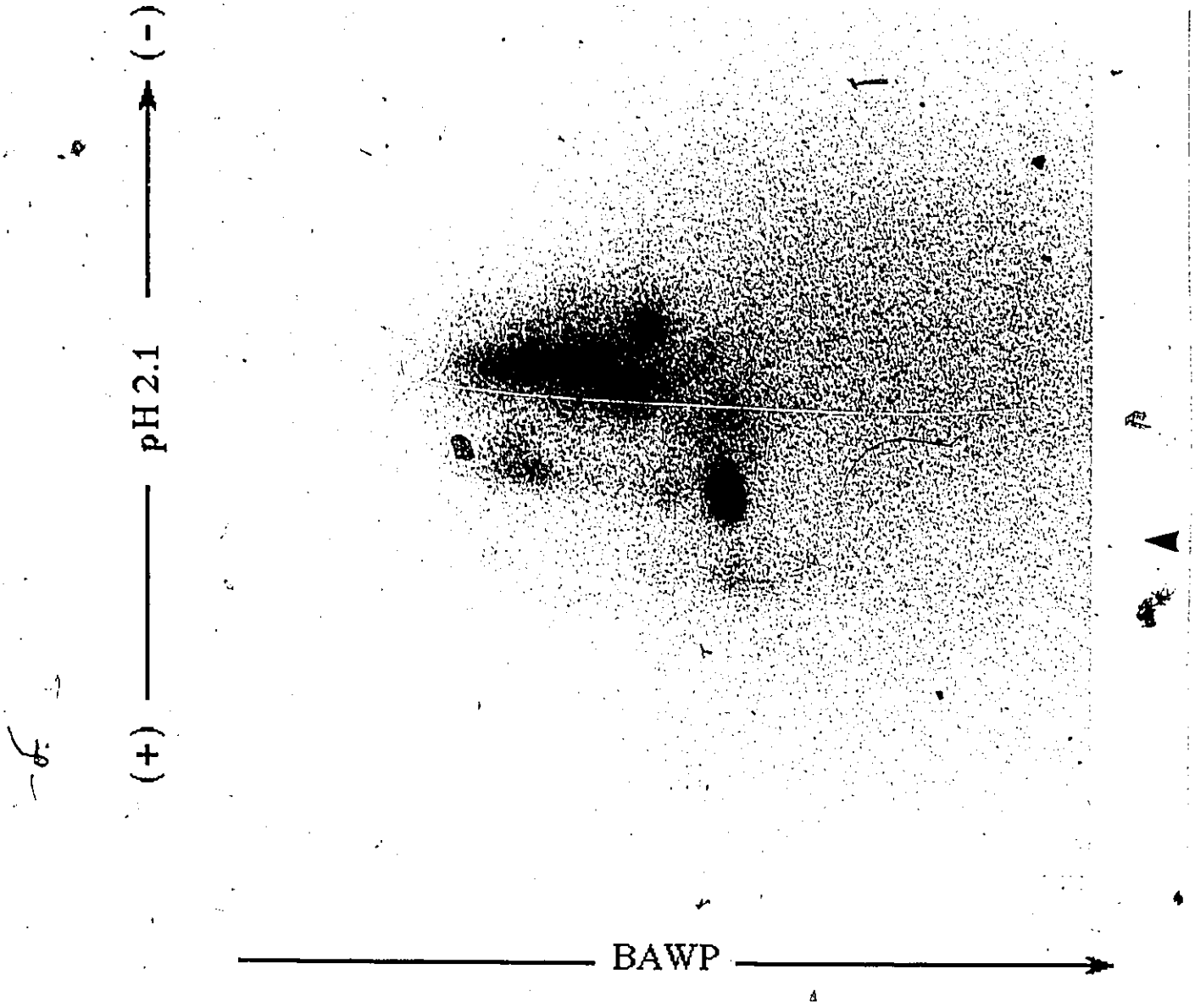


Figure 50. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-III by 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **40**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

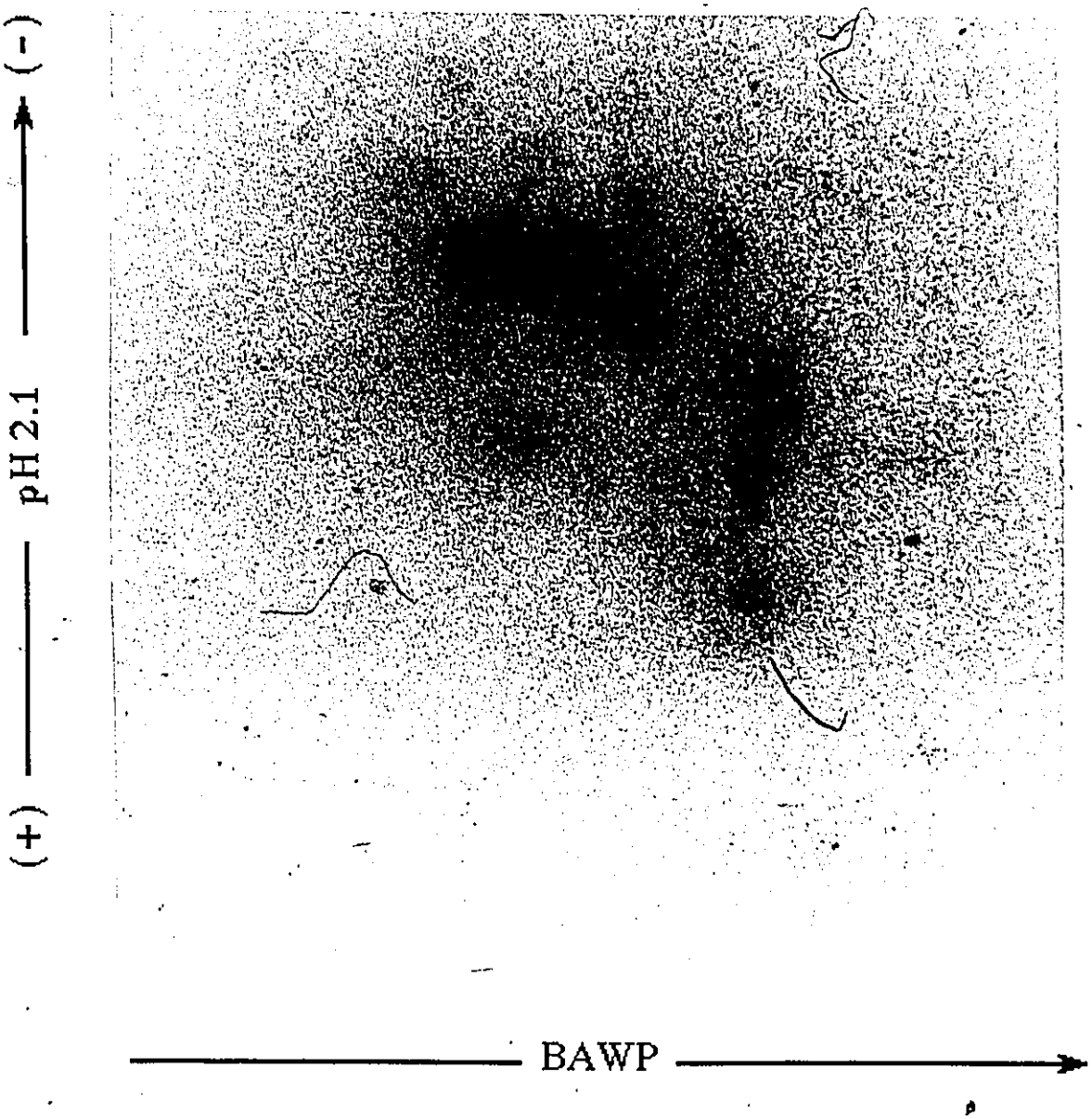


Figure 51. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-III by 17β-Iodo[2'-^{14}C]acetoxyandrost-4-en-3-one **47**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

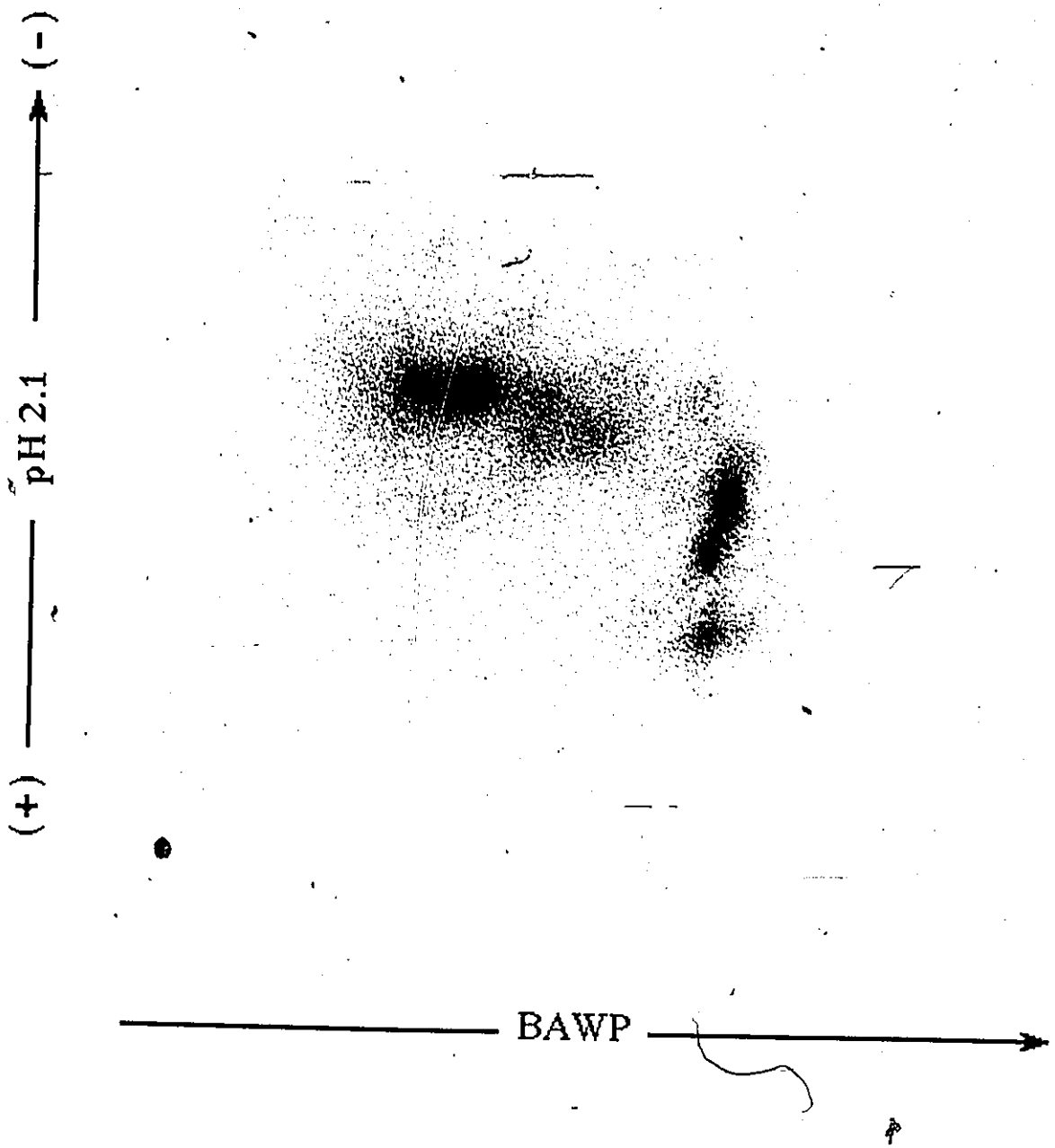


Figure 52. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-III by 17β-Iodo[2'-^{14}C]acetoxy-5β-androstan-3-one **51**.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

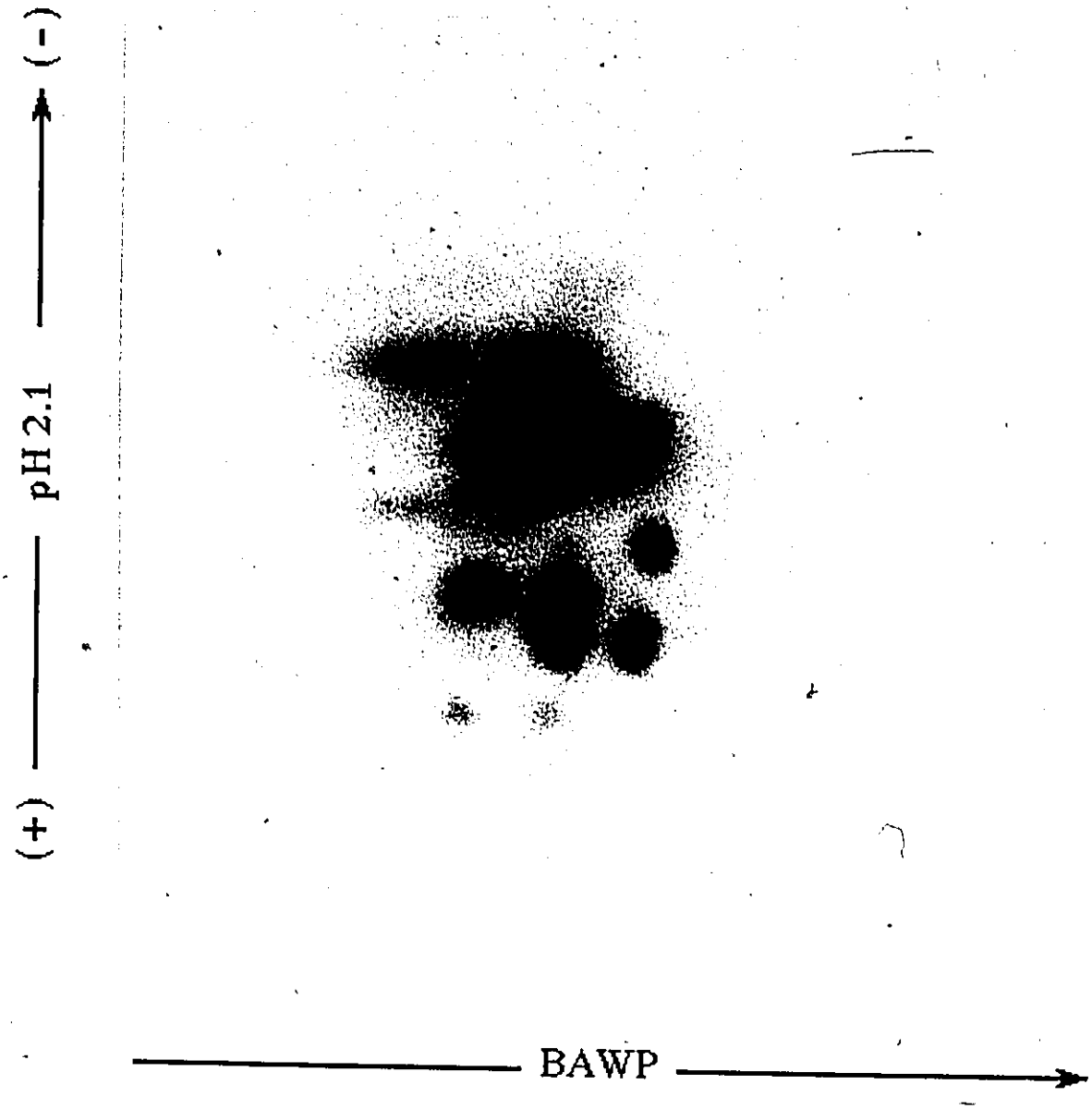


Figure 53. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-I by 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **33** in the Presence of NADP.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

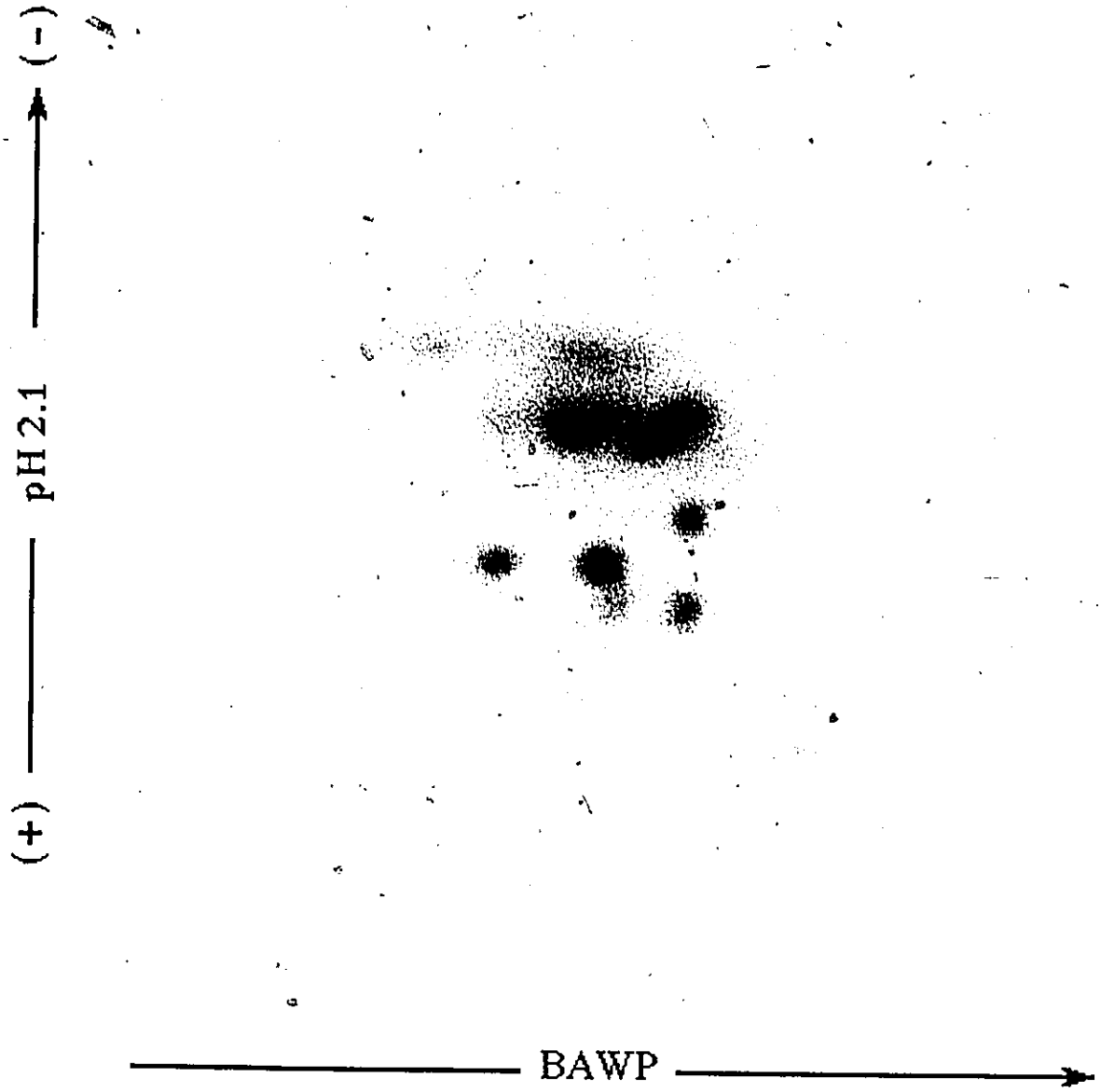


Figure 54. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-I by 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **40** in the Presence of NADP.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

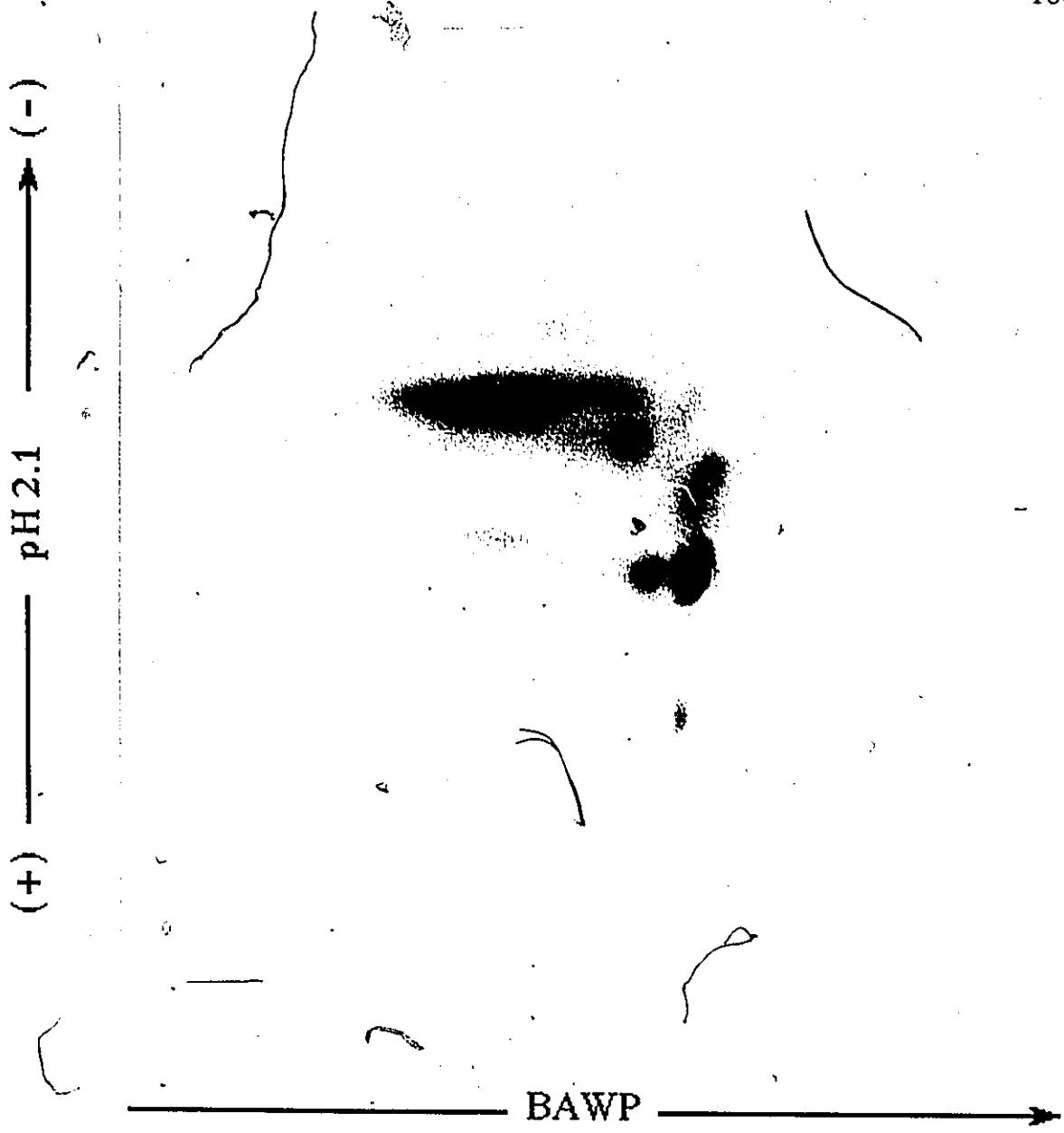


Figure 55. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-III by 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **33** in the Presence of NADP.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

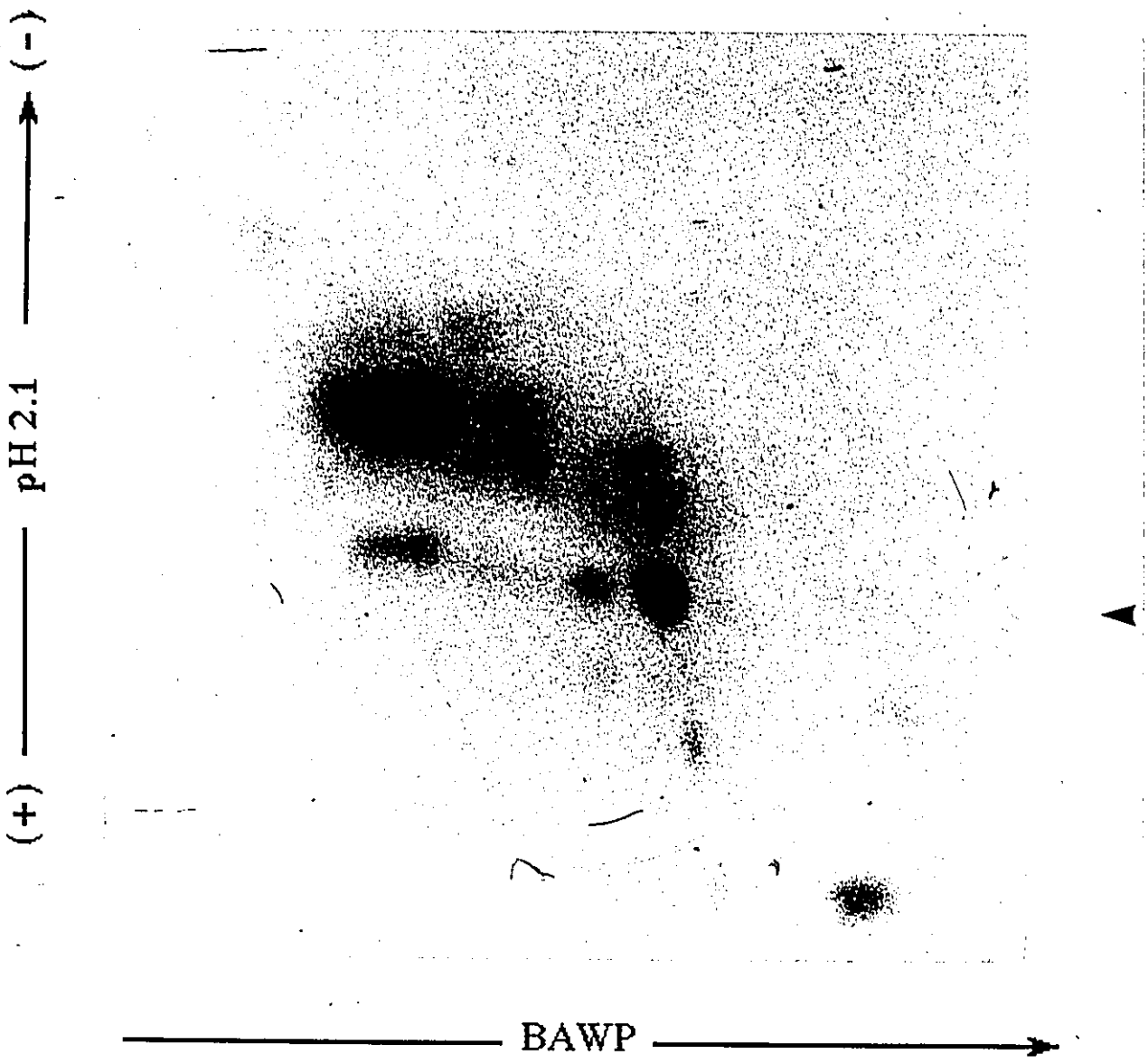


Figure 56. Peptide Map of the Chymotrypsin Digest of 3 h Inactivation of 17β-HSD-III by 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3, 17-dione **40** in the Presence of NADP.

Electrophoresis (450 V, 1 h) was carried out at pH 2.1 in the first dimension. Elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. The peptides were located by autoradiography. The location of the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride is indicated by an arrow.

PART II

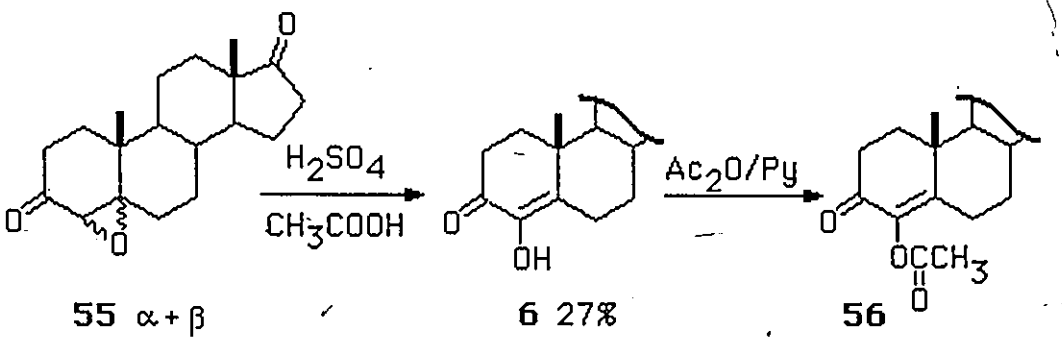
SYNTHESIS AND CHEMISTRY OF C-19 FUNCTIONALIZED STEROIDS.

RESULTS AND DISCUSSION

A. Synthesis of C-4 Substituted Androgens.

1. Preparation of 4-Hydroxyandrost-4-ene-3,17-dione **6** and 4-Acetoxyandrost-4-ene-3,17-dione **56**.

As previously mentioned in Part I, 4-hydroxyandrost-4-ene-3,17-dione **6** was initially synthesized in order that the iodoacetoxy derivative could be made and used to map the A-ring region of the 17β-HSD active site. The synthesis of the 4-hydroxy compound **6** has previously been achieved by acid-catalyzed opening of the 4,5-epoxides **55**[83].

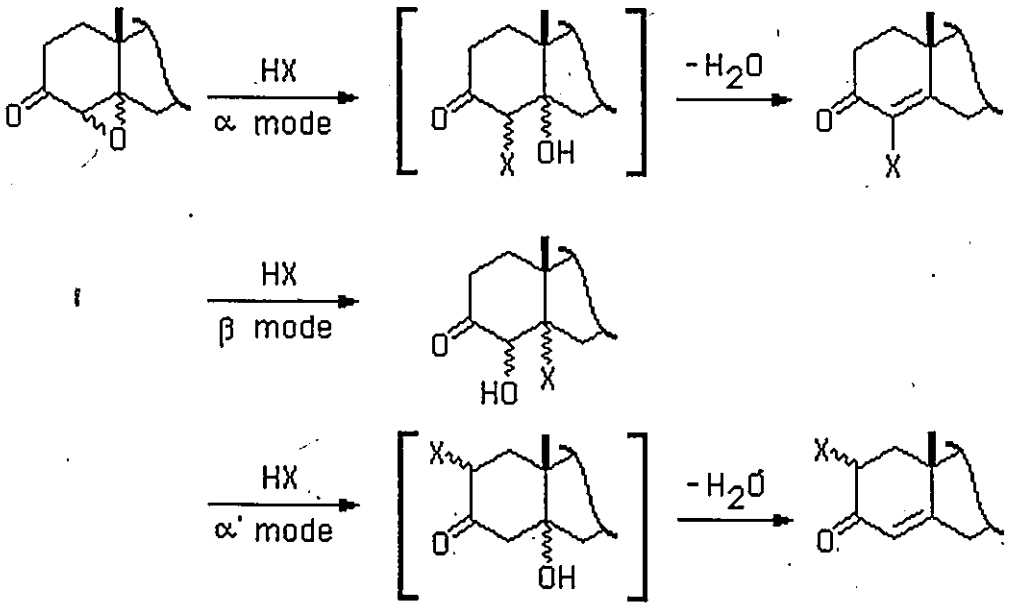


55 α+β **6** 27% **56**

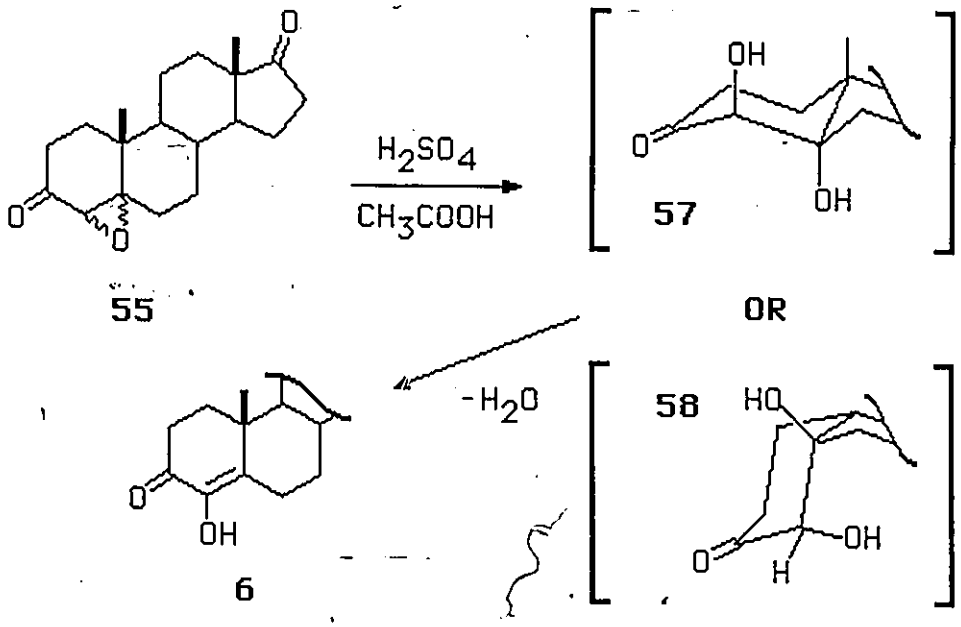
We successfully repeated this reaction involving treatment of the 4,5-epoxides **55** with sulphuric acid in acetic acid. The 4,5-epoxides **55** were obtained as a mixture (*ca*. 4:1, by ^{1}H NMR analysis) of 4β,5β- and 4α,5α-

epoxides by treatment of androst-4-ene-3,17-dione **18** (see p.23) with alkaline hydrogen peroxide. The compound isolated in 27% yield was identical with the known[83,84] 4-hydroxyandrost-4-ene-3,17-dione **6**. The yield is rather low but is consistent with the yield (10-15%) obtained by Brodie and co-workers[83]. The ^{13}C NMR spectrum was also assigned (see details in the appendix). The 4-acetoxy compound identical with the known[85] 4-acetoxyandrost-4-ene-3,17-dione **56** was obtained simply by treating the 4-hydroxy compound **6** with acetic anhydride in pyridine. The reason for making this compound **56** was to use it as a control in the affinity labeling work with the 17β-HSD.

In the presence of nucleophiles, three different modes of epoxide opening have been observed[91] and are illustrated below.



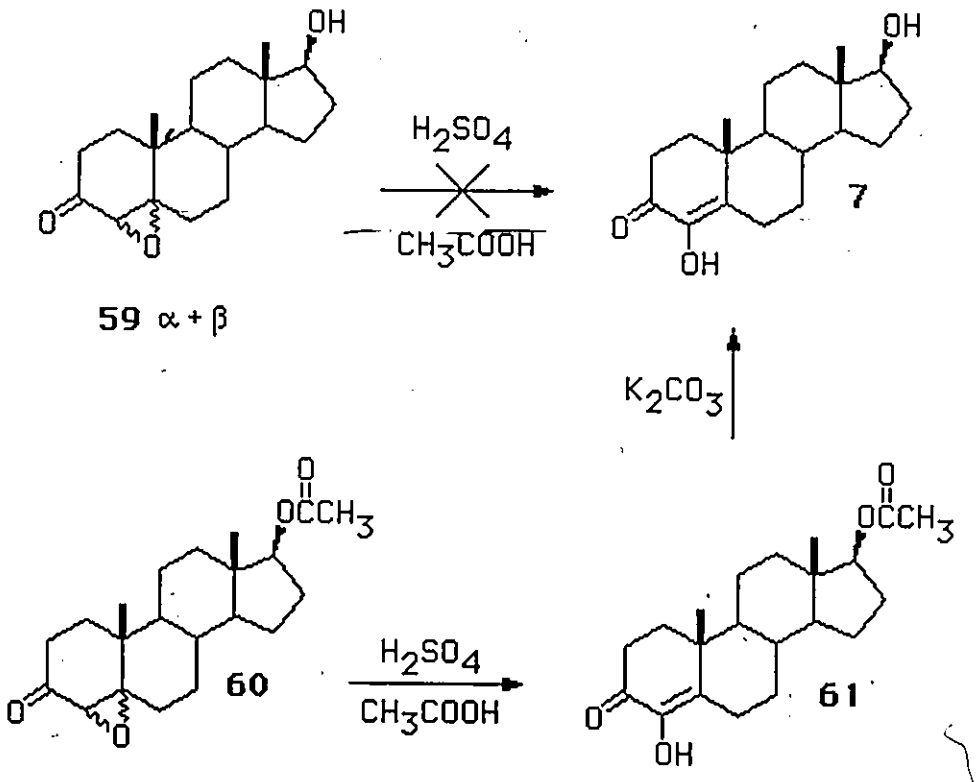
It remains difficult to understand the factors which are responsible for these differences since they seem to include not only the nature of the epoxide but also that of the solvent and the acid employed. It is possible that the diosphenol **6** may have arisen *via* dehydration of the initially formed diol **57** (α mode). However, in this case, the intermediacy of the diol **58** (β mode) cannot be ruled out.



4-Hydroxyandrost-4-ene-3,17-dione **6** and its acetate **56** are both potent inhibitors of the biosynthesis of estrogens. For example, these compounds inhibit the conversion of androst-4-ene-3,17-dione **18** to estrogens in human placental and rat ovarian microsomes[83,86]. These compounds (**6** and **56**) were found to be effective in controlling estrogen-dependent reproductive and neoplastic processes[83,87]. It has also been shown that they act as suicide inhibitors of aromatase[88,89,90].

2. Preparation of 4,17β-Dihydroxyandrost-4-en-3-one 7.

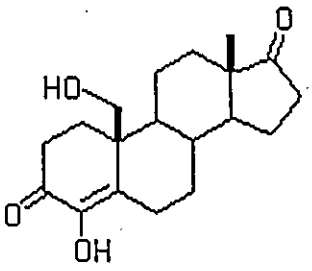
In order to complete the enzymatic work, it was necessary to synthesize 4,17β-dihydroxyandrost-4-en-3-one **7** since this compound would be the product obtained upon incubation of the 4-hydroxy compound **6** with the 17β-HSD. We found that when the 17β-hydroxy-4,5-epoxy compound **59** (which was obtained as a mixture, *ca* . 6.8:1 by ^{1}H NMR analysis, of 4β,5β- and 4α,5α-epoxides) was treated with sulphuric acid in acetic acid, the desired diosphenol **7** was not formed. However, when the 17β-hydroxyl group was acetylated, treatment with sulphuric acid in acetic acid gave a product, isolated in 85% yield, with identical properties[92,93] to the known 17β-acetoxy-4-hydroxyandrost-4-en-3-one **61**. Treatment of the 17β-acetoxy derivative **61** with potassium carbonate afforded the diosphenol **7**, albeit in low yield (16%).



3. Preparation of 4,19-Dihydroxyandrost-4-ene-3,17-dione **63**.

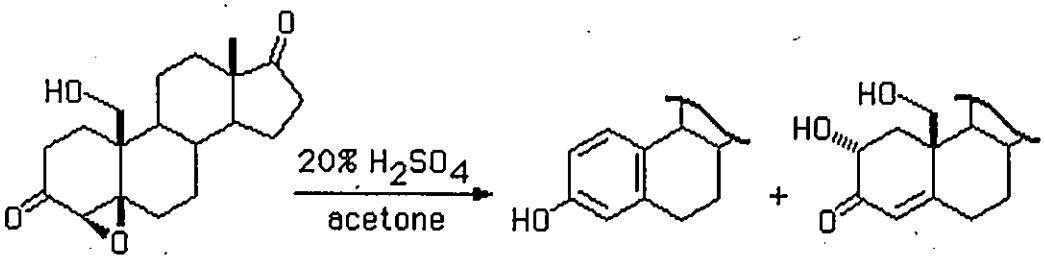
i. Acid-catalyzed Opening of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62**.

We were interested in synthesizing the derivative of compound **6** with a 19-hydroxyl group **63**. The purpose was to eventually synthesize a bifunctional affinity label with iodoacetoxy groups at C-4 and C-19.



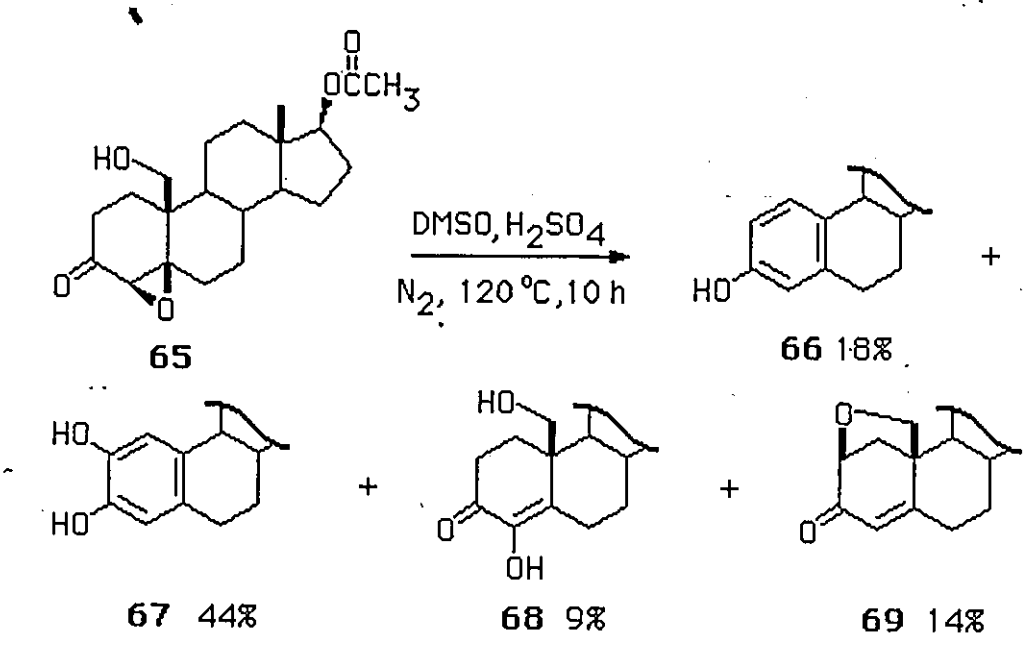
63

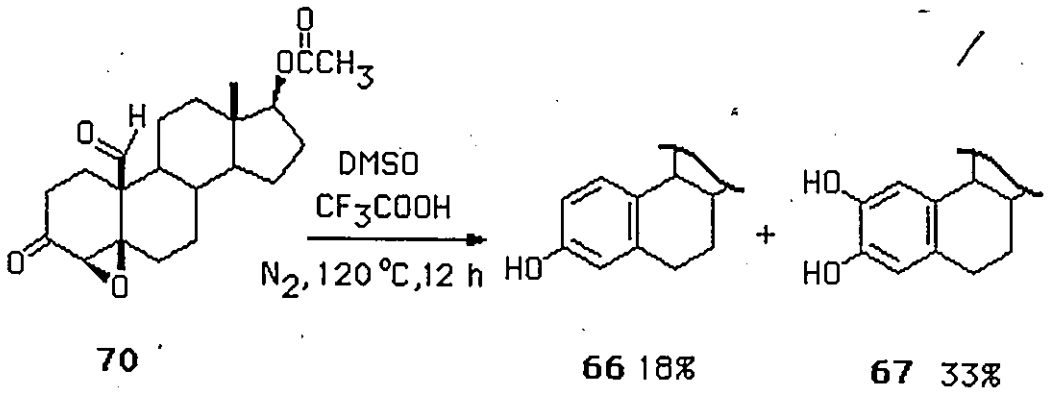
There are few reports on acid-catalyzed reactions of 19-substituted 4,5-epoxy-3-ketones. Hosada and Fishman[94] obtained the 2α-hydroxy compound **64** in 20% yield upon aqueous sulphuric acid treatment of the 19-hydroxy-4β,5β-epoxide **62** (α' mode of ring opening). The same reaction was also found[95] to produce 3-hydroxy-1,3,5(10)-estratrien-17-one **16** (12%) along with the 2α-hydroxy compound **64**.

