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THE CHEMISTRY AND BIOCHEMISTRY OF AROMATASE INHIBITORS.

by

Marc D. Messier

A thesis submitted in partial fulfillment  
of the requirements for the degree of

Doctor of Philosophy

in the

Department of Chemistry  
Ottawa-Carleton Institute of Chemistry, University of Ottawa  
Ottawa, Canada

Candidate: Marc D. Messier

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**A SYLVIE**

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## ABSTRACT

Aromatase is the last enzyme in estrogenesis, and as such is of central importance in estrogen metabolic and reproductive processes. Four central postulates of aromatase active site topology were elaborated:

1) The C-19 cavity postulate predicted that a cavity exists in aromatase. Steroids synthesized to investigate this postulate have confirmed that a 6Å deep pocket exists to accommodate C-19 androgen derivatives.

2) The C-3 chelation postulate suggested that chelating groups at the C-3 position might constitute a new generation of aromatase inhibitors and cause aromatase inactivation. Steroidal derivatives with C-3 ethylenedioxy and C-3 thioketal groups have shown this approach to be invalid.

3) The C-5 nucleophile postulate led to investigate the mechanism of action of known C-4, C-5 and C-6 pharmacophores via C-4,5 and C-5,6 androgen epoxides. No inactivation of the enzyme was reported in the presence of these epoxy-steroids, thus weakening contemporary aromatase mechanistic theory involving mechanisms relying on an incoming C-4 nucleophile.

Topological analysis and computer simulations of known pharmacophoric patterns led to the biochemical investigations of over 80 steroidal test compounds, many exhibiting potent competitive and some inactivating kinetic profiles. The synthesis of hitherto unreported androgen derivatives was described.

4) Bioorganic simulations of the aromatase mechanism have led to a novel approach to the aromatase mechanism of action. These approaches are based on altered A-ring reactivity via thioketal or hemi-thioketal enzyme intermediates. The chemistry of postulated active site generated analogues was investigated and the resulting rearrangement products lend credence to mechanisms requiring altered reactivities during aromatase oxidations.

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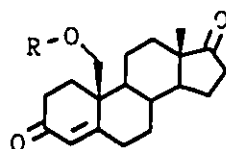
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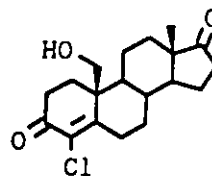


41 R-Ac 37 R-tBDMSi

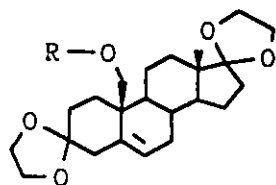
42 R-Ms 38 R-TMSi

43 R-Tr 39 R-MOM

2 R-H 40 R-tBDMSi, 6 $\alpha$ -OH



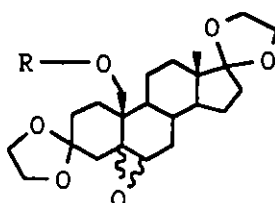
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45 R-H

46 R-tBDMSi

47 R-Ac

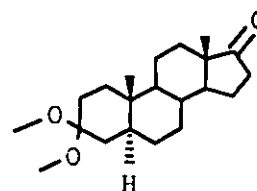


48 R-H, 5,6 $\alpha$ -epoxide

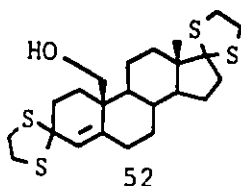
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50 R-tBDMSi, 5,6 $\beta$ -epoxide

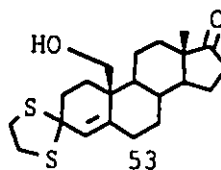
51 R-tBDMSi, 5,6 $\alpha$ -epoxide



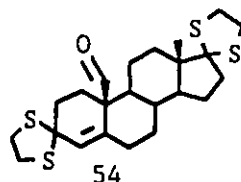
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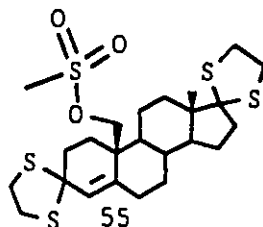
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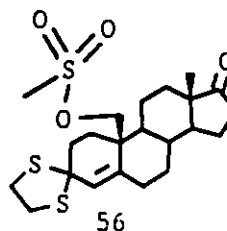
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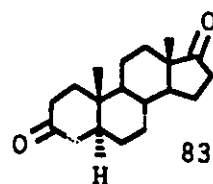
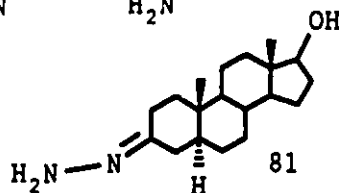
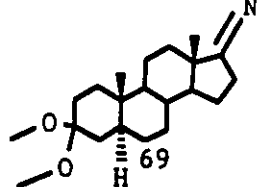
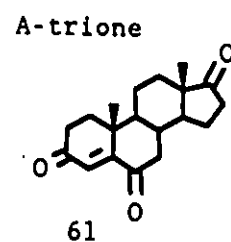
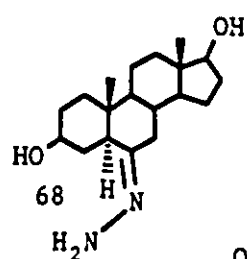
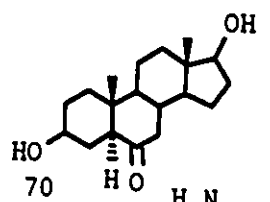
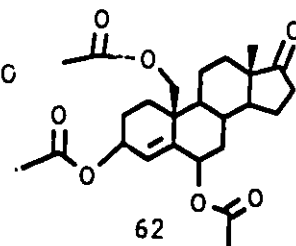
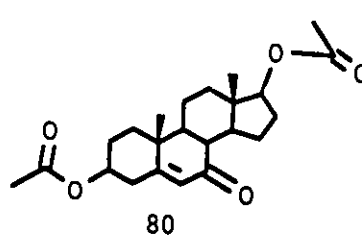
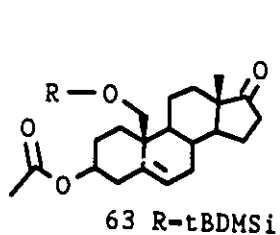
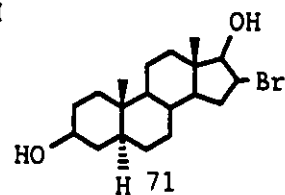
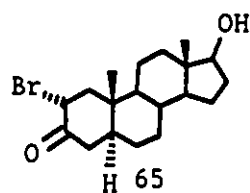
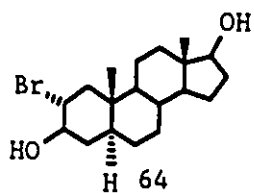
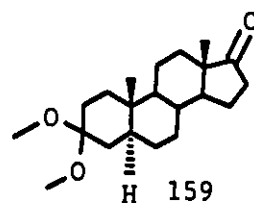
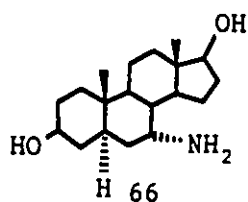
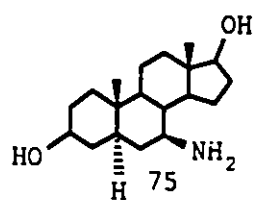
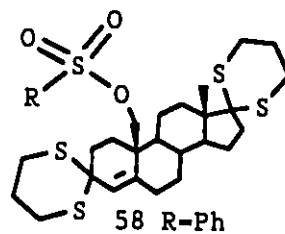
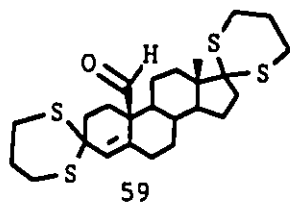
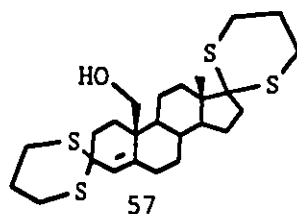
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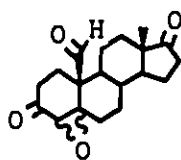


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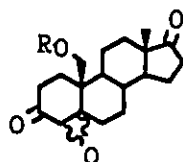


56





77 4,5-β

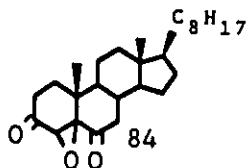


72 R-H, 4,5-β

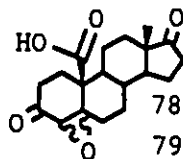
73 R-H, 4,5-α

74 R-tBDMSi, 4,5-β

76 R-tBDMSi, 4,5-α

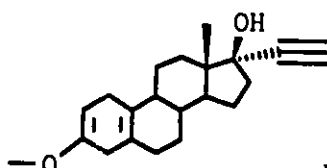


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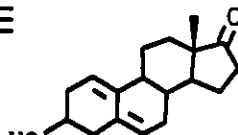


78 4,5-β

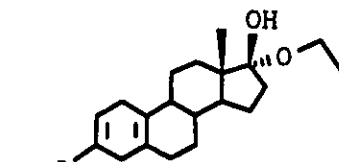
79 4,5-α



90

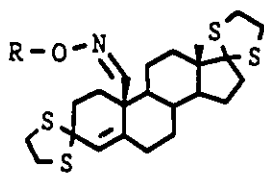


91



89

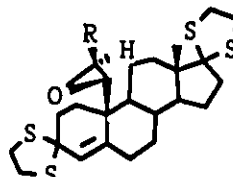
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85 R-H