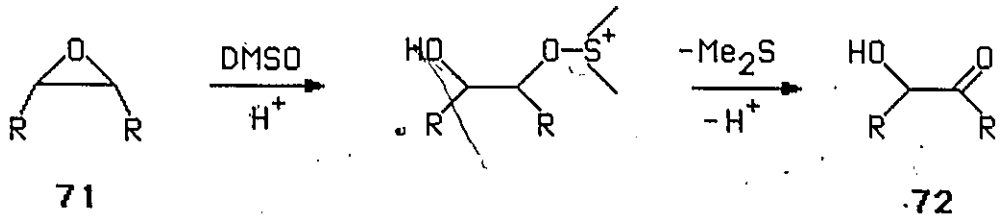


62 **16** 12% **64** 23%

The reaction of the 19-alcohol **65** and the 19-aldehyde **70** with dimethyl sulfoxide in the presence of an acid catalyst has been described by Morisawa and Tanabe[96]. The major product **67** obtained for both **65** and **70**, was suggested to arise *via* the initial acid-catalyzed opening (α' mode) of the epoxide by dimethyl sulfoxide[96]. Under these conditions, an epoxide **71** should normally be converted to an α-hydroxy ketone **72**[97].

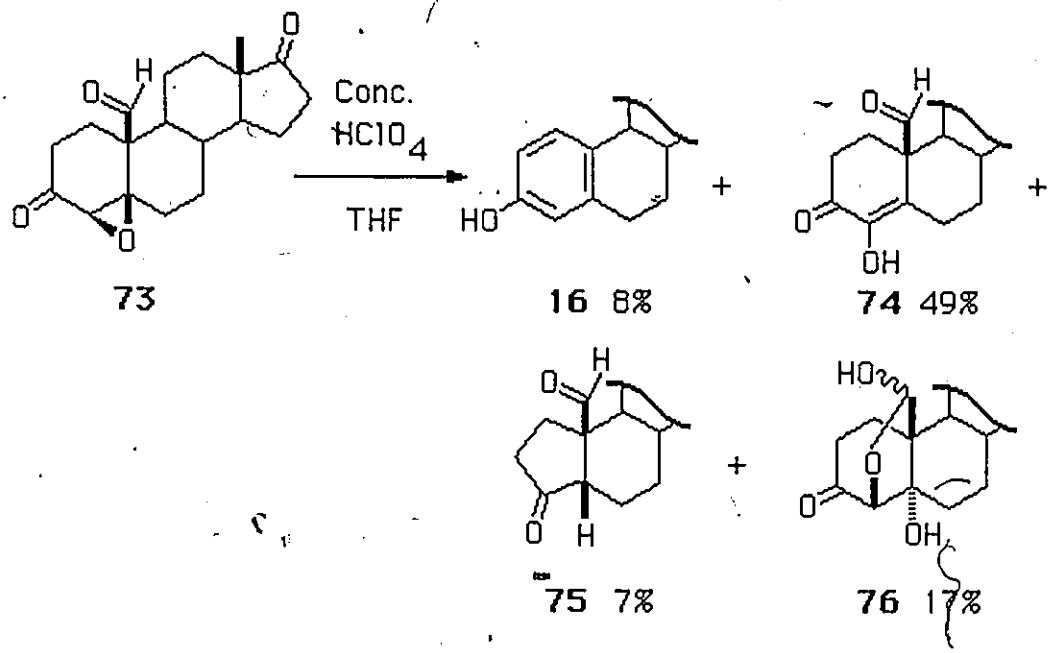






71 72

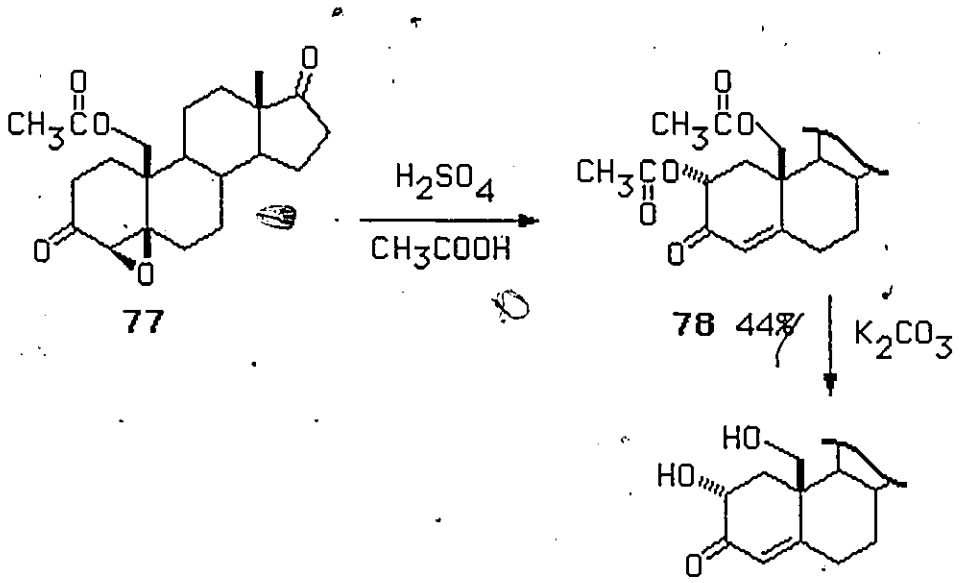
Mastalerz and Morand[98] reported that when the 4β,5β-epoxy-aldehyde **73** was treated with concentrated perchloric acid in tetrahydrofuran, up to 49% of the diosphenol **74** was formed along with other products. According to the authors[98], the nature of the A/B ring junction of compound **75** remains uncertain, but they suggested that the cis-fusion is most likely, by analogy with 1-hydrindanones.



73 **16** 8% **74** 49% **75** 7% **76** 17%

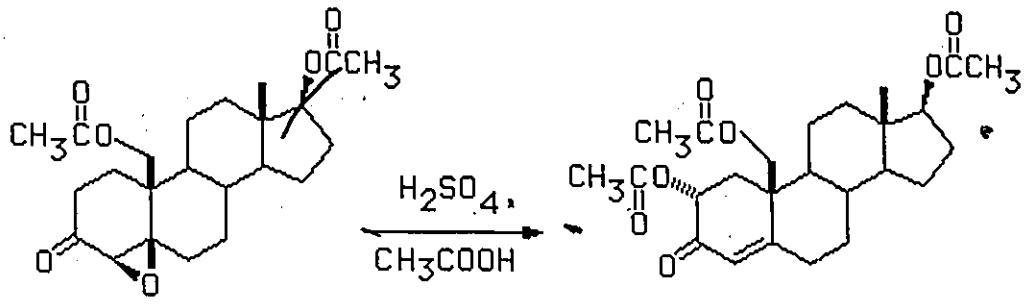
Since the nature of the solvent may affect the mode of epoxide opening, we decided to treat the 19-acetoxy-4β,5β-epoxide **77** with sulphuric acid in acetic

acid, in order to see if the 2α- or the 4-hydroxy derivative would be obtained. The product obtained was the 2α,19-diacetoxy-compound **78** arising from the α' mode of ring opening. This result is similar to that obtained by Hosada and Fishman[94] when compound **62** was treated with aqueous sulphuric acid in acetone. The 2α,19-diacetate **78** was also hydrolyzed to the known[94] 2α,19-diol **64**.



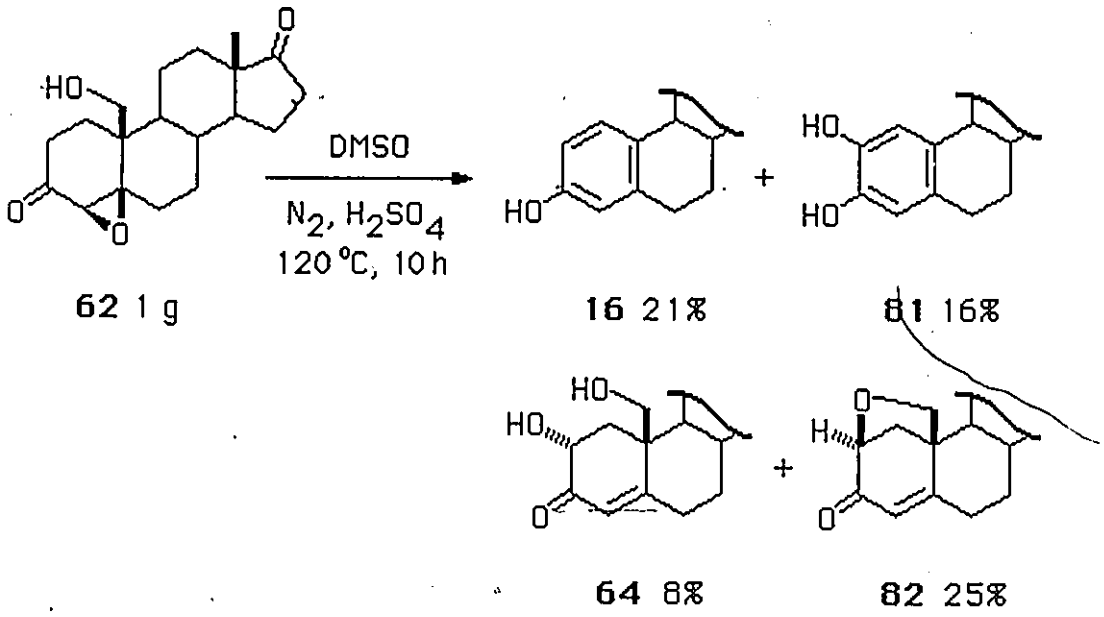
77 **78** 44% **64**

We found that the 2α-acetoxy compound **80** was also obtained when 17β,19-diacetoxy-4β,5-epoxy-5β-androstane-3,17-dione **79** was treated with sulphuric acid in acetic acid. This last reaction indicates that with sulphuric acid in acetic acid, the α' mode of opening of a C-19 substituted steroid occurs whether there is a carbonyl or an acetoxy group at C-17.



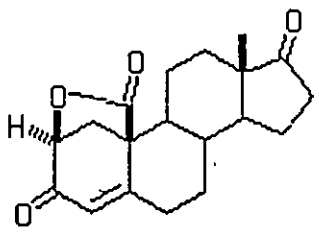
79 80

The sulphuric acid catalyzed reaction of Morisawa and Tanabe[96] was repeated with 19-hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62**. Surprisingly, none of the 4-hydroxy derivative **63** was obtained and the major product was not found to be the 2,3-dihydroxy compound **81** but the cyclized product **82**. Furthermore 8% of the 2α-hydroxy derivative **64** was also isolated. The overall yield of this reaction is low mainly because repeated chromatography (see details in the experimental section) is necessary to purify the mixture of products. 3-Hydroxy-1,3,5(10)-estratrien-17-one **16** was found to be identical (tlc and ^{1}H NMR) with authentic material. Similarly, the proton and ^{13}C NMR spectra obtained for the second compound are in agreement with the compound **81**, 2,3-dihydroxy-1,3,5(10)-estratrien-17-one[99]. In the proton NMR spectrum, the resonances at δ 4.95 and 4.98 both integrating for one proton and exchanging with D_2O are assigned to C-2 and C-3 hydroxyl groups. Two singlets integrating for one proton each at δ 6.58 and 6.79 were assigned to C-1 and C-4 hydrogen atoms. 2α,19-Dihydroxyandrost-4-ene-3,17-dione **64** was identical (^{1}H NMR) with authentic material previously obtained by sulphuric acid opening of the 19-acetoxy-4β,5β-epoxide **77**.



62 1 g **16** 21% **81** 16% **64** 8% **82** 25%

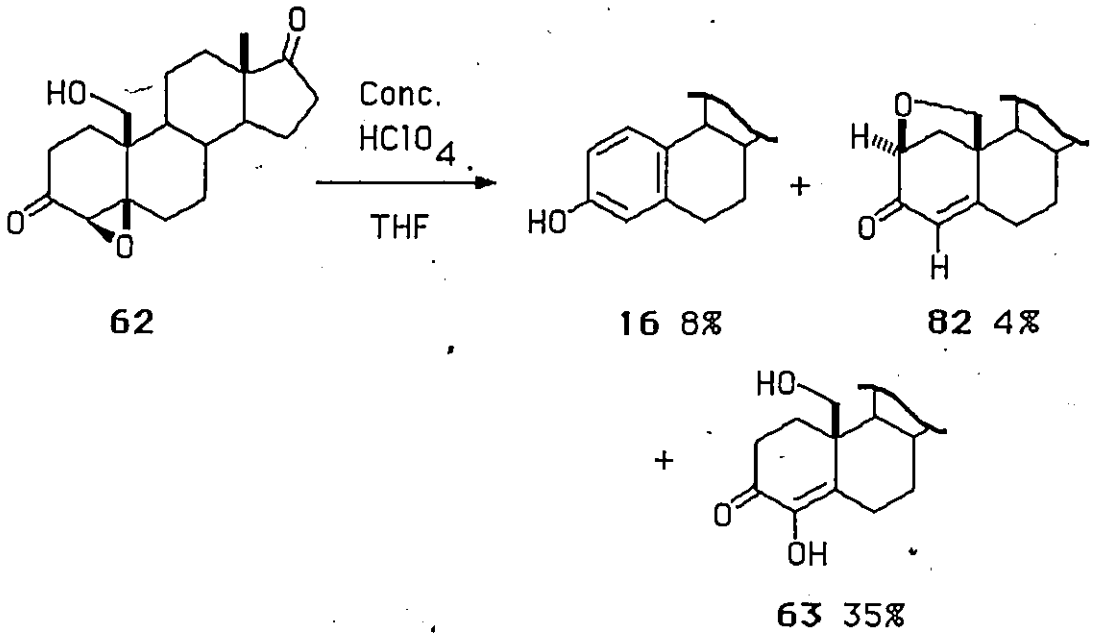
Finally, 2β,19-oxidoandrost-4-ene-3,17-dione **82** has a ^{1}H NMR spectrum {δ 0.90 (s, 3H, 18-CH_3), 3.76 (q, 2H, 19-CH_2), 4.29 (d of d, 1H, 2α-H), and 5.78 (br s, 1H, 4-H)} similar to the spectrum reported[94] for 2β-hydroxy-4-androstene-3,17-dion-19-oic acid 2,19-lactone **83**. The compound **82** was further characterized by the usual techniques (MS, IR, UV) since it has not been reported before. The ^{13}C NMR spectrum was assigned and is summarized in the appendix.



83

The slightly different composition of products obtained in our studies compared to those of Morisawa and Tanabe[96] may be explained by the fact that different starting materials were used in the two studies (carbonyl instead of acetoxy group at C-17). Furthermore, even if we followed the procedure given by Morisawa and Tanabe, the ratio of products obtained may be influenced by the scale (1 g in our case compared to 4 g by Morisawa) on which the reaction is performed.

We examined the behavior of the 19-hydroxy compound **62** when treated with concentrated perchloric acid in tetrahydrofuran in order to compare its behavior with that of the 19-aldehyde-4β,5β-epoxy compound **73** during the same treatment. Furthermore, this reaction with the 19-aldehyde **73** is a good method to obtain a respectable yield (49%) of the 4-hydroxy derivative **74**[98]. Reaction of the 19-hydroxy compound **62** produced the diosphenol **63** in 35% yield. This compound **63** shows the characteristic ultraviolet absorption of diosphenols at 280 nm[100]. The proton on the 4-hydroxyl group resonates at δ 6.31 and is exchanged with D_2O. Two other compounds, **16** and **82**, were isolated and identified by comparison with authentic material. However, at least four other compounds were present in the reaction mixture but could not be purified. Because this method requires long and careful chromatography to obtain pure 4,19-dihydroxy compound **63**, we examined another route to synthesize this compound.

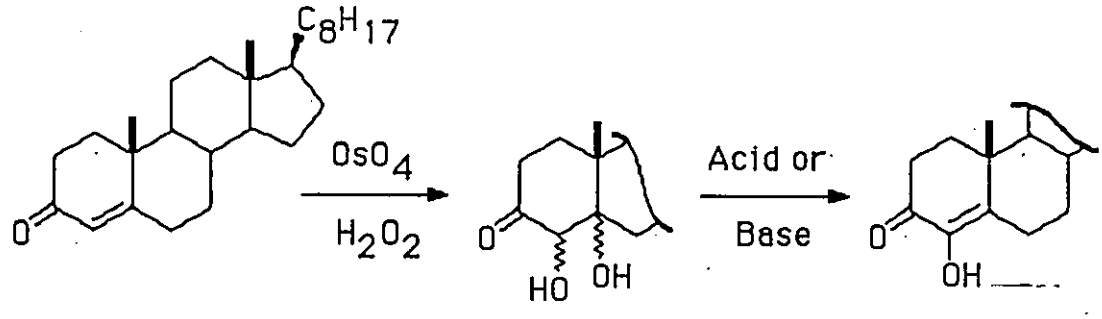


62 16 8% 82 4%

63 35%

ii. Preparation of 4β,5,19-Trihydroxy-5β-androstane-3,17-dione **88** and 4α,5,19-Trihydroxy-5α-androstane-3,17-dione **89**.

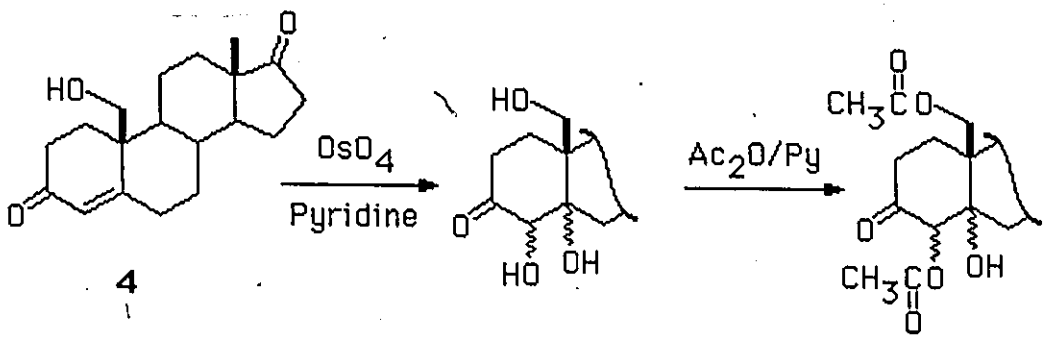
The dehydration of 4,5-diols (**85**, **86**) obtained from the oxidation of an unsaturated compound **84**, may be effected by acid or base, and give the diosphenol **87** as product[101].



84 **85** 4β, 5β **87**

86 4α, 5α

Thus we treated the 19-hydroxy compound **4** with osmic acid in pyridine and obtained the triols **88** and **89**.



88 4β, 5β
89 4α, 5α
90 4β, 5β
91 4α, 5α

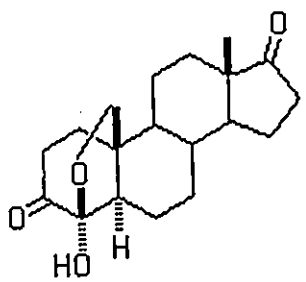
In order to assign the configuration at C-4 and C-5, it was necessary to purify compounds **88** and **89**. Since the latter are not adequately separated on tlc, the mixture of alcohols **88** and **89** was acetylated, in the hope that the R_f values of the acetates formed would be distinct, thus facilitating their purification. However, the mixture of acetates **90** and **91** was homogeneous on the basis of tlc and was found to be a *ca*. 1:2.3 mixture by ^1H NMR. The mixture of triols **88** and **89** was then purified by column chromatography. The first compound eluted from the column, isolated in 29% yield, was assigned the 4β,5β-configuration on the basis of its circular dichroism spectrum. It was observed by Djerassi and Closson[102], that inversion of configuration at C-5 in the steroid skeleton is accompanied by a major change in the Cotton effect[103] associated with the 3-ketone group: the positive Cotton effect of a 3-oxo-5α- steroid (A/B-trans) contrasts with the weakly negative effect of a 3-oxo-5β- steroid (A/B-cis). This difference has been frequently used

for establishing the stereochemistry at C-5 in 3-oxo-steroids[104]. The circular dichroism spectrum of the first compound eluted from the column indicates a maximum at 300 nm with a $\Delta\varepsilon = +5.7$ whereas the circular dichroism spectrum of the more polar compound indicates a maximum at 295 nm with a $\Delta\varepsilon = +8.3$. Therefore the first compound was assigned the 4β,5β-configuration **88** since its $\Delta\varepsilon$ was smaller than the more polar compound which was assigned the 4α,5α-configuration **89**. Similar differences in the maximum of absorption (ORD spectra) were reported by Kubota and co-workers[105]: the 4α,5α-diol **86** had a positive Cotton effect at 297 nm wheras the 4β,5β- diol **85** showed a negative Cotton effect at a higher wavelength, 299 nm.

The more polar compound **89** eluted from the column was not eluted in pure form. As indicated by tlc, it was contaminated with traces of compound **88** which were successfully removed by washing the mixture of compounds with methylene chloride. Thus 4α,5,19-trihydroxy-5α-androstane-3,17-dione **89** was obtained as a homogeneous compound by tlc. However, the proton NMR spectrum of the 4α,19-diacetoxy derivative **91** (also homogeneous on the basis of tlc) indicated, besides the resonances at δ 5.47 (s,1H, 4β-H) and at δ 4.54 (d, 2H, 19-CH_2), a resonance at δ 3.64 integrating for two protons which could not be assigned and which suggested the presence of an impurity. Even after examination of the ^{13}C NMR spectrum of the 4α,19-diacetoxy derivative **91**, we could not identify the impurity. The presence of this impurity explains why the observed values for the elemental analysis of compound **89** are different from the theory. Because we succeeded in purifying only a limited amount of material **89**, we could not recrystallize or try to further purify this compound.

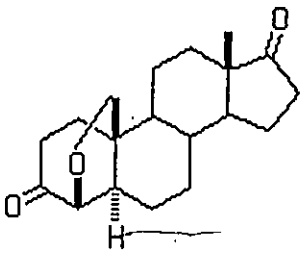
iii. Acid-catalyzed Treatment of 4,5,19-Trihydroxyandrostane-3,17-dione **88** and **89**.

We first tried to dehydrate the mixture of 4β,5β,19- and 4α,5α,19-triols **88** and **89** by dissolving the compounds in acetone in the presence of traces of HCl. The reaction was followed by tlc for a period of 30 min and only one product, less polar than the starting mixture and UV negative, was formed. After 23 h, the starting mixture of alcohols **88** and **89** had been completely converted to two products. The first product was isolated in 56% yield and the mass spectrum indicated a molecular ion at m/z 318 in agreement with the loss of one molecule of water. The product is not the diosphenol **63** since it does not have an ultraviolet absorption in the 275 nm region[100] or a hydroxyl group resonance at δ 6.3 exchangeable with D_2O (*cf.* diosphenol **63**). The proton NMR spectrum indicates that the C-19 methylene group is probably in a ring since the coupling constant is 7.6 Hz, which is similar to the value found for compound **26** (see p.28) The structure **92** is proposed for this compound. The ^{13}C NMR resonance at δ 205.7 is attributed to the C-3 ketone and the resonance at δ 103.6 is assigned to C-4. The stereochemistry at C-5 is suggested to be α, based on the examination of Drieding molecular models. When the A/B ring junction is cis, i.e. with a 5β-H, the 19-alcohol is too far from C-4 for cyclization to occur.



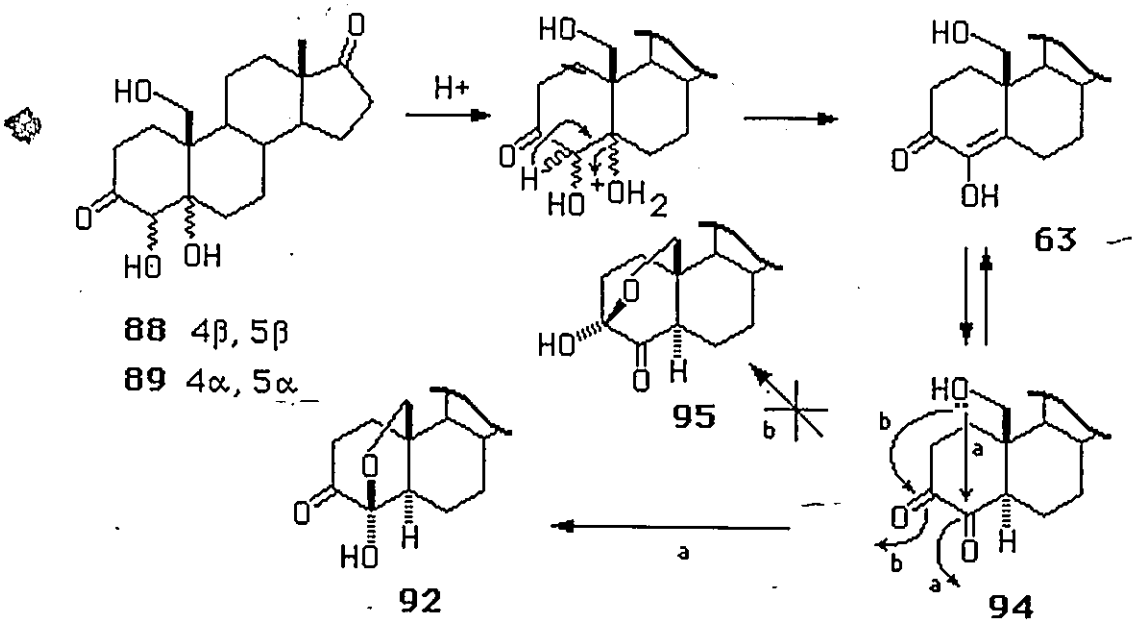
92

We note that the infrared spectrum of compound **92** indicates only one carbonyl absorption at 1730 cm^{-1}. Similarly, only one carbonyl absorption was found[98] for the 3,17-diketone **93**.



93

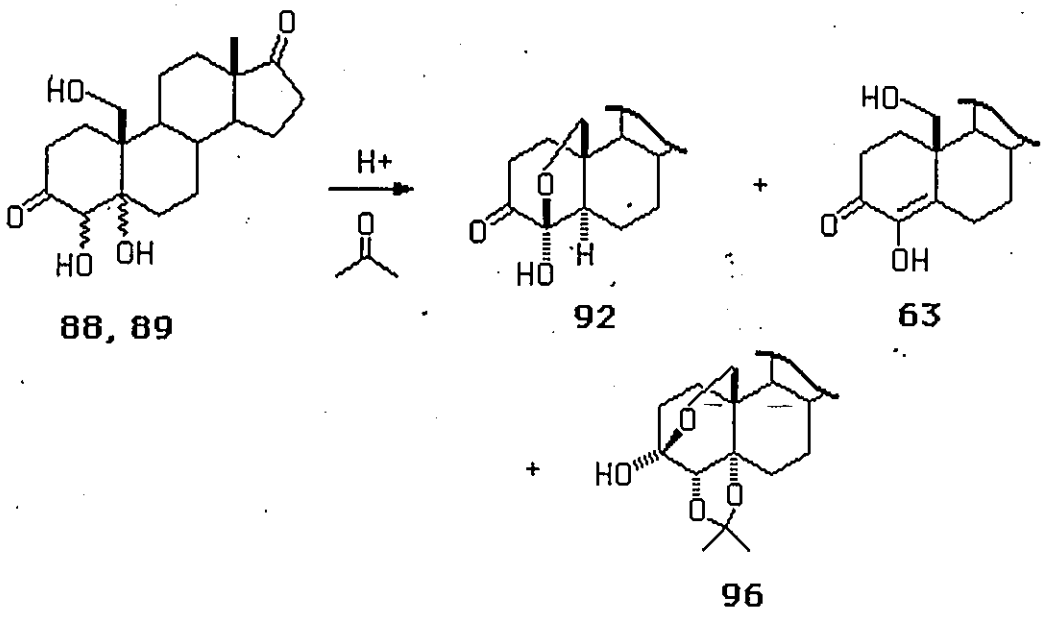
We propose the following mechanism to explain the formation of compound **92**.



88 4β, 5β
89 4α, 5α

According to this mechanism, the C-5 tertiary alcohol is protonated and lost as a water molecule. The diosphenol **63** thus obtained would be in equilibrium with the 3,4-diketone **94**. The 19-hydroxyl group would then cyclize at C-4 affording the compound **92**. When the reaction was repeated with more starting material (290 mg instead of 80 mg) we were able to isolate 4% of the diosphenol **63** which was identical with authentic material. We suggest that the cyclization of the 19-hydroxyl group occurs as illustrated by route "a" rather than route "b" since if compound **95** was the product, the ^{13}C NMR resonance for C-5, a methine group adjacent to a carbonyl group, should be around δ 59[106] rather than at δ 48.1 as we found for **92**.

The second product obtained upon treatment of triols **88** and **89** with HCl in acetone was isolated in 19% yield. The mass spectrum showed a molecular ion at m/z 376. This corresponds to the addition of 40 mass units to the starting material which has a mass of 336. The proton NMR spectrum with two resonances at δ 1.40 and δ 1.47, both integrating for three protons, clearly indicates that one molecule of acetone had been added by forming an isopropylidene derivative. Furthermore, the proton NMR spectrum suggested that the 19-methylene group is in a ring since the coupling constant for the quartet integrating for two protons and resonating at δ 3.94 is 9.3 Hz, as opposed to 11-12 Hz for non-cyclized C-19 methylene protons. That only one ketone was present was confirmed by the ^{13}C NMR spectrum where only the C-17 ketone was found resonating at δ 220.6. Therefore the structure **96** is assigned to the second product obtained from the treatment of the mixture of triols **88** and **89** with HCl in acetone. The stereochemistry of the 4,5-isopropylidene group has to be α as indicated by Drieding molecular models since a β-arrangement prevents the cyclization of the 19-alcohol at C-3.



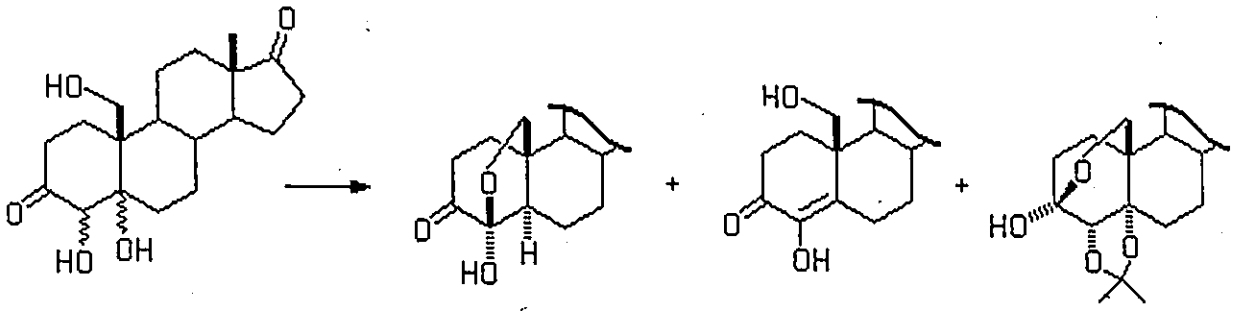
88, 89 92 63 96

In order to eliminate the formation of the isopropylidene derivative **96** and hopefully favour the formation of the diosphenol **63**, different solvents were used in the presence of HCl to effect the dehydration of the triols **88** and **89**. The results are summarized in Table VII. When the reaction is carried out in methanol or tetrahydrofuran the cyclized compound **92** is formed and some unreacted starting material remains but none of the diosphenol **63** is present when the reaction is terminated. However, when the reaction is performed in acetic acid, with the 4β,5β,19-trihydroxy compound **89**, 100% of the 4,19-dihydroxy derivative **63** is obtained.

In summary, the best method to synthesize 4,19-dihydroxyandrost-4-ene-3,17-dione **63** is not *via* the acid-catalyzed opening of the 19-hydroxy-4β,5β-epoxide **62** but by dehydration of the 4β,5β,19-trihydroxy compound **88** with HCl in acetic acid.

TABLE VII

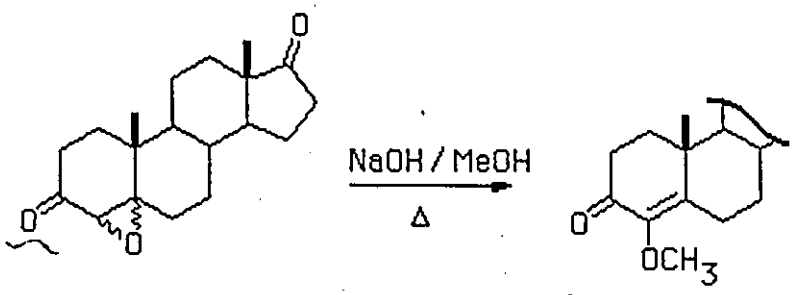
Acid-catalyzed Reactions of 4β,5,19-Trihydroxy-5β-androstane-3,17-dione **88** and 4α,5,19-Trihydroxy-5α-androstane-3,17-dione **89**.



88, 89		**92**	**63**	**96**
88, 89	Conc. HCl Acetone, 20 h	36%	4 %	22%
88, 89	Conc. HCl Acetone, 23 h	56%	–	19%
88, 89	Conc. HCl Methanol, 22 h	20%	–	–
88, 89	Conc. HCl THF, 21 h	35%	–	–
88	Conc. HCl CH_3COOH, 16 h	–	100%	–

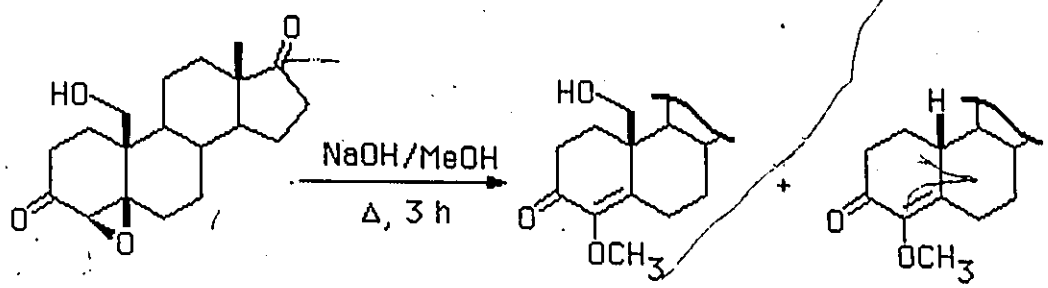
iv. Base-catalyzed Opening of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62**.

The base-catalyzed opening of the 19-hydroxy-4β,5β-epoxide **62** was also examined since Mann and Pietrzak[107] recently reported the formation of 4-methoxyandrost-4-ene-3,17-dione **97** in 62% yield when the 4,5-epoxides **55** were refluxed in alkaline methanol for 3 h.



55 **97**

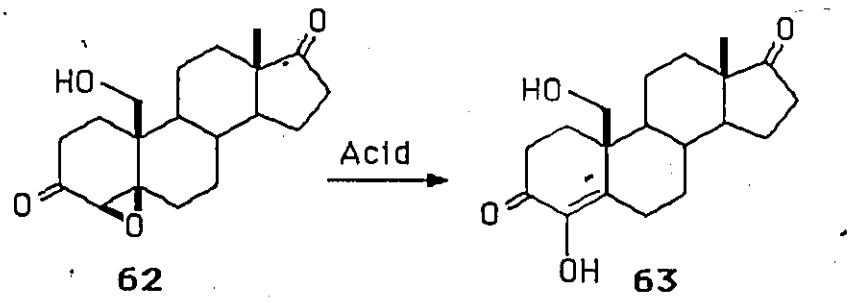
We found that when the 19-hydroxy-4β,5β-epoxide **62** was similarly refluxed in alkaline methanol for 3 h extensive decomposition occurred, giving mainly very polar material which we did not succeeded in purifying. We were able to isolate from this reaction traces of 4-methoxy-19-hydroxyandrost-4-ene-3,17-dione **98** (7%) and 4-methoxyestr-4-ene-3,17-dione **99** (6%). The 4-methoxy compound had a proton NMR spectrum and melting point identical to the known[98] 4-methoxyestr-4-ene-3,17-dione **99**. The proton NMR spectrum of compound **98** has a singlet integrating for three protons at δ 3.63 assigned to the C-4 methoxy group. The methoxy group is also found at δ 60.5 in the ^{13}C NMR spectrum, along with resonances at δ 220.3 (C, C-17), 194.5 (C, C-3), 149.8 and 149.0 (C, C-4 and C-5).



62 98 99

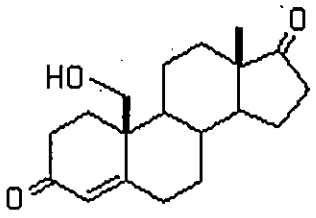
B. Reaction of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** with alkaline peroxide.

As we previously mentioned, the 19-hydroxy-4β,5β-epoxy compound **62** was first synthesized to permit examination of the acid-catalyzed opening of the epoxide to generate the 4-hydroxy derivative **63**.

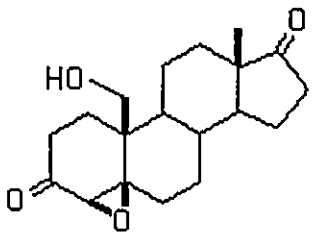


62 **63**

19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** had previously been obtained in 65% yield by Morand and co-workers[108] by treating a solution of 19-hydroxyandrost-4-ene-3,17-dione **4** (200 mg) in methanol with 0.2 N NaOH and 30% hydrogen peroxide at 0-4°C for 16 h. However, we found that the reaction is complete after 60 to 90 min, similar to Fishman and Hosada's finding[94]. When the reaction is performed on a larger scale (~1 g), and left for 12 h or more at 0-4°C, the yield of epoxide **62** decreases drastically and a rearranged product **100** is isolated in ~20% yield. Another rearranged product, different from **100**, was also isolated when the 19-hydroxy compound **4** was treated under slightly different conditions (0.4 N NaOH instead of 0.5 N NaOH, and 15 h 15 min instead of 12 h). The structure of this latter product will be discussed in section C.2.



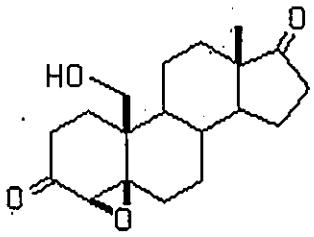
NaOH / 30% H_2O_2 / MeOH



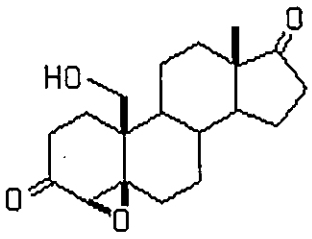
+ **100**

4 **62**

0.2 g	t=16 h[108] 0-4 °C	65%	
1.0 g	t=16 h 0-4 °C	Traces	~20 %



NaOH / 30% H_2O_2 / MeOH



+ **100**

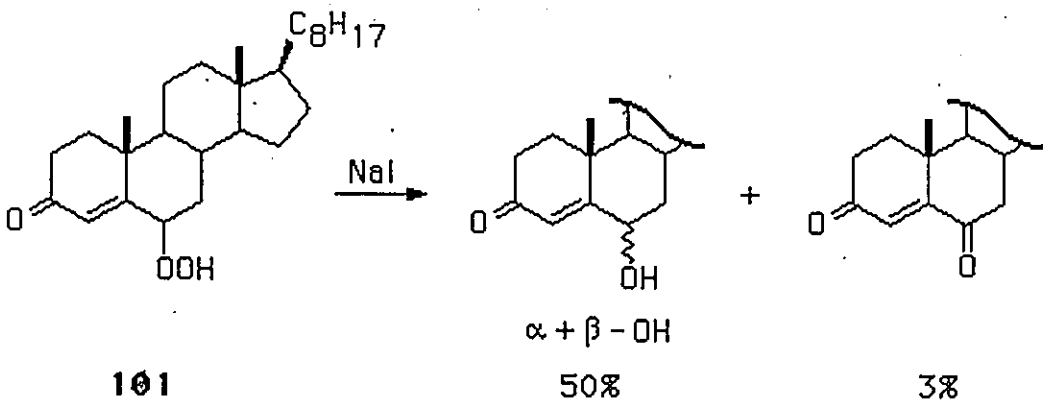
62 **62**

2.0 g	t=12 h 0 °C → 21 °C	3%	85%

We confirmed that the 4β,5β -epoxide **62** is an intermediate in the formation of the rearranged product since, upon treatment with 30% hydrogen peroxide in basic methanol (see experimental section E.1), 85% of the rearranged product **100** is isolated. The mass spectrum of the rearranged product **100** indicates a molecular weight of 350 corresponding to the addition of three atoms of oxygen to the starting alcohol **4**. The fragment at m/z 334 corresponds to the loss of 16(i.e. of one atom of oxygen) which is characteristic of hydroperoxides[109,110,111,112].

The proton NMR spectrum also suggested the presence of a hydroperoxy group with a signal at δ 8.16 which disappears upon addition of D_2O. The spectroscopic data of the rearranged product **100** are summarized in Table VIII as well as those of compounds **4** and **62**. The signal for hydroperoxidic protons are in the range δ 8-9[113]. The proton NMR spectrum of the rearranged product **100** suggests that one atom of oxygen is inserted next to a methylene group because of the presence of a multiplet integrating for two protons at δ 4.12. This is confirmed by the methylene resonance at δ 73.3 in the ^{13}C NMR spectrum. Both the infrared and the ^{13}C NMR spectra suggested that the C-17 ketone had not been modified. However the A-ring is modified as indicated by the resonance at δ 168.2 in the ^{13}C NMR spectrum. This latter resonance is in the range of lactones.

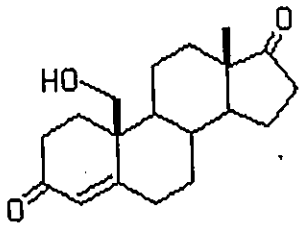
Iodide is known to cleave peroxides. For example, the reduction of 6β-hydroperoxycholest-4-en-3-one **101** gave a mixture of 6α- and 6β-alcohols, isolated in 50% yield along with a very small amount of the Δ^4-3,6-dione[114].



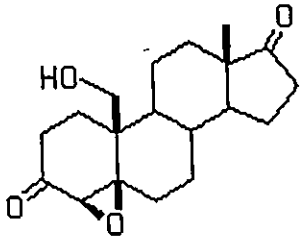
101 50% 3%

TABLE VIII

Comparison of the Spectroscopic Properties of Compounds **4**, **62** and **100**



4



62

100

	4	**62**	**100**
Molecular Formula	$C_{19}H_{26}O_3$	$C_{19}H_{26}O_4$	$C_{19}H_{26}O_6$
IR (cm^{-1})			
17-CO	1735	1735	1735
3-CO	1665	1710-1700sh	1705
			3315
^1H NMR (δ)			
C-19	3.98	3.97	4.32
(J_{ab})	(10.6 Hz)	(11.0 Hz)	(10.5 Hz)
C-18	0.89	0.88	0.88
C-4	5.94	2.90	3.45
			4.12
			(m, 2H)
			8.16
		(br s, 1H, exchanges with D_2O)	
^{13}C NMR (δ)			
C-19	65.5	64.8	68.9
C-18	13.7	13.7	13.7
C-17	220.4	220.0	219.7
C-4	126.5	60.0	55.2
C-5	167.2	68.8	66.7
C-3	200.3	205.9	168.2 (C=O)
			73.3 (CH_2-O)

To verify the presence of a hydroperoxidic group, the rearranged product **100** was dissolved in a solution of potassium iodide in 95% ethanol; the clear solution turned deep yellow (liberation of iodine), thus confirming the presence of a hydroperoxy group. The cleavage of the hydroperoxide group was repeated on a larger scale in order to identify the product formed. The product isolated showed a signal at δ 3.10 in its proton NMR spectrum which disappeared with D_2O and confirmed the conversion of the hydroperoxide group to a hydroxyl group. The proton NMR spectrum of the rearranged product **100** and of the product isolated after the cleavage of the hydroperoxide function are shown in Figures 57 and 58, respectively. The hydroperoxide function of the rearranged product **100** could also be methylated by treatment with methyl iodide and silver oxide[110]. The proton NMR spectrum of the isolated product indicated a singlet at δ 3.80 integrating for 3 protons, in agreement with a methyl peroxy group replacing the hydroperoxide proton resonance at δ 8.16. The rest of the proton NMR spectrum was identical to that of the starting material **100**.