86 R-Me

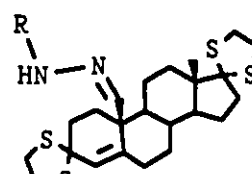
87 R-Bn



93 R-Me

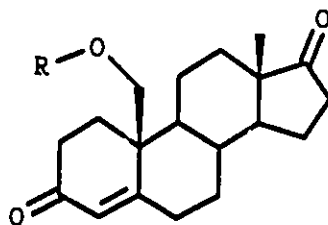
92 R-COOH

88 R-H



94 R-Tos

82 R-H



2 R-H

43 R-Tr

102 R-Bz

103 R-Tf

104 R-tBu(Ph)<sub>2</sub>Si

105 R-p-TOS

37 R-tBDMSi

38 R-TMSi

39 R-MOM

40 R-tBDMSi, 6α-OH

41 R-Ac

42 R-Ms

## Chapter 1

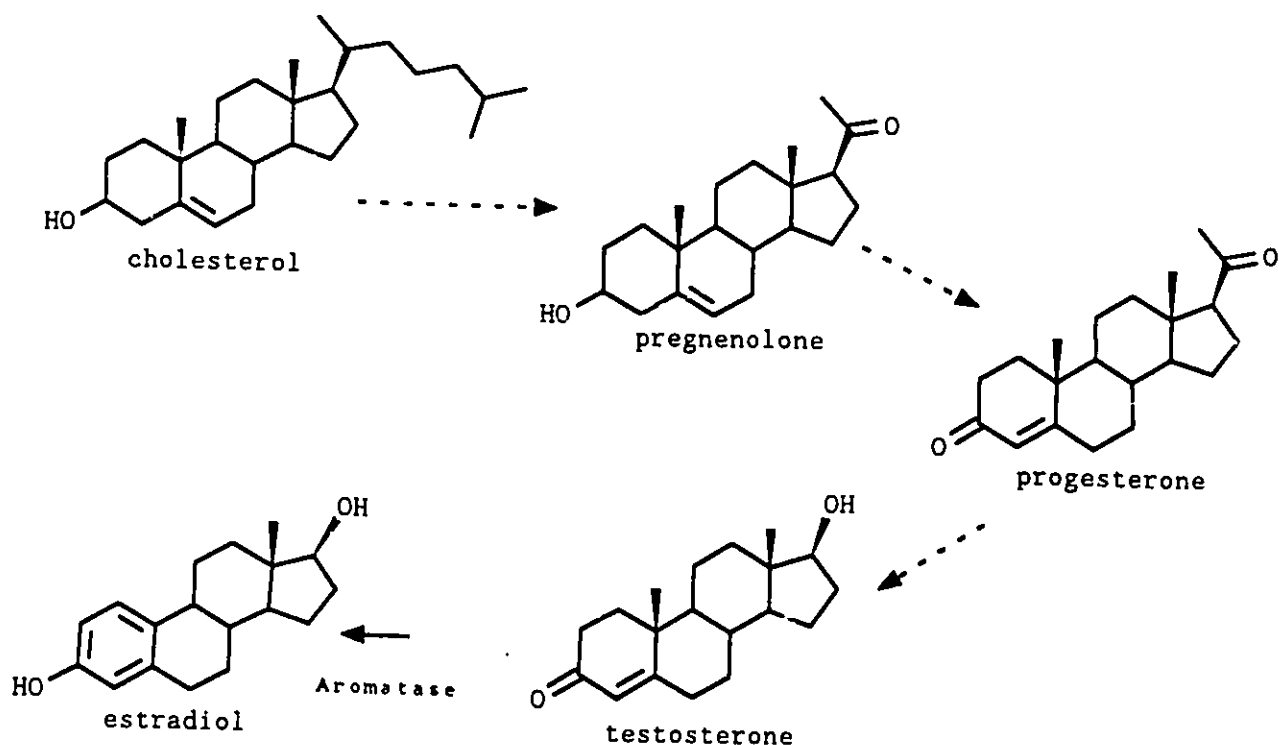
### 1.1 Introduction

Aromatase is the last enzyme in the biosynthesis of estrone and estradiol. In view of the central importance of estrogens in mammalian metabolic and reproductive processes, it is of pharmacological importance to understand and control the actions of this key estrogenic enzyme. (scheme 1.1)

Inhibitors of aromatase find applications in the control of estrogen dependent states<sup>1</sup> such as gynecomastia, endometriosis, endometrial cancer<sup>2d</sup>, one third of the breast cancers in North America, two thirds of prostatic hyperplasia, contraception and maintenance of pregnancy.

In the treatment of estrogen-dependent breast cancer, surgical removal of the main estrogen producing organs lowers the estrogen levels only temporarily<sup>1c</sup>; peripheral tissues eventually take over production. A recent compilation covering 32 years of breast cancer<sup>2</sup> treatment indicate that "all women with newly diagnosed localized invasive breast cancer can and should be offered some form of systemic treatment within and outside of a clinical trial".

Control of estrogen production, particularly important in estrogen-dependent cancers, could be reached chemically via systemic adjuvant therapy. Although general  $P_{450}$  inhibitors can be used, serious side effects occur when other enzymic processes shut down, and it is often desirable to affect only the estrogen pool. Since it holds a terminal position in the biosynthesis of estrogens, aromatase is a designated target.



**Scheme 1.1:** Biosynthesis of estrogens

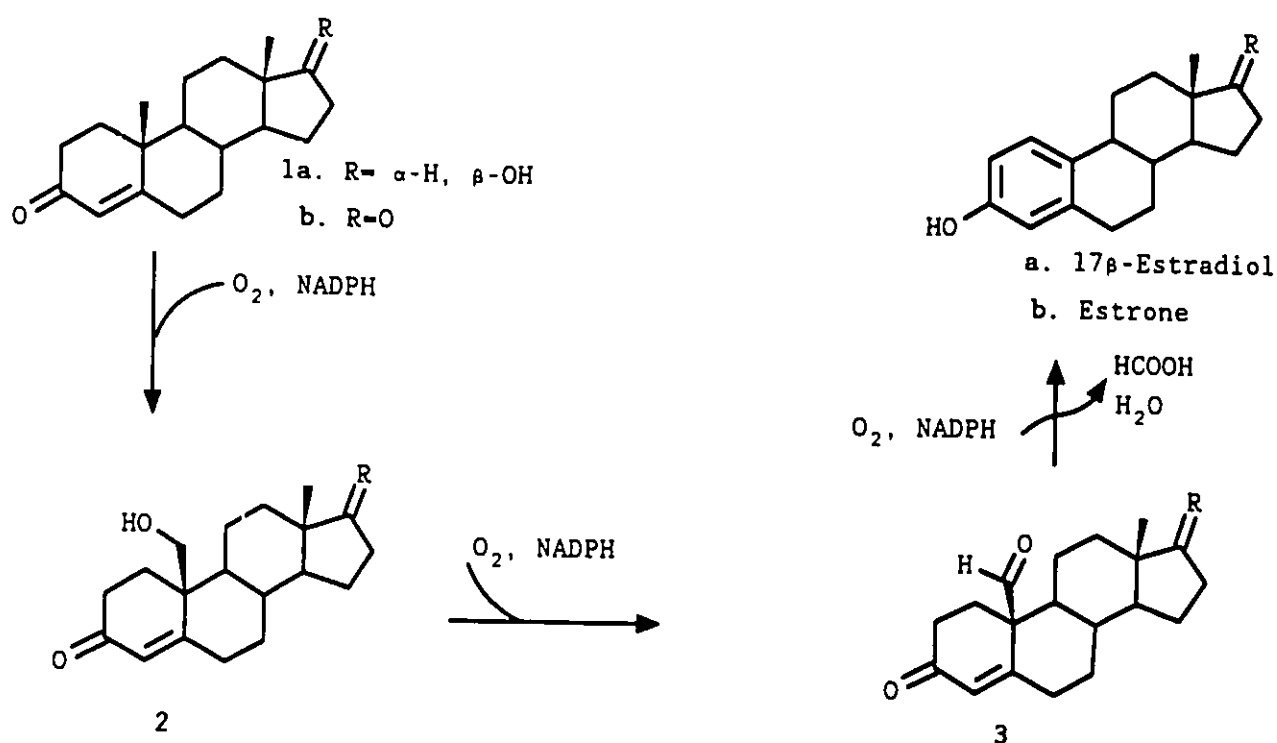
## 1.2 Historical

Aromatase was first isolated by Ryan<sup>3b</sup> in 1959 and is conveniently obtained from the microsomal fraction of fresh human placental tissue. It is a cytochrome P<sub>450</sub> oxidase<sup>3a</sup> which transforms androstenedione to estrone at a single catalytic site. In this thesis and in the literature, the term aromatase usually refers to the crude insoluble microsomal preparation according to the procedure of Thomson and Siiteri<sup>3c</sup> or Vickery's solubilized preparations<sup>3d</sup> rather than the unstable purified isozymes I and II of Osawa<sup>4</sup>.

Aromatase isozymes I and II show different selectivities for 16-hydroxy androgens. Their minimum active form (135 kDa) consists of a 1:1 complex of cytochrome P-450<sup>arom</sup> (52kDa to 55kDa) and NADPH-cytochrome P<sub>450</sub> reductase (83kDa), along with several molecules of phospholipids. Numerous laboratories<sup>5</sup> have reported partial isolation of P-450<sup>arom</sup> with different enzyme activity. The partial amino acid sequence<sup>5e</sup> indicates a cysteine at the site associated with the active center. Cysteine often acts as a ligand for the heme iron in cytochrome P<sub>450</sub>. Simpson<sup>5f</sup> has sequenced the

cDNA encoding for the P-450<sup>arom</sup>.

The mechanism that transform androgens to estrogens is not fully elucidated. It has been shown that the oxidase aromatase requires three moles of oxygen and three moles of NADPH per mole of androgen<sup>6</sup>. The enzyme sequentially oxidizes the C-19 methyl of an androgen to the alcohol, then to the aldehyde stage. A third oxidation produces estrogen with the concurrent release of formate. The final process by which the A ring is aromatized and the oxidized C-19 carbon is expelled as formate requires further study.



**Scheme 1.2:** Androgen oxidation and stoichiometry

No advanced steroid intermediate occurring after the aldehyde oxidation has been isolated. This may indicate that the third oxidation generates a very reactive intermediate which quickly degrades to estrone, or it could signify that a discrete enzyme- steroid intermediate is first formed, transformed to estrone, and then released from the enzyme site. Overall, the demethylation is known to generate an estrogen, water molecules, and a formate. Labeling studies

confirm that the released formate contains: one oxygen from the first oxidation, one oxygen from the third oxidation step, and the C-19 carbon along with its pro-R hydrogen<sup>7</sup>.

Although considerable data is available on the aromatization of androgen to estrogen by aromatase, the exact mechanism is unknown. The importance of this enzyme in medicine warrants further investigations on the mechanism and interactions with inhibitors to allow pharmaceutical modulation.

### **1.3 Kinetic studies of aromatase**

Incubation of labelled androstenedione showed the intermediates 19-OH-A 2 and 19-oxo-A 3 appear in the first four minutes<sup>8</sup> of the incubation period. Although the 19-alcohol appears first, the 19-oxo-A reaches higher concentrations first, then quickly falls to low levels. Thus, there seems to be a "bottleneck" in the initial kinetics. This "bottleneck" would have to occur after the second oxidation, and before the covalent modifications leading to the third intermediate. After the initial "priming" period, the kinetics change; the 19-oxo-androgen concentration drops and the 19-hydroxy-A stabilizes. This sequence of events, determined under sufficient NADPH to simulate biological metabolism, is quite different from that reported by Thompson<sup>9</sup> in 1974.

The experimental facts which have helped in determining the stereochemistry of this important sequence of oxygenations are reviewed below. Mechanisms for the controversial third oxidation will then be analyzed.

#### **1.3.1 Mechanism and Carbon Labels**

In the incubation of 19-<sup>14</sup>C-testosterone the C-19 carbon is mostly incorporated in the released formate<sup>10</sup>, though some radioactivity is found in [<sup>14</sup>C]-CO<sub>2</sub> and [<sup>14</sup>C]-H<sub>2</sub>CO.