An epoxide function was assumed to be present in the rearranged product **100** because of a singlet in the proton NMR spectrum, resonating at δ 3.45 and integrating for one proton (*cf.* δ 2.90 for **62**). In order to find out if the epoxide function was still present, the rearranged product **100** was treated with 2% hydrogen bromide in acetic acid. We had performed this reaction before on the 4β,5β-epoxide **62** and isolated 4-bromo-19-hydroxyandrost-4-ene-3,17-dione **102** in 71% yield.

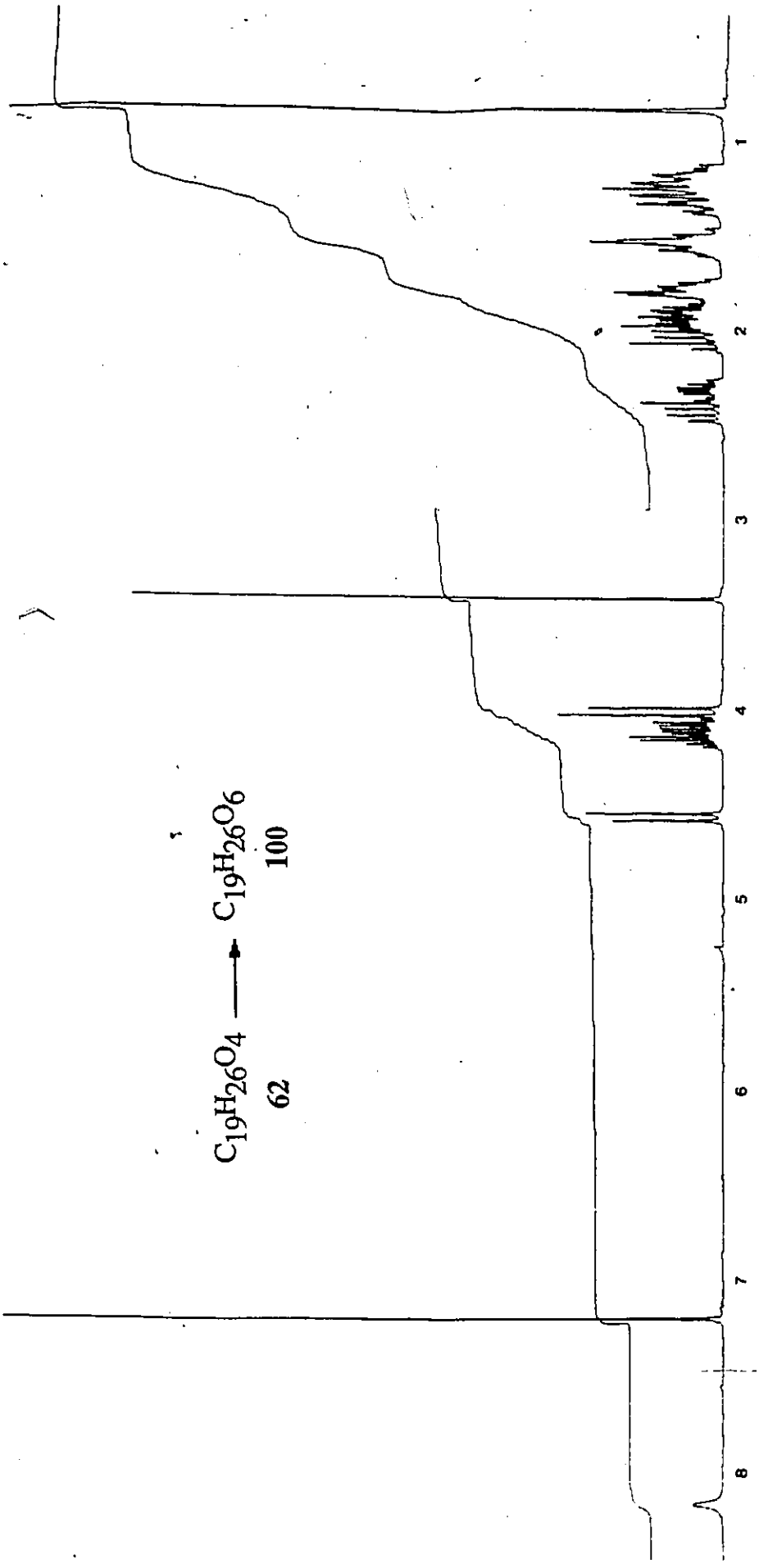


Figure 57. Proton NMR Spectrum of Compound **100**.

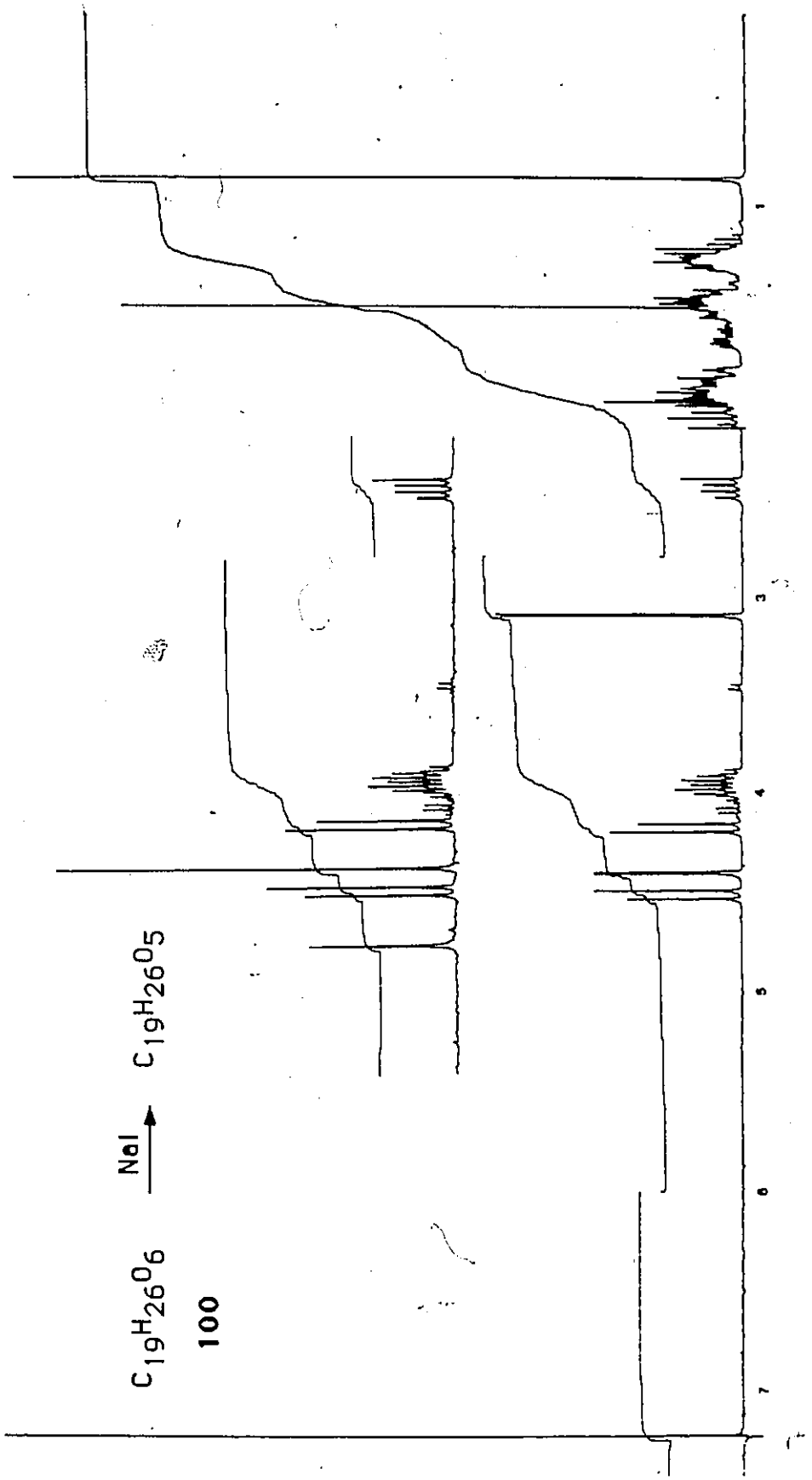
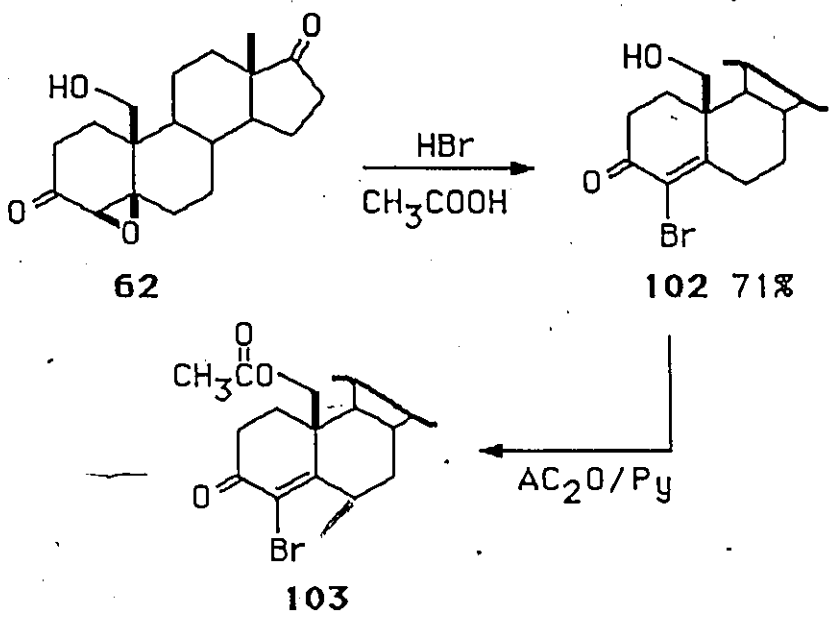
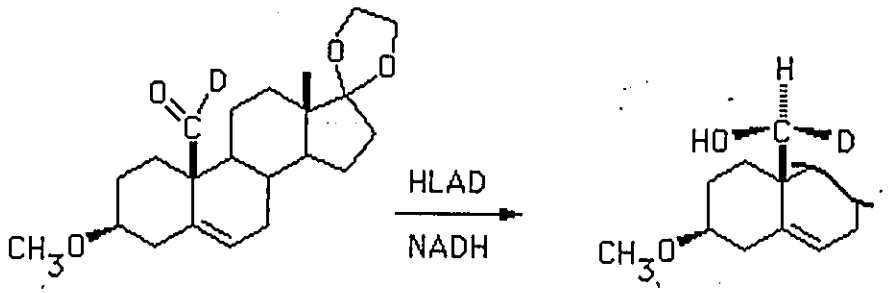


Figure 58. Proton NMR Spectrum of the Product Obtained from the Treatment of the Rearranged Compound **100** with Sodium Iodide (upper trace: addition of D_2O to the $CDCl_3$ solution).



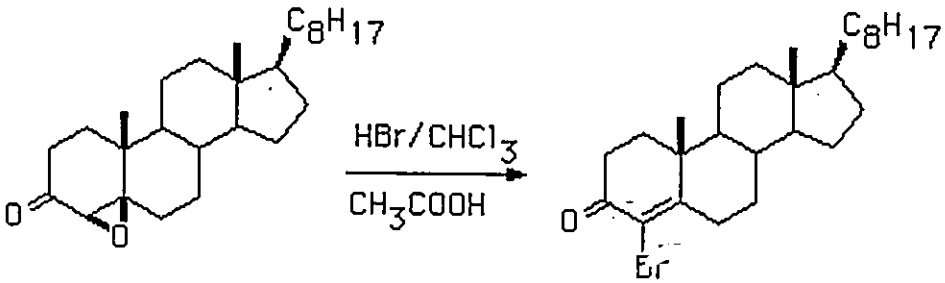
The mass spectrum of **102** indicated the addition of a bromine atom (M+2 peak almost equal in intensity to the molecular ion because of the presence of a molecular ion containing the ^{81}Br isotope) and the ultraviolet absorption at 262 nm confirmed the nucleophilic opening of the protonated epoxide by bromine and elimination of a water molecule to give the unsaturated compound **102**. We observed an uneven coupling of the C-19 methylene protons in chloroform solution: the 19-pro-R hydrogen atom appears as a doublet at δ 4.12 (J=10.3 Hz) whereas the 19-pro-S hydrogen atom appears as a doublet of doublets at δ 3.92 (J=10.8 Hz, J=5.4 Hz). However, upon addition of D_2O, the usual quartet pattern of the C-19 methylene was restored indicating that the uneven coupling was due to coupling to the C-19 alcohol proton. When the proton NMR spectrum was obtained in dimethyl sulfoxide, the uneven coupling was not observed. The assignment of the chemical shift of the 19R- and 19S-hydrogen atoms has been reported by Caspi and co-workers[115]. The assignment was made for the

19S,19^{2}H alcohol **105** which was obtained from the reduction of the aldehyde **104** by the horse liver alcohol dehydrogenase (HLAD).



104 **105**

Compound **102** was subsequently treated with acetic anhydride to afford the acetate **103**. The mass spectrum indicated the addition of the acetoxy group which was also confirmed in the proton NMR spectrum by the signal at δ 2.00 integrating for three protons. Similar opening of the 4β,5β-epoxide **106** with 40% hydrogen bromide in glacial acetic acid and chloroform was reported by Shaw and Stevenson[116] to give the 4-bromo derivative **107**.



106 **107**

When the rearranged compound **100** was treated with hydrogen bromide, two products were formed and neither of them contained a bromine atom as indicated by the absence of the characteristic M+2 peak in their mass spectra.

The major product isolated had a molecular weight of 334, corresponding to the cleavage of the peroxide group. Furthermore, the proton and ^{13}C NMR spectra of this product were identical to those obtained for the product generated by the reduction of the peroxide by sodium iodide.

Acetylation of the rearranged product **100** was also performed. Two products were formed and the mass spectrum of the major product isolated indicated a molecular weight of 374, corresponding to the loss of one molecule of water and addition of an acyl group.

In summary, we were not able to identify the rearranged product **100** with the information available from the various spectra. Furthermore, the reactions that we performed to confirm the presence of a hydroperoxy group and an epoxide gave products which we were unable to identify. The rearranged product is an amorphous solid which is not very soluble in common organic solvents. The prospect of getting a suitable crystal for X-ray analysis seemed highly hampered by this low solubility of the rearranged compound. With patience, we were able to obtain small needles of the rearranged material from a 95% ethanol solution. An appropriate crystal was obtained upon very slow cooling of a solution of the compound in 95% ethanol. The structure was determined by Dr.E.J. Gabe at the National Research Council of Canada and the crystallographic parameters will be reported in due course. The structure of **100**, 19a-hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione, is illustrated in Figure 59 . The compound possesses a 6-membered A-ring and a 19a-hydroperoxy group. The B-,C- and D-rings are not modified compared to the 19-hydroxy-4β,5β-epoxide **62** . A mechanism is proposed to explain the formation of compound **100** and is illustrated below.

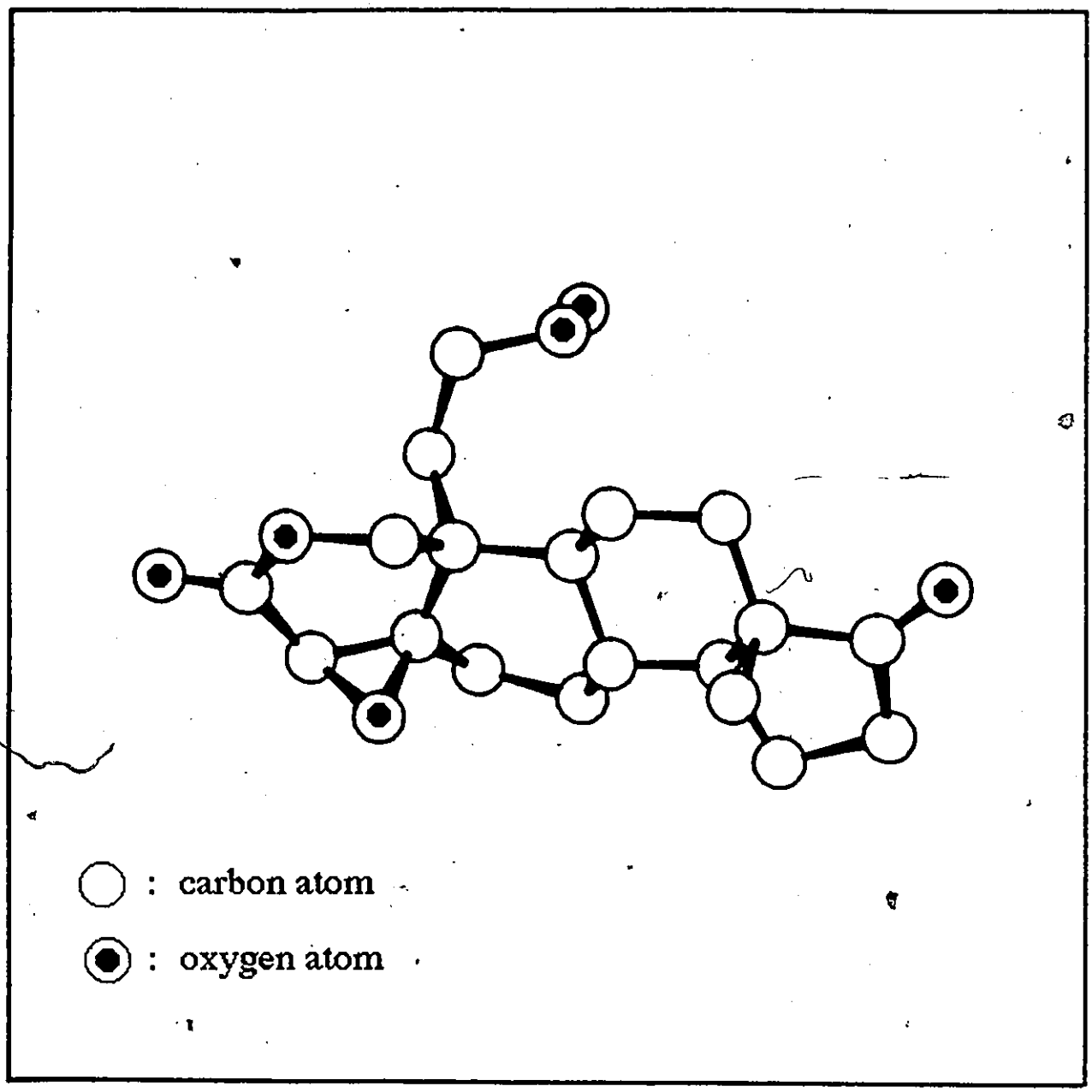
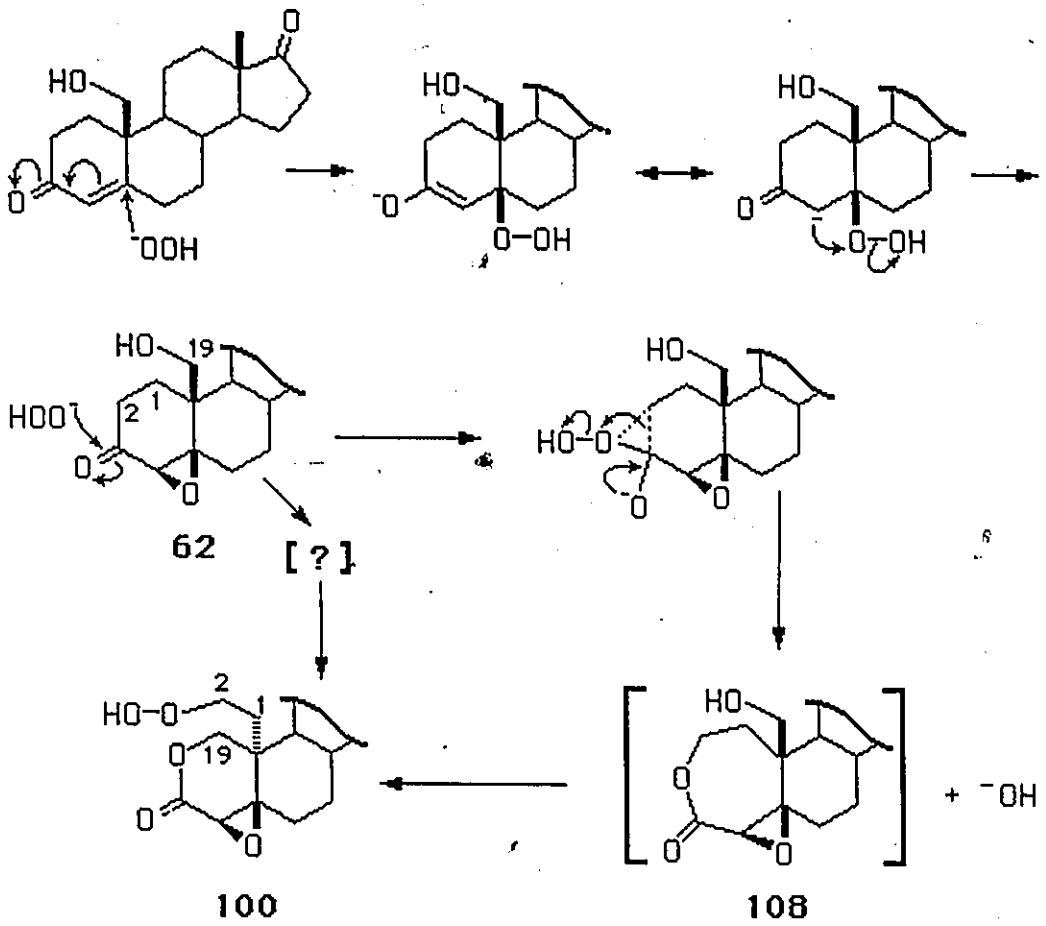
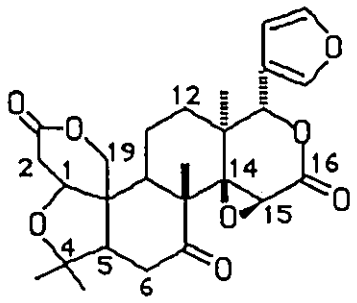


Figure 59. Structure of Compound **100** Determined by X-ray Crystallography.

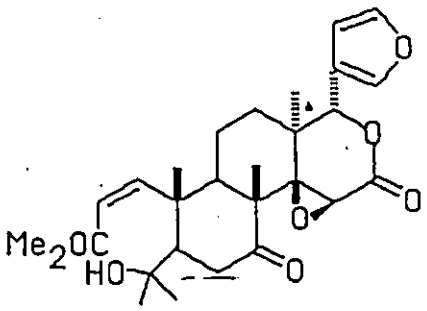


The epoxidation of the polarized double bond with hydrogen peroxide in aqueous alkaline media, first reported by Weitz and Scheffer[117], would be the initial step since it was found that the 4β,5β-epoxide **62** is cleanly converted to the rearranged product **100**. The nucleophilic addition by a Michael type mechanism, involving the attack of a hydroperoxy-carbanionic intermediate was proposed by Bunton and Minkoff[118]. A second attack of the hydroperoxy nucleophile on the C-3 carbonyl group of **62** may rearrange, *via* **108** or other intermediates, to give the hydroperoxide **100**.

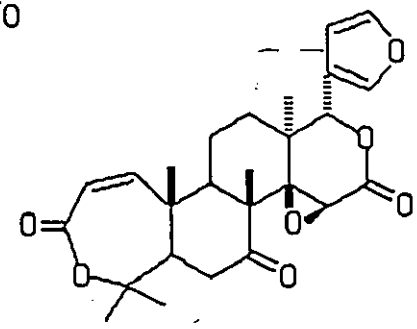
The characteristic arrangement of the A-ring in **100** occurs in natural products, for example in limonoids. Limonoids are a series of degraded C_{26} triterpenes which have been isolated from various plants belonging to the families *Rutaceae* and *Meliaceae* [119]. Limonin **109**, the characteristic bitter principle of citrus species, is one of the most extensively studied limonoids[120]. The proton and ^{13}C NMR spectrums of other limonoids, for example, methylobacunoate **110** and obacunone **111** were reported by Druyer and co-workers[121].



109

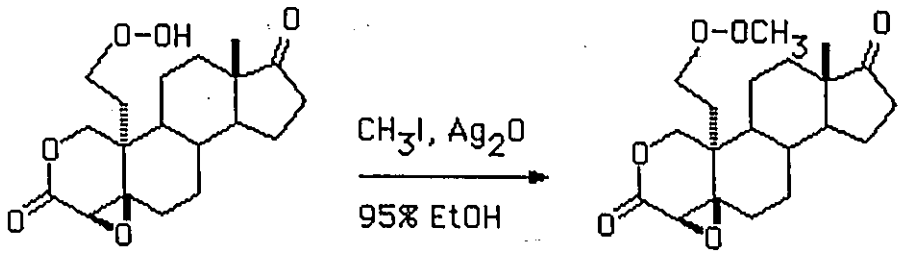


110



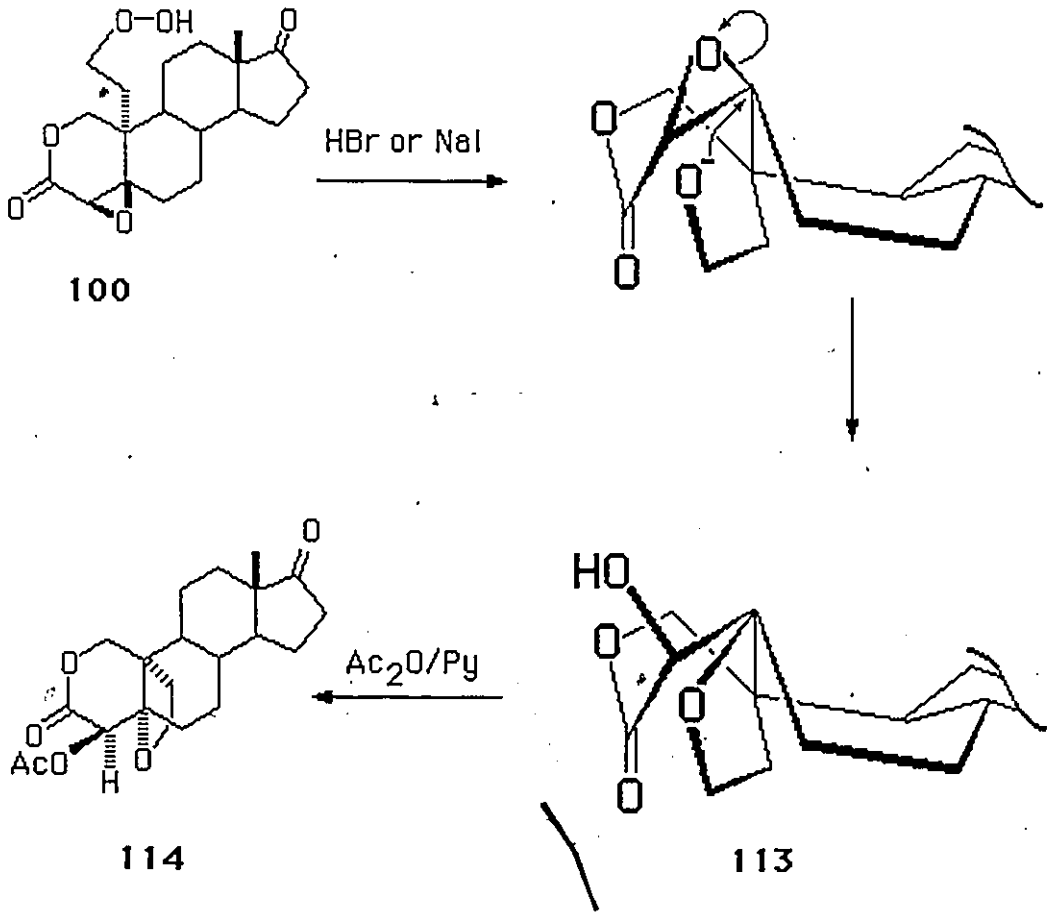
111

Knowing the structure of the rearranged product **100**, it is now possible to understand what happened on treatment with methyl iodide. Simply, the hydroperoxy group was methylated and 19a-hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **112** was obtained. The proton NMR spectrum of **112** was identical to the starting material **100** except for the signal at δ 3.80 integrating for three protons which replaced the hydroperoxide proton resonance at δ 8.16 in **100.** The ^{13}C NMR spectrum indicates the methyl peroxide resonance at δ 62.2.



100 **112** 57%

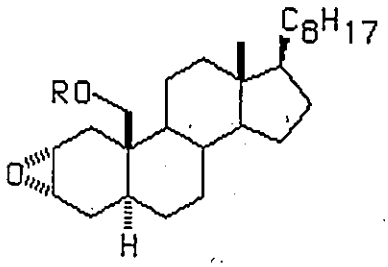
Similarly, the product obtained when the peroxide **100** was treated with sodium iodide or with hydrogen bromide is identified as **113**. The peroxide function is first cleaved. Then follows a cyclization of the 19a-oxygen atom at C-5, thus opening the epoxide and generating a 4β-hydroxyl group. This 4β-hydroxyl group is acetylated to give **114**.



100 **114** **113**

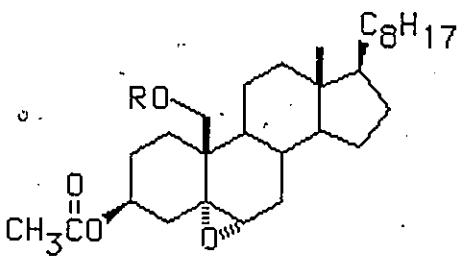
The ^{13}C NMR data (summarized in the appendix) of compound **113** are in agreement with the proposed structure, namely a methine resonance at δ 70.4, which is attributed to C-4 and a quaternary carbon at δ 85.4, which is assigned to C-5.

Such participation of the 19-oxygen atom has previously been observed by Kocovsky and Cerny[122,123]. They investigated the behavior of the 19-methoxy or 19-acetoxy epoxides **115**, **116** and **117**, **118**.



115 : R = CH_3

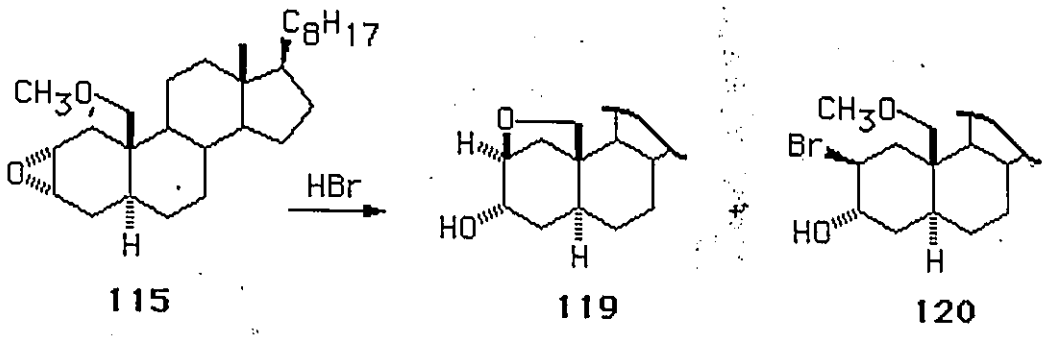
116 : R = CH_3CO



117 : R = CH_3

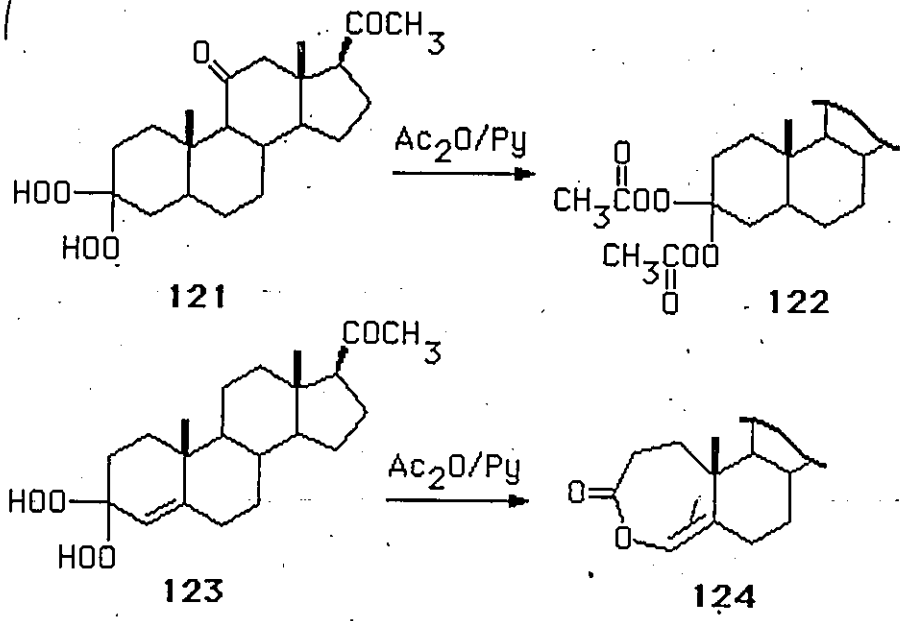
118 : R = CH_3CO

They found that the cleavage of the 2α,3α-epoxide **115** with hydrobromic acid yielded a mixture of the cyclic ether **119** (48%) as a product of methoxyl group participation and the bromohydrin **120** (52%), arising by normal diaxial opening of the epoxide ring by bromide anion as external nucleophile. Similar products were also obtained when the 19-acetoxy derivative **116** was treated with hydrobromic acid, albeit in this case, the proportion of bromohydrin was now 85%. In the case of the 5α,6α-epoxides **117** and **118**, only the bromohydrin was obtained upon treatment with hydrobromic acid.



115 119 120

Finally, there is still the reaction of the hydroperoxide **100** with acetic anhydride for which the products are not identified. A product was isolated which was homogeneous by tlc but shown by ^{13}C NMR to be a mixture of products. As indicated by the methine resonances at δ 114.1 (111.5) and δ 137.1 (134.5), a double bond is formed in the products. The mass spectrum indicates a molecular ion of 374 which corresponds to the addition of one acyl group (42) to the hydroperoxide **100**, but which is also accompanied by the loss of one molecule of water (18): 374=350+42-18. Velluz and co-workers[124] reported that unsaturated bis-hydroperoxides rearrange to lactones when they are treated with acetic anhydride in pyridine. The acetylation of the saturated bis-hydroperoxide **121** afforded the diester **122** wheras treatment of the unsaturated bis-hydroperoxide **123** with acetic anhydride gave the lactone **124.** The identification of the products obtained upon acetylation of the hydroperoxide **100** must await further work.



In summary, we found an easy method to convert an α,β-unsaturated ketone to a hydroperoxy-oxa-epoxy derivative, in one step and high yield. Furthermore, this reaction opens the door to steroids of unnatural configuration. Such "retro" steroids (9β,10α) have previously been synthesized by sequences requiring many steps[125].

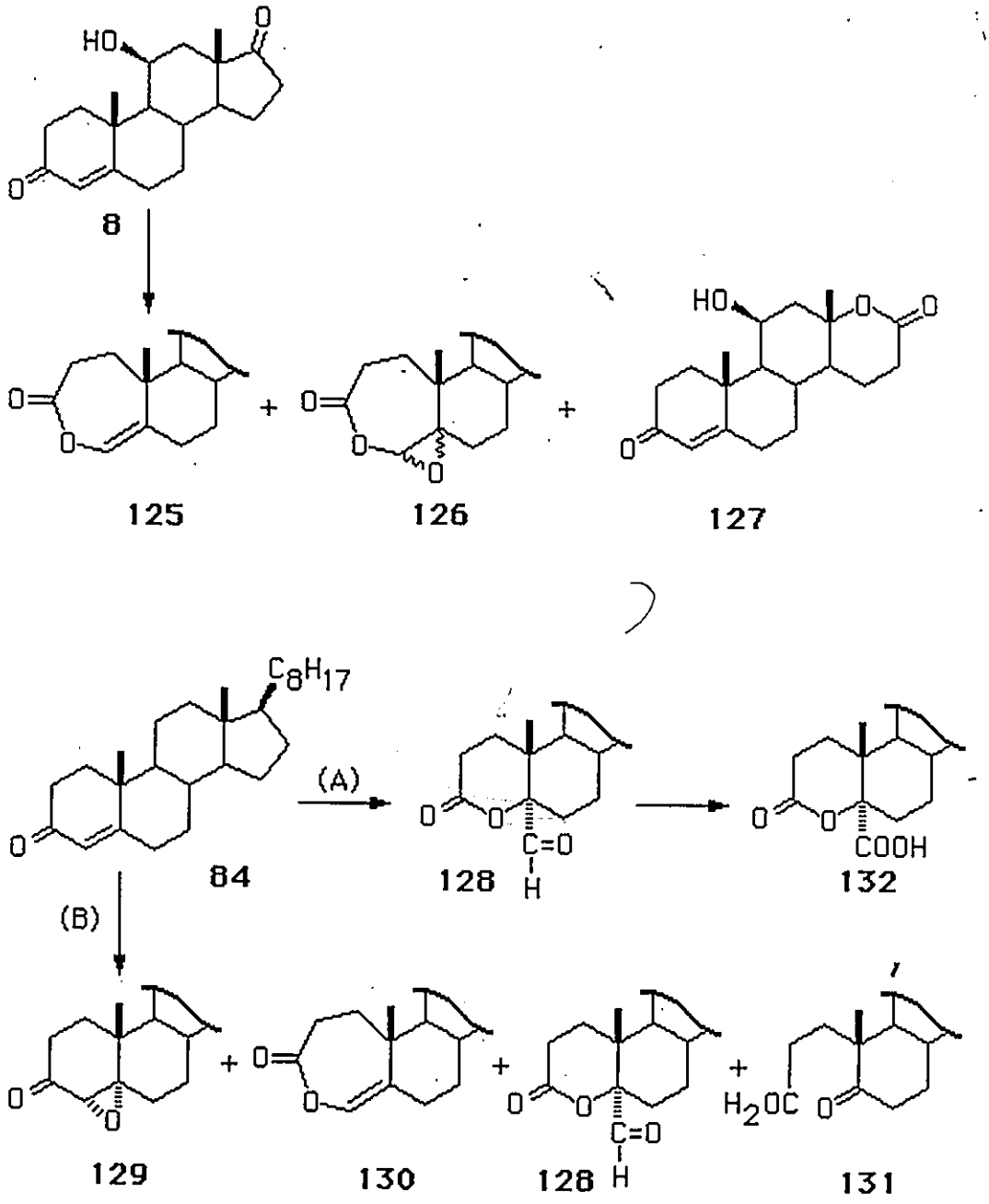
C. Baeyer-Villiger Rearrangement.

1. Introduction.

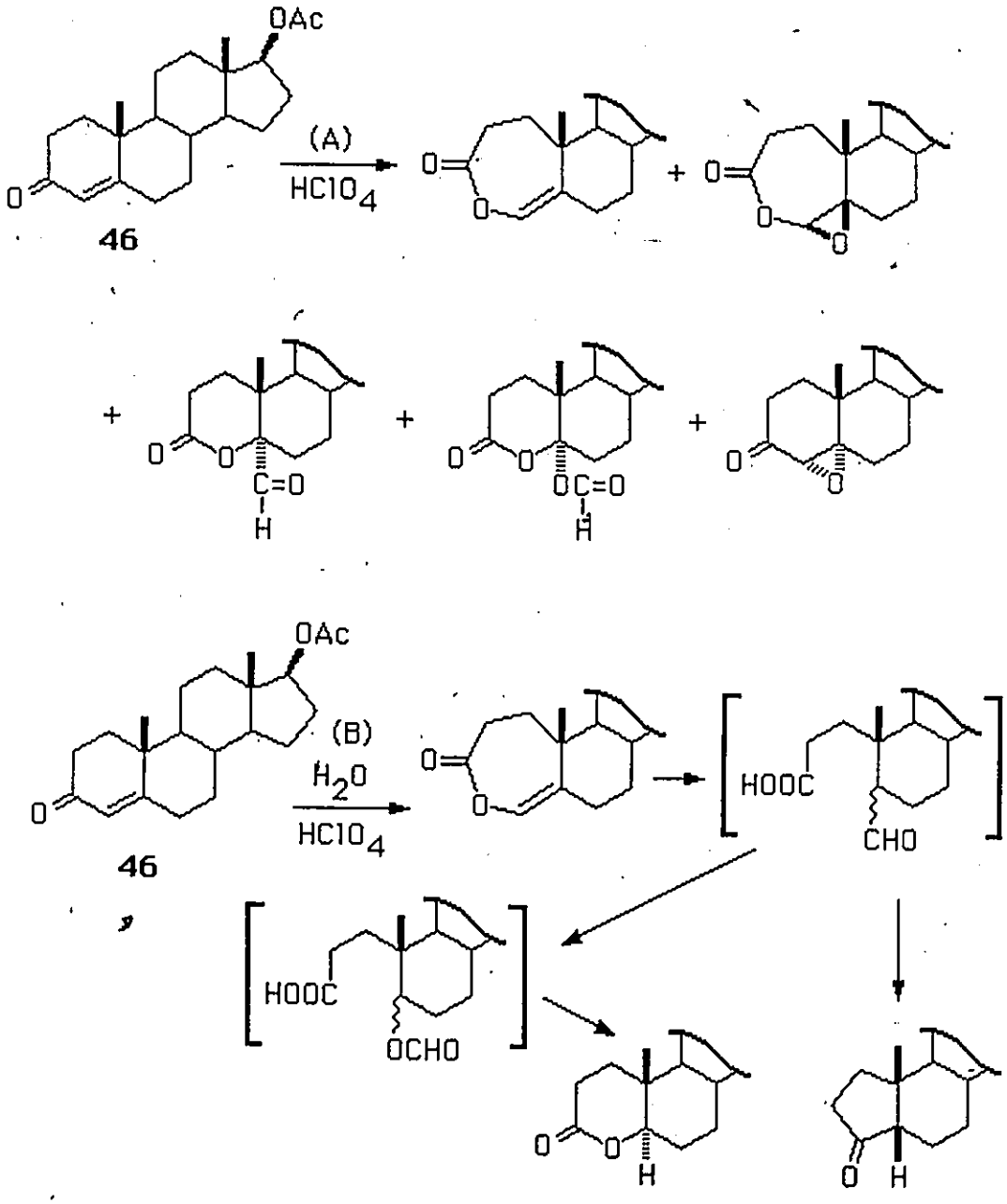
In our first attempts to identify the structure of the rearranged product **100**, we scrutinized the literature to find out what was known about Baeyer-Villiger rearrangements related to steroids. The second approach was to apply conditions of Baeyer-Villiger rearrangement to the 19-hydroxy-4β,5β-epoxy compound **62** in the hope of isolating products which would help us to elucidate the structure of the hydroperoxide **100**.