### 1.3.2 Mechanism and Hydrogen Labels

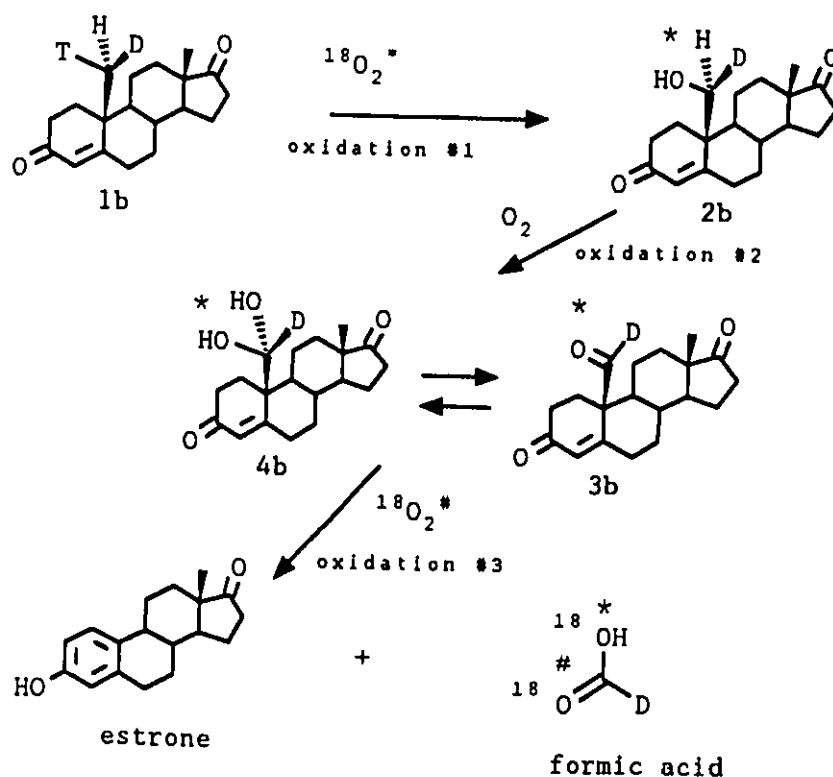
The fate of the three C-19 hydrogen atoms was determined by incubating deuterium and tritium labeled precursors. The C-19 hydrogen atoms can be differentiated as they are pro-chiral. In 1969 Akhtar<sup>11</sup> showed preferential loss of the 19-(S)-tritium in the aromatization of 19-hydroxy-testosterone. The tritium is found in a water/formate ratio of 25/75 from the 19-S-alcohol **2a**. The 19-[<sup>3</sup>H]-oxo-4-androstene-3,17-dione **3b** retained 96% of the tritium in the formic acid. Work combining <sup>18</sup>O and tritium or deuterium at the C-19 position<sup>6</sup> support the previous conclusions; 99.5 % of the deuterium on the aldehyde group ending up in the formic acid produced. Related C-19 labelled steroid precursors also lose the pro-S-hydrogen atom<sup>21</sup> predominantly. In addition to the 19-pro-S-hydrogen, aromatase stereospecifically eliminates the 1 $\beta$  and 2 $\beta$  hydrogen atoms. The 1 $\alpha$ -H, 2 $\alpha$ -H, 4-H, and 6 $\beta$ -hydrogen<sup>22</sup> atoms are retained in the released estrogen.

### 1.3.3 Mechanism and Oxygen Labels

For every mole of androgen transformed to estrogen, the enzyme requires 3 moles of oxygen gas. Labeling studies indicate the fate of these oxygen atoms. The first and third oxygen atoms are found in formic acid. The 19-hydroxide **2** resulting from the first oxidation has been isolated. The second oxidation yields an intermediate gem diol **4** or aldehyde **3** which has also been isolated. Since aromatase will oxidize advanced intermediates and release an estrogen, re-incubating labeled steroids would yield information concerning the stereospecificity of the subsequent steps. An assumption is that the advanced intermediate re-integrates the enzyme cavity and behaves stereochemically as if it had never left. Another crucial assumption is that the enzyme follows approximately the same pathway and mechanism whether oxidizing an advanced intermediate or an enzyme-bound intermediate. Hollander<sup>13</sup> demonstrated that

19-hydroxy-4-androstene-3,17-dione is not a true intermediate of aromatase, as it did not equilibrate with an exogenous source. Rather, it is suggested that the steroid is released fortuitously. This would explain the low concentrations of these metabolites.

Further, Kelly<sup>14</sup> showed that both 19-hydroxy and 19-oxo-4-androstene-3,17-dione are competitive inhibitors of aromatase, suggesting that the structure of the steroid itself is sufficiently close to the corresponding enzyme-intermediate to re-integrate the enzyme cavity and be oxidized.



**Scheme 1.3.1:** Summary of different radiolabel experiments performed on aromatase.

### 1.3.3.1 Fate of the 19 Oxygen

Oxidation regiospecificity can be measured either by incubating labeled intermediates or by incubating cold substrate in an isotopically enriched medium. In this fashion, incubating 19-oxo-4-androstene-3,17-dione with  $^{18}\text{O}_2$

yields 90% incorporation in the formate. Alternatively, by labeling the substrate aldehyde, 82% of the aldehyde oxygen is retained in the re-isolated formic acid. In a similar set of experiments, there was 87%  $^{18}\text{O}$  incorporation in the formate obtained from the incubation of  $^{18}\text{O}_2$  and 19-hydroxy-4-androstene-3,17-dione. Similarly, 90% of the label from 19- $^{18}\text{O}$ -alcohol group was re-isolated in formic acid. These data<sup>6</sup> indicate that oxygen atoms resulting from oxidation #1 and #3 are found in the re-isolated formate, whereas the oxygen resulting from oxidation #2 is not released on the formate or the steroid. This is represented in scheme 1.3.1.

#### 1.3.3.2 Fate of the 3 and 18 Oxygens

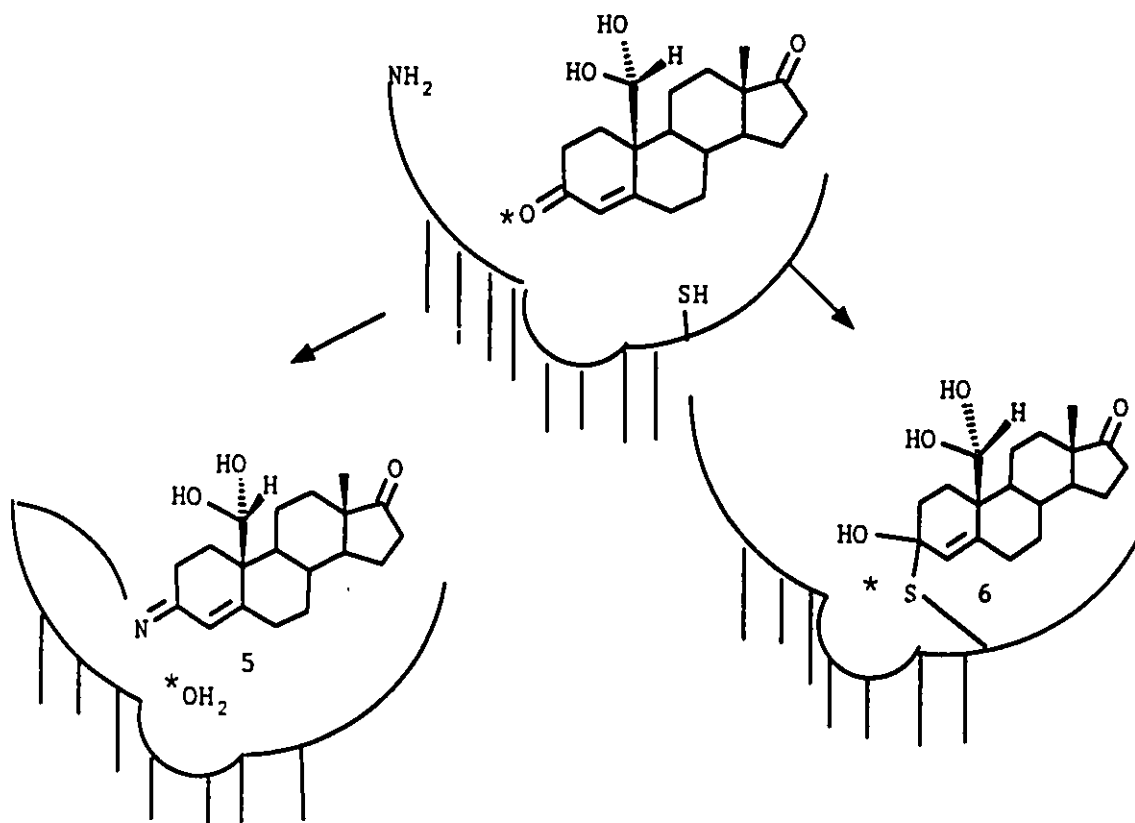
Beusen and Covey<sup>15</sup> show over 90% label retention in the estrogen fraction and in the re-isolated starting androgen by incubating 3- $^{18}\text{O}$ -labeled androstenedione and 3- $^{18}\text{O}$ -testosterone. This corroborates Holland's<sup>16</sup> work and has been used to argue against a Shiff's base intermediate at C-3 or C-17. These arguments assume that reaction at the C-3 or C-17 oxygen would result in obligatory loss to the medium and that once lost, the same oxygen atoms could not selectively interact with the steroid and be incorporated back in the original position. This would be an unusual enzymic behavior for aromatase which is considered highly regio and stereoselective in other processes. For example, oxidation #2 is thought to generate a C-19 gem-diol group 4. In solution, such species quickly<sup>17</sup> equilibrate with the C-19 aldehyde 3; a solution equilibrium of this C-19-gem-diol with the tautomeric aldehyde would thus scramble the oxygen label at C-19. Yet this scrambling is not observed on the enzyme. Further, as demonstrated by incubating 19- $^{18}\text{O}$ -oxo-4-androstene-3,17-dione, even free C-19-aldehyde, capable of generating both gem-diol tautomers, re-integrates the enzyme cavity to yield highly labeled formate, adopting the proper enzyme-substrate complex at the pro-chiral aldehyde.

Therefore, when C-3 or C-17- $^{18}\text{O}$ -androgens retain the label throughout the process of aromatization, the conservative interpretation is that the enzyme retained the label. It certainly does not prove that no reaction occurs at these carbon centers. The stereoselectivity usually exhibited by aromatase warrants caution in refuting a mechanism based on analyzing substrate and products only.

Indeed it is quite reasonable that the enzyme does modify the C-3-carbonyl group as a Schiff's base 5, then re-hydrolyzes the imine with the original oxygen retained in the enzyme cavity.

Alternatively, a C-3-hemithioketal intermediate 6 could operate in the active site; this type of intermediate has been postulated<sup>18a</sup> for P-450<sup>c<sub>am</sub></sup>, and Simons<sup>18b</sup> has invoked such an intermediate species to explain the absolute requirement for the C-20 keto in glucocorticoids.

The importance stems from the knowledge that nucleophiles react with  $\alpha,\beta$ -unsaturated ketones at positions corresponding to C-3 and C-5 on the steroid A ring. By modifying the carbonyl at C-3, the two possible intermediates 5 or 6 offer a C-4 position susceptible to nucleophilic attack. As will be seen, this reactivity plays a part in the current theory on aromatase inhibitors.



**Scheme 1.3.2:** Possible hemithioketal and Schiff's base intermediates

#### 1.4 Mechanisms: Introduction

It is reasonable to assume that the active site of aromatase presents an oxidized ferric species to the  $\beta$  face of the A ring, near the C-19 position. This would generate the first two oxidized intermediates, the 19-hydroxide and 19-aldehyde. Where the third oxidation occurs is unknown, though it is reasonable to assume a third oxidizing

equivalent above the A ring. The possible targets are then:  
 the carbonyls at C-19 and C-3  
 the double bond at C-4,5  
 the C-H (by insertion) at C-1, C-2, C-6 or C-19  
 the C-C (by insertion) at C-10,19

All of these positions can be oxidized and could lead to aromatization. Chemically, there are four positions where oxidation would readily occur: the C-19 aldehyde, the [C-2 $\beta$ H] enolizable position, the [C-4,5] alkene and the [C-10,19] tertiary aldehyde.