It is known that the outcome of the Baeyer-Villiger oxidation of α,β-unsaturated ketones is dependent upon the nature of the ketone and the reaction conditions used[126]. Several Δ^4-3-keto steroids have been subjected to Baeyer-Villiger oxidation reactions. In 1962, Caspi and co-workers[127] found that the reaction of peroxybenzoic acid with 11β-hydroxyandrost-4-ene-3,17-dione **8** in the presence of anhydrous perchloric acid in chloroform gave A-homo-3a-oxa-11β-hydroxyandrost-4-ene-3,17-dione **125** in 60% yield, which was accompanied by the A-homo-epoxide **126** and traces of a compound tentatively assigned as the D-homo-lactone **127**. In contrast, Pinhey and Schaffner[128] reported that the oxidation of cholest-4-en-3-one **84** with trifluoroperoxyacetic acid (A) gave only the aldehyde **128**. When the same reaction was repeated using peroxybenzoic acid in the presence of anhydrous perchloric acid (B)[124], low yields of 4α,5-epoxy-5α-cholestan-3-one **129**, 4-oxa-A-homocholest-4a-en-3-one **130**, 5α-formyl-4-oxa-cholestan-3-one **128** and of Windaus acid **131**[129] (a known degradation product of Δ^4-3 -ketones) were isolated together with some unchanged starting material **84**.

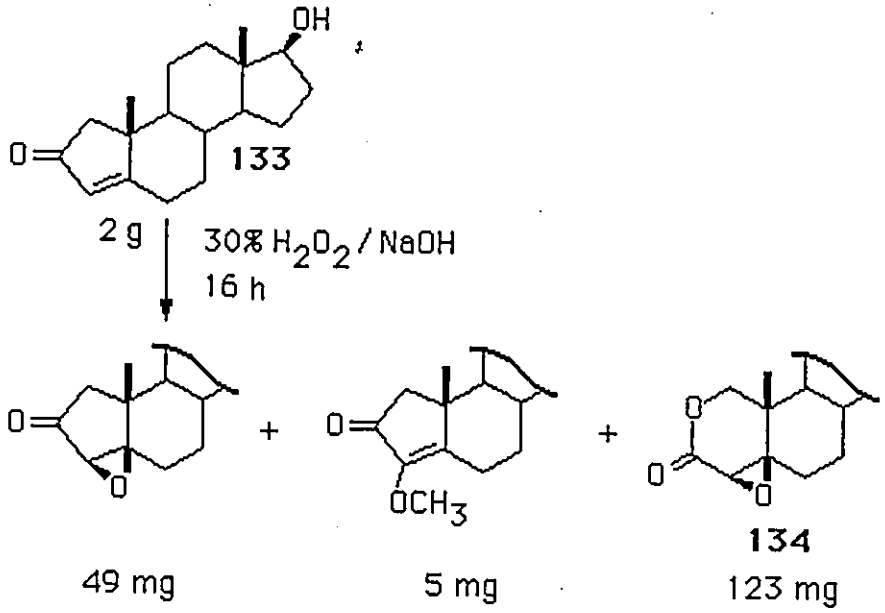
The compound **128** was oxidized to the acid **132** and found to be identical with authentic material thus confirming the stereochemistry at C-5.



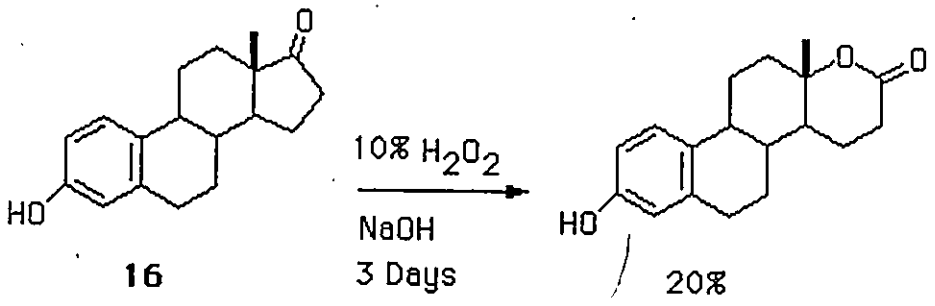
It was also shown by Pinhey and Schaffner[130] that the aldehyde lactone **128** resulted from the acid-catalyzed rearrangement of an intermediate epoxy lactone. Mazur and co-workers[131] obtained a similar result by performing the oxidation of 17β-acetoxyandrost-4-en-3-one **46** with peroxybenzoic acid with dry (A) and aqueous perchloric acid (B).



Levine[132] reported the formation of the epoxy lactone **134**, obtained from the alkaline hydrogen peroxide oxidation of A-nor-17β-hydroxyandrost-4-en-3-one **133**, presumably *via* the epoxy ketone.



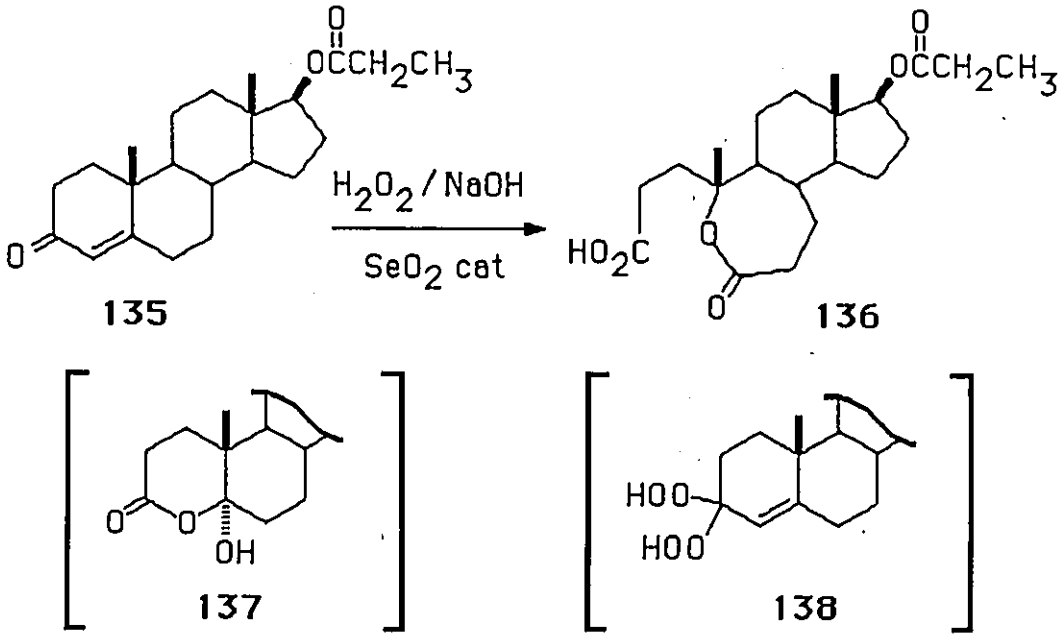
As pointed out by Levine[132], the insertion of an oxygen atom into a 5-membered ring with alkaline hydrogen peroxide is not surprising since Westerfeld[133] reported a similar oxidation with 3-hydroxy-1,3,5(10)-estratrien-17-one **16**. Insertion of oxygen in the steroid D-ring with acidic hydrogen peroxide was also reported by Jacobsen and co-workers[134,135,136].



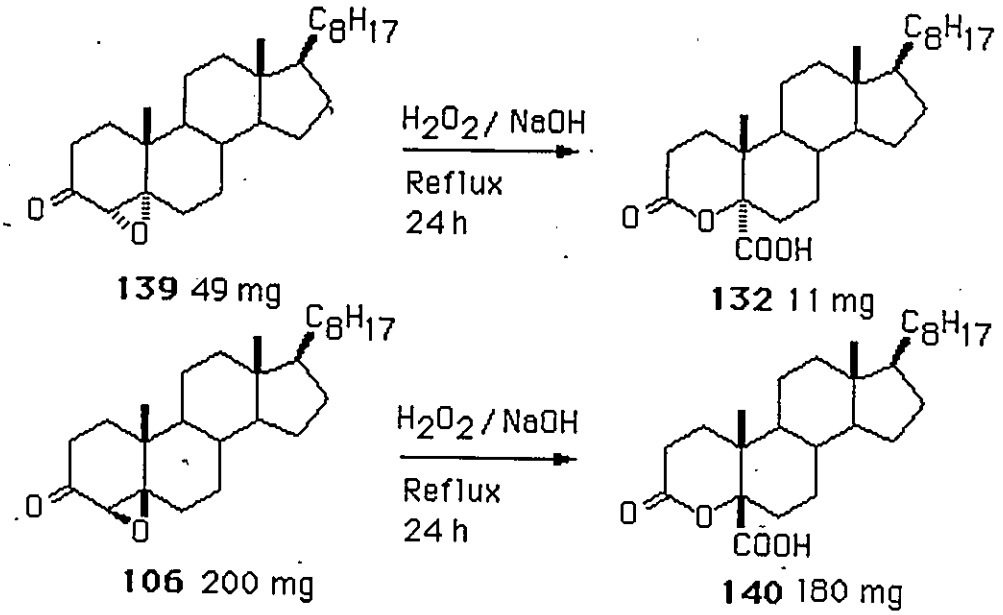
Oxidation of the propionate ester of 17β-hydroxyandrost-4-en-3-one **135** with hydrogen peroxide in the presence of catalytic amounts of selenium dioxide yielded a 7-membered lactone **136**[137]. Two assumptions concerning the mechanism leading to **136** were made[137]:

a) the 4-oxa compound **137** is an intermediate in the formation of **136**;
b) attack of hydrogen peroxide on the C-3 ketone of **135** leads to *gem*-3,3-dihydroperoxide **138**.

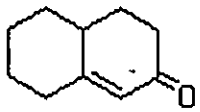
In fact, the 4-oxa compound **137** was converted to **136** with hydrogen peroxide in the presence of selenium dioxide. Evidence for the second assumption arises from observations of Velluz and co-workers[124,138,139] that the *gem*-3,3-dihydroperoxide **123** (see p.139) rearranges to the enol lactone **124**.



Finally, Reusch and LeMahieu[140] reported that the oxidation of 4α,5-epoxy-5α-cholestan-3-one **139** or 4β,5-epoxy-5β-cholestan-3-one **106** with alkaline hydrogen peroxide gave the lactone carboxylic acids **132** and **140**, respectively.



The Baeyer-Villiger oxidation of simple α,β-unsaturated cyclic ketones **141** and **142** has been investigated by DeBoer and Ellwanger[141] using trifluoroperoxyacetic acid and *m*-chloroperoxybenzoic acid under a variety of reaction conditions (e.g. temperature, reaction time, acidity, amount of oxidizing agent). Oxidation reactions with *m*-chloroperoxybenzoic acid gave the simplest product mixtures (α- and β- epoxy ketones, enol lactone). In contrast, oxidation using trifluoroperoxyacetic acid gave a variety of products resulting from reactions of the initially formed products with more trifluoroperoxyacetic acid or with trifluoroacetic acid which was produced during the reaction.



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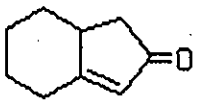
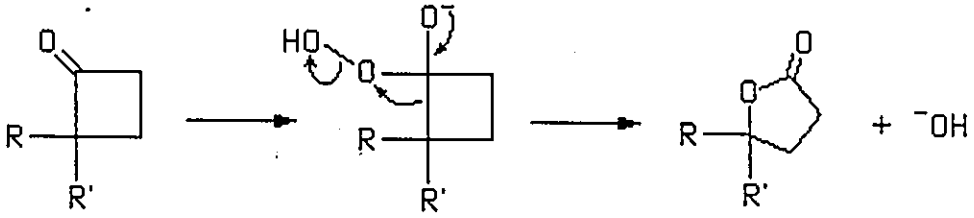


142

Finally, Trost and co-workers[142] found that treatment of a *gem* - disubstituted cyclobutanone **143** with basic hydrogen peroxide in methanol solution at room temperature allowed smooth conversion to the γ-butyrolactone **144** which could be isolated in 82-100% yield.



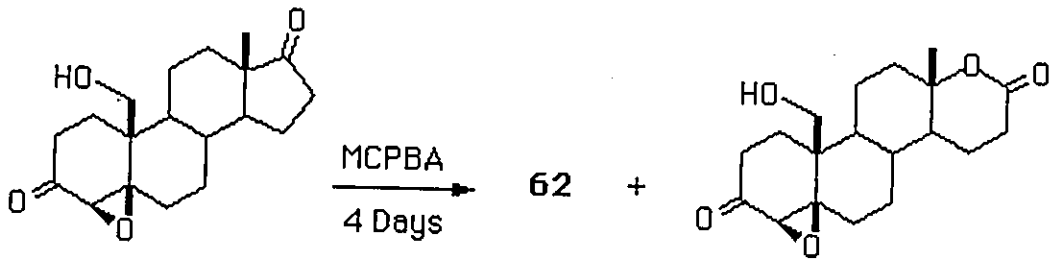
143

144

Since all these reports indicate that the Baeyer-Villiger reaction is highly dependent on the nature of the ketone and of the oxidizing agent, we decided to study the behavior of 19-hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** with *m*-chloroperoxybenzoic acid.

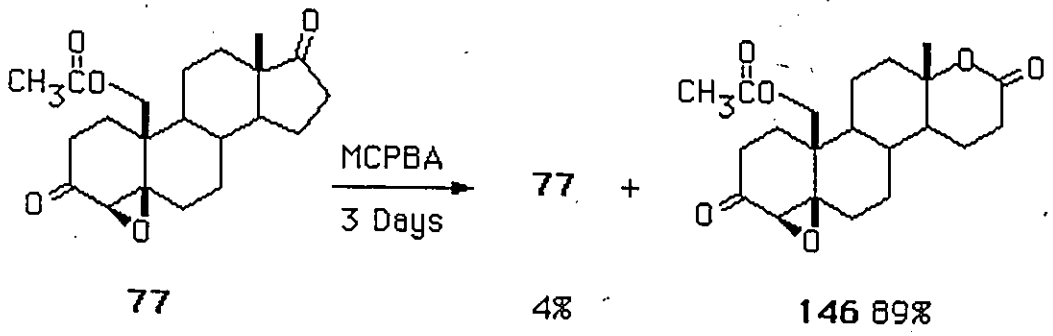
2. Reaction of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** with MCPBA.

After treating the 4β,5β -epoxy diketone **62** with *m*-chloroperoxy-benzoic acid for 4 days, only one product (40%yield) was formed and it was identified as 19-hydroxy-4β,5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **145**. Also, 29% of unreacted starting material was isolated. The evidence for the insertion of oxygen in the D-ring is the following: the C-18 methyl resonance, in the proton NMR spectrum, is shifted downfield at δ 1.32, in agreement with the insertion of an oxygen atom at the 17a position[143]. Similarly, the C-18 methyl resonance in the ^{13}C NMR spectrum, is shifted downfield at δ 20.1 again in agreement with published data[144]. All 19 carbon resonances were assigned and they are summarized in the appendix. As pointed out by Dave, Stothers and Warnhoff[145] melting point, tlc, and even proton NMR spectra are unreliable criteria of purity of any ring expansion products. The best criterion of purity for ring expansion products is the ^{13}C NMR spectrum[145].



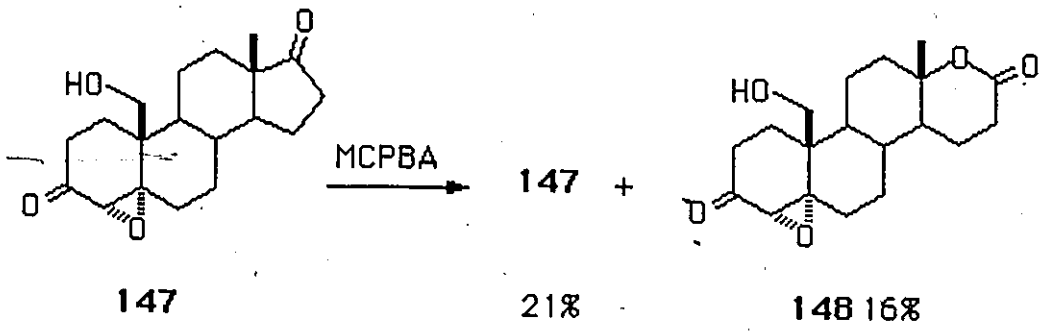
62 29% **145** 40%

Similarly, treatment of 19-acetoxy-4β,5-epoxy-5β-androstane-3,17-dione **77** with *m*-chloroperoxybenzoic acid afforded 19-acetoxy-4β,5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **146** in 89% yield. The acetate group of compound **146** was cleaved by treatment with potassium carbonate and the resulting alcohol was identical (mass spectrum, ^{1}H NMR spectrum, melting point and R_f on tlc) to the previously synthesized alcohol **145**. The yield of the product **146** was higher than that obtained for compound **145** and, along with compound **146**, it was possible to isolate traces of a mixture of two overlapping spots by tlc which was not investigated further.

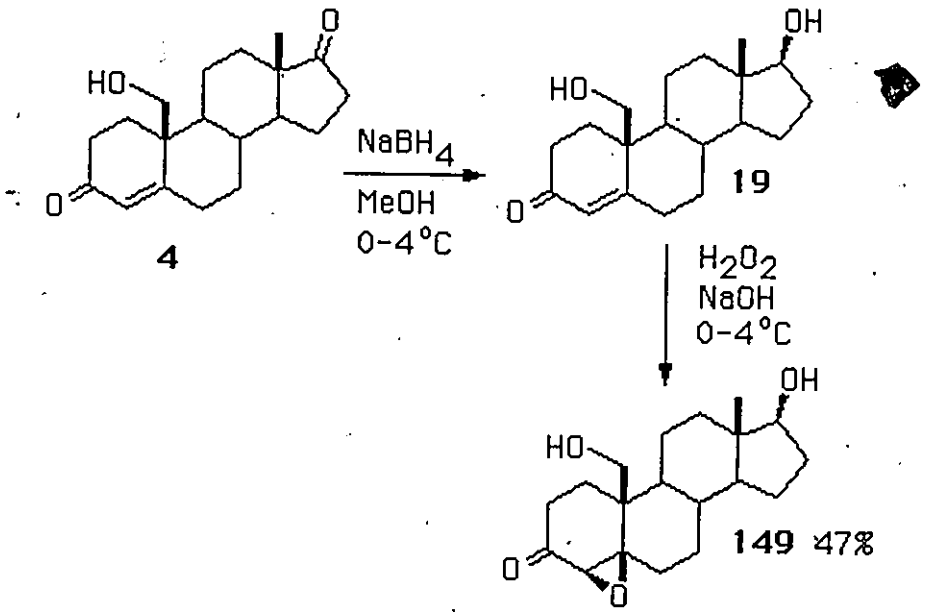


77 4% **146** 89%

The insertion of an oxygen atom in the D-ring was also observed when 19-hydroxy-4α,5-epoxy-5α-androstane-3,17-dione **147** was treated with *m*-chloroperoxybenzoic acid. In this case, 19-hydroxy-4α,5-epoxy-17a-oxa-D-homo-5α-androstane-3,17-dione **148** is isolated in only 16% yield. Again the proton NMR spectrum indicated a singlet at δ 1.31, integrating for three protons and assigned to the C-18 methyl group next to the 17a-oxygen atom.

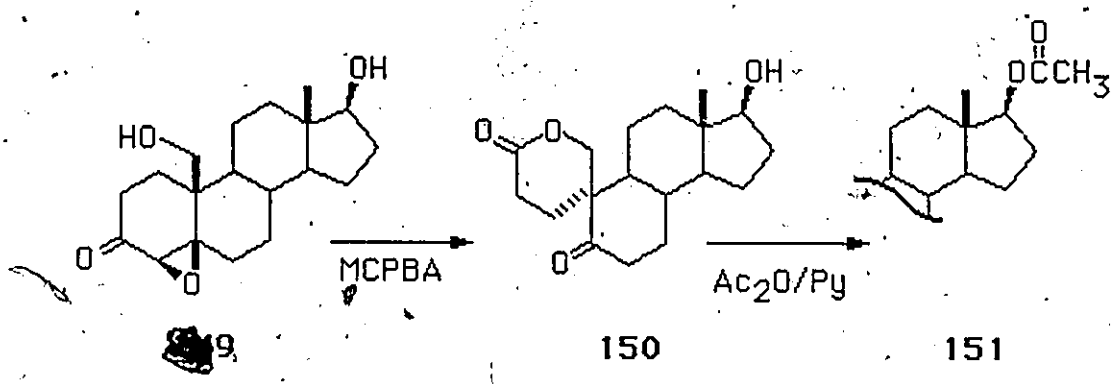


The above results indicate that the Baeyer-Villiger rearrangement is specific to the D-ring with the isomeric epoxides **62** and **147**. It was then decided to repeat the same reaction on 17β,19-dihydroxy-4β,5-epoxy-5β-androstan-3-one **149** which does not have a C-17 carbonyl group. This compound was obtained in 47% yield from 19-hydroxyandrost-4-ene-3,17-dione **4** by treatment with sodium borohydride and subsequent epoxidation with alkaline hydrogen peroxide.



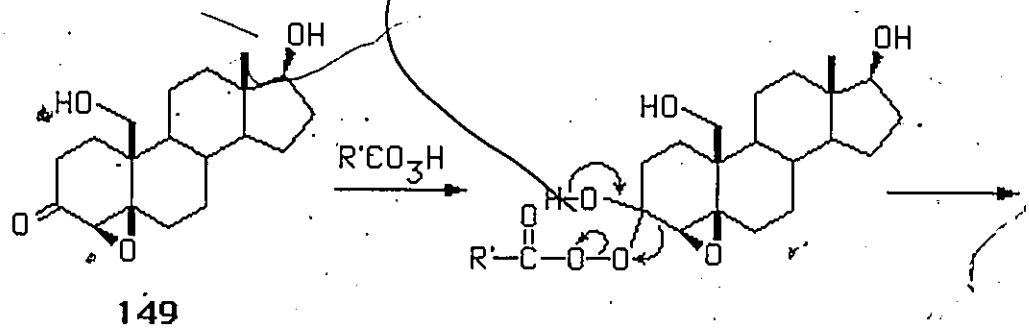
It was found that compound **149** reacted very slowly with *m*-chloro-peroxybenzoic acid. After 11 days, three products were formed and some

starting material was still present. The least polar compound was isolated after two column chromatographies (see experimental section F.1.iv). The compound appeared homogeneous on the basis of tlc but was found by ^{13}C NMR to be a mixture of isomers (*ca.* 10 : 1). The proton NMR spectrum indicated that the epoxide function was lost because of the absence of a resonance at δ 2.88. Furthermore an oxygen atom had been introduced next to a methylene group as indicated by the multiplet integrating for two protons at δ 4.12. However, the structure of this substande could not be assigned. Similarly, we were unsuccessful in identifying the most polar compound. The compound of intermediate polarity was found, by mass spectrometry, to have a molecular weight of 306. The elemental analysis agreed with loss of CH_2 from the starting epoxide **149**, thus indicating an empirical formula of $C_{18}H_{26}O_4$. The proton NMR spectrum indicated the C-18 methyl group at δ 0.81, the 17α-H at δ 3.63 and a quartet integrating for two protons centred at δ 4.44. The infrared spectrum was consistent with a lactone (1745 cm^{-1}) and a saturated ketone (1705 cm^{-1}). The presence of a saturated ketone and of a lactone were confirmed by resonances at δ 210.6 and δ 172.1, respectively in the ^{13}C NMR spectrum. Based on these data, the second product is proposed to be A-nor-3,5-seco-5-oxo-17β,19-dihydroxy-androstane-3-carboxylic acid 3,19-lactone **150**. In agreement with this structure, only one acetate group was introduced when **150** was treated with acetic anhydride in pyridine. The 17β-acetoxy derivative **151** shows a triplet resonating at δ 4.58 and integrating for one proton, in the proton NMR spectrum, which is assigned to the 17α-hydrogen. And, as expected, the quartet integrating for two protons, assigned to the 19-methylene, is centred at δ 4.42 as in the starting material **150**.

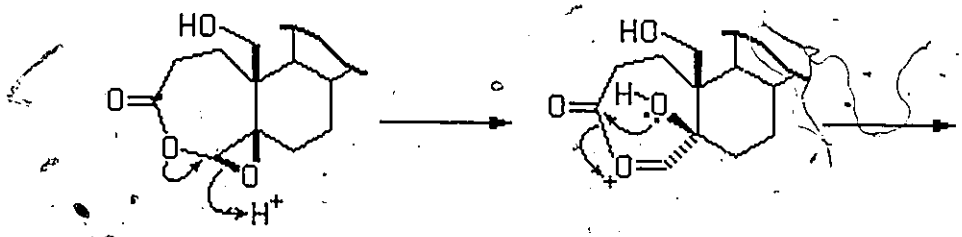


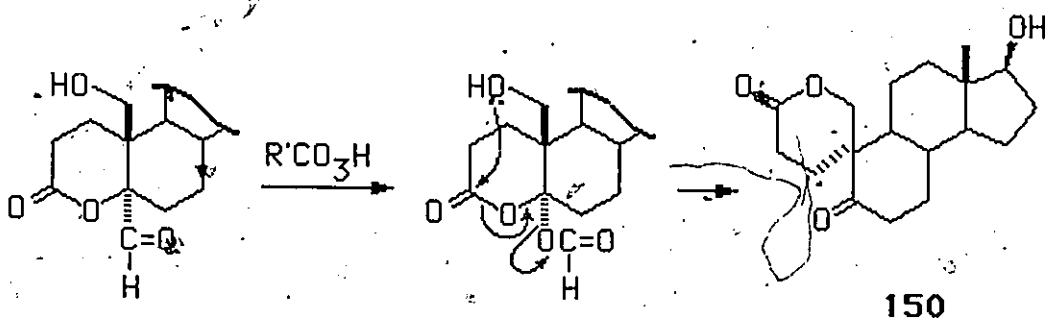
149 150 151

The formation of compound **150** may be explained by the following mechanism:



149

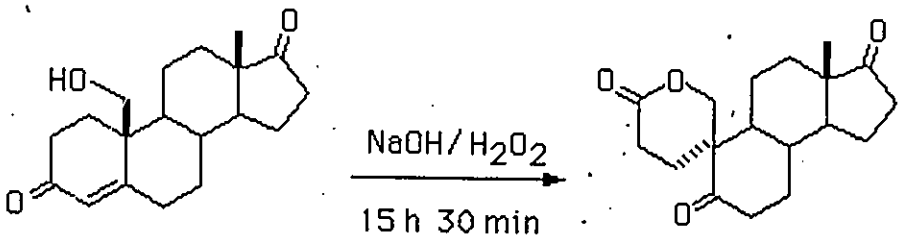




150

Initial Baeyer-Villiger rearrangement occurs with insertion of an oxygen atom at position 3a, according to the mechanism proposed by Criegee[146]. Then follows the opening of the epoxide, generating a 6-membered ring lactone with a formyl group at C-5. A second insertion of oxygen atom occurs. Participation of the 19-hydroxyl group triggers the elimination of the formyl group to give **150**. A similar sequence of reactions was initially proposed by Pinhey and Schaffner[130] in order to explain the formation of Windaus acid **131** (see p.141).

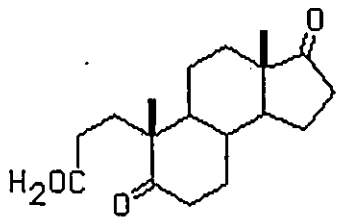
As previously mentioned, a rearranged product, different from the hydroperoxide **100** was isolated when the 19-hydroxy compound **4** was treated with hydrogen peroxide and base in methanol under slightly different conditions of time and amount of sodium hydroxide used to generate the hydroperoxide **100**. This product is identified as A-nor-3,5-seco-5,17-dioxoandrostane-3-carboxylic acid 3,19-lactone **152** based on the following evidence.



4 **152** 26%

The high resolution mass spectrum and the elemental analysis indicated a formula of $C_{18}H_{24}O_4$ corresponding to the loss of one carbon and two hydrogen atoms and the addition of one atom of oxygen. The presence of a saturated ketone was deduced from the carbonyl absorption at 1710 cm^{-1} in the infrared spectrum as well as from the resonance at δ 210.1 in the ^{13}C NMR spectrum. The proton NMR spectrum indicated a resonance at δ 0.94 unchanged from the C-18 resonance of the starting material **4**. That one methylene group was next to an oxygen atom was indicated by a quartet at δ 4.48 integrating for two protons. Also in agreement with the proposed structure, the compound did not react when treated under acetylating conditions. The isolation of this compound **152** occurred when we repeated the reaction with the unsaturated compound **4** in order to get more of the rearranged product **100**. This fact illustrates the sensitivity of the Baeyer-Villiger reaction to small variations in reaction time and concentration of base, for example.

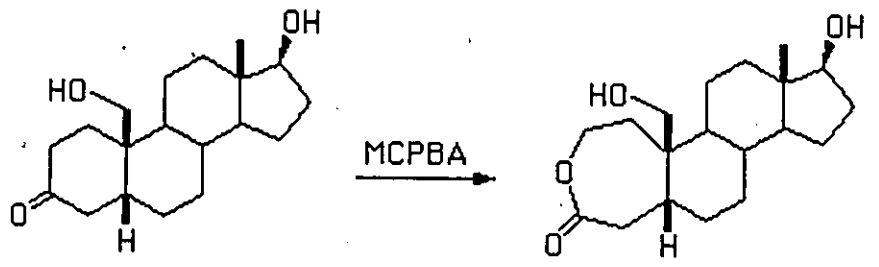
The keto-acid functionality, as illustrated in compound **153**, has also been obtained by treatment of a 5β-3-ketone with potassium superoxide[147] and by treatment of androst-4-ene-3,17-dione **18** with lithium peroxide and water[148]. Milewich and Axelrod[149] published a large scale preparation of A-nor-3,5-seco acid which is based on $NaIO_4$-$KMnO_4$ oxidation of a derivative of 17β-hydroxy-androst-4-en-3-one **5**. Such keto acids may be prepared in a number of ways[150] (chromium trioxide cleavage of double bonds, ozonolysis of enol lactones with periodic acid, etc.) and they have been used to prepare hetero-steroids[150,151].



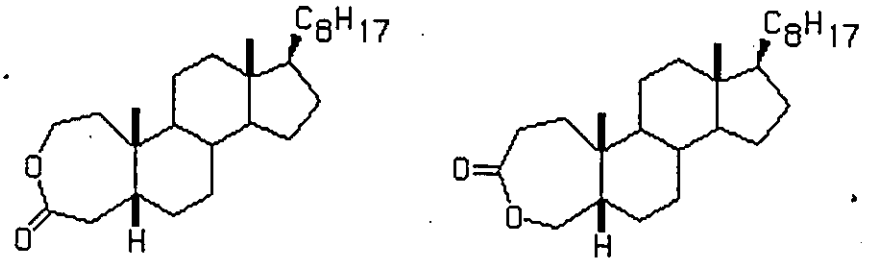
153

3. Reaction of 17β,19-Dihydroxy-5β-androstan-3-one **24** with MCPBA.

The reaction of 17β,19-dihydroxy-5β-androstan-3-one **24** with *m*-chloroperoxybenzoic acid was studied in order to find out if again the insertion of an oxygen atom at the 3a position would initiate a rearrangement involving the cyclization of the C-19 alcohol, as in compound **150**. Thus treatment of the diol **24** with *m*-chloroperoxybenzoic acid afforded two products in 46% and 35% yield, respectively. The elemental analysis of the first compound indicated the addition of one atom of oxygen. The proton NMR data indicated that this oxygen was introduced in the A-ring next to a methylene group because of the resonance of a multiplet at δ 4.16 integrating for two protons. The C-19 methylene group was still present as indicated by the quartet centred at δ 3.72. From comparison of the ^{13}C NMR chemical shifts with data obtained by Dave, Stothers and Warnhoff[145] for A-homo-2a-oxa-5β-cholestan-3-one **155** and A-homo-3a-oxa-5β-cholestan-3-one **156**, we concluded that the first product isolated in 46% yield is A-homo-17β,19-dihydroxy-2a-oxa-5β-androstan-3-one **154**. (The ^{13}C NMR data are listed in the appendix.)



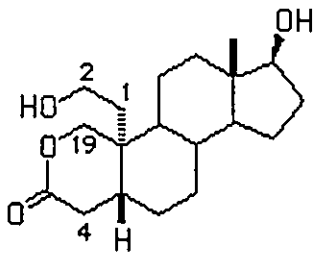
24 154



155 156

The elemental analysis of the second product also indicated the addition of one atom of oxygen. The presence of a lactone was indicated by the infrared absorption at 1730 cm^{-1}. A quartet centred at δ 4.37 and integrating for two protons suggested a methylene group next to an oxygen atom. Also, a broad doublet at δ 3.72 integrating for two protons was present. The ^{13}C NMR data confirmed the presence of a lactone with resonances at δ 172.8 for the carbonyl group and δ 71.4 for the methylene group next to the oxygen atom. However the methylene group at δ 60.9 was at higher field than normally found for the C-19 alcohol (δ 64-66). Two hydroxyl groups were still present in the second product since upon treatment with acetic anhydride in pyridine, the product obtained showed two singlet resonances integrating for three protons each at δ 2.01 and

δ 2.04 in its proton NMR spectrum. Based on the ^{13}C NMR data, we propose two possible structures for the second product, **157** or **158**. The structure **157** would be obtained following insertion of an oxygen atom at the 2a position and cyclization of the 19-alcohol. Structure **158** would necessitate the insertion of the oxygen at the 3a position and cyclization of the 19-alcohol.



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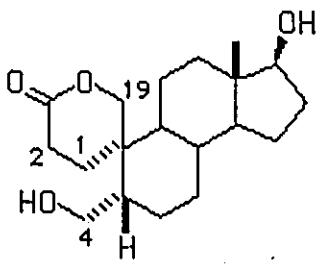
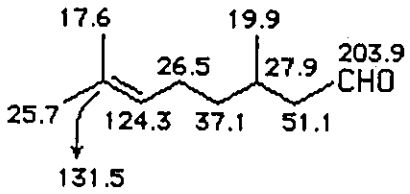


158

In order to differentiate between the two structures, oxidation of the second product was performed according to Ratcliffe's method[152](chromium trioxide-pyridine complex). The reaction was done on a small scale (40 mg) and, after column chromatography, only 9 mg of product was obtained. The mass spectrum showed a molecular ion at m/z 318 indicating that the 17β-alcohol had been oxidized jointly with the other free alcohol. An aldehyde function was present as indicated by the resonance at δ 9.76 in the proton NMR spectrum. It is expected that if an aldehyde is formed in **157** that a methylene group would be adjacent to it whereas in structure **158** a methine group would be adjacent to the aldehyde group. The chemical shifts of a methylene group adjacent to an aldehyde function were reported around δ 50 in the monoterpenes **159** and **160**[153], and at δ 45.9 in **161**[154].



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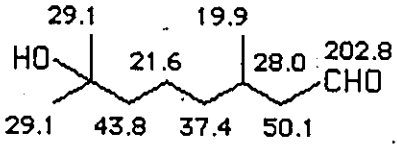

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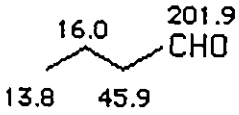
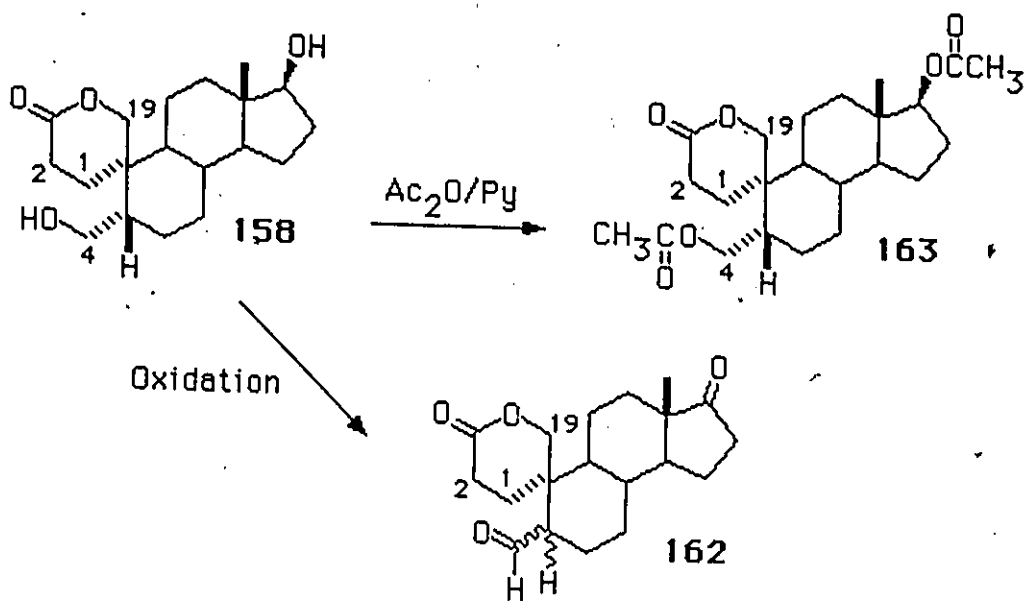
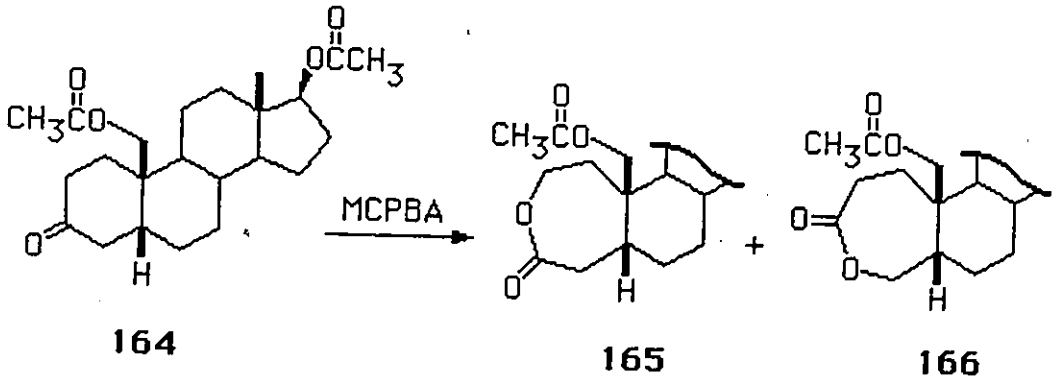


161

The ^{13}C NMR spectrum obtained did not indicate the presence of any methylene groups in the δ 40-50 region except for a resonance at δ 35.6 attributed to the C-16 methylene group. Even if the compound isolated was homogeneous on the basis of tlc and proton NMR, some resonances were doubled in the ^{13}C NMR spectrum. For instance, the C-19 methylene group appeared at δ 69.4 and 69.0. This latter information confirms that structure **162** must be assigned to the product since C-5 is an asymmetric carbon α to the aldehyde group, thus an epimerizable centre. Such an epimerization cannot occur with a structure like **157**. Evidence in favor of the structure **158** also comes from the fact that saturated 5α- or 5β-3-keto steroids generally give a ~1:1 ratio of isomeric ring products (2a-oxa and 3a-oxa)[145,155]. Since the first compound was identified as the A-homo-2a-oxa derivative **154**, it is more plausible that the second product derives from the A-homo-3a-oxa derivative as is the case for the structure **158**.



The reaction of 17β,19-diacetoxy-5β-androstan-3-one **164** with *m*-chloroperoxybenzoic acid was then studied to confirm that when the 19-alcohol is not available, two products should be formed, in approximately equal ratio and correspond to the insertion of an oxygen atom at the 2a and 3a positions. The product formed was homogeneous on tlc but the ^{13}C NMR spectrum indicated that a 1:1 mixture of both isomers **165** and **166** had been formed, in agreement with what was expected. The ^{13}C resonances were assigned in comparison with known values for 2a and 3a-oxa steroids[145] and they are reported in the appendix.



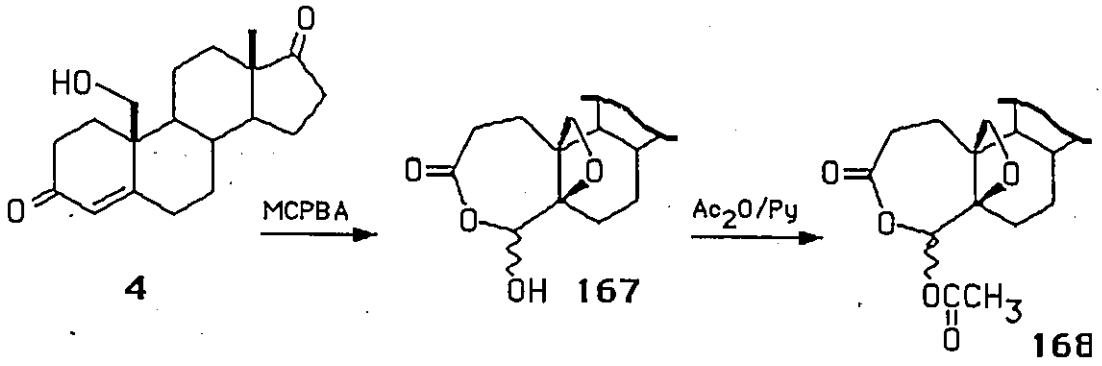
In summary, we have documented the participation of the C-19 hydroxyl group upon insertion of an oxygen atom at the 3a position when the steroid has a 5β-configuration, i.e. preferential lactonization involving C-3 and C-19.

4. Reaction of 19-Hydroxyandrost-4-ene-3,17-dione **4** with MCPBA.

Keeping in mind this tendency of the C-19 hydroxyl group to participate in Baeyer-Villiger rearrangements, the reaction of the unsaturated C-19 alcohol **4** with *m*-chloroperoxybenzoic acid was studied. Three compounds were isolated from this reaction. The least polar compound could not be identified. The high resolution mass spectrum of the compound of intermediate polarity indicated a molecular formula of $C_{19}H_{26}O_5$ corresponding to the addition of two atoms of oxygen to the starting material **4** ($C_{19}H_{26}O_3$). Examination of the proton NMR spectrum showed a quartet at δ 4.57, integrating for two protons and a singlet at δ 3.60 integrating for one proton and exchanging with D_2O. A lactone absorption band was also present in the infrared spectrum at 1740 cm^{-1}. However, based on these data alone, we cannot offer a structure for this second compound. The small amount of material isolated (7 mg) is the limiting factor for not doing more elucidation work at this moment.

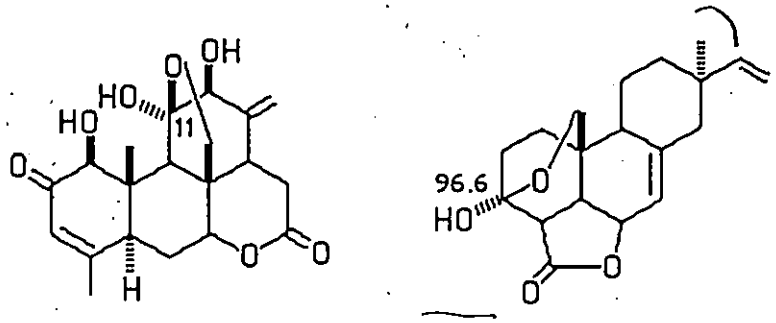
The more polar compound was found to have the same molecular formula as the second compound, indicating the addition of two atoms of oxygen. The presence of a lactone was indicated by the infrared absorption at 1730 cm^{-1} and by ^{13}C NMR resonance at δ 171.4. Besides a quartet centred at δ 3.88, integrating for two protons and assigned to a methylene group next to an oxygen

atom, the proton NMR spectrum showed a doublet at δ 3.27, integrating for one proton and exchanging upon addition of D_2O , which was assigned to a secondary alcohol. Finally, a very fine doublet at δ 4.97 became a singlet after the addition of D_2O, thus indicating that it was coupling with the hydroxyl proton. From these data and the ^{13}C NMR spectrum, the structure of this third compound is proposed to be A-homo-3a-oxa-19,5-oxido-4ξ-hydroxy-5β-androstane-3,17-dione **167**. The stereochemistry of the hydroxyl group at C-4 has not been determined.