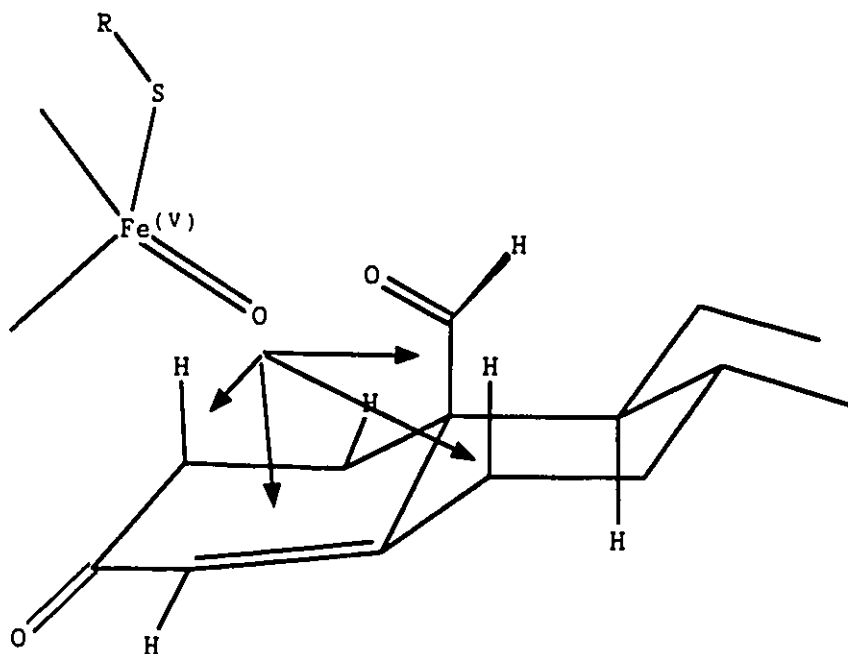
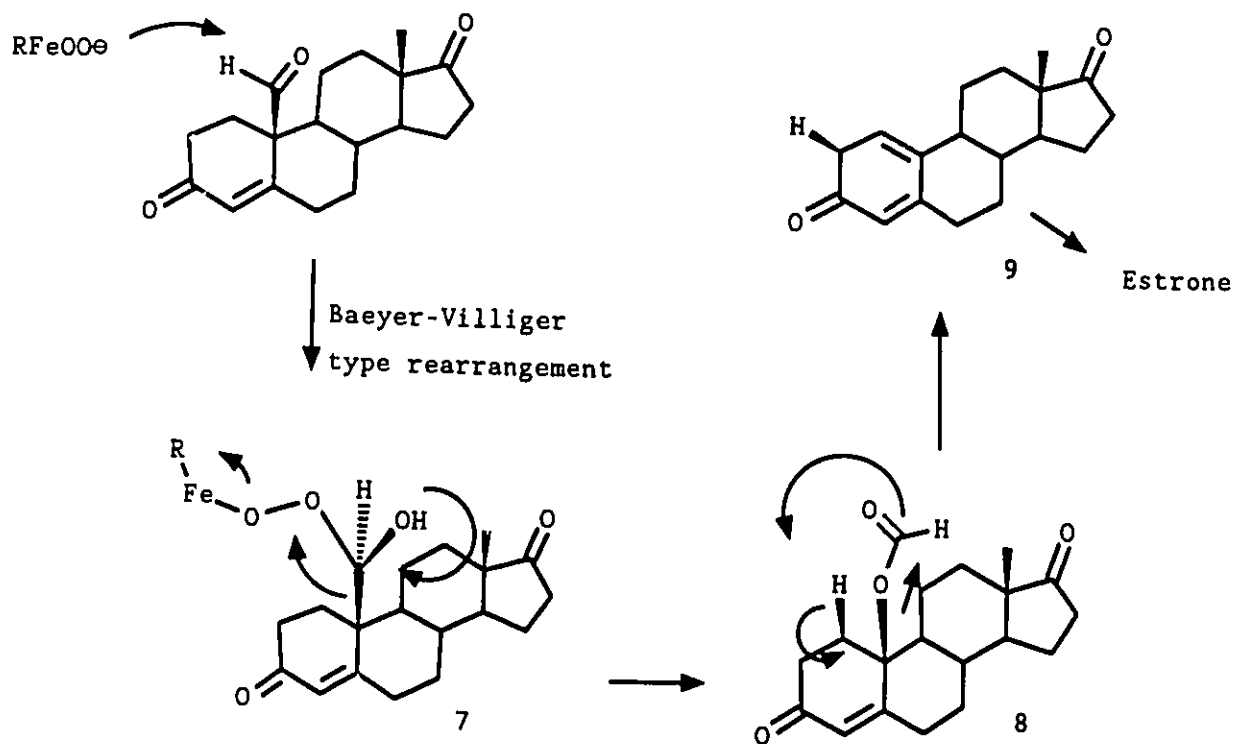


Diagram 1.4.1: Possible Sites of Androgen Oxidation

#### 1.4.1 Oxidation at C-10

The third oxidation of aromatase could proceed through a C-19-gem-hydroxy-ferric-hydroperoxide. This intermediate, produced by hydroperoxide addition to an aldehyde, would be susceptible to rearrangements. Aldehydes undergoing the Baeyer-Villiger rearrangement usually<sup>19</sup> yield a carboxylic acid by hydrogen migration. An early postulate involved the intermediacy of a C-19 acid, since tertiary carboxylic acids decarboxylate easily. By incubating 19-carboxy-4-androstene-3,17-dione, decarboxylation to the nor-steroid, followed by aromatase mediated oxidation of the resulting nor-androgen yields estrone and CO<sub>2</sub>. A labeling experiment showed the amount of labeled CO<sub>2</sub> released during the incubation of 19-[<sup>14</sup>C]-4-androstene-3,17-dione is small and insufficient for this mechanism to be a major pathway of estrogen biosynthesis.