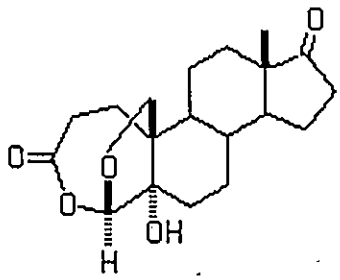
4 167 168

The methine resonance at δ 101.1 is attributed to C-4, in good agreement for values of alcohols next to oxygen atoms. For example, the resonance of C-11 in **169** was reported at δ 108.9[156] and the resonance for C-3 in compound **170** was reported at δ 96.6[157].



169 170

Finally, the acetylation of compound **167** was performed and the product **168** obtained is in agreement with the proposed structure **167** rather than the other possible structure **172.** The tertiary hydroxy group of the structure **172** would not be acetylated with acetic anhydride in pyridine. The product obtained after treatment of **167** with acetic anhydride in pyridine is homogeneous on the basis of tlc but is a mixture of epimers (4α- and 4β-acetates) by proton NMR analysis (*ca* . 1:1).



172

In summary, we examined some reactions of C-19 substituted androgens and participation of the C-19 hydroxyl group has been documented. The lactones synthesized could be modified to methylene lactones. The α-methylene-δ-lactone group which is found in several naturally occurring terpenoids is a highly efficient biological alkylator[158]. Several estrogen derivatives containing the α-methylene-δ-lactone group as part of the D-ring were prepared as anti-tumor agents and found highly toxic towards carcinoma cells in culture[159].

EXPERIMENTAL

A. Materials

1. Radioactive Compounds.

19-Hydroxy[6,7-^{3}H]androst-4-ene-3,17-dione **4** [^{3}H] (sp. radioactivity 45.0 Ci/mmol), 3α-hydroxy[1,2-^{3}H]5β-androstan-17-one **20** [^{3}H] (sp. radioactivity 45.0 Ci/mmol), 17β-hydroxy[1,2-^{3}H]androst-4-en-3-one **5** [^{3}H] (sp. radioactivity 51.5 Ci/mmol), iodo[2-^{14}C]acetic acid (sp. radioactivity 56.0 Ci/mmol), iodo[2-^{3}H]acetic acid (sp. radioactivity 58 mCi/mmol), 17α-hydroxy-[1,2-^{3}H]androst-4-en-3-one (sp. radioactivity 50.0 Ci/mmol) were purchased from New England Nuclear.

2. Non-radioactive Steroids.

19-Hydroxyandrost-4-ene-3,17-dione **4** was a generous gift from Dr. J.M. Koekenoer, Africans University, Johannesburg, South Africa. 11β-Hydroxyandrost-4-ene-3,17-dione **8**, 6β-hydroxyandrost-4-ene-3,17-dione **10**, 17β-hydroxyandrost-4-en-3-one **5**, androst-4-ene-3,17-dione **18**, 3β-hydroxy-5β-androstan-17-one **22**, 5β-androstane-3,17-dione **21**, 3α-hydroxy-5β-androstan-3-one **20** and 3-hydroxy-1,3,5(10)-estratrien-17-one **16** were from Steraloids or Sigma Chemical Company.

3. Coenzymes.

Pyridine nucleotides (NADP and NADPH) were purchased from Boehringer Mannheim Company.

4. Chromatographic and Electrophoretic Chemicals.

Polybuffer exchanger PBE 94 and Polybuffer 74 for chromatofocusing were obtained from Pharmacia Inc. Matrex Gel Red A was purchased from Amicon Company. Ampholyte pH range 5-7 from LKB was obtained from Fisher Scientific Company, as well as the ammonium persulfate. Acrylamide, bisacrylamide, TEMED and mixed bed resin AG 50-X8 CD, 20-50 mesh were from Bio-Rad Laboratories. Urea, 99.5% was from BDH Chemical Company. The urea solution was purified by mixing with mixed bed resin AG 50-X8 CD. The resin was removed by filtration and the treatment was repeated. Silica gel N, for thin layer and preparative chromatography, was from Machery Nagel und Co. Fluorescent indicator, activated zinc silicate was from Baker Chemical Company. Silica gel for column chromatography, flash chromatography type 20-45 microns, was from Terochem Laboratories. Davisil silica gel (Fischer Scientific Company), 60-240 mesh, was used occasionally for column chromatography. Thin layer chromatography was performed on Merck pre-coated silica gel 60 F-254 plates. Cellulose sheets 13255 were from Eastman Kodak Company. N-Epsilon-monodinitrophenyl-L-lysine monohydrochloride was from Mann Research Laboratories.

5. Scintillation Fluids.

2,5-Diphenyloxazole (PPO) was purchased from BDH Chemical Company and ACS, aqueous counting scintillant was from Amersham Company.

6. Buffer Salts, Solvents and Other Chemicals.

All salts, solvents and chemicals were high purity or reagent grade.

B. Methods.

1. General.

Melting points were determined on a Hoover Uni-melt apparatus and are uncorrected. Optical rotations were measured with a Perkin-Elmer 241 polarimeter and are for chloroform solutions unless otherwise specified. Circular dichroism spectra were obtained with 95% ethanol solutions on a Cary 61 spectrophotometer. Ultraviolet spectra were obtained with 95% ethanol solutions on a Varian DMS 90 spectrophotometer. Infrared spectra were recorded on a Perkin-Elmer 783 infrared spectrophotometer using chloroform solutions unless otherwise specified. Proton magnetic resonance spectra were recorded on a Varian XL-300 instrument using deuterochloroform as solvent, unless otherwise specified and chloroform as an internal standard (7.240 ppm). ^{13}C nuclear magnetic resonance spectra were obtained on a Varian FT-80 instrument with deuterochloroform as solvent and internal standard. The ^{13}C nuclear magnetic resonance spectra (125 MHz) of compounds **91** and **162** were recorded by Dr. J.-R. Brisson on a Bruker AM-500 instrument using deuterochloroform as solvent. Mass spectra were obtained from a V.G. 7070-E double focusing instrument. Microanalyses were carried out by M-H-W Laboratories, Phoenix AZ, U.S.A. All solvents were distilled prior to utilization. The light petroleum used was of boiling range 30-60°C. The water for all the manipulations involving the enzymes was deionized and glass distilled.

2. Work up.

Work up involved pouring the reaction mixture into water, extracting with methylene chloride and washing the extracts with water (and with 5% HCl if pyridine was present). The extracts were then dried with anhydrous sodium sulphate and the solvent removed on a rotary evaporator.

3. Chromatography.

Chromatography refers to medium pressure column chromatography using *ca.* 30 g of adsorbant for every 100 mg of steroid being separated. The column was packed with a slurry of silica gel in methylene chloride. The material to be separated was introduced onto the column as a solution in methylene chloride and eluted with the appropriate solvent.

4. Acetylation (Method A).

The general procedure for the acetylation was to dissolve the steroid in pyridine (for example 50 mg in 1 ml of pyridine) and to add acetic anhydride (2 ml) and stir overnight at room temperature. Work up was as described in 2.

5. Haloacetylation (Method B).

The procedure for the bromo- or iodo-acetylation was accomplished by sequential addition of cooled (0-4^{o}C) methylene chloride solutions of iodoacetic acid, dicyclohexylcarbodiimide and of 4-dimethylaminopyridine to a stirred solution of steroid in dry methylene chloride in an ice-water bath. The reaction mixture was stirred for 1 to 5 h. The progress of the reaction was followed by tlc

and terminated when all the starting material was consumed. The product was directly applied on a column of silica gel and purified.

6. Iodo[2-^{14}C]acetylation (Method C).

The iodo[2-^{14}C]acetoxy steroids were synthesized as described in Method B. The steroid (1.5×10^{-5} mol) was dissolved in dry methylene chloride (300 µl). Iodo[2-^{14}C]acetic acid (50 to 200 µCi), dicyclohexylcarbodiimide (1.9×10^{-5} mol) and 4-dimethylaminopyridine (2.5×10^{-5} mol) all in dry methylene chloride (300 µl) were used as described previously. The purification was on preparative tlc (20 cm x 20 cm x 0.75 mm) previously washed with methanol and dried in the oven at 110°C. The purity of the radioactive product was verified by thin layer chromatography and was greater than 99%.

7. Stability of the Iodoacetoxy Steroids in pH 7.0 Buffer.

The iodo[2'-^{14}C]acetoxy steroids (5×10^{-9} mol, in 95% ethanol) were added to 20 mM phosphate buffer, pH 7.0 (1 ml). The solution was incubated at 25°C for 180 min, then the steroid was extracted with 2 ml of ethyl acetate. The ethyl acetate fraction was separated, dried under a stream of nitrogen, diluted with non-radioactive iodoacetoxy steroid and applied on a tlc plate (20 cm x 10 cm x 0.25 mm). The solvent systems used for the elution of the tlc are specified for each derivative in the corresponding Figures 5-10. After elution the derivative was visualized under UV or by spraying with 5% sulfuric acid in methanol and heating. The silica gel was divided into sections of 1.0 or 0.5 cm. The silica gel of each section was scraped and the radioactivity measured.

8. Preparation of Radioactive Substrates.

The purity of the radioactive steroids was verified by tlc in the solvent system benzene : acetone (4 : 1 , v/v) for 17β-hydroxy[1,2-^{3}H]androst-4-en-3-one **5** [^{3}H] and for 3α-hydroxy[1,2-^{3}H]-5β- androstan-17-one **20** [^{3}H]. Methylene chloride : acetone (6 :4, v/v) was used for 19-hydroxy[6,7-^{3}H]androst-4-ene-3,17-dione **4** [^{3}H]. For enzyme assays, the stock solutions of the radioactive steroids were diluted with non-radioactive steroids in methanol to a final specific activity of 4.5 μCi/μmol. The final steroid concentration was 0.1 μmol/ml. The solutions were stored at -20°C.

9. Assay for Dehydrogenase Activity.

For assays with hydroxysteroid substrates, the incubation mixtures contained the following: radioactive steroid substrate, 3 nmol in 30 μl of methanol; $NADP^+$, 0.5 μmol in 0.7 ml of 0.1 M glycine-NaOH buffer, pH 9.5 and enzyme fraction, 1-3 μl. The final volume was made up to 1 ml by the addition of 0.15 M KCl. Samples were incubated at 37°C for 30 min. The amount of enzyme added to each incubation mixture was adjusted so that the extent of substrate oxidation was less than 40%. Incubations in which the enzyme was omitted were carried out as control. For assays with ketosteroid substrates, the conditions and the concentrations were the same as above except that NADPH was the cofactor and 0.1 M Tris-maleate buffer, pH 7.6 was used instead of the glycine buffer. The incubations were terminated by extraction of the steroid substrate and product from the incubation medium with 2 ml of ethyl acetate. The ethyl acetate was removed and evaporated to dryness under a stream of nitrogen. Non- radioactive steroid standards in methanol were added and the mixture was applied on tlc (20 cm x 10 cm x 0.25 mm), previously dried at 110°C, and the elution was in

benzene : acetone (4 : 1, v/v). The steroids were detected under an ultraviolet lamp. The spots were scraped off the plate into scintillation vials containing 0.4 ml of methanol and 8 ml of scintillation fluid (8 g of 2,5-diphenyloxazole in 2 liters of toluene) was added to each vial. The radioactivity was measured in a Searle Mark III liquid scintillation counter.

For assays with non-radioactive steroids, the incubation mixture contained the following: NADPH, 0.5 µmol in 0.7 ml of 0.1 M Tris-maleate, pH 7.6; 0.3 ml of 0.15 M KCl and 45 nmol of steroid in 95% ethanol. The enzyme, 6-20 µl (0.14 nmol in 10 mM Tris-HCl, pH 8.0, 20% glycerol, 0.5 mM DTT) was then added. Separate incubations (15) were performed for each steroid to be tested as substrate and incubated at 37°C for 90 min. The incubations were terminated by extraction of the steroid substrate and product from the incubation medium with 2 ml of distilled ethyl acetate. The organic layers were combined and the solvent removed under reduced pressure. The residual material was diluted with methanol and methylene chloride and applied on preparative tlc plates (20 cm x 20 cm x 0.75 mm) previously washed with distilled methanol and dried at 110°C. The solvent systems used for different steroids are summarized in Table IX. A mixture of starting material and product was applied on one side of the tlc plate, 3 cm away from the applied incubation mixture, as a reference. The reference steroids were visualized under ultraviolet light or were sprayed with a 5% solution of sulfuric acid in methanol (protecting the rest of the silica gel with a glass plate) and the plate was then carefully heated on a heating plate. The silica gel corresponding to the starting material and to the product was separately scraped and extracted twice with methylene chloride : methanol (6 ml : 1 ml) and centrifuged. The solvent was evaporated under nitrogen, diluted in approximately 1 ml of methylene chloride : methanol (1 : 1) and filtered using a microfilter adapted on a syringe. The samples were again dried under nitrogen and analyzed by mass spectrometry.

TABLE IX

Solvent Systems Used for the Various Steroids.

Steroid	Methylene Chloride : Ethyl Acetate
6β-Hydroxyandrost-4-ene-3,17-dione **10**	40 : 60
11β-Hydroxyandrost-4-ene-3,17-dione **8**	40 : 60
3-Hydroxy-1,3,5(10)-estratrien-17-one **16**	40 : 60
4-Hydroxyandrost-4-ene-3,17-dione **6**	50 : 50
3α,19-Dihydroxy-5β-androstane-3,17-dione **23**	60 : 40
3β,19-Dihydroxy-5β-androstane-3,17-dione **15**	60 : 40
17β-Hydroxy-5β-androstan-3-one **12**	30 : 70
19-Hydroxy-5β-androstane-3,17-dione **14**	20 : 80

10. Expression of Enzyme Activity and Protein Determination.

The percent conversion of substrate (S) to product (P) was calculated according to the formula [P/(S + P)]100. The enzyme activity is expressed as units/ml. One unit of activity is defined as the amount of enzyme catalyzing the oxidation or reduction of 1 μmol of steroid substrate per min under the specific condition of the assay. The protein concentration was determined by the Coomassie Blue dye binding method[160].

11. Crude Enzyme Preparation.

All procedures for the purification of the 17β-HSD were carried out

at 0-4°C. A female New Zealand white rabbit was sacrificed by cervical dislocation, the liver was removed and immersed in an ice cold solution of 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT. The gallbladder was removed and the liver was minced with scissors and a 20% (w/v) homogenate was prepared in the same buffer using a Sorval Omni-Mixer for one min at maximum speed. The homogenate was centrifuged at 10 000 g for 30 min in a Sorval RC2-B centrifuge and the supernatant obtained was centrifuged at 105 000 g for 90 min in a Beckman ultracentrifuge. This supernatant was then concentrated by ultrafiltration in an Amicon Diaflo apparatus equipped with a YM-10 membrane.

12. Affinity Chromatography.

An affinity column (1.5 cm x 40 cm) of agarose immobilized Procion Red HE3B was equilibrated by washing with at least ten bed volumes of 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT. The concentrated enzyme fraction (50 ml to 80 ml) was centrifuged prior to application on the column to remove any insoluble material. The flow rate of application and of subsequent elution was of 18 ml/h. The column was eluted first with 150 ml of 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT containing 1 mM EDTA and 5 mM $MgCl_2$. Then followed a 600 ml linear gradient of NaCl from 0 M to 1 M in 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT, containing 1 mM EDTA and 5 mM $MgCl_2$. The elution was continued with a 200 ml linear gradient of NaCl from 1 M to 2 M in 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT containing 5 mM $MgCl_2$. Finally the column was eluted with 400 ml of 2.5 M NaCl in 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT containing 10 mM $MgCl_2$. Fractions (5 ml) were collected and assayed for 17α- and 17β-HSD activities. The fractions having 17β-HSD activity (usually present in the 2.5 M NaCl) were

pooled. Cold glycerol was added to a final concentration of 20% (v/v) and the fraction was concentrated by ultrafiltration. The concentrated sample was dialyzed against 8 liters (2 x 4 liters) of 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 20% glycerol. About 50 ml of 0.025 M histidine buffer, pH 6.0, 0.5 mM DTT was added to the enzyme fraction and the fraction was centrifuged to remove the insoluble material. The fraction was again concentrated by ultrafiltration.

13. Chromatofocusing.

The previously concentrated sample (~10 ml) was applied to a column (1 cm x 30 cm) packed with Polybuffer Exchanger PBE 94 that had been previously equilibrated with 0.025 M histidine buffer, pH 6.0, 0.5 mM DTT. The column was eluted with a solution of Polybuffer 74 (30 ml of Polybuffer 74 was adjusted to pH 5.0 with 1 N HCl and diluted to 300 ml with deareated distilled water, DTT was added to give a final concentration of 0.5 mM) at a flow rate of 18 ml/h and 1.0 ml fractions were collected and assayed for 17β-HSD activity. The pH gradient developed during the elution of the column was monitored by placing fractions of the eluant in an ice bath and measuring the pH within 2 h of collection using a Fischer Accumet pH meter, model 520. The fractions corresponding to each peak of enzyme activity were pooled, diluted with an equal volume of 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 10% glycerol and concentrated by ultrafiltration. Each enzyme fraction was further dialyzed against 100 ml of 10 mM Tris-HCl, pH 8.0, 0.5 mM DTT, 10% glycerol. The final concentration of glycerol was adjusted to 20%. The pure enzyme fractions were stored at -78°C. Enzyme purity was assessed by polyacrylamide-gel electrophoresis and by isoelectric focusing polyacrylamide-gel electrophoresis.

14. Polyacrylamide-Gel Electrophoresis.

Polyacrylamide-gel electrophoresis was carried out as described by Davis[54]. The acrylamide monomer concentration was 10.5%. The gels were subjected to a current of 3 mA/tube for 45 min prior to the addition of the enzyme. The buffer used for electrophoresis was 0.02 M Tris, 0.2 M glycine, pH 8.3. Bromophenol blue (0.01%) was used as the tracking dye. Electrophoresis was carried out at 4°C at an initial current of 2 mA/tube for 15 min, then at 3 mA/tube.

15. Protein Staining.

The gels were first fixed in 12.5% trichloroacetic acid for 30 min in a water bath at 60°C and then stained with 0.2% aqueous Coomassie Brilliant Blue R-250 in 45% ethanol-10% acetic acid for 30 min at 60°C. Destaining was carried out first in 25% ethanol-10% acetic acid at 60°C for 30 min and then at room temperature overnight. The gels were stored in 10% acetic acid.

16. Isoelectric Focusing Polyacrylamide-Gel Electrophoresis.

Isoelectric focusing in polyacrylamide gel was carried out by a modification of the method of Hayes and Wellner[55]. Polyacrylamide gels (0,6 cm x 9 cm) were prepared from a solution containing acrylamide (8%,w/v). N,N-methylene bisacrylamide (0.24%,w/v), LKB ampholytes 40%, w/v, pH 5-7 (5%, v/v), ammonium persulfate (0.024%, w/v) and N,N,N',N'-tetramethyl-ethylene diamine (0.24%, v/v). The cathode solution was 0.02 M NaOH and the anode solution was 0.01 M phosphoric acid. Before the application of the protein samples the gels were run at 4°C for 15 min at 200 V, then for 30 min at 300 V and finally for 30 min at 400 V. The NaOH solution was removed from the cathode compartment and the gel surfaces were rinsed with distilled water. Protein

samples containing glycerol (20%, w/v) were applied on the gels and overlaid with the NaOH cathode solution. Isoelectric focusing was carried out at 4°C for 30 min at 450 V followed by 4 h at 500 V. Staining was as described above.

17. Urea Isoelectric Focusing Polyacrylamide-Gel Electrophoresis.

The procedure was similar to the one described for isoelectric focusing polyacrylamide-gel with the following modification. A solution of urea, previously purified by treating the solution twice with mixed-bed resin, was added to the acrylamide solution. The final concentration of urea was 8 M. The electrophoresis, staining and destaining was accomplished as described above.

18. Carboxymethylation of Amino Acids.

The carboxymethylation of L-histidine was performed according to the method of Crestfield[161]. Acetyl-L-histidine (225 mg) was dissolved in 7 ml of water and iodoacetic acid (270 mg) dissolved in 0.03 N NaOH (3.5 ml) was added. The pH was adjusted to 8.1 by the addition of 1 N NaOH and the volume was adjusted to about 25 ml with water. Solid sodium bicarbonate (113 mg) was added and the solution was heated under reflux for 3 h. Hydrolysis of the acetyl group was achieved by adding 25 ml of concentrated HCl and 70 ml of 6 N HCl and refluxing for 6 h. The solvent were removed under reduced pressure. The carboxymethylation of L-cysteine, L-lysine and L-methionine was done in a similar manner but without the hydrolytic step.

19. Inactivation of 17β-HSD by Haloacetoxy Steroids.

Inactivation with the various steroid derivatives was carried out in

20 mM phosphate buffer, pH 7.0. In a typical experiment, the purified enzyme (4.5 nmol, in 10 mM Tris-HCl, pH 8.0, 10 mM DTT, 20% glycerol) was applied on a Sephadex G-25 medium column previously equilibrated in 20 mM phosphate buffer, pH 7.0. Phosphate buffer, 20 mM, pH 7.0 was added to have a final volume of 750 µl and centrifugal gel filtration[79] was performed at 4°C in order to remove the DTT. The resulting enzyme solution was equilibrated 15 min at 25°C and a fraction of this enzyme (0.75 nmol, 125 µl) was mixed with 3.75 nmol of haloacetoxy steroid in 3 µl of ethylene glycol monomethyl ether and 2 µl of 20 mM phosphate buffer, pH 7.0. Aliquots were removed (at 15 or 30 min intervals) and assayed for enzyme activity as previously described. DTT was present in the incubation mixture when the enzyme activity was measured in order to stop further alkylation. Incubations in which the haloacetoxy steroid was replaced by the appropriate acetoxy steroid served as controls.

20. Inactivation of 17β-HSD by Iodoacetoxy Steroids for Amino Acid Analysis or for Peptide Mapping.

Inactivations were performed as described above. Enzyme (10 nmol) was inactivated with a 5-fold excess of [^{14}C]iodoacetoxy steroids (50 nmol, in ethylene glycol monomethyl ether) in 20 mM phosphate buffer, pH 7.0 at 25°C. The reaction was stopped by the addition of 2-mercaptoethanol (2×10^{-4} mol) and incubated for 1 h at 25°C. The reaction mixture was then dialyzed against 1N NaOH (8 liters) containing 2-mercaptoethanol (3 mM) for 4 h to facilitate the cleavage of the steroid ester bond and against water containing 2-mercaptoethanol (3 mM) until the radioactivity in the dialyzate had returned to background values.

For amino acid analysis, the dialyzed inactivated enzyme was lyophilized and acid hydrolysis was performed in 6N HCl containing 2-mercaptoethanol in evacuated sealed tubes at 110°C for 22 h. The HCl was

evaporated under vacuum and the sample was dissolved in 10 mM HCl and centrifuged prior to amino acid analysis. Standard carboxymethyl-L-histidine and S-carboxymethyl-L-cysteine were added to the hydrolysis products obtained from 5 nmol of inactivated enzyme and amino acid analysis of this mixture was performed with a Waters cation exchange amino acid analysis column (P/W 80002, sulfonated styrene-divinylbenzene, sodium form particle) connected to a Varian 5400 liquid chromatograph and a Varian UV-visible 100 detector. The eluting gradient was 0.2 M sodium citrate, pH 3.27 (from Pierce concentrate) to 1M, pH 7.4 (Varian #2 sodium buffer). The ninhydrin solution was prepared from Pierce lithium acetate buffer. The analyzer effluent was collected at 0.2 min intervals following the ninhydrin reaction and counted in 10 ml of ACS aqueous counting scintillant. The counts were corrected for ninhydrin quenching.

For peptide mapping, the inactivated enzyme solution was dialyzed against 8 M urea (previously treated with mixed bed resin) in 0.01 M Tris-HCl, pH 8.0, 3 mM 2-mercaptoethanol overnight. The resulting denatured enzyme solution was flushed with nitrogen for 20 min. Iodoacetic acid (1×10^{-5} mol) in 1N NaOH (20 µl) was added and the reaction was left under nitrogen for 80 min, in the dark. The sample was extensively dialyzed against water containing 3 mM 2-mercapto-ethanol and lyophilized. The carboxymethylated enzyme was treated with 4% (w/w) chymotrypsin in 0.1 M NH_4HCO_3 (1 ml) for 16 h at 37°C. After lyophilization, the chymotryptic digest was dissolved in 120 µl of pH 2.1 buffer (acetic acid : formic acid : water, 64 : 16 : 720, v/v/v) and centrifuged to remove the insoluble material. Chymotryptic digest (70 µl) and the tracking dye N-epsilon-mono-dinitrophenyl-L-lysine monochloride were applied on a cellulose sheet. Electrophoresis was performed for 1 h at 450 volts. The cellulose sheet was dried and the elution in the second dimension was done in isobutanol : acetic acid : water : pyridine, 60 : 12 : 48 : 40. Peptides were located by autoradiography.

21. Performic Acid Oxidation.

Performic acid was prepared by mixing 5 volumes of 30% H_2O_2 with 95 volumes of 99% formic acid and incubating the mixture at 25°C for 2 h. Oxidation of amino acids was done by adding performic acid (250 µl) to a solution of amino acids in 99% formic acid (125 µl) and incubating at 37°C for 3 h. The formic acid was evaporated under vacuum and amino acids analysis was performed as previously described.

22. Stoichiometry of ^{14}C-Carboxymethyl Incorporation.

Enzyme (10 nmol) was inactivated with 19-iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33** under the conditions described in section B.20. The reaction was stopped at time intervals and dialyzed against water until the radioactivity in the dialyzate had returned to background values. A control was done by incubating the steroid **33** with 2-mercaptoethanol for 1 h at 25°C before adding the enzyme. After the addition of the enzyme, the incubation was continued for 3 h at 25°C. The dialyzed inactivated enzyme was lyophilized and dissolved in 70% formic acid. The radioactivity and protein concentration were determined.

C. Preparation of Steroid Derivatives of Part I.

1. Preparation of 19-Hydroxy-5β-androstane-3,17-dione **14**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (2.00 g, 6.62 x 10^{-3} mol) was dissolved in 40 ml of 95% ethanol and was added to a solution of 10% palladium on charcoal (0.59 g) in 95% ethanol (5 ml) previously saturated with hydrogen. The reaction was stirred under an atmosphere of hydrogen at room

temperature. After 3 h, the intake of hydrogen stopped. The solution was filtered and concentrated under reduce pressure to a clear oil. The product could be crystallized in methanol but it was still impure. Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded 3α-ethoxy-3β,19-oxido-5α-androstan-17-one **26** (6%). mp 137-138°C (lit.[69] mp 137-138°C); IR γ_{max} 2940, 2870, 1735 (17-CO) cm^{-1}; ^1H NMR δ 0.80 (s, 3H, 18-CH_3), 1.15 (tr, 3H, $OCH_2\underline{CH_3}$), 3.61 (q, 2H, $O\underline{CH_2}CH_3$), 4.01 (q, 2H, δ_a 3.87, δ_b 4.15, J_{ab}=9 Hz, 19-CH_2); ^{13}C NMR δ 13.6 (CH_3, C-18), 15.6 (CH_3, $OCH_2\underline{CH_3}$), 21.0 (CH_2, C-11), 21.6 (CH_2, C-15), 29.9 (CH_2, [C-4]), 30.0 (CH_2, C-6), 30.6 (CH_2, C-7), 31.5 (CH_2, C-12), 31.7 (CH_2, [C-2]), 33.8 (C, C-10), 35.5 (CH, C-8), 35.7 (CH_2, C-16), 38.8 (CH_2, [C-1]), 39.3 (CH, [C-9]), 47.5 (C, C-13), 49.2 (CH, [C-5]), 51.4 (CH, C-14), 57.1 (CH_2, $O\underline{CH_2}CH_3$), 67.0 (CH_2, C-19), 97.9 (C, C-3), 220.7 (C, C-17); MS m/z 332 (M^+), 317 (M-15, CH_3), 303 (M-29, CH_2CH_3), 287 (M-45, OCH_2CH_3); HRMS calcd for $C_{21}H_{32}O_3$: 332.2343, found: 332.2331. Anal. Calcd for $C_{21}H_{32}O_3$: C, 75.85; H, 9.71. Found: C, 76.06; H, 9.63. The column was then eluted with methylene chloride : ethyl acetate, 70 : 30 and with methylene chloride : ethyl acetate, 50 : 50 to afford 19-hydroxy-5β-androstane-3,17-dione **14** (84%). mp 201-204°C (large transparent crystals from slow evaporation of methylene chloride) (lit.[69] mp 208-209°C); IR γ_{max} 3630 (sharp, OH), 3460 (broad, OH), 2940, 2860, 1735 (17-CO), 1710 (3-CO) cm^{-1}; C.D. λ_{max}= 300 nm, $\Delta\varepsilon$ = +5.8 ; ^1H NMR δ 0.86 (s, 3H, 18-CH_3), 3.81 (q, 2H, δ_a 3.68, δ_b 3.94,

J_{ab}=10.8 Hz, 19-CH_2); ^{13}C NMR δ 13.7 (CH_3, C-18), 20.4 (CH_2, C-11), 21.6 (CH_2, C-15), 24.3 (CH_2, C-6), 25.8 (CH_2, C-7), 30.6 (CH_2, [C-1]), 31.8 (CH_2, C-12), 35.0 (CH, C-8), 35.7 (CH_2, C-16), 36.1 (CH, [C-5]), 36.7 (CH_2, [C-2]), 39.0 (C, C-10), 41.1, (CH, [C-9]), 41.4 (CH_2, C-4), 47.7 (C, C-13), 51.7 (CH, C-14), 64.5 (CH_2, C-19), 212.8 (C, C-3), 220.7 (C, C-17); MS m/z 304 (M^+), 289 (M-15, CH_3), 286 (M-18, H_2O), 274 (M-30, CHOH).

2. Preparation of 17β,19-Dihydroxyandrost-4-en-3-one **19**.

To a cooled solution (0-4°C, ice-water bath) of 19-hydroxyandrost-4-ene-3,17-dione **4** (1.14 g, 3.77 x 10^{-3} mol) in methanol (200 ml) sodium borohydride (284 mg, 7.5 x 10^{-3} mol) was added. After stirring for 1 h at 0-4°C, the reaction was neutralized with acetic acid. Following the work up, 17β,19-dihydroxyandrost-4-en-3-one **19** , slightly contaminated with starting material **4**, was isolated (1.18 g) and characterized as its diacetate. The acetylation was performed with acetic anhydride (Method A) and column chromatography (using Davisil silica gel) eluted with ether afforded 17β,19-diacetoxyandrost-4-en-3-one **172** (807 mg, 55%) as a clear oil. ^{1}H NMR δ 0.81 (s,3H, 18-CH_3), 1.98 (s, 3H, $COCH_3$), 2.02 (s, 3H, $CO\underline{CH_3}$), 4.39 (q, 2H, δ_a 4.14, δ_b 4.64, J_{ab}= 11.2 Hz, 19-CH_2), 4.56 (tr, 1H, 17α-H), 5.89 (s, 1H, 4-H); ^{13}C NMR δ 12.0 (CH_3, C-18), 20.9 (CH_3, $CO\underline{CH_3}$), 20.9 (CH_2, C-11), 21.0 (CH_3, $CO\underline{CH_3}$), 23.3 (CH_2, C-15), 27.3 (CH_2, C-16), 31.6 (CH_2, C-7), 32.9* (CH_2, C-6), 33.5* (CH_2, C-1), 34.5

(CH_2, C-2), 35.9 (CH, C-8), 36.8 (CH_2, C-12), 41.7 (C, C-10), 42.4 (C, C-13), 50.5 (CH, C-14), 53.9 (CH, C-9), 66.7 (CH_2, C-19), 82.1 (CH, C-17), 126.7 (CH, C-4), 165.4 (C, C-5), 170.6 (C, $\underline{C}OCH_3$). 171.0 (C, $\underline{C}OCH_3$). 199.2 (C, C-3); MS m/z 388 (M^+), 346 (M-42, $COCH_3$), 328 (M-60), 316 (M-72).

3. Preparation of 17β,19-Dihydroxy-5β-androstan-3-one **24**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (2.00 g, 6.6 x10^{-3} mol) was treated with sodium borohydride as previously described in part C.2. The crude product, 17β,19-dihydroxyandrost-4-en-3-one was hydrogenated as described in part C.1. Column chromatography eluted with methylene chloride : ethyl acetate, 60 : 40 afforded 17β,19-dihydroxy-5β-androstan-3-one **24** (1.31 g, 65%). mp 145°C (recrystallized from ethyl acetate) (lit.[69] mp 183-185°C); $[\alpha]_D$ +25° (c 0.24) (lit.[69] $[\alpha]_D$ +26°); IR γ_{max} 3620 (OH), 3360 br (OH), 2940, 2860, 1710 (3-CO) cm^{-1}; ^1H NMR δ 0.73 (s, 3H, 18-CH_3), 3.64 (tr, 1H, 17α-H), 3.80 (q, 2H, δ_a 3.65, δ_b 3.95, J_{ab}=10.7 Hz, 19-CH_2); MS m/z 306 (M^+), 288 (M-18, H_2O), 276 (M-30, HOCH); HRMS calcd for $C_{19}H_{30}O_3$: 306.2187, found: 306.2201.

4. Preparation of 17β-Hydroxy-5β-androstan-3-one **12**.

17β-Hydroxyandrost-4-en-3-one **5** (3.00 g, 1.04 x 10^{-2} mol) in pyridine (10 ml) was added to a suspension of palladium on charcoal (10%) (0.31 g) in pyridine (5 ml) previously saturated with hydrogen. After stirring for 7 h at

room temperature under hydrogen, the intake of hydrogen stopped. Work up afforded 17β-hydroxy-5β-androstan-3-one **12** (89%). mp 141°C (needles from acetone : hexane, 1 : 1) (lit.[162] mp 142-142.5°C); IR γ_{max} 3600 (sharp, OH), 3450 (br, OH), 2940, 2870, 1705 (3-CO) cm^{-1}; MS m/z 290 (M^+), 275 (M-15, CH_3), 272 (M-18, H_2O); HRMS calcd for $C_{19}H_{30}O_2$: 290.2238, found: 290.2239.

5. Preparation of 3β,19-Dihydroxy-5β-androstan-17-one **15** and 3α,19-Dihydroxy-5β-androstan-17-one **23**.

To a cooled solution (0-4°C, ice-water bath) of 19-hydroxy-5β-androstane-3,17-dione **14** (0.20 g, 6.58 x 10^{-4} mol) sodium borohydride (25 mg) was added. The reaction was terminated after 2 h 45 min by adding a few drops of acetic acid and water. The products were extracted with methylene chloride and purified by column chromatography. Elution with methylene chloride : ethyl acetate, 60 : 40 afforded 3β,19-dihydroxy-5β-androstan-17-one **15** (73 mg, 36%). mp 179-180°C (recrystallized in ethyl acetate); $[\alpha]_D$ +148° (c 0.23, methanol); IR γ_{max} 2840, 2960, 1730 (17-CO) cm^{-1}; ^1H NMR δ 0.82 (s, 3H, 18-CH_3), 3.70 (q, 2H, δ_a 3.54, δ_b 3.86, J_{ab}=10.9 Hz, 19-CH_2), 3.61 (m, 1H, w/2 *ca.* 7.6 Hz); MS m/z 306 (M^+), 288 (M-18, H_2O), 276 (M-30, HOCH); HRMS calcd for $C_{19}H_{30}O_3$: 306.2187, found: 306.2167. Further elution with methylene chloride : ethyl acetate, 50 : 50 afforded 3α,19-dihydroxy-5β-androstan-17-one **23** (45 mg, 23%). mp 190-191°C (recrystallized in ethyl acetate); $[\alpha]_D$ +98° (c 0.15, methanol); IR γ_{max} 2940, 2860, 1730 (17-CO) cm^{-1}; ^1H NMR δ 0.84 (s, 3H, 18-CH_3), 3.72 (q, 2H, δ_a 3.56, δ_b 3.89,

J_{ab}=10.9 Hz, 19-CH_2), 3.61 (m, 1H, w/2 *ca.* 16.7 Hz); MS m/z 306 (M^+), 288 (M-18, H_2O), 276 (M-30, HOCH); HRMS calcd for $C_{19}H_{30}O_3$: 306.2187, found: 306.2160.

6. Preparation of 19-Iodoacetoxyandrost-4-ene-3,17-dione **29**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (0.20 g, 6.74 x 10^{-4} mol) was treated with iodoacetic acid (0.20 g, 1.1 x 10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded 19-iodoacetoxyandrost-4-ene-3,17-dione **29** (63%). mp 127°C (colorless or light yellow plates from methylene chloride : ether); $[\alpha]_D$ +162° (c 0.21); UV λ_{max} 238 nm (ε 9 139); IR γ_{max} 2940, 2860, 1735 (17-CO and 19-$\underline{CO}CH_2I$), 1670 (3-CO), 1625 (sh) cm^{-1}; ^1H NMR δ 0.91 (s, 3H, 18-CH_3), 3.62 (s, 2H, 19-CO$\underline{CH_2}$I), 4.46 (q, 2H, δ_a 4.23, δ_b 4.69, J_{ab}=11.3 Hz, 19-CH_2), 5.93 (s, 1H, 4-H); MS m/z 470 (M^+), 343 (M-127, I), 301 (M-169, $COCH_2I$ + H), 284 (M-186), 271 (M-199, 169 + CHOH); HRMS calcd for $C_{21}H_{27}O_4I$: 470.0947, found: 470.0969.

7. Preparation of 19-Bromoacetoxyandrost-4-ene-3,17-dione **30**.

19-Hydroxyandrost-4-en-3,17-dione **4** (0.20 g, 6.62 x 10^{-4} mol) was treated with bromoacetic acid (0.20 g, 1.44 x 10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded

19-bromoacetoxyandrost-4-ene-3,17-dione **30** (96%) as a clear oil. $[\alpha]_D$ +164° (c 0.26); UV λ_{max} 238 nm, (ε 9 009); IR γ_{max} 2940, 2860, 1735 (17-CO and 19-COCH$_2$Br), 1665 (3-CO), 1620 (sh) cm^{-1}; ^{1}H NMR δ 0.91 (s, 3H, 18-CH$_3$), 3.76 (s, 2H, 19-COCH$_2$Br), 4.50 (q, 2H, δ_a 4.26, δ_b 4.74, J_{ab}=11.3 Hz, 19-CH$_2$), 5.93 (s, 1H,4-H); MS m/z 424/422 (M^+, ^{81}Br-^{79}Br), 284, 271 (M-151, CHOCOCH$_2$Br); HRMS calcd for $C_{21}H_{27}O_4Br$: 422.1085, found: 422.1086.

8. Preparation of 19-Acetoxyandrost-4-ene-3,17-dione **31**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (0.10 g, 3.31 x 10^{-4} mol) was treated with acetic anhydride (Method A). Work up afforded 19-acetoxy-androst-4-ene-3,17-dione **31** (100%) as a clear oil. This compound was previously crystallized by Edwards and co-workers[69]. ^{1}H NMR δ 0.90 (s, 3H, 18-CH$_3$), 2.00 (s, 3H, 19-COCH$_3$), 4.41 (d, 2H, δ_a 4.15, δ_b 4.66, J_{ab}=11.3 Hz, 19-CH$_2$), 5.91 (s, 1H, 4-H); ^{13}C NMR δ 13.9 (CH$_3$, C-18), 20.9 (CH$_3$, COCH$_3$), 21.0 (CH$_2$, C-11), 21.7 (CH$_2$, C-15), 31.0 (CH$_2$, C-12), 31.6 (CH$_2$, C-7), 32.9* (CH$_2$, C-6), 33.6* (CH$_2$, C-1), 34,6 (CH$_2$, C-2), 35.7 (CH$_2$, C-16), 35.8 (CH, C-8), 41.9 (C, C-10), 47.5 (C, C-13), 51.2 (CH, C-14), 54.1 (CH, C-9), 66.6 (CH$_2$, C-19), 126.9 (CH, C-4), 164.6 (C, C-5), 170.4 (C, COCH$_3$), 198.8 (C, C-3), 219.5 (C, C-17); MS m/z 344 (M^+), 302 (M-42, COCH$_3$),272 (M-73, CHOCOCH$_3$).

9. Preparation of 19-Iodo[2'-^{3}H]acetoxyandrost-4-ene-3,17-dione **32**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (10 mg, 3.31 x 10^{-5} mol) was treated with iodoacetic acid (6 mg, 3.33 x 10^{-5} mol) and iodo[2-^{3}H]acetic acid (5 mCi) (Method C). Purification by column chromatography using chloroform : ether, 1 : 1 as eluant afforded 19-iodo[2'-^{3}H]acetoxyandrost-4-ene- 3,17-dione **32** (64%, sp. radioactivity 45 mCi/mmol). MS m/z 470 (M^{+}).

10. Preparation of 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (4 mg, 1.32 x 10^{-5} mol) was treated with iodo[2-^{14}C]acetic acid (200 μCi) and with iodoacetic acid (0.8 mg, 3.8 x 10^{-6} mol) (Method C). Preparative tlc eluted with methylene chloride : ethyl acetate, 60 : 40 afforded 19-iodo[2-^{14}C]acetoxyandrost-4-ene-3,17-dione **33** (41%, sp. radioactivity 13 mCi/mmol). MS m/z 472/470 (M^{+}, ^{14}C-^{12}C), 404/402 (M-68).

11. Preparation of 19-Iodoacetoxy[6,7-^{3}H]androst-4-ene-3,17-dione **29** [^{3}H].

19-Hydroxy[6,7-^{3}H]androst-4-ene-3,17-dione **4** [^{3}H] (100 μCi) and non-radioactive steroid (1.66 x 10^{-5} mol) were treated with iodoacetic acid (1.6 x 10^{-5} mol) (Method C). Purification by column chromatography using chloroform : ether, 50 : 50 as eluant afforded 19-iodoacetoxy[6,7-^{3}H]androst-4-ene-3,17-dione **29** [^{3}H] (75%, sp. radioactivity 32 mCi/mmol). m/z 470 (M^{+}).

12. Preparation of 19-Iodoacetoxy-5β-androstane-3,17-dione **34**.

19-Hydroxy-5β-androstane-3,17-dione **14** (203 mg, 6.68 x 10^{-4} mol) was treated with iodoacetic acid (200 mg, 1.07 x 10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded 19-iodoacetoxy-5β-androstane-3,17-dione **34** (71 mg, 23%) as a light yellow oil. $[\alpha]_D$ +59° (c 0.37); IR γ_{max} 2930, 2860, 1730 (17-CO and $\underline{CO}CH_2I$), 1710 (sh, 3-CO) cm^{-1}; ^1H NMR δ 0.88 (s, 3H, 18-CH_3), 3.69 (d, 2H, J=1.9 Hz, 19-$COCH_2I$), 4.49 (q, 2H, δ_a 4.45, δ_b 4.52, J_{ab}=12.7 Hz, 19-CH_2); MS m/z 472 (M^+), 454 (M-18, H_2O), 345 (M-127, I), 327 (M-145), 304; HRMS calcd for $C_{21}H_{29}O_4I$: 472.1102, found: 472.1109.

13. Preparation of 19-Bromoacetoxy-5β-androstane-3,17-dione **35**.

19-Hydroxy-5β-androstane-3,17-dione **14** (201 mg, 6.66 x 10^{-4} mol) was treated with bromoacetic acid (200 mg, 1.4 x 10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded 19-bromoacetoxy-5β-androstane-3,17-dione **35** (245 mg, 96%) as a clear oil. IR γ_{max} 2640, 2879, 1740 (17-CO and 19-$\underline{CO}CH_2Br$), 1715 (3-CO) cm^{-1}; ^1H NMR δ 0.88 (s,3H, 18-CH_3), 3.83 (d, 2H, J=2.9 Hz, $COCH_2Br$), 4.35 (q, 2H, δ_a 4.20, δ_b 4.51, J_{ab}=11.4 Hz, 19-CH_2); MS m/z 426/424 (M^+, ^{81}Br-^{79}Br); HRMS calcd for $C_{21}H_{29}O_4Br$: 424.1241, found: 424.1265.

14. Preparation of 19-Acetoxy-5β-androstane-3,17-dione **36**.

19-Hydroxy-5β-androstane-3,17-dione **14** (88 mg, 2.95 x 10^{-4} mol) was treated with acetic anhydride (Method A). Work up afforded 19-acetoxy-5β-androstane-3,17-dione **36** (100%) as a clear oil (lit.[69] mp 165-167°C, crystallized with methanol); $[\alpha]_D$ +72° (c 0.18); IR γ_{max} 2940, 2860, 1735 (17-CO and 19-$\underline{CO}CH_3$), 1710 (sh, 3-CO); ^{1}H NMR δ 0.87 (s, 3H, 18-CH_3), 2.06 (s, 3H, 19-$COCH_3$), 4.25 (q, 2H, δ_a 4.09, δ_b 4.41, J_{ab}=11.2 Hz, 19-CH_2); MS m/z 346 (M^+), 273 (M-73, CH_2OCOCH_3); HRMS calcd for $C_{21}H_{30}O_4$: 346.2136, found: 346.2144.

15. Preparation of 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37**.

19-Hydroxy-5β-androstane-3,17-dione **14** (5 mg, 1.64 x 10^{-5} mol) was treated with iodo[2-^{14}C]acetic acid (50 μCi) and with iodoacetic acid (0.9 mg, 4.83 x 10^{-6} mol) (Method C). Preparative tlc eluted with methylene chloride : ethyl acetate, 90 : 10 afforded 19-iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37** (4%, sp. radioactivity 50 mCi/mmol). MS m/z 474/472 (M^+, ^{14}C-^{12}C), 347/345 (M-127, I).

16. Preparation of 6β-Iodoacetoxyandrost-4-ene-3,17-dione **38**.

6β-Hydroxyandrost-4-ene-3,17-dione **10** (20 mg, 6.62 x 10^{-5} mol) was treated with iodoacetic acid (20 mg, 1.07 x 10^{-4} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 85 : 15 as eluant afforded 6β-iodoacetoxyandrost-4-ene-3,17-dione **38** (18 mg, 57%). mp 160-161°C (small crystals from ether : hexane); $[\alpha]_D$ +43° (c 0.09); UV λ_{max} 233 nm (ε 15 897); IR γ_{max} 2950, 2860, 1735 (17-CO and 6β-$\underline{CO}CH_2I$), 1680 (3-CO) cm^{-1}; ^1H NMR δ 0.94 (s, 3H, 18-CH_3), 1.34 (s, 3H, 19-CH_3), 3.67 (q, 2H, δ_a 3.63, δ_b 3.72, J_{ab}=10.5 Hz, 6β-$COCH_2I$), 5.45 (tr, 1H, 6α-H), 5.94 (s, 1H, 4-H); MS m/z 470 (M^+), 344 (M-126, I), 302 (M-168, $COCH_2I$); HRMS calcd for $C_{21}H_{27}O_4I$: 470.0947, found: 470.0999.

17. Preparation of 6β-Acetoxyandrost-4-ene-3,17-dione **39**.

6β-Hydroxyandrost-4-ene-3,17-dione **10** (10 mg, 3.31 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded 6β-acetoxy-androst-4-ene-3,17-dione **39** (100%). mp 195-196°C, decomposes (lit.[73] mp 200-204°C); UV λ_{max} 234 nm (ε 11 914); IR γ_{max} 2950, 2920, 2860, 1735 (17-CO and 6β-$\underline{CO}CH_3$), 1675 (3-CO) cm^{-1}; ^1H NMR δ 0.94 (s, 3H, 18-CH_3), 1.29 (s, 3H, 19-CH_3), 2.05 (s, 3H, 6β-$COCH_3$), 5.46 (tr, 1H, 6α-H), 5.95 (s,1H, 4-H); MS m/z 344 (M^+), 302 (M-42, $COCH_3$), 287 (M-57).

18. Preparation of 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **40**.

6β-Hydroxyandrost-4-ene-3,17-dione **10** (5 mg, 1.65×10^{-5} mol) was treated with iodo[2-^{14}C]acetic acid (50 μCi) (Method C). Column chromatography eluted with methylene chloride : ethyl acetate, 85 : 15 afforded 6β-iodo-[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **40** (22%, sp. radioactivity 56 Ci/mmol). MS m/z 472/470 (M^+, ^{14}C-^{12}C).

19. Preparation of 11β-Iodoacetoxyandrost-4-ene-3,17-dione **41**.

11β-Hydroxyandrost-4-ene-3,17-dione **8** (20 mg, 6.65×10^{-5} mol) was treated with iodoacetic acid (20 mg, 1.07×10^{-4} mol) (Method B). Column chromatography eluted with methylene chloride : ethyl acetate, 75 : 25 afforded 11β-iodoacetoxyandrost-4-ene-3,17-dione **41** (7 mg, 21%). mp 168°C (crystals from ether : methylene chloride); $[\alpha]_D$ +132° (c 0.12); IR γ_{max} 2940, 2860, 1740 (17-CO and 11β-COCH$_2$I), 1665 (3-CO) cm^{-1}; ^{1}H NMR δ 1.08 (s, 3H, 18-CH$_3$), 1.31 (s, 3H, 19-CH$_3$), 3.65 (s, 2H, 11β-COCH$_2$I), 5.51 (br d, 1H, J=2.9 Hz, 11α-H), 5.69 (s, 1H, 4-H); MS m/z 470 (M^+), 302 (M-168, COCH$_2$I), 284 (302-18, H$_2$O); HRMS calcd for $C_{21}H_{27}O_4I$: 470.0947, found: 470.0969.

20. Preparation of 11β-Acetoxyandrost-4-ene-3,17-dione **42**.

11β-Hydroxyandrost-4-ene-3,17-dione **8** (10 mg, 3.31 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up followed by column chromatography afforded 11β-acetoxyandrost-4-ene-3,17-dione **42** (8 mg, 67%). mp 190°C (lit.[74] mp 193-194°C); IR γ_{max} 2980-2940, 1740 (17-CO and 11β-$\underline{CO}CH_3$), 1670 and 1640 (3-CO) cm^{-1}; ^1H NMR δ 1.03 (s, 3H, 18-CH_3) 1.27 (s,3H, 19-CH_3), 2.03 (s, 3H, 11β-$COCH_3$), 5.47 (br d, 1H, J=3.1 Hz, 11α-H), 5.69 (s, 1H, 4-H); MS m/z 344 (M^+), 302 (M-42, $COCH_3$).

21. Preparation of 17β-Iodoacetoxyandrost-4-en-3-one **44**.

17β-Hydroxyandrost-4-en-3-one **5** (0.50 g, 1.7 x 10^{-3} mol) was treated with iodoacetic acid (522 mg, 2.8 x 10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 afforded 17β-iodoacetoxyandrost-4-en-3-one **44** (0.40 g, 51%). mp 138-139°C (crystals in ether : petroleum ether); $[\alpha]_D$ +82° (c 0.27); UV λ_{max} 240 nm (ε 15 960); IR γ_{max} 2950, 1730 (17-CO and $\underline{CO}CH_2I$), 1665 (3-CO),1620 cm^{-1}; ^1H NMR δ 0.86 (s, 3H, 18-CH_3), 1.17 (s,3H, 19-CH_3), 3.67 (d, 2H, J=5.5 Hz, 17β-$COCH_2I$), 4.64 (tr, 1H, 17α-H), 5.71 (s, 1H, 4-H); MS m/z 456 (M^+), 441 (M-15, CH_3), 414 (M-42), 371 (M-85), 288 (M-168, $COCH_2I$); HRMS calcd for $C_{21}H_{29}O_3I$: 456.1153, found: 456.1140.

22. Preparation of 17β-Bromoacetoxyandrost-4-en-3-one **45**.

17β-Hydroxyandrost-4-en-3-one **5** (0.50 g, 1.7 x 10^{-3} mol) was treated with bromoacetic acid (0.50 g, 3.6 x 10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded 17β-bromoacetoxyandrost-4-en-3-one **45** (497 mg, 70%). mp 137°C (crystals from acetone : hexane) (lit.[75] mp 142°C); $[\alpha]_D$ +87° (c 0.23); UV λ_{max} 239 nm (ε 11 016); IR γ_{max} 2940, 2860, 1730 (17-CO and $\underline{CO}CH_2Br$), 1660 (3-CO), 1610 cm^{-1}; ^1H NMR δ 0.85 (s, 3H, 18-CH_3), 1.17 (s, 3H, 19-CH_3), 3.81 (d, 2H, J=1.6 Hz, 17β-$COCH_2Br$), 4.66 (tr, 1H, 17α-H), 5.71 (s, 1H, 4-H); MS m/z 410/408 (M^+, ^{81}Br-^{79}Br), 368/366 (M-42), 325/323 (M-85); HRMS calcd for $C_{21}H_{29}O_3Br$: 408.1292, found: 408.1305.

23. Preparation of 17β-Acetoxyandrost-4-en-3-one **46**.

17β-Hydroxyandrost-4-en-3-one **5** (5.00 g, 1.74 x 10^{-2} mol) in pyridine (50 ml) was treated with acetic anhydride (100 ml) (Method A). Work up afforded 17β-acetoxyandrost-4-en-3-one **46** (5.56 g, 97%). mp 138-139°C (lit.[76] mp 138.5-140°C); $[\alpha]_D$ +88° (c 0.6); ^1H NMR δ 0.82 (s,3H, 18-CH_3), 1.17 (s,3H, 19-CH_3), 2.02 (s,3H, 17β-$COCH_3$), 4.58 (tr, 1H, 17α-H), 5.71 (s, 1H, 4-H); ^{13}C NMR δ 11.9 (CH_3, C-18), 17.3 (CH_3, C-19), 20.4 (CH_2, C-11), 21.1 (CH_3, 17β-$CO\underline{CH_3}$), 23.4 (CH_2, C-15), 27.4 (CH_2, C-16), 31.4 (CH_2, C-7), 32.7 (CH_2,

C-6), 33.8 (CH_2, C-2), 35.3(CH, C-8), 35.6 (CH_2, C-1), 36.5 (CH_2, C-12), 38.5 (C, C-10), 42.4 (C, C-13), 50.1 (CH, C-14), 53.6 (CH, C-9), 82.4 (CH, C-17), 123.8 (CH, C-4), 170.9 (C,17- $\underline{CO}CH_3$), 171.0 (C, C-5), 199.4 (C, C-3); MS m/z 330 (M^+), 288 (M-42).

24. Preparation of 17β-Iodo[2'-^{14}C]acetoxyandrost-4-en-3-one **47**.

17β-Hydroxyandrost-4-en-3-one **5** (5 mg, 1.74 x 10^{-5} mol) was treated with iodo[2-^{14}C]acetic acid (52 μCi) and iodoacetic acid (1.5 mg, 8.06 x 10^{-6} mol) (Method C). Preparative tlc eluted with methylene chloride : ethyl acetate, 97 : 3 afforded 17β-iodo[2'-^{14}C]acetoxyandrost-4-en-3-one **47** (2%, sp. radioactivity 14 mCi/mmol). MS m/z 458/456 (M^+, ^{14}C-^{12}C), 416/414 (M-42).

25. Preparation of 17β-Iodoacetoxy-5β-androstan-3-one **48**.

17β-Hydroxy-5β-androstan-3-one **12** (210 mg, 7.24 x 10^{-4} mol) was treated with iodoacetic acid (200 mg, 1.07 x 10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded 17β-iodoacetoxy-5β-androstan-3-one **48** (248 mg, 75%). mp 112°C (needles from ether); $[\alpha]_D$ +28° (C 0.29); IR γ_{max} 2940, 2870, 1715 (3-CO and 17β-$\underline{CO}CH_2I$) cm^{-1}; ^{1}H NMR δ 0.83 (s, 3H, 18-CH_3), 1.01 (s, 3H, 19-CH_3), 3.67 (q, 2H, J=9.6 Hz, 17β-$COCH_2I$), 4.64 (tr, 1H. 17α-H); ^{13}C NMR δ -5.0 (17β-$CO\underline{CH_2}I$), 12.0 (C-18), 20.6 (C-11), 22.6 (C-19), 23.4 (C-15), 25.3 (C-6),

26.4 (C-7), 27.0 (C-16), 34.9 (C-10), 35.3 (C-8), 36.9 (C-12), 37.0* (C-2), 37.1* (C-1), 40.7 (C-9), 42.3 (C-13), 43.1 (C-4), 44.2 (C-5), 50.7 (C-14), 84.1 (C-17), 168.9 ($\underline{CO}CH_2I$), 213.0 (C-3); MS m/z 458 (M^+), 443 (M-15, CH_3), 289 (M-169, $COCH_2I$); HRMS calcd for $C_{21}H_{31}O_3I$: 458.1310, found: 458.1310.

26. Preparation of 17β-Bromoacetoxy-5β-androstan-3-one **49**.

17β-Hydroxy-5β-androstan-3-one **12** (203 mg, 7.0×10^{-4} mol) was treated with bromoacetic acid (215 mg, 1.55×10^{-3} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant afforded 17β-bromoacetoxy-5β-androstan-3-one **49** (86%). mp 111°C (crystals in ether : hexane); $[\alpha]_D$ +126° (c 0.33); IR γ_{max} 2940, 2860, 1730 (17β-$\underline{CO}CH_2Br$), 1710 (3-CO) cm^{-1}; ^1H NMR δ 0.82 (s, 3H, 18-CH_3), 1.01 (s, 3H, 19-CH_3), 3.81 (s, 2H, 17β-$COCH_2Br$), 4.67 (tr, 1H, 17α-H); MS m/z 412/410 (M^+, ^{81}Br-^{79}BR), 397/395 (M-15, CH_3), 342/340 (M-70), 290 (M-120, $COCH_2Br$), 272 (290-18, H_2O); HRMS calcd for $C_{21}H_{31}O_3Br$: 410.1448, found: 410.1468.

27. Preparation of 17β-Acetoxy-5β-androstan-3-one **50**.

17β-Hydroxy-5β-androstan-3-one **12** (100 mg, 3.45×10^{-4} mol) was treated with acetic anhydride (Method A). Work up afforded 17β-acetoxy-5β-androstan-3-one **50** (98%). mp 141-142°C (lit.[77] mp 142-144°C);

$[\alpha]_D$ +22° (c 0.24); IR γ_{max} 2940, 2860, 1730-1710 (17β-$\underline{CO}CH_3$ and 3-CO) cm^{-1}; ^1H NMR δ 0.79 (s, 3H, 18-CH_3), 1.01 (s, 3H, 19-CH_3), 2.02 (s, 3H, 17β-$COCH_3$), 4.59 (tr, 1H, 17α-H); MS m/z 332 (M^+), 317 (M-15, CH_3), 290(M-42, $COCH_3$), 272 (290-18, H_2O); HRMS calcd for $C_{21}H_{32}O_3$: 332.2343, found: 332.2319.

28. Preparation of 17β-Iodo[2-^{14}C]acetoxy-5β-androstan-3-one **51**.

17β-Hydroxy-5β-androstan-3-one **12** (5 mg, 1.72 x 10^{-5} mol) was treated with iodo[2-^{14}C]acetic acid (140 μCi) and iodoacetic acid (0.9 mg, 4.84 x 10^{-6} mol) (Method C). Preparative tlc eluted with methylene chloride : ethyl acetate, 97 : 3 afforded 17β-iodo[2'-^{14}C]acetoxy-5β-androstan-3-one **51** (6%, sp. radioactivity 46 mCi/mmol); MS m/z 460/458 (M^+, ^{14}C-^{12}C).

29. Preparation of 3-Iodoacetoxy-1,3,5(10)-estratrien-17-one **52**.

3-Hydroxy-1,3,5(10)-estratrien-17-one **16** (10 mg, 3.7 x 10^{-5} mol) was treated with iodoacetic acid (10 mg, 5.38 x 10^{-5} mol) (Method B). Column chromatography using methylene chloride : ethyl acetate, 95 : 5 as eluant afforded 3-iodoacetoxy-1,3,5(10)-estratrien-17-one **52** (40%). mp 145-146°C (needles in ether : hexane) (lit.[19] mp 134-135°C); $[\alpha]_D$ +112° (c 0.33); IR γ_{max} 2940, 2860, 1735 (3-$\underline{CO}CH_2I$ and 17-CO); ^1H NMR δ 0.89 (s, 3H, 18-CH_3), 3.87 (s, 2H. 3-$COCH_2I$), 6.82 (s, 1H, 4-H), 6.83 (d, 1H, J=8 Hz, 1-H), 7.29 (d, 1H, J=8 Hz,2-H);

MS m/z 438 (M^+), 270 (M-168, $COCH_2I$); HRMS calcd for $C_{20}H_{23}O_3I$: 438.0685, found: 438.0682.

30. Preparation of 3-Acetoxy-1,3,5(10)-estratrien-17-one **53**.

3-Hydroxy-1,3,5(10)-estratrien-17-one **16** (0.3 g, 1.1 x 10^{-3} mol) was treated with acetic anhydride (Method A). Work up afforded 3-acetoxy-1,3,5(10)-estratrien-17-one **53** (94%). mp 125-126°C (lit.[78] mp 125-127°C); ^1H NMR δ 0.89 (s, 1H, 18-CH_3), 2.27 (s, 3H, 3-$COCH_3$), 6.79 (s, 1H, 4-H), 6.83 (d, 1H, J=8.5 Hz, 1-H), 7.27 (d, 1H, J=8.5 Hz, 2-H).

31. Preparation of 3-Iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54**.

3-Hydroxy-1,3,5(10)-estratrien-17-one **16** (5 mg, 1.85 x 10^{-5} mol) was treated with iodo[2-^{14}C]acetic acid (187 μCi) and iodoacetic acid (1.1 mg, 5.9 x 10^{-6} mol) (Method C). Preparative tlc eluted with methylene chloride : ethyl acetate, 99 : 1 afforded 3-iodo[2'-^{14}C]acetoxy-1,3,5(10)-estratrien-17-one **54** (4%, sp. radioactivity 46 mCi/mmol). MS m/z 438 (M^+), 270 (M-168, $COCH_2I$).

D. Preparation of Steroid Derivatives of Part II.

1. Preparation of 4-Hydroxyandrost-4-ene-3,17-dione **6** and 4-Acetoxyandrost-4-ene-3,17-dione **56**.

Androst-4-ene-3,17-dione **18** (1.00 g, 3.50×10^{-3} mol) was dissolved in 40 ml of methanol and cooled in an ice-water bath. Ice-cold 20% sodium hydroxide (2 ml) and 30% hydrogen peroxide (3.4 ml) were added and the mixture was stirred for 30 min at 0-4°C. Then the mixture was left for 22 h at 5-7°C. The reaction mixture was added to ice-cold water. After 15 min, the product was filtered and washed with water. The product was a mixture of 4β,5-epoxy-5β-androstane-3,17-dione and 4α,5-epoxy-5α-androstane-3,17-dione (*ca.* 4:1, respectively, by ^{1}H NMR) (60%). ^{1}H NMR δ 0.88 (0.89) (s, 3H, 18-CH_3), 1.17 (s, 3H, 19-CH_3), 2.98 (3.04) (s, 1H, 4α-H (4β-H)); MS m/z 302 (M^+), 287 (M-15, CH_3), 284 (M-18, H_2O), 274 (M-28). The mixture of epoxides was then dissolved in a cooled solution of sulphuric acid in acetic acid (2 : 98) and the reaction was stirred at room temperature for 45 min. The reaction mixture was then poured in ice-cold water. The product was filtered and dried. Recrystallization of the product in ethyl acetate was not successful to remove the more polar impurities. Purification by column chromatography (using Davisil silica gel) eluted with hexane : ether, 50 : 50 afforded 4-hydroxyandrost-4-ene-3,17-dione **6** (27%). mp 202°C (lit.[83] mp 203.5-206°C, lit.[84] mp 199-202°C); ^{1}H NMR δ 0.90 (s, 3H, 18-CH_3), 1.18 (s, 3H, 19-CH_3), 6.07 (s, 1H, 4-OH); ^{13}C NMR δ 13.7 (CH_3, C-18), 17.1 (CH_3, C-19), 20.2 (CH_2, C-11), 21.7 (CH_2, C-15), 22.7 (CH_2, C-1), 29.8 (CH_2, C-12), 31.3 (CH_2, C-7), 31.7 (CH_2, C-6), 34.6 (CH_2, C-16), 34.7 (CH, C-8), 35.7 (C, C-10), 37.8 (CH_2, C-2), 47.4 (C, C-13),

50.9 (CH, C-14), 54.2 (CH, C-9), 139.0 (C, C-4), 141.3 (C, C-5), 193.4 (C, C-3), 220.6 (C, C-17); MS m/z 302 (M^+), 287 (M-15, CH_3), 274 (M-28), 260 (M-42); HRMS calcd for $C_{19}H_{26}O_3$: 302.1875, found: 302.1870. 4-Hydroxyandrost-4-ene-3,17-dione **6** (30 mg, 9.93 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded 4-acetoxyandrost-4-ene-3,17- dione[87] **56** (100%). ^1H NMR δ 0.90 (s, 3H, 18-CH_3), 1.25 (s, 3H, 19-CH_3), 2.22 (s, 3H, 4-$COCH_3$); ^{13}C NMR δ 13.7 (C-18), 17.6 (C-19), 20.3 (4-CO<u>CH</u>$_3$), 20.3 (C-11), 21.7 (C-15), 23.9 (C-1), 29.7 (C-12), 31.2 (C-7), 33.3 (C-6), 34.6 (C-16), 34.7 (C-8), 35.7 (C-10), 39.1 (C-2), 47.5 (C-13), 50.7 (C-14), 53.7 (C-9), 139.2 (C-4), 155.0 (C-5), 168.6 (4-<u>CO</u>CH_3), 190.5 (C-3), 220.3 (C-17); MS m/z 344 (M^+), 302 (M-24, $COCH_3$), 287, 274, 260.

2. <u>Preparation of 17β-Acetoxy-4-hydroxyandrost-4-en-3-one **61** and of 4,17β-Dihydroxyandrost-4-en-3-one 7</u>.

17β-Hydroxyandrost-4-en-3-one **5** (1.00 g, 3.47 x 10^{-3} mol) was first epoxidized with 30% hydrogen peroxide in alkaline methanol as previously described in section D.1. The resulting mixture of 4β,5β- and 4α,5α-epoxides (83%, *ca.* 6.8:1, by ^1H NMR) {^1H NMR δ 0.75 (0.76) (s, 3H, 18-CH_3), 1.14 (s, 3H, 19-CH_3), 2.96 (3.02) (s, 1H, 4α-H (4β-H)), 3.63 (tr, 1H, 17α-H)}; MS m/z 304 (M^+), 289 (M-15, CH_3), 286 (M-18, H_2O), 276 (M-28)} was treated with acetic anhydride (Method A). Treatment of the acetoxy derivative (0.30 g, 8.67 x 10^{-4} mol) with sulphuric acid in acetic acid for 47 min, as described in section D.1,

afforded 17β-acetoxy-4-hydroxyandrost-4-en-3-one **61** (254 mg, 85%). mp 192-193°C (lit[92] mp 194-196°C; lit[93] mp 184-186°C); ^{1}H NMR δ 0.81 (s, 3H, 18-CH_3), 1.16 (s, 3H, 19-CH_3), 2.03 (s, 3H, 17β-$COCH_3$), 4.58 (tr, 1H, 17α-H), 6.05 (s, 1H, exchanges with D_2O, 4-OH); MS m/z 346 (M^+), 331 (M-15, CH_3), 318 (M-28), 304 (M-42, $COCH_3$), 302. 17β-Acetoxy-4-hydroxyandrost-4-en-3-one **61** (99 mg, 2.86 x 10^{-4} mol) was dissolved in 95% ethanol (8 ml) and potassium carbonate (255 mg) was added. The reaction mixture was stirred at room temperature for 2 days. Work up and column chromatography eluted with methylene chloride : ethyl acetate, 80 : 20 afforded 4,17β-dihydroxyandrost-4-en-3-one **7** (16%). ^{1}H NMR δ 0.77 (s, 3H, 18-CH_3), 1.16 (s, 3H, 19-CH_3), 3.62 (tr, 1H, 17α-H), 6.05 (s, 1H, exchanges with D_2O, 4-OH); MS m/z 304 (M^+), 289 (M-15, CH_3), 286 (M-18, H_2O).

3. Preparation of 4,19-Dihydroxyandrost-4-ene-3,17-dione **63**.

i. Acid-catalyzed Opening of 19-Hydroxy-4β,5-epoxy-5β-androstan-3,17-dione **62**.

a. Preparation of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (5.00 g, 1.65 x 10^{-2} mol) was dissolved in methanol (150 ml) and the solution was cooled in an ice-water bath. Ice-cold 10% NaOH (18 ml) and ice-cold 30% H_2O_2 (30 ml) were added

to the stirred solution. After stirring 1 h 15 min at 0-4°C, the reaction was poured in water and extracted with methylene chloride. Evaporation of the solvent afforded 19-hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** which was recrystallized in methanol (4.20 g, 80%). mp 202°C (lit.[94] mp 201-203°C); IR γ_{max} 2945, 2860, 1735 (17-CO), 1710-1700 (3-CO); ^{1}H NMR δ 0.88 (s, 3H, 18-CH_3), 2.90 (s, 1H, 4α-H), 3.97 (q, 2H, δ_a 3.78, δ_b 4.15, J_{ab}=11 Hz, 19-CH_2); ^{13}C NMR δ 13.7 (CH_3, C-18), 21.4 (CH_2, C-11), 21.4 (CH_2, C-1), 21.6 (CH_2, C-15), 29.2 (CH_2, C-12), 30.0 (CH_2, C-6), 31.3 (CH_2, C-7), 32.1 (CH_2, C-2), 34.6 (CH, C-8), 35.6 (CH_2, C-16), 41.6 (C, C-10), 46.8 (CH, C-9), 47.6 (C, C-13), 51.0 (CH, C-14), 60.0 (CH, C-4), 64.8 (CH_2, C-19), 68.8 (C, C-5), 206.0 (C, C-3), 220.0 (C, C-17); MS m/z 318 (M^+), 300 (M-18, H_2O), 288 (M-30, HOCH), 272, 271.

b. Preparation and Treatment with Sulphuric Acid in Acetic Acid of 19-Acetoxy-4β,5-epoxy-5β-androstane-3,17-dione **77**.

19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** (2.00 g, 6.29 x 10^{-3} mol) was treated with acetic anhydride (Method A). Work up afforded 19-acetoxy-4β,5-epoxy-5β-androstane-3,17-dione **77** as an oil which was crystallized in methanol (2.15 g, 95%). mp 145-146°C; $[\alpha]_D$ +189° (c 0.43); IR γ_{max} 2940, 2860, 1735 (17-CO and 19-$\underline{CO}CH_3$), 1710-1700 sh (3-CO) cm^{-1}; ^{1}H NMR δ 0.87 (s, 3H, 18-CH_3), 2.11 (s, 3H, 19-$COCH_3$), 2.90 (s, 1H, 4α-H), 4.40

(q, 2H, δ_a 4.22, δ_b 4.57, J_{ab}=11.5 Hz, 19-CH_2); ^{13}C NMR δ 13.7 (CH_3, C-18), 21.0 (CH_3, CO$\underline{CH_3}$), 21.4 (CH_2, C-11), 21.5 (CH_2, C-15), 21.6 (CH_2, C-1), 29.2 (CH_2, C-12), 29.6 (CH_2, C-6), 31.2 (CH_2, C-7), 31.8 (CH_2, C-2), 34.6 (CH, C-8), 35.5 (CH_2, C-16), 40.8 (C, C-10), 46.6 (CH, C-9), 47.6 (C, C-13), 51.1 (CH, C-14), 60.3 (CH, C-4), 65.3 (CH_2, C-19), 68.3 (C, C-5), 171.0 (C, $\underline{CO}CH_3$), 205.5 (C, C-3), 219.5 (C, C-17); MS m/z 360 (M^+), 318 (M-42, CH_3CO); HRMS calcd for $C_{21}H_{28}O_5$: 360.1929, found: 360.1911. 19-Acetoxy-4β,5-epoxy-5β-androstane **77** (0.82 g, 2.27 x 10^{-3} mol) was treated for 10 min with a cooled mixture of sulphuric acid and acetic acid (2 : 98, 19 ml). Work up and column chromatography (Davisil silica gel) eluted with hexane : ether, 50 : 50 afforded 2α,19-diacetoxyandrost-4-ene-3,17-dione **78** (402 mg, 44%). mp 140-141°C (needles from ether) (lit.[94] mp 141-142°C); ^{1}H NMR δ 0.89 (s, 3H, 18-CH_3), 2.05 (s, 3H) and 2.14 (s, 3H) 2α- and 19-$COCH_3$; 4.46 (q, 2H, δ_a 4.22, δ_b 4.71, J_{ab}=11.4 Hz, 19-CH_2), 5.74 (q, 1H, J=6.6 Hz, J=13.6 Hz, 2β-H), 5.93 (s, 1H, 4-H); ^{13}C NMR δ 13.7 (CH_3, C-18), 20.5 (CH_2, C-11), 20.8 (CH_3, CO$\underline{CH_3}$), 20.9 (CH_3, CO$\underline{CH_3}$), 21.5 (CH_2, C-15), 30.8 (CH_2, C-12), 31.4 (CH_2, C-7), 32.1 (CH_2, C-6), 35.3 (CH, C-8), 35.5 (CH_2, C-16), 39.7 (CH_2, C-1), 44.2 (C, C-10), 47.3 (C, C-13), 51.0 (CH, C-14), 54.9 (CH, C-9), 66.3 (CH_2, C-19), 70.9 (CH, C-2), 125.1 (CH, C-4), 164.4 (C, C-5), 170.1 (C, $\underline{CO}CH_3$), 170.6 (C, $\underline{CO}CH_3$), 193.6 (C, C-3), 219.5 (C, C-17); MS m/z 402 (M^+), 360 (M-42, $\overline{CO}CH_3$); HRMS calcd for $C_{23}H_{30}O_6$: 402.2034, found: 402.2043.

2α,19-Diacetoxyandrost-4-ene-3,17-dione **78** (106 mg, 2.63 x 10^{-4} mol) was dissolved in 95% ethanol (5 ml) and potassium carbonate (355 mg) was added. The reaction was left stirring at room temperature for 15 h 30 min. Work up and column chromatography eluted with methylene chloride : ethyl acetate, 50 : 50 afforded 2α,19-dihydroxyandrost-4-ene-3,17-dione[94] **64** (39 mg, 47%). ^{1}H NMR δ 0.88 (s, 3H, 18-CH_3), 3.47 (br s, 1H, exchanges with D_2O, 2α-OH), 4.05 (q, 2H, δ_a 3.98, δ_b 4.12, J_{ab}=10.4 Hz, 19-CH_2), 4.68 (q, 1H, J=6.2 Hz, J=13.3 Hz, 2β-H), 5.99 (s, 1H, 4-H); ^{13}C NMR δ 13.7 (CH_3, C-18), 20.5 (CH_2, C-11), 21.6 (CH_2, C-15), 30.8 (CH_2, C-12), 31.5 (CH_2, C-7), 32.9 (CH_2, C-6), 35.5 (CH, C-8), 35.6 (CH_2, C-16), 42.7 (CH_2, C-1), 46.2 (C, C-10), 47.5 (C, C-13), 51.0 (CH, C-14), 54.9 (CH, C-9), 65.7 (CH_2, C-19), 69.6 (CH, C-2), 123.5 (CH, C-4), 167.9 (C, C-5), 200.1 (C, C-3), 220.0 (C, C-17); MS m/z 318 (M^+), 300 (M-18, H_2O), 288 (M-30, HOCH), 274, 270; HRMS calcd for $C_{19}H_{26}O_4$: 318.1824, found: 318.1823.

c. Preparation and Treatment with Sulphuric Acid in Acetic Acid of 17β,19-Diacetoxy-4β,5-epoxy-5β-androstan-3-one **79**.

17β,19-Dihydroxyandrost-4-en-3-one **19** (6.64 x 10^{-3} mol) was treated with 30% hydrogen peroxide in alkaline methanol as described in section D.3.a. The resulting 4β,5β-epoxide was treated with acetic anhydride (Method A). Work up afforded 17β,19-diacetoxy-4β,5-epoxy-5β-androstan-3-one **79**. ^{1}H NMR δ 0.77 (s, 3H, 18-CH_3), 2.02 (s, 3H), and 2.11 (s, 3H) 17β- and 19-$COCH_3$,

2.88 (s, 1H, 4-H), 4.20 (d, 1H, J=11.5 Hz, 19-CH_2), 4.56 (br d, 2H, 19-CH_2 and 17α-H); MS m/z 404 (M^+). 17β,19-Diacetoxy-4β,5-epoxy-5β-androstan-3-one **79** (726 mg, 1.80×10^{-3} mol) was dissolved in a mixture of sulphuric acid in acetic acid (2 : 98, 15 ml) and the solution was stirred for 50 min. A sticky yellow precipitate slowly formed after the reaction mixture was poured on ice. This oily precipitate crystallized in methanol affording 2α,17β,19-triacetoxyandrost-4-en-3-one **80** (85 mg, 14%). mp 192-193°C; $[\alpha]_D$ +98° (c 0.24); UV λ_{max} 239 nm (ε 10 189); IR γ_{max} (KBr) 2960, 2870, 1735(17β- and 19-$\underline{CO}CH_3$), 1685 (3-CO) cm^{-1}; ^1H NMR δ 0.81 (s, 3H, 18-CH_3), 2.02 (s, 3H), 2.05 (s, 3H), 2.14 (s, 3H) 2β-, 17β- and 19-$COCH_3$, 4.46 (q, 2H, δ_a 4.22, δ_b 4.69, J_{ab}=11.2 Hz, 19-CH_2), 4.57 (tr, 1H, 17α-H), 5.75 (q, 1H, J=6.5 Hz, 2β-H), 5.91 (s, 1H, 4-H); ^{13}C NMR δ 12.1 (CH_3, C-18), 20.8 (CH_2, C-11), 20.9 (CH_3, $CO\underline{CH_3}$), 20.9 (CH_3, $CO\underline{CH_3}$), 21.1 (CH_3, $CO\underline{CH_3}$), 23.2 (CH_2, C-15), 27.3 (CH_2, C-16), 31.6 (CH_2, C-7), 32.3 (CH_2, C-6), 35.6 (CH, C-8), 36.7 (CH_2, C-12), 39.8 (CH_2, C-1), 42.4 (C, C-10), 44.2 (C, C-13), 50.5 (CH, C-14), 54.8 (CH, C-9), 66.6 (CH_2, C-19), 71.1 (CH, C-2), 82.1 (CH, C-17), 125.0 (CH, C-4), 165.0 (C, C-5), 170.2 (C, $\underline{CO}CH_3$), 170.7 (C, $\underline{CO}CH_3$), 171.1 (C, $\underline{CO}CH_3$), 193.7 (C, C-3); MS m/z 446 (M^+), 404 (M-42, $COCH_3$), 386, 344, 326, 318, 314, 302.

d. Treatment of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** with Sulphuric Acid in Dimethyl Sulfoxide.

19-Hydroxyandrost-4-ene-3,17-dione **4** (1.00g, 3.31 x 10^{-3} mol) was dissolved in dimethyl sulfoxide (70 ml) and concentrated sulphuric acid (3 µl) was added. The reaction was heated at 120°C, under nitrogen for 10 h. The reaction mixture was poured into water (~150 ml) and the sticky precipitate was filtered (347 mg) and the remaining aqueous phase was extracted with methylene chloride. The methylene chloride extracts were combined and the solvent was first removed with a rotary evaporator, then the remaining dimethyl sulfoxide was removed under vacuum. At this point, it was necessary to heat the solution with a boiling water bath in order to remove the solvent. A first chromatography of the residue thus obtained using a Chromatotron (Model 7924 T, Harrison Research Inc.) equipped with a 4 mm plate and eluted with petroleum ether : ether, 60 : 40 afforded 3-hydroxy-1,3,5(10)-estratrien-17-one **16** (6%). ^{1}H NMR δ 0.89 (s, 3H, 18-CH_3), 4.48 (s, 1H, exchanges with D_2O, 3-OH), 6.56 (s, 1H, 4-H), 6.62 (d, 1H, J=8.2 Hz, 1-H), 7.13 (d, 1H, J=8.2 Hz, 2-H); MS m/z 270 (M^+). Further elution of this plate with ether : methylene chloride, 90 : 10, followed by methylene chloride afforded 2α,19-dihydroxyandrost-4-ene-3,17-dione **64** (identified by ^{1}H NMR and MS, which were identical to those reported for **64** in section D.3.i.b.). Further purification of the fraction obtained when the plate was eluted with petroleum ether : ether, 20 : 80 was done again using the Chromatotron equipped with a 2 mm plate. Elution with petroleum ether : ether, 40 : 60 afforded 2β,19-oxidoandrost-4-ene-3,17-dione **82** (24%). mp 162-163°C (recrystallized from ether); $[\alpha]_D$ +281° (c 0.52); UV λ_{max} 242 nm (ε 16 255); IR γ_{max} (KBr) 2940, 2850, 1735 (17-CO), 1670 (3-CO), 1600 cm^{-1};

^{1}H NMR δ 0.90 (s, 3H, 18-CH_3), 3.76 (q, 2H, δ_a 3.49, δ_b 4.04, J_{ab}=7.5 Hz, 19-CH_2), 4.29 (d of d, 1H, J=1.9 Hz, J=6.3 Hz, 2α-H), 5.78 (br s 1H, 4-H); ^{13}C NMR δ 13.8 (CH_3, C-18), 21.0 (CH_2, C-11), 21.7 (CH_2, C-15), 28.1 (CH_2, [C-6, C-7]), 31.4 (CH_2, [C-12]), 35.7 (CH_2, C-16), 35.7 (CH, C-8), 42.3 (CH_2, C-1), 44.5 (CH, C-9), 47.4 (C,C-13), 50.6 (C, C-10), 51.3 (CH, C-14), 69.5 (CH_2, C-19), 80.9 (CH, C-2), 123.4 (CH, C-4), 167.3 (C, C-5), 196.8 (C, C-3), 219.3 (C, C-17); MS m/z 300 (M^+), 271, 270 (M-30), 357, 243; HRMS calcd for $C_{19}H_{24}O_3$: 300.1719, found: 300.1736. The sticky precipitate first obtained was purified on the Chromatotron equipped with a 2 mm plate. Elution with petroleum ether : ether, 70 : 30 afforded 3-hydroxy-1,3,5(10)-estratrien-17-one **16** (15%) and elution with petroleum ether : ether, 60 : 40 and 55 : 45 afforded 2,3-dihydroxy-1,3, 5(10)-estratrien-17-one[163] **81** (16%). ^{1}H NMR δ 0.89 (s, 3H, 18-CH_3), 4.95 and 4.98 (d, 2H, exchanges with D_2O, 2- and 3-OH), 6.58 and 6.79 (s, 2H, 1- and 4-H); ^{13}C NMR δ 13.8 (CH_3, C-18), 21.6 (CH_2, C-15), 26.0 (CH_2, C-11), 26.6 (CH_2, C-7), 28.7 (CH_2, C-6), 31.5 (CH_2, C-12), 35.9 (CH_2, C-16), 38.2 (CH, C-8), 44.0 (CH, C-9), 48.1 (C, C-13), 50.4 (CH, C-14), 112.5 and 115.5 (CH, C-1 and C-4), 129.0 and 132.3 (C, C-5 and C-10), 141.5 and 141.6 (C, C-2 and C-3), 221.6 (C, C-17); MS m/z 286 (M^+).

e. Treatment of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** with Perchloric Acid in Tetrahydrofuran.

19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** (333 mg, 1.05 x 10^{-3}) was dissolved in tetrahydrofuran (10 ml) and 35% perchloric acid

(1 ml) was added. The solution was stirred at room temperature for 21 h. Work up and column chromatography eluted successively with the following mixtures of methylene chloride : ethyl acetate: 95 : 5; 90 : 10; 75 : 25; 70 : 30; 60 : 40; 50 : 50; 40 : 60 and 30 : 70 first afforded 3-hydroxy-1,3,5(10)-estratrien-17-one **16** (8%) (^{1}H NMR identical to the one reported in section D.3.i.d.). 2β,19-Oxido-androst-4-ene-3,17-dione **82** was isolated in 4% yield (spectral data identical to those reported for **82** in section D.3.i.d.). 4,19-Dihydroxyandrost-4-ene-3,17-dione **63** was isolated in 35% yield. mp 213-214 ^{o}C (needles from ethyl acetate); $[\alpha]_D$ +212^{o} (c 0.25); UV λ_{max} 280 nm (ε 9 046); IR γ_{max} 3450 (OH), 2940, 2860, 1730 (17-CO), 1670 (3-CO), 1640 cm^{-1}; ^{1}H NMR δ 0.89 (s, 3H, 18-CH_3), 3.95 (q, 2H, δ_a 3.87, δ_b 4.03, J_{ab}=10.5 Hz, 19-CH_2), 6.31 (s, 1H, exchanges with D_2O, 4-OH); MS m/z 318 (M^+), 300 (M-18, H_2O), 288 (M-30, HOCH), 287.

ii. Preparation of 4β,5,19-Trihydroxy-5β-androstane-3,17-dione **88** and 4α,5,19-Trihydroxy-5α-androstane-3,17-dione **89** and their Acetates.

19-Hydroxyandrost-4-ene-3,17-dione **4** (300 mg, 9.93 x 10^{-4} mol) was dissolved in pyridine (1 ml) and benzene (5 ml). Osmic acid (250 mg, 9.83 x 10^{-4} mol) in benzene (5 ml) was added to the solution. The reaction was stirred at room temperature for 24 h. The color of the solution rapidly changed from dark yellow to dark orange and finally dark brown. The osmate ester was cleaved by adding a solution of sodium bisulphite (1.50 g) in water (20 ml) and pyridine (3 ml). The resulting two-phases solution was vigorously stirred for 30 min. The color of the solution changed from dark brown to orange. Work up and column

chromatography eluted with methylene chloride : ethyl acetate, 63 : 37 afforded starting material **4** (22 mg, 7%). The second compound eluted was 4β,5,19-trihydroxy-5β-androstane-3,17-dione **88** (96 mg, 29%). mp 195-196°C; $[\alpha]_D$ +96° (c 0.20); CD λ_{max} 300 nm (Δε +5.7); IR γ_{max} (KBr) 3440(br, OH), 2940, 1725 (3-CO and 17-CO) cm^{-1}; ^1H NMR δ 0.86 (s, 3H, 18-CH_3), 2.89 (s, 1H, exchanges with D_2O, 5β-OH), 3.27 (d, 1H, exchanges with D_2O, 19-OH), 3.59 (tr, 1H, becomes a d with D_2O, J=11.2 Hz, 19-pro(R)-H), 3.76 (d, 1H, exchanges with D_2O, J=2.6 Hz, 4β-OH), 4.34 (d, 1H, J=11.5 Hz, 19-pro(S)-H), 4.44 (d, 1H, becomes a s with D_2O, J=2.6 Hz, 4α-H); ^{13}C NMR δ 13.8 (CH_3, C-18), 20.8 (CH_2, C-11), 21.8 (CH_2, C-15), 25.8 (CH_2, C-6), 27.6 (CH_2, C-7), 30.8 (CH_2, [C-1]), 31.6 (CH_2, C-12), 34.0 (CH, C-8), 34.0 (CH_2, [C-2]), 35.7 (CH_2, C-16), 43.6 (CH, C-9), 43.9 (C, C-10), 47.5 (C, C-13), 52.1 (CH, C-14), 63.6 (CH_2, C-19), 73.6 (CH, C-4), 83.3 (C, C-5), 209.4 (C, C-3), 219.6 (C, C-17); MS m/z 336 (M^+), 318 (M-18, H_2O), 306 (M-30, HOCH); HRMS calcd for $C_{19}H_{28}O_5$: 336.1929, found: 336.1912. Anal. calcd for $C_{19}H_{28}O_5$: C, 67.81; H, 8.39. Found: C, 67.71; H, 8.33. Further elution of the column with methylene chloride : ethyl acetate, 60 : 40 afforded mainly 4α,5,19-trihydroxy-5α-androstane-3,17-dione **89** which was contaminated with the 4β,5,19-triol **88**. Addition of methylene chloride to this fraction removed all the traces of 4β,5β,19-triol **88** and left 4α,5,19-trihydroxy-5α-androstane-3,17-dione **89** (11 mg , 3%) which was homogeneous on the basis of tlc. mp 223-224°C; $[\alpha]_D$ +128° (c 0.165,

methanol); IR γ_{max} (KBr) 3400 (br, OH), 2940, 1740 (3-CO and 17-CO) cm^{-1}; CD λ_{max} 295 nm ($\Delta\varepsilon$ +8.3); ^{1} NMR δ (pyridine-d_5) 0.76 (s, 3H, 18-CH_3), 3.60 (s, 1H), 3.82 (s, 1H), 4.21 (q, 2H, δ_a 4.13, δ_b 4.29, J_{ab}=9.0 Hz, 19-CH_2), 4.83 (br s, 2H, exchanges with D_2O, 4α- and 5α-OH), 5.03 (s, 1H, 4β-H); MS m/z 336 (M^+), 318 (M-18, H_2O), 306 (M-30, HOCH), 302, 287; HRMS calcd for $C_{19}H_{28}O_5$: 336.1929, found: 336.1930. Anal. Calcd for $C_{19}H_{28}O_5$: C, 67.81; H, 8.39. Found: C, 69.02; H, 8.63.

4β,5,19-Trihydroxy-5β-androstane-3,17-dione **88** (26 mg, 7.74 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded 4β,19-diacetoxy-5β-hydroxyandrostane-3,17-dione **90** (100%). ^{1}H NMR δ 0.87 (s; 3H, 18-CH_3), 2.20$_7$ and 2.21 (s, 3H, 4β- and 19-$COCH_3$), 4.46 (s, 2H, 19-CH_2), 5.64 (s, 1H, 4α-H); MS m/z 420 (M^+).

4α,5,19-Trihydroxy-5α-androstane-3,17-dione **89** (2.4 mg, 7.14 x 10^{-6} mol) was treated with acetic anhydride (Method A). Work up afforded 4α,19-diacetoxy-5α-hydroxyandrostane-3,17-dione **91** (100%). ^{1}H NMR δ 0.86 (s, 3H, 18-CH_3), 2.12 and 2.20 (s, 3H, 4α- and 19-$COCH_3$), 3.64 (br s , 2H), 4.54 (d, 2H, J=6.8 Hz, 19-CH_2), 5.47 (s, 1H, 4β-H); ^{13}C NMR δ 13.8 (CH_3, C-18), 20.6 (CH_3, $CO\underline{CH_3}$), 20.9 (CH_2, C-11), 21.2 (CH_3, $CO\underline{CH_3}$), 21.6 (CH_2, C-15), 24.5 (CH_2, C-6), 28.8 (CH_2, C-7), 29.1 (CH_2, [C-1]), 29.7 (CH_2), 31.7 (CH_2,

C-12), 34.2 (CH, C-8), 35.7 (CH_2, C-16), 37.1 (CH_2), 43.9 (C, C-10), 45.5 (CH, C-9), 47.6 (C, C-13), 51.2 (CH, C-14), 64.3 (CH_2, C-19), 70.6 (CH, C-4), 78.5 (C, C-5), 79.4 (CH), 169.7 (C, $\underline{C}OCH_3$), 170.5 (C, $\underline{C}OCH_3$), 202.7 (C, C-3), 220.3 (C, C-17); MS m/z 420 (M^+), 402 (M-18, H_2O), 378 (M-42, $COCH_3$), 360.

Treatment of 19-hydroxyandrost-4-ene-3,17-dione **4** (130 mg, 4.30 x 10^{-4} mol) with osmic acid (125 mg) in pyridine (0.8 ml) and benzene (15 ml) was repeated as previously described. A mixture of 4α,5,19-trihydroxy-5α-androstane-3,17-dione **89** and 4β,5,19-trihydroxy-5β-androstane-3,17-dione **88** was isolated (124 mg, 86%). The mixture of triols **88** and **89** (104 mg, 3.08 x 10^{-4} mol) was treated with acetic anhydride (Method A). Work up afforded a homogeneous compound on the basis of tlc but which was a *ca.* 1 : 2.3 mixture (by ^{1}H NMR) of 4β,19-diacetoxy-5β-hydroxyandrostane3,17-dione **90** and 4α,19-di-acetoxy-5α-hydroxyandrostane-3,17-dione **91**. ^{1}H NMR δ (0.86) 0.87 (s, 3H, 18-CH_3), 2.05 (2.06) (s, 3H) and (2.07) 2.08 (s, 3H) 4α- and 4β,19-CO$\underline{CH_3}$, 4.46 (s, 2H, 19-CH_2) (4.54, d, 19-CH_2), (5.47) 5.63 (s, 1H, 4α-H); MS m/z 420 (M^+), 378 (M-42, $COCH_3$), 360, 344, 318, 305.

iii. Acid-catalyzed Treatment of 4β,5,19-Trihydroxy-5β-androstane-3,17-dione **88** and 4α,5,19-Trihydroxy-5α-androstane-3,17-dione **89**.

a. Treatment of **88** and **89** with HCl in Acetone.

A mixture of triols **88** and **89** (290 mg, 8.63 x 10^{-4} mol) was dissolved in acetone (10 ml) containing 2 drops of concentrated HCl. The reaction was stirred at room temperature for 20 h. The solvent was evaporated under a stream of nitrogen and the residue was purified by column chromatography. Elution with methylene chloride : ethyl acetate, 85 : 15 afforded 4α-hydroxy-4β,19-oxido-5α-androstane-3,17-dione **92** (100 mg, 36%). mp 188-189°C (recrystallized in ether); $[\alpha]_D$ +34° (c 0.16); IR γ_{max} 3490 (br, OH), 2940, 2890, 1730 (3-CO and 17-CO) cm^{-1}; ^1H NMR δ 0.87 (s, 3H, 18-CH_3), 4.03 (q, 2H, δ_a 3.80, δ_b 4.26, J_{ab}=8.3 Hz, 19-CH_2), 4.33 (s, 1H, exchanges with D_2O, 4α-OH); ^{13}C NMR δ 14.1 (CH_3, C-18), 21.1 (CH_2, C-11), 21.8 (CH_2, C-15), 22.4 (CH_2, [C-6]), 28.1 (CH_2, [C-7]), 31.7 (CH_2, C-12), 32.9 (CH_2, [C-2]), 35.2 (CH_2, [C-1]), 35.8 (CH_2, C-16), 36.0 (CH, C-8), 46.6 (C, C-10), 47.9 (C, C-13), 48.1* (CH, C-5), 49.9* (CH, C-9), 50.5 (CH, C-14), 70.1 (CH_2, C-19), 103.6 (C, C-4), 205.7 (C, C-3), 220.0 (C, C-17); MS m/z 318 (M^+), 290 (M-28), 287, 275, 272. Anal. Calcd for $C_{19}H_{26}O_4$: C, 71.65; H, 8.24. Found: C, 71.70; H, 8.47. Further elution with methylene chloride : ethyl acetate, 80 : 20 afforded 4α,5-isopropylidenedioxy-3α-hydroxy-3β,19- oxido-5α-androstan-17-one **96** (73 mg, 22%). mp 225-226°C; $[\alpha]_D$ +70° (c 0.16); IR γ_{max} 3580 and 3310 (OH), 2995, 2940, 2880, 2860, 1730 (17-CO) cm^{-1}; ^1H NMR δ 0.82 (s, 3H, 18-CH_3), 1.40 (s, 3H, 4α,5α -isopropylidene-CH_3) and 1.47 (s, 3H, 4α,5α -isopropylidene-CH_3) 3.66 (d, 1H, J=1.6 Hz, 4β-OH), 3.94 (q, 2H, δ_a 3.90, δ_b 3.98, J_{ab}=9.3 Hz, 19-CH_2); ^{13}C NMR δ 13.7 (CH_3, C-18), 21.0 (CH_2, C-11),

21.5 (CH_2, C-15), 23.3 (CH_2, [C-1]), 25.4 (CH_2, C-6), 26.6 (CH_2, C-7), 26.8 (CH_3) and 27.2 (CH_3) 4α,5α -isopropylidene-CH_3, 31.5 (CH_2, C-12), 33.7 (CH_2, [C-2]), 34.9 (CH, C-8), 35.7 (CH_2, C-16), 39.7 (C, C-10), 43.4 (CH, C-9), 47.6 (C, C-13), 50.8 (CH, C-14), 67.6 (CH_2, C-19), 83.3 (CH, C-4), 97.2 (C, C-5), 109.7 (C, C-3), 220.6 (C, C-17); MS m/z 376 (M^+), 361 (M-15, CH_3), 318, 301, 288, 283, 272. The last compound eluted was 4,19-dihydroxyandrost-4-ene-3,17-dione **63** (12 mg, 4%). The ^1H NMR and MS were identical to those reported for **63** in section D.3.i.e. When the reaction was performed as described above but for 23 h with a mixture of triols **88** and **89** (80 mg, 2.38 x 10^{-4} mol), the yields for the products **92** and **96** were 56% and 19%, respectively.

b. Treatment of **88** and **89** with HCl in Methanol.

A mixture of triols **88** and **89** (200 mg, 5.95 x 10^{-4} mol) was dissolved in methanol (6 ml) containing 1 drop of concentrated HCl. The solution was stirred at room temperature for 22 h. Work up and column chromatography were as described in section D.3.iii.a. 4α-Hydroxy-4β,19-oxido-5α-androstane-3,17-dione **92** was isolated in 20% yield.

c. Treatment of **88** and **89** with HCl in Tetrahydrofuran.

A mixture of triols **88** and **89** (160 mg, 4.76 x 10^{-4} mol) was dissolved in tetrahydrofuran (6 ml) and 1 drop of concentrated HCl was added. The solution was stirred at room temperature for 21 h. Work up and column chromatography were as described in section D.3.iii.a. 4α-Hydroxy-4β,19-oxido-5α-androstane-3,17-dione **92** was isolated in 35% yield.

d. Treatment of **88** with HCl in Acetic Acid.

4β,5,19-Trihydroxy-5β-androstane-3,17-dione **88** (33 mg, 9.82 x 10^{-5} mol) was dissolved in 5 ml of a mixture of acetic acid : HCl (20.0 ml : 0.5 ml). The solution was stirred at room temperature for 16 h. Work up afforded 4,19-dihydroxyandrost-4-ene-3,17-dione **63** (100%). The ^{1}H NMR and MS were identical to those reported for **63** in section D.3.i.e.

iv. Base-catalyzed Opening of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62**.

19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** (1.00 g, 3.15 x 10^{-3} mol) was dissolved in methanol (125 ml) and sodium hydroxide (1.50 g in 10 ml of water) was added. The solution was heated at reflux for 3 h. Most of the solvent was removed under reduced pressure. Water was added to the reaction mixture and extracted with methylene chloride. The residue obtained after the evaporation of the solvent was purified on a Chromatotron with a 4 mm plate. A gradient of petroleum ether : ether was used to elute the plate (100% petroleum ether : 0% ether to 100% ether, the amount of ether was increased in increments of 5%). The first compound eluted was 4-methoxyestr-4-ene-3,17-dione **99** (60 mg, 6%). mp 155-156°C (needles from ether : hexane) (lit.[98] mp 154-156°C); ^{1}H NMR δ 0.91 (s, 3H, 18-CH_3), 3.61 (s, 3H, 4-OCH_3); ^{13}C NMR δ 13.9 (CH_3, C-18), 21.7 (CH_2, C-15), 25.7 (CH_2, C-11), 26.0 (CH_2, C-6), 26.5 (CH_2, C-1), 29.4 (CH_2, C-12), 31.4 (CH_2, C-7), 35.8 (CH_2, C-16), 37.0 (CH_2, C-2), 39.7 (CH, C-8), 42.5 (CH, C-10), 47.7 (C, C-13), 49.7* (CH, C-9), 50.2* (CH, C-14), 60.2 (CH_3, 4-OCH_3), 147.3 (C, [C-4]), 149.1 (C, [C-5]), 194.2 (C, C-3),

220.1 (C, C-17); MS m/z 302 (M^+); HRMS calcd for $C_{19}H_{26}O_3$: 302.1875, found: 302,1879. The second conpound eluted was 4-methoxy-19-hydroxy-androst-4-ene-3,17-dione **98** (7%). mp 191-192°C (recrystallized form ether); $[\alpha]_D$ +148° (c 0.105); UV λ_{max} 256 nm (ε 12648); IR γ_{max} 2940, 2850, 1730 (17-CO), 1670 (3-CO) cm^{-1}; ^1H NMR δ 0.89 (s, 3H, 18-CH_3), 3.63 (s, 3H, 4-OCH_3), 3.96 (q, 2H, δ_a 3.89, δ_b 4.02, J_{ab}=10.8 Hz, 19-CH_2); ^{13}C NMR δ 13.8 (CH_3, C-18), 20.7 (CH_2, C-11), 21.6 (CH_2, C-15), 23.8 (CH_2, C-6), 30.4 (CH_2, C-12), 31.6 (CH_2, C-7), 32.5 (CH_2, [C-1]), 35.2 (CH_2, [C-2]), 35.4 (CH, C-8), 35.7 (CH_2, C-16), 43.8 (C, C-10), 47.5 (C, C-13), 51.2 (CH, C-14), 54.2 (CH, C-9), 60.5 (CH_3, 4-OCH_3), 65.8 ($\overline{CH}_2$, C-19), 149.0 (C, [C-4]), 149.8 (C, [C-5]), 194.5 (C, C-3), 220.3 (C, C-17); MS m/z 332 (M^+), 302 (M-30, HOCH), 301. Anal. Calcd for $C_{20}H_{28}O_4$: C, 72.24; H; 8.49. Found: C, 71.25; H, 8.34.

E. Preparation and Chemistry of 19a-Hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100**.

1. Conversion of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** to 19a-Hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100**.

19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** (2.00 g, 6.29×10^{-3} mol) was dissolved in methanol (70 ml) and the solution was cooled in an ice-water bath. Ice-cold 4 N NaOH (15 ml) and ice-cold 30% H_2O_2 (44 ml) were added to the stirred solution. The solution was kept in ice for 4 h then left stirring without adding more ice, overnight (12 h). The reaction was terminated by the addition of water (50 ml) and extracted with methylene chloride to afford 19-hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** (63 mg, 3%). Following acidification, the aqueous phase was extracted with methylene chloride to afford 19a-hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100** (1.86 g, 85%). mp 187-188°C (needles from 95% ethanol); $[\alpha]_D$ +27° (c 0.79); IR γ_{max} 3315 (OOH), 2940, 2865, 1735 and 1705 (3-CO and 17-CO) cm^{-1}; ^1H NMR δ 0.88 (s, 3H, 18-CH_3), 3.45 (s, 1H, 4α-H), 4.32 (q, 2H, δ_a 4.04, δ_b 4.60, J_{ab}=10.5 Hz, 19a-CH_2), 4.15 (m, 2H, 1-CH_2), 8.16 (br s, 1H, exchanges with D_2O, 19a-OOH); ^{13}C NMR δ 13.7 (CH_3, C-18), 21.3 (CH_2, C-11), 21.7 (CH_2, C-15), 23.7 (CH_2, [C-6]), 24.8 (CH_2, [C-7]), 31.4 (CH_2, C-12), 33.3 (CH, C-8), 34.4 (CH_2, C-19), 35.5 (CH_2, C-16), 38.8 (C, C-10), 47.3 (C, C-13), 48.5 (CH, C-9), 51.6 (CH, C-14), 55.2 (CH, C-4), 66.7 (C, C-5), 68.9 (CH_2, C-19a), 73.3

(CH_2, C-1), 168.2 (C, C-3), 219.7 (C, C-17); MS m/z 350 (M^+), 334 (M-16, O), 332 (M-18, H_2O), 289, 288, 278, 277. Anal. Calcd for $C_{19}H_{26}O_6$: C, 65.10; H, 7.48. Found: C, 65.20; H, 7.52.

2. Treatment of **100** with Methyl Iodide. Formation of 19a-Methylperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **112**.

19a-Hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100** (102 mg, 2.91 x 10^{-4} mol) was dissolved in 10 ml of 95% ethanol. It was necessary to heat gently the ethanol solution to get all the steroid into solution. Methyl iodide (540 mg, 3.80 x 10^{-3} mol) and silver oxide (500 mg, 2.16 x 10^{-3} mol) were added and the solution was stirred at room temperature for 16 h. The solution was again heated gently and the gray-brown precipitate was filtered. The solvent was removed under reduced pressure. The residue was purified by chromatography using methylene chloride : ethyl acetate, 90 : 10 as eluant. 19a-Methylperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **112** was obtained in 57% yield. mp 127-128°C; $[\alpha]_D$ +47° (c 0.52); IR γ_{max} (KBr) 2940, 2960, 2870, 1735 and 1725 sh (17-CO and 3-OCO) cm^{-1}; ^1H δ 0.88 (s, 3H, 18-CH_3), 3.40 (s, 1H, 4α-H), 3.80 (s, 3H, 19a-$OOCH_3$), 4.27 (q, 2H, δ_a 4.02, δ_b 4.52, J_{ab}=10.9 Hz, 19a-CH_2), 4.12 (m, 2H, 1-CH_2); ^{13}C NMR δ 13.6 (CH_3, C-18), 21.6 (CH_2, C-11), 21.6 (CH_2, C-15), 23.7 (CH_2, [C-6]), 24.5 (CH_2, [C-7]), 31.3 (CH_2, C-12), 33.5 (CH, C-8), 33.9 (CH_2, C-19), 35.4 (CH_2, C-16), 38.4 (C, C-10), 47.2 (C, C-13), 47.5 (CH, C-9), 51.4 (CH, C-14), 54.6 (CH, C-4),

62.2 (CH_3, $OOCH_3$), 66.7 (C, C-5), 67.7 (CH_2, C-19a), 69.8 (CH_2, C-1), 167.3 (C, C-3), 219.6 (C, C-17); MS m/z (364 is absent, M^+), 334 (M+H-31, OCH_3), 332, 289, 288, 278, 277. Anal. Calcd for $C_{20}H_{28}O_6$: C, 65.89; H, 7.75. Found: 65.76; H, 7.62.

3. Treatment of **100** with Sodium Iodide. Formation of 4β-Hydroxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **113**.

19a-Hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100** (320 mg, 9.14×10^{-4} mol) was dissolved in a solution of 95% ethanol (10 ml) and ether (8 ml). To the resulting milky solution, sodium iodide (800 mg, 5.34×10^{-3} mol) and 2 drops of acetic acid were added. The solution turned dark yellow and was stirred at room temperature for 7 h. After the work up, the residue was purified by column chromatography. Elution with methylene chloride : ethyl acetate, 90 : 10 afforded a first compound (3 mg). mp 178-180°C decomposes; ^{1}H NMR δ 0.87 (s, 3H, 18-CH_3), 3.53 (s, 1H, 4-H), 4.29 (q, 2H, δ_a 3.99, δ_b 4.58, J_{ab}=11.0 Hz), 5.22 (q, 2H, δ_a 5.19, δ_b 5.26, J_{ab}=11.4 Hz), 5.82 (q, 1H, δ_a 5.80, δ_b 5.85, J_{ab}=11.4 Hz); MS m/z 316 (M^+); HRMS calcd for $C_{19}H_{24}O_4$: 316.1628, found: 316.1678. Elution with methylene chloride : ethyl acetate, 85 : 15, afforded A-nor-3,5-seco-5,17-dioxo-19-hydroxyandrostane-3-carboxylic acid 3,19- lactone **152** (9 mg). The physical properties of **152** are described in section F.1.v. Finally, elution with methylene chloride : ethyl acetate, 80 : 20, gave 4β-hydroxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **113** (47 mg, 16%).

mp 74-75°C; $[\alpha]_D$ +9° (c 0.32); IR γ_{max} (KBr) 3450 (br, OH), 2940, 2870, 1735 (17-CO and 3-OCO) cm^{-1}; ^1H NMR δ 0.88 (s, 3H, 18-CH_3), 3.10 (d, 1H, exchanges with D_2O, J=2.9 Hz, 4β-OH), 3.97 (m, 2H, 1-CH_2), 4.35 (q, 2H, δ_a 4.19, δ_b 4.52, J_{ab}=12.6 Hz, 19a-CH_2), 4.41 (d, 1H, becomes a s upon addition of D_2O, 4α-H); ^{13}C NMR δ 13.7 (CH_3, C-18), 21.5 (CH_2, C-11), 21.6 (CH_2, C-15), 22.1 (CH_2, [C-6]), 24.6 (CH_2, [C-7]), 31.4 (CH_2, C-12), 33.2 (CH, C-8), 35.6 (CH_2, C-16), 37.1 (CH_2, C-19), 46.0 (CH, C-9), 46.9 (C, C-13), 47.9 (C, C-10), 51.6 (CH, C-14), 64.1 (CH_2, C-19a), 68.6 (CH_2, C-1), 70.4 (CH, C-4), 85.4 (C, C-5), 174.1 (C, C-3), 219.9 (C, C-17); MS m/z 334 (M^+), 277. Anal. Calcd for $C_{19}H_{26}O_5$: C, 68.22; H, 7.84. Found: C, 68.02; H, 8.00.

4. Preparation of 4β-Acetoxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **114**.

4β-Hydroxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **113** (42 mg, 1.26 x 10^{-4} mol) was acetylated (Method A). 4β-Acetoxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **114** (39 mg, 9.84 x 10^{-5} mol) was isolated in 78% yield. IR γ_{max} (KBr) 2960, 2920, 2860, 1760, 1740 cm^{-1}; ^1H NMR δ 0.88 (s, 3H, 18-CH_3), 2.22 (s, 3H, 4β-$COCH_3$), 3.95 (q, 2H, 1-CH_2), 4.37 (q, 2H, δ_a 4.26, δ_b 4.47, J_{ab}=12.6 Hz, 19a-CH_2), 5.58 (s, 1H, 4α-H); ^{13}C NMR δ 13.9 (CH_3, C-18), 20.7 (CH_3, CO$\underline{CH_3}$), 21.6 (CH_2, C-11), 21.7 (CH_2, C-15),

22.7 (CH_2, [C-6]), 25.9 (CH_2, [C-7]), 31.6 (CH_2, C-12), 33.7 (CH, C-8), 35.7 (CH_2, C-16), 36.4 (CH_2, C-19), 45.9 (CH, C-9), 47.3 (C, C-13), 47.9 (C, C-10), 51.4 (CH, C-14), 64.1 (CH_2, C-19a), 68.7 (CH_2, C-1), 70.0 (CH, C-4), 84.0 (C, C-5), 168.1• (C, C-3), 169.8• (C, $\underline{C}OCH_3$). 219.4 (C, C-17); MS m/z 376 (M^+), 334 (M-42), 333, 277.

5. Treatment of **100** with Hydrogen Bromide. Formation of 4β-Hydroxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **113**.

19a-Hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100** (391 mg, 1.12×10^{-3} mol) was dissolved in acetic acid (1.5 ml) and placed in an ice-water bath. A solution of hydrogen bromide (0.1 ml) in acetic acid (6 ml) was added and the reaction was stirred for 1 h. Water (30 ml) was added and the pale yellow residue obtained after the work up was purified by column chromatography. The first compound (36 mg) was eluted with methylene chloride : ethyl acetate, 80 : 20. mp 215-216°C (recrystallized in ether : ethyl acetate); IR γ_{max} 1733, 1710 sh cm^{-1}; ^1H NMR δ 0.94 (s, 3H, 18-CH_3), 4.40 (tr, 1H), 4.49 (d, 1H, J=12.2 Hz), 4.88 (q, 1H, J=6 Hz). Anal. Found: C, 60.98; H, 6.63. Elution with methylene chloride : ethyl acetate, 78 : 22 afforded 4β-hydroxy-5, 19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **113** (43%). The physical data are described in section E. 3.

6. Treatment of **100** with Acetic Anhydride in Pyridine.

19a-Hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100** (50 mg, 1.43 x 10^{-4} mol) was treated with acetic anhydride (Method A). Work up followed by column chromatography eluted with methylene chloride : ethyl acetate, 92 : 8 and 90 : 10, afforded two compounds. The least polar compound (31 mg) was a clear oil, homogeneous on tlc but a mixture of isomers (*ca*. 1:1.8) by ^{1}H NMR. IR γ_{max} 3500 br, 2940, 2860, 1740 br, 1670 cm^{-1}; ^{1}H NMR δ 0.87 (0.88) (s, 3H, 18-CH_3), 2.11 (2.14) (s, 3H, $COCH_3$), 3.51 (3.53) (s, 1H, 4α-H), 3.95 (4.06) (d, 1H, J=10.0 Hz), 4.59 (d of d, 2H, J=10.1 Hz), 4.71 (d, 1H, J=7.2 Hz), 5.46 (d, 1H, J=13.5 Hz), 7.15 (d, 1H, J=7.2 Hz), 7.28 (s, 1H); ^{13}C NMR δ 13.5 (CH_3), 20.5 (20.1) (CH_2), 21.7 (20.6) (CH_3), 23.4 (22.8) (CH_2), 25.1 (24.8) (CH_2), 31.2 (31.1) (CH_2), 32.8 (32.0) (CH), 35.4 (CH_2), 40.5 (C), 46.4 (CH), 48.3 (47.3) (C), 52.1 (51.9) (CH), 56.7 (55.6) (CH), 65.8 (65.5) (C), 69.0 (68.4) (CH_2), 114.1 (111.5) (CH), 137.1 (134.5) (CH), 166.9 (C), 219.4 (C); MS m/z 374 (M^+), 333, 332. Further elution afforded 14 mg of a second compound. IR γ_{max} 3500, 2940, 1735 br cm^{-1}; ^{1}H NMR δ 0.87 (s, 3H, 18-CH_3), 2.05 (s, 3H, $COCH_3$), 2.08 (s, 3H, $COCH_3$), 3.39 (s, 1H), 4.27 (q, 2H, δ_a 4.03, δ_b 4.51, J_{ab}=11.4 Hz), 6.86 (tr, 1H); MS m/z 434 (M^+), 374, 333, 332, 331.

7. Preparation of 4-Bromo-19-hydroxyandrost-4-ene-3,17-dione **102** and 4-Bromo-19-acetoxyandrost-4-ene-3,17-dione **103**.

19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** (200 mg, 6.29×10^{-4} mol) dissolved in acetic acid (3 ml) was cooled in an ice-water bath. A solution of HBr in acetic acid (0.2 ml of HBr in 12 ml of acetic acid) was added to the stirred steroid solution. After 15 min, the reaction was terminated by adding cold water (100 ml). 4-Bromo-19-hydroxyandrost-4-ene-3,17-dione **102** (171 mg, 71%) was isolated as a white precipitate. mp 163°C; $[\alpha]_D$ +214° (c 0.285); UV λ_{max} 262 nm (ε 12 136); IR γ_{max} 2940, 2860, 1735 (17-CO), 1680 (3-CO) cm^{-1}; ^1H NMR δ 0.90 (s, 3H, 18-CH_3), 4.02 (q, 2H, δ_a 3.92, δ_b 4.12, J_{ab}=10.6 Hz, 19-CH_2); ^{13}C NMR δ 13.8 (CH_3, C-18), 21.1 (CH_2, C-11), 21.6 (CH_2, C-15), 30.1 (CH_2, C-12), 31.6 (CH_2, C-7), 32.2* (CH_2, C-6), 33.2* (CH_2, C-1), 34.9 (CH_2, C-2), 35.4 (CH, C-8), 35.7 (CH_2, C-16), 47.4• (C, C-10), 47.6• (C, C-13), 51.0 (CH, C-14), 54.0 (CH, C-9), 65.8 (CH_2, C-19), 124.1 (C, C-4), 163.7 (C, C-5), 191.2 (C, C-3), 220.1 (C, C-17); MS m/z 382/380 (M^+, ^{81}Br-^{79}Br), 352/350 (M-30, HOCH), 301 (M-81, Br), 300 (M-82, Br+H); HRMS calcd for $C_{19}H_{25}O_3Br$: 380.0980, found: 380.0987. 4-Bromo-19-hydroxyandrost-4-ene-3,17-dione **102** (30 mg, 7.89×10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded 4-bromo-19-acetoxyandrost-4-ene-3,17-dione **103** as an oil (23 mg, 67%). mp 155°C (crystal from methanol); UV λ_{max} 260 nm (ε 8 607); ^1H NMR δ 0.91 (s, 3H, 18-CH_3), 2.00 (s, 3H, 19-$COCH_3$), 4.42 (q, 2H, δ_a 4.15, δ_b 4.69, J_{ab}=11.0 Hz, 19-CH_2); ^{13}C NMR δ 13.8 (CH_3, C-18),

20.9 (CH_3, $CO\underline{CH_3}$), 21.1 (CH_2, C-11), 21.6 (CH_2, C-15), 30.1 (CH_2, C-12), 31.5 (CH_2, C-7), 32.3* (CH_2, C-6), 32.9* (CH_2, C-1), 34.5 (CH_2, C-2), 35.4 (CH, C-8), 35.6 (CH_2, C-16), 45.5 (C, C-10), 47.5 (C, C-13), 51.0 (CH, C-14), 54.1 (CH, C-9), 66.5 (CH_2, C-19), 124.4 (C, C-4), 162.1 (C, C-5), 170.5 (C, $\underline{CO}CH_3$), 190.4 (C, C-3), 219.5 (C, C-17); MS m/z 424/422 (M^+, ^{81}Br-^{79}Br), 364/362 (M-60), 353/351 (M-71), 352/350 (M-72), 336/334 (M-88); HRMS calcd for $C_{21}H_{27}O_2Br$: 422.1085, found: 422.1085.

F. Baeyer-Villiger Rearrangement.

1. Reaction of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** with MCPBA.

i. Treatment of 19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** with MCPBA.

19-Hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** (180 mg, 5.66×10^{-4} mol) was dissolved in chloroform (4 ml), MCPBA (180 mg) was added and the reaction was stirred at room temperature. After 32 h, 60 mg of MCPBA were added and the reaction was terminated after 4 days. Work up was followed by column chromatography using successively the following solvents: methylene chloride : ethyl acetate, 85 : 15, 80 : 20 and 70 : 30. Starting material **62** (53 mg, 29%) was recovered and 19-hydroxy-4β,5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **145** (77 mg, 40%) was isolated. mp 252-253°C (transparent plates from slow evaporation of ethyl acetate); $[\alpha]_D$ +62° (c 0.18);

IR γ_{max} 3660 (OH), 3440 br (OH), 2950, 2870, 1720 (17-OCO and 3-CO) cm^{-1}; ^1H NMR δ 1.32 (S, 3H, 18-CH_3), 2.93 (s, 1H, 4α-H), 3.90 (q, 2H, δ_a 3.76, δ_b 4.04; J_{ab}=11.4 Hz, 19-CH_2); ^{13}C δ 19.8 (CH_2, C-15), 20.1 (CH_3, C-18), 21.6 (CH_2, C-1), 22.9 (CH_2, C-11), 28.4* (CH_2, C-6), 28.9* (CH_2, C-16), 29.8 (CH_2, C-7), 32.1 (CH_2, C-2), 37.5 (CH, C-8), 39.2 (CH_2, C-12), 41.3 (C, C-10), 45.8• (CH, C-9), 46.0• (CH, C-14), 60.2 (CH, C-4), 64.8 (CH_2, C-19), 68.5 (C, C-5), 82.4 (C, C-13), 171.0 (C, C-17), 205.5 (C, C-3); MS m/z 334 (M^+), 319 (M-15, CH_3), 316 (M-18, H_2O), 306 (M-28, CO), 304 (M-30, HOCH); HRMS calcd for $C_{19}H_{26}O_5$: 334.1773, found: 334.1774. Anal. Calcd for $C_{19}H_{26}O_5$: C, 68.62; H, 7.84. Found: C, 68.42; H, 7.84.

ii. Treatment of 19-Acetoxy-4β,5-epoxy-5β-androstane-3,17-dione 77 with MCPBA.

19-Acetoxy-4β,5-epoxy-5β-androstane-3,17-dione **77** (211 mg, 5.87 x 10^{-4} mol) was dissolved in chloroform (8 ml) and MCPBA (254 mg) was added. After stirring for 70 h at room temperature, 50 mg of MCPBA were added. The reaction was stopped after 6 days. Column chromatography using successively the following mixture of solvents: methylene chloride : ethyl acetate, 90 : 10, 85 : 15 and 80 : 20 afforded 9 mg of starting material **77** (4%) , 16 mg of a mixture of product (overlapping spots on tlc) and 196 mg (89%) of 19-acetoxy-4β, 5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **146**. mp 69°C; $[\alpha]_D$ +48° (c 0.21); ^1H NMR δ 1.31 (s, 3H, 18-CH_3), 2.10 (s, 3H, 19-CO$\underline{CH_3}$),

2.92 (s, 1H, 4α-H), 4.34 (q, 2H, δ_a 4.18, δ_b 4.49, J_{ab}=11.4 Hz, 19-CH_2); MS m/z 376 (M^+), 348 (M-28, CO), 335 (M-41), 321 (M-55), 316 (M-60). Anal. Calcd for $C_{21}H_{28}O_6$: C, 66.98; H, 7.50. Found: C, 66.84; H, 7.28. 19-Acetoxy-4β,5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **146** (40 mg, 1.06 x 10^{-4} mol) dissolved in 95% ethanol (7 ml) was stirred with K_2CO_3 (190 mg) at room temperature for 18 h. Work up followed by column chromatography eluted with methylene chloride : ethyl acetate, 55 : 45 afforded 19-hydroxy-4β,5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **145** (16 mg, 46%) identical (MS and ^1H NMR) to **145** (section F. 1. i.).

iii. Treatment of 19-Hydroxy-4α,5-epoxy-5α-androstane-3,17-dione **147** with MCPBA.

19-Hydroxy-4α,5-epoxy-5α-androstane-3,17-dione **147**[98] (56 mg, 1.77 x 10^{-4} mol) was dissolved in chloroform (3.5 ml). MCPBA (81 mg) was added and the clear solution was stirred at room temperature. After 1 day, 61 mg of MCPBA were added. The reaction was terminated after 6 days. Column chromatography eluted with methylene chloride : ethyl acetate, 70 : 30 afforded 12 mg (24%) of starting material **147**. Further elution with methylene chloride : ethyl acetate, 50 : 50 afforded 19-hydroxy-4α,5-epoxy-17a-oxa-D-homo-5α-androstane-3,17-dione **148** (16%). mp 229-230°C; IR γ_{max} 3620 (OH), 3420 br (OH), 2940, 2880, 1710 (3 and 17-CO) cm^{-1}; ^1H NMR δ 1.31 (s, 3H, 18-CH_3), 3.15 (s, 1H, 4β-H), 3.90 (q, 2H, δ_a 3.84, δ_b 3.95, J_{ab}=10.3 Hz, 19-CH_2);

MS m/z 334 (M^+), 316 (M-18, H_2O), 306 (M-28, CO), 304 (M-30, HCOCH).

Anal. Calcd for $C_{19}H_{26}O_5$: C, 68.22; H, 7.84. Found: C, 67.81; H, 7.71.

iv. Preparation and Treatment with MCPBA of 17β,19-Dihydroxy-4β,5-epoxy-5β-androstan-3-one **149**. Isolation and Acetylation of A-Nor-3,5-seco-5-oxo-17β,19-dihydroxyandrostane-3-carboxylic acid 3,19-lactone **150**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (2.10 g, 6.95 x 10^{-3} mol) dissolved in methanol (80 ml) was cooled in an ice-water bath. Sodium borohydride (0.33 g) was added. The reaction was terminated after 90 min by the addition of acetic acid (5 drops). The residue obtained after the removal of the solvent was dissolved in methanol (30 ml) and cooled in an ice-water bath. Ice-cold 10% NaOH (10 ml) and 30% hydrogen peroxide (12 ml) were successively added and the reaction was stirred for 30 min at 0-4°C. Work up and column chromatography eluted with methylene chloride : ethyl acetate, 65 : 35 afforded 17β,19-dihydroxy-4β,5-epoxy-5β-androstan-3-one **149** (1.05 g, 47%). mp 155-156°C (crystals from ether) (Lit.[96] mp 150-153°C); $[\alpha]_D$ +136° (c 0.275); IR γ_{max} 3600 (OH), 2940, 1710 (3-CO) cm^{-1}; ^1H NMR δ 0.74 (s, 3H, 18-CH_3), 2.88 (s, 1H, 4α-H), 3.62 (tr, 1H, 17α-H), 3.96 (q, 2H, δ_a 3.73, δ_b 4.18, J_{ab}=11.4 Hz, 19-CH_2); ^{13}C NMR δ 11.1 (CH_3, C-18), 21.3 (CH_2, C-11), 21.8 (CH_2, C-1), 23.2 (CH_2, C-15), 30.0 (CH_2, C-16), 30.1 (CH_2, C-6), 30.3 (CH_2, C-7), 32.2 (CH_2, C-2), 35.1 (CH, C-8), 36.5 (CH_2, C-12), 41.7 (C, C-10), 43.0 (C, C-13), 46.8 (CH, C-9),

50.8 (CH, C-14), 59.9 (CH, C-4), 64.9 (CH_2, C-19), 69.1 (C, C-5), 81.4 (CH, C-17), 206.2 (C, C-3); MS m/z 320 (M^+), 302 (M-18, H_2O), 290 (M-30, HOCH), 289. 17β,19-Dihydroxy-4β,5-epoxy-5β-androstan-3-one **149** (114 mg, 3.56 x 10^{-4} mol) was dissolved in chloroform (5 ml) and stirred at room temperature with MCPBA (120 mg). The reaction was stopped after 11 days and purified by column chromatography. Elution with methylene chloride : ethyl acetate, 70 : 30 and 68.5 : 32.5 afforded a mixture of two compounds by tlc (63 mg) which was purified again by column chromatography eluted with methylene chloride : ethyl acetate, 73 : 27 and 72 : 28. The least polar compound isolated was not identified and was found by ^{13}C NMR to be a mixture of isomers (*ca* .10:1) despite the fact it was homogeneous on tlc. mp 69-70°C; ^1H NMR δ 0.73 (s, 3H, 18-CH_3), 3.27 (s, 1H, exchanges with D_2O), 3.73 (q, 2H, δ_a 3.57, δ_b 3.89, J_{ab}=8 Hz, 19-CH_2), 3.62 (tr, 1H, 17α-H), 4.12 (m, 2H). The second compound, more polar, was not obtained in a pure form. The first column was further eluted with methylene chloride : ethyl acetate, 65 : 35 to afford a third compound (20 mg) homogeneous on tlc but that could not be identified . Finally, the second compound was obtained when the reaction was repeated with **149** (342 mg, 1.07 x 10^{-3} mol). Column chromatography eluted with methylene chloride : ethyl acetate, 73 : 27 afforded A-nor-3,5-seco-5-oxo-17β,19-dihydroxyandrostane-3-carboxylic acid 3,19-lactone **150** (68 mg, 21%). mp 162-163°C (recrystallized in ethyl acetate : ether); IR γ_{max} 2940, 2860, 1745 (3-OCO), 1705 (5-CO); ^1H NMR δ 0.81 (s, 3H, 18-CH_3), 3.63 (tr, 1H, 17α-H), 4.44 (q, 2H, δ_a 4.39, δ_b 4.48, J_{ab}=11.8 Hz, 19-CH_2); ^{13}C NMR δ 11.3 (CH_3, C-18), 22.9 (CH_2, C-11), 23.2 (CH_2, [C-7]), 23.3 (CH_2, C-15), 28.2 (CH_2, [C-16]), 30.3 (CH_2, [C-12]), 31.1 (CH_2, [C-1]),

36.0 (CH, C-8), 36.4 (CH_2, C-2), 38.4 (CH_2, C-6), 43.0 (C, C-13), 50.4 (CH, C-14), 51.5 (CH, C-9), 51.7 (C, C-10), 69.0 (CH_2, C-19), 81.1 (CH, C-17), 172.1 (C, C-3), 210.6 (C, C-5); MS m/z 306 (M^+), 288 (M-18, H_2O), 279, 278 (M-28, CO); Anal. Calcd for $C_{18}H_{26}O_4$: C, 70.54; H, 8.56. Found: C, 70.38; H, 8.46.

A-Nor-3,5-seco-5-oxo-17β,19-dihydroxyandrostane-3-carboxylic acid 3,19-lactone **150** (22 mg, 7.19 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded A-nor-3,5-seco-5-oxo-17β-acetoxy-19-hydroxy-androstane-3-carboxylic acid 3,19-lactone **151** (23 mg, 91%). mp 183-184°C; $[\alpha]_D$ -16° (c 0.095); IR γ_{max} (KBr) 2960, 2910, 2880, 2860, 1735 (3-OCO and 17β-$COCH_3$), 1705 (5-C0) cm^{-1}; ^1H NMR δ 0.86 (s, 3H, 18-CH_3), 2.03 (s, 3H, 17β-$COCH_3$),4.42 (q, 2H, δ_a 4.38, δ_b 4.47, J_{ab}=11.8 Hz, 19-CH_2), 4.58 (tr, 1H, 17α-H); MS m/z 348 (M^+), 330 (M-18, H_2O), 320 (M-28, CO), 306 (M-42, $COCH_3$); HRMS calcd for $C_{20}H_{28}O_5$: 348.1957, found: 348.1932.

v. Preparation of A-Nor-3,5-seco-5,17-dioxoandrostane-3-carboxylic acid 3,19-lactone **152**.

19-Hydroxyandrost-4-ene-3,17-dione **4** (1.00 g, 3.31 x 10^{-3} mol) was dissolved in methanol (17 ml) and cooled in an ice-water bath. Ice-cold 4 N NaOH (2 ml) and 30% H_2O_2 (4 ml) were successively added and the reaction was stirred in an ice-water bath, without adding more ice, for 15 h 30 min. Water (30 ml) was added to the reaction mixture which was extracted with methylene chloride to afford 19-hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62**

(60 mg, 6%). The remaining aqueous phase was acidified with HCl and extracted with methylene chloride. The residue obtained after evaporation of the solvent was purified by column chromatography. Elution with methylene chloride : ethyl acetate, 85 : 15 afforded A-nor-3,5-seco-5,17-dioxoandrostane-3-carboxylic acid 3,19-lactone **152** (265 mg, 26%). mp 223-224°C; $[\alpha]_D$ +50° (c 0.415); IR γ_{max} 2940, 2860, 1735 (17-CO and 3-OCO), 1710 (5-CO) cm^{-1}; ^1H NMR δ 0.94 (s, 3H, 18-CH_3), 4.43 (q, 2H, δ_a 4.38, δ_b 4.48, J_{ab}=13.2 Hz, 19-CH_2); ^{13}C NMR δ 13.8 (CH_3, C-18), 21.6 (CH_2, C-11), 22.5 (CH_2, C-15), 23.1 (CH_2, [C-7]), 28.1 (CH_2, [C-1]), 30.1 (CH_2, [C-2]), 31.2 (CH_2, C-12), 35.4 (CH_2, C-16), 35.5 (CH, C-8), 38.1 (CH_2, C-6), 47.5 (C, C-13), 50.6 (CH, C-9), 51.4 (CH, C-14), 51.6 (C, C-10), 68.8 (CH_2, C-19), 172.0 (C, C-3), 210.1 (C, C-5), 219.0 (C, C-17); MS m/z 304 (M^+), 286 (M-18, H_2O), 277, 276 (M-28, CO); HRMS calcd for $C_{18}H_{24}O_4$: 304.1668, found: 304.1660. Anal. Calcd for $C_{18}H_{24}O_4$: C, 71.01; H, 7.95. Found: C, 70.71; H, 7.91.

2. Reaction of 17β,19-Dihydroxy-5β-androstane-3-one **24** with MCPBA.

i. Treatment of 17β,19-Dihydroxy-5β-androstane-3-one **24** with MCPBA; Formation of A-Homo-17β,19-dihydroxy-2a-oxa-5β-androstan-3-one **154** and A-Nor-3,4-seco-4,17β,19-trihydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **158**.

To a solution of 17β,19-dihydroxy-5β-androstan-3-one **24** (0.10 g, 3.27 x 10^{-4} mol) dissolved in chloroform (3 ml) was added MCPBA (0.10 g) and the reaction was stirred at room temperature for 2 days. Purification by column chromatography, eluted successively with methylene chloride : ethyl acetate, 65 : 35, 60 : 40, 55 : 45 and 50 : 50 first afforded A-homo-17β,19-dihydroxy-2a-oxa-5β-androstan-3-one **154** (48 mg, 46%). mp 199-200°C; $[\alpha]_D$ +57° (c 0.12); IR γ_{max} 2940, 1725 (3-OCO) cm^{-1}; ^1H NMR δ 0.72 (s, 3H, 18-CH_3), 3.72 (q, 2H, δ_a 3.51, δ_b 3.94, J_{ab}=11.1 Hz, 19-CH_2), 3.64 (tr, 1H, 17α-H), 4.16 (m, 2H, 2-CH_2); ^{13}C NMR δ 11.2 (CH_3, C-18), 20.9 (CH_2, C-11), 23.3 (CH_2, C-15), 25.1 (CH_2, C-7), 29.0 (CH_2, C-16), 30.5 (CH_2, C-6), 33.6 (CH, C-8), 33.7 (CH_2, [C-1]), 35.6 (CH, C-9), 36.4 (CH_2, C-4), 37.1 (CH_2, C-12), 40.9 (C, C-10), 41.1 (CH, C-5), 43.0 (C, C-13), 51.3 (CH, C-14), 62.3 (CH_2, C-2), 65.2 (CH_2, C-19), 81.6 (CH, C-17), 176.2 (C, C-3); MS m/z 322 (M^+), 304 (M-18, H_2O), 294 (M-28, CO), 292 (M-30, HOCH). Anal. Calcd for $C_{19}H_{30}O_4$: C, 70.75; H, 9.38. Found: C, 70.88; H, 9.34. The second compound eluted was A-nor-3,4-seco-4, 17β,19-trihydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **158** (37 mg, 35%). mp 108-110°C; $[\alpha]_D$ +4° (c 0.10); IR γ_{max} 1730 br (3-OCO) cm^{-1}; ^1H NMR δ 0.71 (s, 3H, 18-CH_3), 3.61 (tr, 1H, 17α-H), 3.72 (br d, 2H, 4-CH_2), 4.37 (q, 2H, δ_a 4.28, δ_b 4.46, J_{ab}=11.8 Hz, 19-CH_2); ^{13}C NMR δ 11.1 (CH_3, C-18), 21.3 (CH_2, C-11), 22.4 (CH_2, [C-1]), 23.1 (CH_2, C-15), 25.4 (CH_2, [C-6]), 27.4 (CH_2, [C-7]), 27.8 (CH_2, [C-16]), 30.4 (CH_2, C-12), 35.8 (CH, C-8),

36.7 (CH_2, C-2), 37.0 (C, C-10), 39.8 (CH, C-5), 42.7 (C, C-13), 45.8 (CH, C-9), 51.2 (CH, C-14), 60.9 (CH_2, C-4), 71.4 (CH_2, C-19), 81.5 (CH, C-17), 172.8 (C, C-3); MS m/z 322 (M^+), 304 (M-18, H_2O), 294 (M-28, CO), 292 (M-30, HOCH), 291, 279, 274; HRMS calcd for $C_{19}H_{30}O_4$: 322.2136, found: 322.2119. Anal. Calcd for $C_{19}H_{30}O_4$: C, 70.75; H, 9.38. Found: C, 70.61; H, 9.39.

ii. Acetylation of A-Homo-17β,19-dihydroxy-2a-oxa-5β-androstan-3-one **154**.

A-Homo-17β,19-dihydroxy-2a-oxa-5β-androstan-3-one **154** (4 mg, 1.24 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded A-homo-17β,19-diacetoxy-2a-oxa-5β-androstan-3-one (100%) as a sticky material. ^1H NMR δ 0.77 (s, 3H, 18-CH_3), 2.02 (s, 3H) and 2.06 (s, 3H) 17β-and 19-$COCH_3$, 4.15 (q, 2H, δ_a 3.91, δ_b 4.39, J_{ab}=11.5 Hz, 19-CH_2), 4.18 (m, 2H, 2-CH_2), 4.58 (tr, 1H, 17α-H); MS m/z 406 (M^+, absent), 364 (M-42, $COCH_3$), 346, 304, 291, 286.

iii. Oxidation of A-Nor-3,4-seco-4,17β,19-trihydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **158**.

The oxidation was carried out as described by Ratcliffe[152] with A-nor-3,4-seco-4,17β,19-trihydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **158** (40 mg, 1.24 x 10^{-4} mol) dissolved in 10 ml of methylene

chloride and to which was added to the following solution: pyridine (0.32 ml) in methylene chloride (1 ml) was cooled in an ice-water bath; Cr(VI)oxide (0.204 g) was added in one portion and the solution was stirred for 5 min then allowed to warm at room temperature over a period of 60 min. After stirring this latter solution with the steroid solution for 20 min , anhydrous diethyl ether (50 ml) was added and the suspension was filtered through a short column of Florisil and washed with methylene chloride. The combined ether and methylene chloride extracts were washed with 10% aqueous HCl, dried over sodium sulfate and the solvent removed under reduced pressure. Column chromatography eluted with methylene chloride : ethyl acetate, 90 : 10 afforded A-nor-3,4-seco-4,17-dioxo-19-hydroxy-5α,β-androstane-3-carboxylic acid 3,19-lactone **162** (9 mg, 22%), homogeneous on the basis of tlc. IR γ_{max} (KBr) 2940, 2880, 2860, 1735 br cm^{-1}; ^{1}H NMR δ 0.85 (s, 3H, 18-CH_3), 4.34 (q, 2H, δ_a 4.31, δ_b 4.37, J_{ab}=11.7 Hz, 19-CH_2), 9.76 (s, 1H, 4-CHO); ^{13}C NMR δ 13.7 (CH_3, C-18), 20.9 (CH_2, C-11), 21.1 (CH_2), 21.5 (CH_2, C-15), 24.0 (CH_2), 25.4 (CH_2), 26.1 (CH_2), 28.2 (CH_2), 28.8 (CH_2), 29.4 (CH_2), 31.5 (CH_2, C-12), 35.2 (CH, C-8), 35.6 (CH_2, C-16), 37.3 (C, C-10), 44.4 (C), 46.8 (CH, C-9), 47.4 (C, C-13), 51.1 (CH, C-14), 52.0 (CH, C-5), 69.0 (69.4) (CH_2, C-19), 202.8 (CH, C-4), 220.0 (C, C-17); MS m/z 318 (M^+), 300 (M-18, H_2O), 290 (M-28, CO), 272; HRMS calcd for $C_{19}H_{26}O_4$: 318.1824, found: 318.1835.

iv. Acetylation of A-Nor-3,4-seco-4,17β,19-trihydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **158**.

A-Nor-3,4-seco-4,17β,19-trihydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **158** (5 mg, 1.49 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded A-nor-3,4-seco-4,17β-diacetoxy-19-hydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **163** (100%) as a clear oil. ^{1}H NMR δ 0.75 (s, 3H, 18-CH_3), 2.01 (s, 3H) and 2.04 (s, 3H) 4- and 17β-$COCH_3$, 4.16 (d, 2H, 4-CH_2), 4.37 (q, 2H, δ_a 4.27, δ_b 4.47, J_{ab}=11.8 Hz, 19-CH_2), 4.55 (tr, 1H, 17α-H); MS m/z 406 (M^+), 364 (M-42), 346 (M-60), 318.

v. Preparation and Treatment with MCPBA of 17β,19-Diacetoxy-5β-androstan-3-one **164**.

17β,19-Dihydroxy-5β-androstan-3-one **24** (150 mg, 4.90 x 10^{-4} mol) was treated with acetic anhydride (Method A). Work up afforded 17β,19-diacetoxy-5β-androstan-3-one **164** (100%) as a sticky material. (lit.[69] mp 127-128°C); ^{1}H NMR δ 0.79 (s, 3H, 18-CH_3), 2.02 (s, 3H) and 2.05 (s, 3H), 17β- and 19-$COCH_3$, 4.24 (q, 2H, δ_a 4.07, δ_b 4.40, J_{ab}=11.1 Hz, 19-CH_2), 4.59 (tr, 1H, 17α-H); MS m/z 390 (M^+), 331, 330, 317, 302. 17β,19-Diacetoxy-5β-androstan-3-one **164** (197 mg, 5.05 x 10^{-4} mol) was dissolved in chloroform (5 ml). MCPBA (250 mg) was added and the reaction was stirred for 2 days at

room temperature. Column chromatography eluted with methylene chloride : ethyl acetate, 92 : 8 afforded a homogeneous compound by tlc which was however a *ca* . 1:1 mixture (by ^{13}C NMR) of A-homo-17β,19-diacetoxy-2a-oxa-5β-androstan-3-one **165** and A-homo-17β,19-diacetoxy-3a-oxa-5β-androstan-3-one **166** (64%). mp 72-73°C; $[\alpha]_D$ +28° (c 0.27); IR γ_{max} 2940, 2860, 2840, 1735 br (3-OCO and 17β,19-COCH$_3$); ^1H NMR δ 0.77 (s, 3H, 18-CH$_3$), 2.02 (s, 3H) and 2.06 (s, 3H) 17β- and 19-COCH$_3$, 4.11 (m, 2H), 4.26 (br q, 2H, δ_a 3.93, δ_b 4.59, 19-CH$_2$), 4.42 (tr, 1H, 17α-H); ^{13}C NMR δ for **165** 12.2 (CH$_3$, C-18), 20.7 (CH$_2$, C-11), 24.9 (CH$_2$, C-15), 26.5* (CH$_2$, C-7), 27.6 (CH$_2$, C-16), 27.7* (CH$_2$, C-6), 34.1 (CH, C-5), 35.4 (CH, C-8), 36.3 (CH$_2$, C-4), 37.1 (CH$_2$, C-1), 37.1 (CH$_2$, C-12), 39.7 (C, C-10), 40.7 (CH, C-9), 42.6 (C, C-13), 51.1 (CH, C-14), 62.7 (CH$_2$, C-2), 66.8 (CH$_2$, C-19), 82.3 (CH, C-17), 175.7 (C, C-3); ^{13}C NMR for **166** 12.1 (CH$_3$, C-18), 20.5 (CH$_2$, C-11), 23.3 (CH$_2$, C-15), 26.2* (CH$_2$, C-7), 27.5 (CH$_2$, C-16), 27.7 * (CH$_2$, C-6), 28.8 (CH$_2$, C-1), 34.1 (CH$_2$, C-2), 35.4 (CH, C-8), 37.1 (CH$_2$, C-12), 38.5 (CH, C-5), 39.6 (C, C-10), 40.9 (CH, C-9), 42.6 (C, C-13), 51.1 (CH, C-14), 66.7 (CH$_2$, C-19), 69.9 (CH$_2$, C-4), 82.3 (CH, C-17), 175.5 (C, C-3); MS m/z 406 (M^+), 346, 333, 304. Anal. Calcd for $C_{23}H_{34}O_6$: C, 67.93; H, 8.43. Found: C, 68.13; H, 8.66.

3. Treatment of 19-Hydroxyandrost-4-ene-3,17-dione **4** with MCPBA.

19-Hydroxyandrost-4-ene-3,17-dione **4** (0.20 g, 6.62 x 10^{-4} mol) was dissolved in chloroform (4 ml, previously mixed with 4 ml of 35% $HClO_4$ and

separated). MCPBA (0.20 g) was added and the solution was stirred at room temperature for 3 days. Work up was followed by column chromatography eluted successively with methylene chloride : ethyl acetate 90 : 10 and 85 : 15 to afford 4 mg of a homogeneous compound by tlc which could not be identified. IR γ_{max} 2940, 2880, 2860, 1730 cm^{-1}; ^1H NMR δ 0.87 (s, 3H, 18-CH_3), 3.50 (m, 1H), 3.72 (m, 1H), 3.83 (q, 2H, δ_a 3.69, δ_b 3.97, J_{ab}~ 9 Hz). Further elution with methylene chloride : ethyl acetate, 82 : 18 afforded a second compound (7 mg, 3%). mp 218-219°C (crystals from ether); IR γ_{max} 2940, 2860, 1740 (17-CO and 3-OCO),1718 sh cm^{-1}; ^1H NMR δ 0.93 (s, 3H, 18-CH_3), 3.60 (s, 1H, exchanges with D_2O), 4.57 (q, 2H, δ_a 4.50, δ_b 4.65, J_{ab}=12.1 Hz); MS m/z 334 (M^+), 306 (M-28, CO), 305, 288, 287, 276; HRMS calcd for $C_{19}H_{26}O_5$: 334.1773, found: 334.1789. The last compound eluted was A-homo-3a-oxa-5β, 19-oxido-4ξ-hydroxy-5β-androstane-3,17-dione **167** (36 mg, 16%). mp 248-249°C (crystals from ethyl acetate); $[\alpha]_D$ +75° (c 0.32); IR γ_{max} 3350 br (OH), 2940, 2880, 2860, 1730 (17-CO, 3-OCO) cm^{-1}; ^1H NMR δ 0.88 (s, 3H, 18-CH_3), 3.27 (d, 1H, J=4 Hz, exchanges with D_2O, 4ξ-OH), 3.88 (q, 2H, δ_a 3.77, δ_b 3.99, J_{ab}=8.5 Hz, 19-CH_2), 4.97 (d, 1H, J=4 Hz, becomes a s upon addition of D_2O, 4ξ-H); ^{13}C NMR δ 13.9 (CH_3, 18-CH_3), 21.5 (CH_2, C-15), 22.1 (CH_2, C-11), 23.8* (CH_2, C-7), 25.3* (CH_2, C-6), 26.2* (CH_2, C-1), 31.7 (CH_2, C-12), 32.6 (CH_2, C-2), 35.6 (CH_2, C-16), 35.7 (CH, C-8), 39.7 (C, C-10),

42.3 (CH, C-9), 47.8 (C, C-13), 50.3 (CH, C-14), 73.4 (CH_2, C-19), 87.4 (C, C-5), 101.1 (CH, C-4), 171.4 (C, C-3), 219.9 (C, C-17); MS m/z 334 (M^+), 316 (M-18, H_2O), 306 (M-28, CO); HRMS calcd for $C_{19}H_{26}O_5$: 334.1773, found: 334.1753. Anal. Calcd for $C_{19}H_{26}O_5$: C, 68.22; H, 7.84. Found: C, 68.28; H, 7.94.

A-Homo-3a-oxa-5,19-oxido-4ξ-hydroxy-5β-androstane-3,17-dione **167** (13 mg, 3.8 x 10^{-5} mol) was treated with acetic anhydride (Method A). Work up afforded an epimeric mixture (*ca* . 1:1, by ^1H NMR) of A-homo-3a-oxa-5,19- oxido-4α- and β-acetoxy-5β-androstane-3,17-dione **168**. mp 211-212°C; IR γ_{max} (KBr) 2940, 2860, 1740-1735 (17-CO, 4-$\underline{CO}CH_3$) cm^{-1}; ^1H NMR δ 0.87 (0.89) (s, 3H, 18-CH_3), 2.06 (2.11) (s, 3H, 4-$COCH_3$), 3.76 (4.14) (q, 2H, δ_a 3.73, δ_b 3.79, J_{ab}=8.7 Hz, 19-CH_2) (q, 2H, δ_a 4.05, δ_b 4.24, J_{ab}=8.6 Hz, 19-CH_2), 5.84 (6.19) (s, 1H, 4-H); MS m/z 376 (M^+), 335, 334 (M-42, $COCH_3$), 333, 306 (M-42-28, CO); HRMS calcd for $C_{21}H_{28}O_6$: 376.1878, found: 376.1910.

CLAIMS TO ORIGINAL RESEARCH

A. Two forms of a 17β-hydroxysteroid dehydrogenase have been purified and the topography of their binding sites was studied using the technique of affinity labeling.

B. The following new steroid derivatives were prepared, characterized and used in the affinity labeling studies:

1. 3β,19-Dihydroxy-5β-androstan-17-one **15**
2. 3α,19-Dihydroxy-5β-androstan-17-one **23**
3. 19-Iodoacetoxyandrost-4-ene-3,17-dione **29**
4. 19-Bromoacetoxyandrost-4-ene-3,17-dione **30**
5. 19-Iodo[2'-^{3}H]acetoxyandrost-4-ene-3,17-dione **32**
6. 19-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **33**
7. 19-Iodoacetoxy[6,7-^{3}H]androst-4-ene-3,17-dione **29** [^{3}H]
8. 19-Iodoacetoxy-5β-androstane-3,17-dione **34**
9. 19-Bromoacetoxy-5β-androstane-3,17-dione **35**
10. 19-Iodo[2'-^{14}C]acetoxy-5β-androstane-3,17-dione **37**
11. 6β-Iodoacetoxyandrost-4-ene-3,17-dione **38**
12. 6β-Iodo[2'-^{14}C]acetoxyandrost-4-ene-3,17-dione **40**
13. 11β-Iodoacetoxyandrost-4-ene-3,17-dione **41**
14. 17β-Iodoacetoxyandrost-4-en-3-one **44**
15. 17β-Iodo[2'-^{14}C]acetoxyandrost-4-en-3-one **47**
16. 17β-Iodoacetoxy-5β-androstan-3-one **48**
17. 17β-Bromoacetoxy-5β-androstan-3-one **49**
18. 17β-Iodo[2'-^{14}C]acetoxy-5β-androstan-3-one **51**

C. 3α-Ethoxy-3β,19-oxido-5α-androstan-17-one **26** and not 3α-hydroxy-3β,19-oxido-5α-androstan-17-one **25** (as reported by Edwards and co-workers[69]) was isolated from the hydrogenation of 19-hydroxyandrost-4-ene-3,17-dione **4** along with 19-hydroxy-5β-androstane-3,17-dione **14**.

D. The acid- and base-catalyzed opening of 19-hydroxy-4β,5-epoxy-5β-androstane-3,17-dione **62** was examined.

E. 4β,5,19-Trihydroxy-5β-androstane-3,17-dione **88** and 4α,5,19-trihydroxy-5α-androstane-3,17-dione **89** were prepared. Acid-catalyzed treatment of **88** and **89** was examined.

F. 19a-Hydroperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **100** was synthesized. The reactions of sodium iodide, hydrogen bromide, methyl iodide and acetic anhydride with **100** were investigated.

G. The Baeyer-Villiger rearrangements of 19-hydroxy-4,5-epoxy steroids were examined.

H. The following new compounds were prepared and characterized:

1. 4,19-Dihydroxyandrost-4-ene-3,17-dione **63**
2. 19-Acetoxy-4β,5-epoxy-5β-androstane-3,17-dione **77**
3. 17β,19-Diacetoxy-4β,5-epoxy-5β-androstan-3-one **79**
4. 2α,17β,19-Triacetoxyandrost-4-en-3-one **80**
5. 2β,19-Oxidoandrost-4-ene-3,17-dione **82**
6. 4α-Hydroxy-4β,19-oxido-5α-androstane-3,17-dione **92**
7. 4α,5-Isopropylidenedioxy-3α-hydroxy-3β,19-oxido-5α-androstan-17-one **96**
8. 4-Methoxy-19-hydroxyandrost-4-ene-3,17-dione **98**
9. 19a-Methylperoxy-4β,5-epoxy-2-oxa-5β,10α-androstane-3,17-dione **112**
10. 4β-Hydroxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **113**
11. 4β-Acetoxy-5,19a-oxido-2-oxa-5α,10α-androstane-3,17-dione **114**
12. 4-Bromo-19-hydroxyandrost-4-ene-3,17-dione **102**
13. 4-Bromo-19-acetoxyandrost-4-ene-3,17-dione **103**
14. 19-Hydroxy-4β,5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **145**

15. 19-Acetoxy-4β,5-epoxy-17a-oxa-D-homo-5β-androstane-3,17-dione **146**
16. 19-Hydroxy-4α,5-epoxy-17a-oxa-D-homo-5α-androstane-3,17-dione **148**
17. A-Nor-3,5-seco-5-oxo-17β,19-dihydroxyandrostane-3-carboxylic acid 3,19-lactone **150**
18. A-Nor-3,5-seco-5-oxo-17β-acetoxy-19-hydroxyandrostane-3-carboxylic acid 3,19-lactone **151**
19. A-Nor-3,5-seco-5,17-dioxoandrostane-3-carboxylic acid 3,19-lactone **152**
20. A-Homo-17β,19-dihydroxy-2a-oxa-5β-androstan-3-one **154**
21. A-Nor-3,4-seco-4,17β,19-trihydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **158**
22. A-Homo-17β,19-diacetoxy-2a-oxa-5β-androstan-3-one **154** (diacetate)
23. A-Nor-3,4-seco-4,17-dioxo-19-hydroxy-5α- and β-androstane-3-carboxylic acid 3,19-lactone **162**
24. A-Nor-3,4-seco-4,17β-diacetoxy-19-hydroxy-5β-androstane-3-carboxylic acid 3,19-lactone **163**
25. The ^{13}C NMR assignments of A-homo-17β,19-diacetoxy-2a-oxa-5β-androstan-3-one **165** and A-homo-17β,19-diacetoxy-3a-oxa-5β-androstan-3-one **166** were made.
26. A-Homo-3a-oxa-5,19-oxido-4α- and β-acetoxy-5β-androstane-3,17-dione **168**

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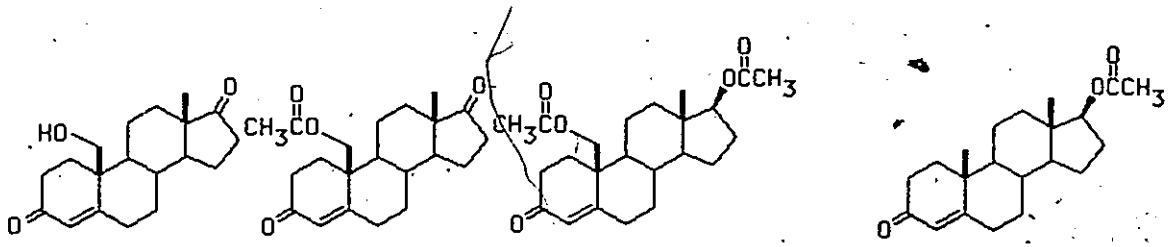
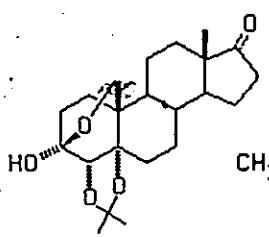
APPENDIX

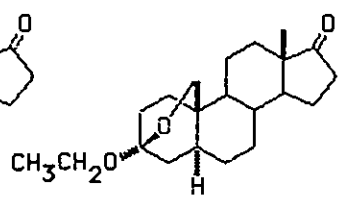
In this appendix, the ^{13}C NMR spectra of the steroids synthesized are summarized. The assignment of ^{13}C peaks in terms of number of attached hydrogen atoms has been made using the pulse sequence DEPT[164] (Distortionless Enhancement of NMR Signals by Polarization Transfer). Several reviews[165,166,167] and publications[144,145,168,169,170,171,172,173,174] in the field of ^{13}C nuclear magnetic resonance of steroids facilitated the assignment of the ^{13}C NMR spectra. Brackets are used to indicate carbon resonances which are tentatively assigned; an asterisk is used to indicate carbon resonance assignments which can be interchanged (when more than one pair is involved the symbol • is used).

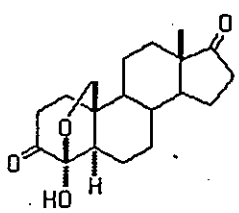
Compound **174** was obtained following treatment of 19-hydroxy-androst-4-ene-3,17-dione **4** with lead tetraacetate[175] and 19-nor-androst-4-ene-3,17-dione **173** was obtained after treatment of androst-4-ene-3,17,19-trione with potassium hydroxide in methanol.

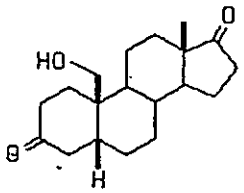


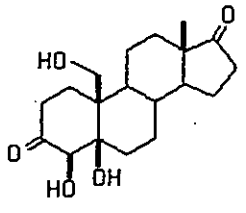
	4	4[171]	31	172	46	46[165] (CD_3CL_3-DIOXAN)
1	33.3*	33.6	33.6*	33.5*	35.6	36.1
2	34.8	35.0	34.6	34.5	38.8	34.2
3	200.3	199.6	198.8	199.2	199.4	197.8
4	126.5	127.3	126.9	126.7	123.8	124.3
5	167.2	165.6	164.6	165.4	171.0	170.4
6	33.2*	33.6	32.9*	32.9*	32.7	32.9
7	31.5	31.9	31.6	31.6	31.4	32.0
8	35.6	36.1	35.8	35.9	35.3	35.8
9	53.9	54.3	54.1	53.9	53.6	54.3
10	43.8	43.9	41.9	41.7	38.5	38.9
11	20.7	21.0	21.0	20.9	20.4	21.0
12	30.8	31.0	31.0	36.8	36.5	37.1
13	47.5	47.7	47.5	42.4	42.4	42.9
14	51.1	51.5	51.2	50.5	50.1	50.8
15	21.6	21.8	21.7	23.3	23.4	23.8
16	35.6	35.8	35.7	27.3	27.4	27.9
17	220.4	220.0	219.5	82.1	82.4	82.5
18	13.7	13.9	13.9	12.0	11.9	12.2
19	65.5	66.1	66.6	66.7	17.3	17.6
$CO\underline{C}H_3$			20.9	20.9	21.1	21.0
$CO\underline{C}H_3$				21.0		
$\underline{C}OCH_3$			170.4	170.6	170.9	170.0
$\underline{C}OCH_3$				171.0		

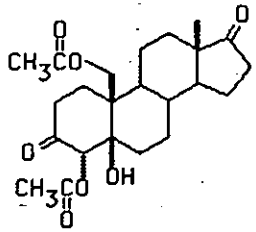




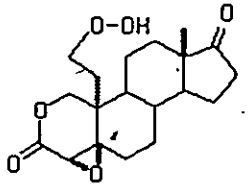


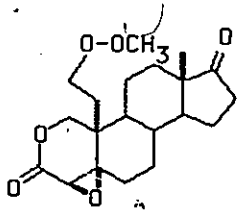


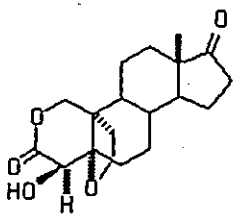


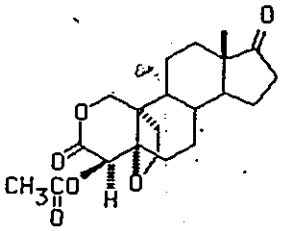


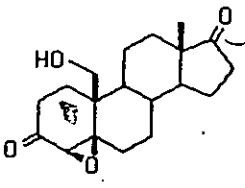
	96	26	92		14	88	91
1	[23.3]	[38.8]	[35.2]	1	[30.6]	[30.8]	[29.1]
2	[33.7]	[31.7]	[32.9]	2	[36.7]	[34.0]	
3	109.7	97.9	205.7	3	212.8	209.4	202.7
4	83.3	[29.9]	103.6	4	41.4	73.6	70.6
5	97.2	[49.2]	48.1*	5	[36.1]	83.3	78.5
6	25.4	30.0	[22.4]	6	24.3	25.8	24.5
7	26.6	30.6	[28.1]	7	25.8	27.6	28.8
8	34.9	35.5	36.0	8	35.0	34.0	34.2
9	43.4	[39.3]	49.9*	9	[41.1]	43.6	45.5
10	39.7	33.8	46.6	10	39.0	43.9	43.9
11	21.0	21.0	21.1	11	20.4	20.8	20.9
12	31.5	31.5	31.7	12	31.8	31.6	31.7
13	47.6	47.5	47.9	13	47.7	47.5	47.6
14	50.8	51.4	50.5	14	51.7	52.1	51.2
15	21.5	21.6	21.8	15	21.6	21.8	21.6
16	35.7	35.7	35.8	16	35.7	35.7	35.7
17	220.6	220.7	220.0	17	220.7	219.6	220.3
18	13.7	13.6	14.1	18	13.7	13.8	13.8
19	67.6	67.0	70.1	19	64.5	63.6	64.3
CH_3	27.2			$CO\underline{CH_3}$			20.6
CH_3	26.8			$CO\underline{CH_3}$			21.2
$O\underline{CH_2}CH_3$		15.6		$\underline{CO}CH_3$			169.7
$O\underline{CH_2}CH_3$		57.1		$\underline{CO}CH_3$			170.5
							(29.7, 37.1, CH_2)

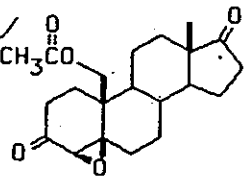




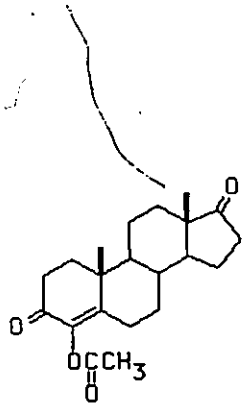


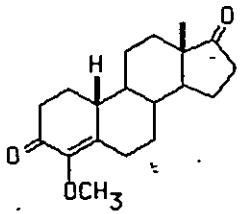


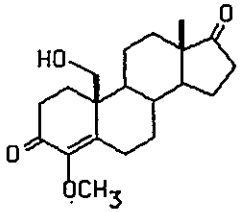


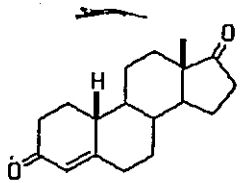


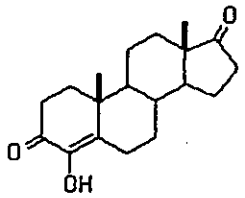
	100	112	113		114	62	77
1	73.3	69.8	68.6	1	68.7	21.4	21.6
2				2		32.1	31.8
3	168.2	167.3	174.1	3	168.1*	206.0	205.5
4	55.2	54.6	70.4	4	70.0	60.0	60.3
5	66.7	66.7	85.4	5	84.0	68.8	68.3
6	[23.7]	[23.7]	[22.1]	6	[22.7]	30.0	29.6
7	[24.8]	[24.5]	[24.6]	7	[25.9]	31.3	31.2
8	33.3	33.5	33.2	8	33.7	34.6	34.6
9	48.5	47.5	46.0	9	45.9	46.8	46.6
10	38.8	38.4	47.9	10	47.9	41.6	40.8
11	21.3	21.6	21.5	11	21.6	21.4	21.4
12	31.4	31.3	31.4	12	31.6	29.2	29.2
13	47.3	47.2	46.9	13	47.3	47.6	47.6
14	51.6	51.4	51.6	14	51.4	51.0	51.1
15	21.7	21.6	21.6	15	21.7	21.6	21.5
16	35.5	35.4	35.6	16	35.7	35.6	35.5
17	219.7	219.6	219.9	17	219.4	220.0	219.5
18	13.7	13.6	13.7	18	13.9	13.7	13.7
19	34.4	33.9	37.1	19	36.4	64.8	65.3
19a	68.9	67.7	64.1	19a	64.1		
$OOCH_3$		62.2		$CO\underline{CH_3}$	20.7		21.0
				$\underline{CO}CH_3$	169.8*		171.0



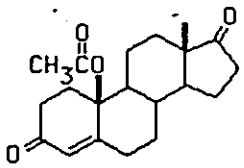


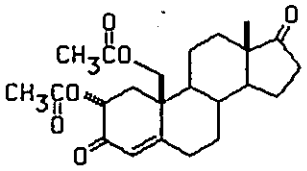


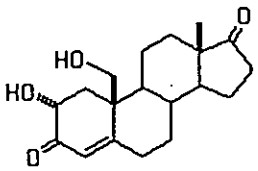


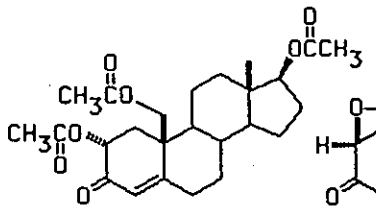


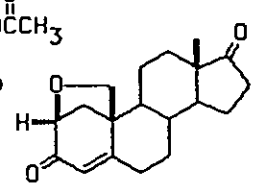
	56	**99**	**98**	**173**[165]	**173**	**6**
1	23.9	26.5	[32.5]	27.1	26.5	22.7
2	39.1	37.0	[35.2]	36.7	36.4	37.8
3	190.5	194.2	194.5	198.0	199.6	193.4
4	139.2	[147.3]	[149.0]	125.0	124.8	139.0
5	155.0	[149.1]	[149.8]	165.4	165.6	141.3
6	33.3	26.0	23.8	35.6	35.6	31.7
7	31.2	31.4	31.6	32.0	31.2	31.3
8	34.7	39.7	35.4	40.3	39.8	34.7
9	53.7	49.7*	54.2	50.1	49.4	54.2
10	35.7	42.5	43.8	42.7	42.3	35.7
11	20.3	25.7	20.7	26.1	25.6	20.2
12	29.7	29.4	30.4	30.5	29.8	29.8
13	47.5	47.7	47.5	47.7	47.6	47.4
14	50.7	50.2*	51.2	50.5	50.0	50.9
15	21.7	21.7	21.6	21.9	21.6	21.7
16	34.6	35.8	35.7	35.4	35.2	34.6
17	220.3	220.1	220.3	218.6	220.3	220.6
18	13.7	13.9	13.8	13.8	13.7	13.7
19	17.6		65.8			17.1
OCH_3		60.2	60.5			
$COCH_3$	20.3					
$\underline{C}OCH_3$	168.6					

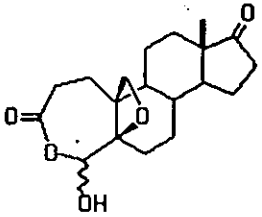




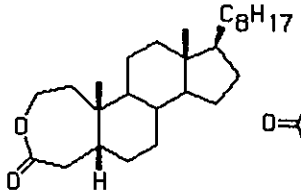




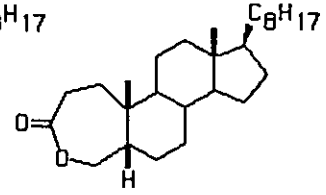




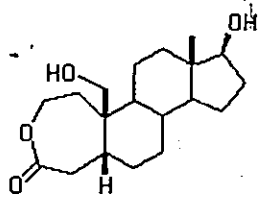
	174	78	64		80	82	167
1	[32.6]	39.7	42.7	1	39.8	42.3	[26.2]
2	[34.9]	70.9	69.6	2	71.1	80.9	32.6
3	197.8	193.6	200.1	3	193.7	196.8	171.4
4	124.8	125.1	123.5	4	125.0	123.4	101.1
5	164.1	164.4	167.9	5	165.0	167.3	87.4
6	[32.5]	32.1	32.9	6	32.3	[28.1]	[25.3]
7	[28.5]	31.4	31.5	7	31.6	[28.1]	[23.8]
8	35.1	35.3	35.5	8	35.6	37.7	35.7
9	52.7	54.9	54.9	9	54.8	44.5	42.3
10	80.4	44.2	46.2	10	42.4	50.6	39.7
11	21.0	20.5	20.5	11	20.8	21.0	22.1
12	30.8	30.8	30.8	12	36.7	[31.4]	31.7
13	47.7	47.3	47.5	13	44.2	47.4	47.8
14	50.2	51.0	51.0	14	50.5	51.3	50.3
15	21.6	21.5	21.6	15	23.2	21.7	21.5
16	35.7	35.5	35.6	16	27.3	35.7	35.6
17	219.9	219.5	220.0	17	82.1	219.3	219.9
18	13.7	13.7	13.7	18	12.1	13.8	13.9
19		66.3	65.7	19	66.6	69.5	73.4
$CO\underline{C}H_3$	21.8	20.8		$CO\underline{C}H_3$	20.9		
$CO\underline{C}H_3$		20.9		$CO\underline{C}H_3$	20.9		
$\underline{C}OCH_3$	169.4	170.1		$CO\underline{C}H_3$	21.1		
$\underline{C}OCH_3$		170.6		$\underline{C}OCH_3$	170.2		
				$\underline{C}OCH_3$	170.7		
				$\underline{C}OCH_3$	171.1		



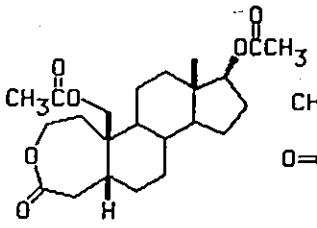
155[145]



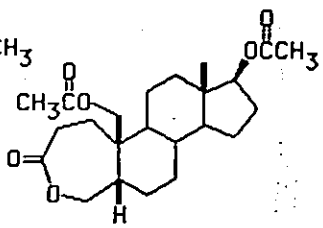
156[145]



154

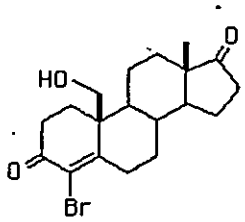


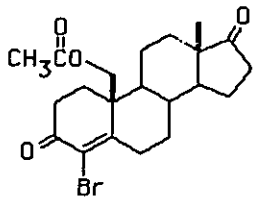
165

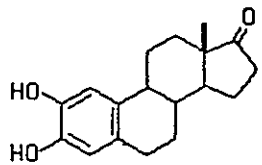


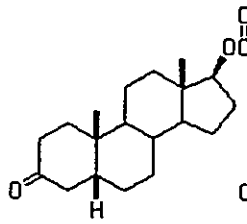
166

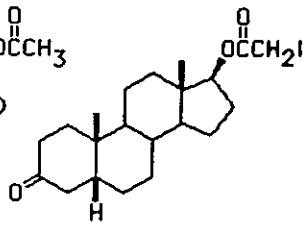
	155[145]	156[145]		154	165	166
1	40.1	28.3	1	33.7	37.1	28.8
2	63.3	33.3	2	62.3	62.7	34.1
3	176.3	176.2	3	176.2	175.7	175.5
4	36.9	70.5	4	36.4	36.3	69.9
5	41.3	45.5	5	41.1	34.1	38.5
6	29.7	27.0*	6	30.5	27.7*	27.7*
7	25.8	27.4*	7	25.1	26.5*	26.2*
8	35.7	35.8	8	33.6	35.4	35.4
9	41.3	41.5	9	35.6	40.7	40.9
10	36.7	36.5	10	40.9	39.7	39.6
11	21.3	21.0	11	20.9	20.7	20.5
12	39.7	40.1	12	37.1	37.1	37.1
13	42.7	42.7	13	43.0	42.6	42.6
14	56.4	56.4	14	51.3	51.1	51.1
15			15	23.3	24.9	23.3
16			16	29.0	27.6	27.5
17			17	81.6	82.3	82.3
18			18	11.2	12.2	12.1
19	24.2	23.9	19	65.2	66.8	66.7
			$CO\underline{CH_3}$		20.1	20.1
			$CO\underline{CH_3}$		20.9	20.9
			$\underline{CO}CH_3$		171.1	171.1
			$\underline{CO}CH_3$		171.1	171.1



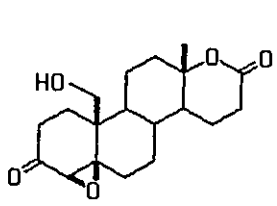


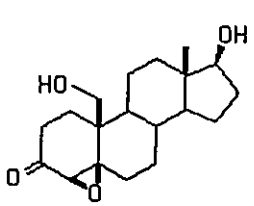


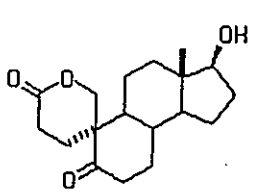


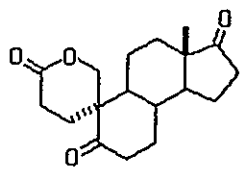


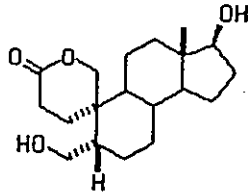
	102	**103**	**81**		**50**		**48**
1	33.2*	32.9*	[115.5]	1	37.1*		37.1*
2	34.9	34.5	[141.6]	2	37.0*		37.0*
3	191.2	190.4	[141.5]	3	212.5		213.0
4	124.1	124.4	[112.5]	4	42.3		43.1
5	163.7	162.1	[129.0]	5	44.2		44.2
6	32.2*	32.3*	28.7	6	25.4		25.3
7	31.6	31.5	26.6	7	26.5		26.4
8	35.4	35.4	38.2	8	35.4		35.3
9	54.0	54.1	44.0	9	40.8		40.7
10	47.4•	45.5	[131.3]	10	35.0		34.9
11	21.1	21.1	26.0	11	20.7		20.6
12	30.1	30.1	31.5	12	37.0		36.9
13	47.6•	47.5	48.1	13	42.7		42.3
14	51.0	51.0	50.4	14	50.8		50.7
15	21.6	21.6	21.6	15	23.5		23.4
16	35.7	35.6	35.9	16	27.6		27.0
17	220.1	219.5	221.6	17	82.5		84.1
18	13.8	13.8	13.8	18	12.1		12.0
19	65.8	66.5		19	22.6		22.6
$CO\underline{C}H_3$		20.9		$CO\underline{C}H_3$	21.1	$CO\underline{C}H_2I$	-5.0
$\underline{C}OCH_3$		170.5		$\underline{C}OCH_3$	170.8	$\underline{C}OCH_2I$	168.9

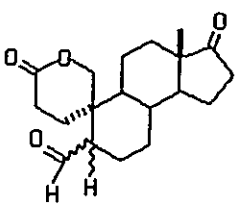












	145	149	150		152	158	162
1	21.6	21.8	[31.1]	1	[28.1]	[22.4]	
2	32.1	32.2	36.4	2	[30.1]	36.7	
3	205.5	206.2	172.1	3	172.0	172.8	
4	60.2	59.9		4		60.9	202.8
5	68.5	69.1	210.6	5	210.1	39.8	52.0
6	28.4*	30.1	38.4	6	38.1	[25.4]	
7	29.8	30.3	[23.2]	7	[23.1]	[27.4]	
8	37.5	35.1	36.0	8	35.5	35.8	35.2
9	45.8•	46.8	51.5	9	50.6	45.8	46.8
10	41.3	41.7	51.7	10	51.6	37.0	37.3 (44.4)
11	22.9	21.3	22.9	11	21.6	21.3	20.9
12	39.2	36.5	[30.3]	12	31.2	30.4	31.5
13	82.4	43.0	43.0	13	47.5	42.7	47.4
14	46.0•	50.8	50.4	14	51.4	51.2	51.1
15	19.8	23.2	23.3	15	22.5	23.1	21.5
16	28.9*	30.0	[28.2]	16	35.4	[27.8]	35.6
17	171.0	81.4	81.1	17	219.0	81.5	220.0
18	20.1	11.1	11.3	18	13.8	11.1	13.7
19	64.8	64.9	69.0	19	68.8	71.4	69.4 (69.0)