Oxygen insertion in the C-10,C-19 bond has been suggested. Chemically, formate formation via such a Baeyer-Villiger oxidation usually requires reaction of the oxygen donor with an aromatic<sup>20</sup> aldehyde, a species more reactive than the C-19 aldehyde. Nevertheless, to assess the biochemical feasibility of his postulate, Akhtar<sup>6</sup> synthesized and incubated the potential intermediate analogue 10 $\beta$ -hydroxy-[G-<sup>3</sup>H]-4-estrene-3,17-dione formate **8**; it did not aromatize during a 10 minute incubation with aromatase.



Scheme 1.4.1: Akhtar et al.

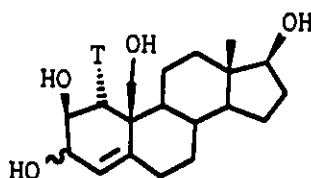
This conclusion relies on the chromatographic behavior of extracted radio-labeled starting material. The authors concluded that the compound is not converted to estrone or estradiol, although 5% of the radioactivity was found in the estrogenic bands and despite the extremely active enzyme preparation which exhibits 62% conversion of 19-oxo-4-androstene-3,17-dione. Under these circumstances, a 5% conversion of the 10-formate after 10 minutes could be interpreted as success, not failure. It is not possible to determine interaction of the compound with the enzyme active site since no kinetic data was reported, and no inhibitor data was collected. Thus, all that can be said about this compound is that a band corresponding to starting material (by comparing  $R_f$  values) was found to retain 82% of the radioactivity in the starting steroid, and 5% label was found in the estrogenic band. No formal attempt to quantify the aromatization was made.

This "intermediate-analogue" does not appear to aromatize. The apparent inability to interact with an enzyme originates from many factors such as slower kinetics of

aromatization, inability to re-integrate the enzyme cavity due to steric or electronic parameters, transport, partition coefficients or solubility constants, requirements for tight binding or even covalent binding between the substrate and amino acids in the active center. For example, the co-incubation of labeled and unlabelled 19-oxo or 19-hydroxy-4-androstene-3,17-dione (2 or 3), indicates that exogenous pools are not equilibrated with the enzyme-generated intermediate, yet both sources are eventually aromatized by the enzyme. These compounds resemble the enzyme intermediate sufficiently to allow appropriate binding and eventually aromatization, but are not the exact species which exist in the active center. The same arguments may be extended to all new enzyme "intermediate-analogues", and certainly a mechanism may not be ruled out when a postulated analogue is not aromatized.

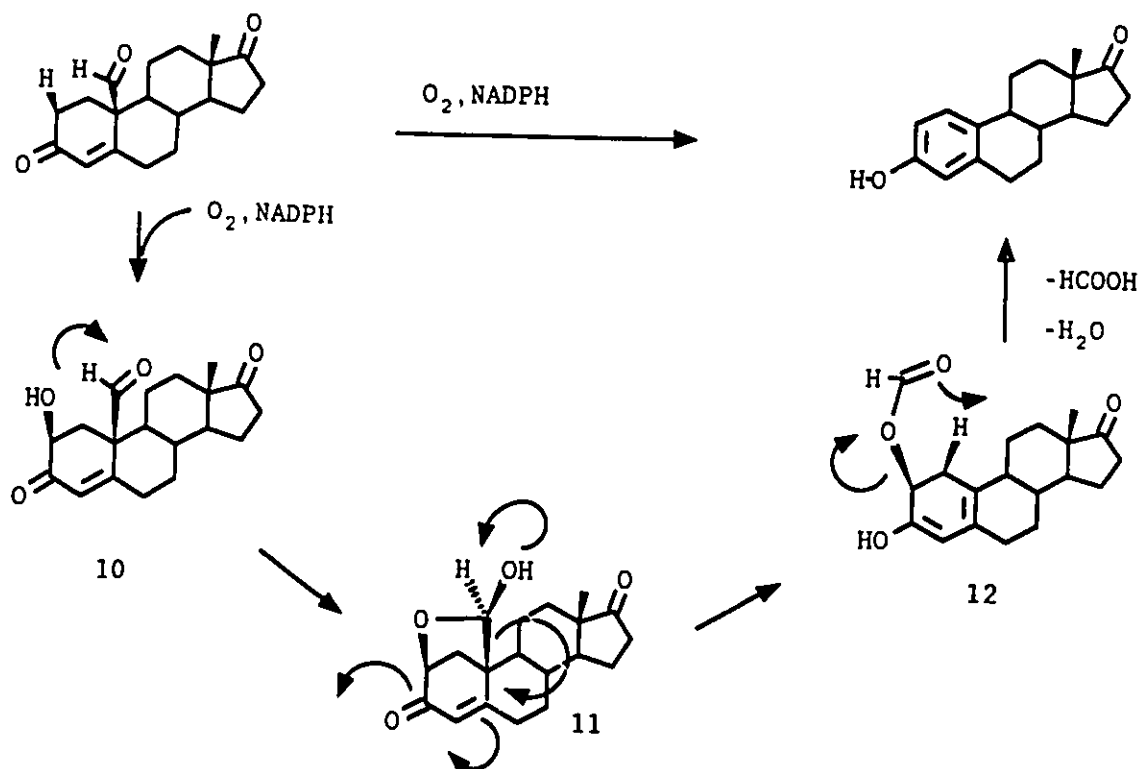
#### 1.4.2 Oxidation at C-2

Chemically,  $\alpha$ -hydroxylation of a ketone can be achieved by treating the enolate with a source of "[O<sup>+</sup>]", such as m-CPBA, O<sub>2</sub>, OsO<sub>4</sub> or o-iodosobenzoic acid<sup>21</sup>. Incubation of microsomal placental aromatase with [1 $\alpha$ -<sup>3</sup>H]-androst-4-ene-3,17-dione, followed by reduction of the medium and isolation yield 0.13% of compound 13.



13

This led Fishman<sup>22</sup> to suggest 2 $\beta$ -hydroxy-19-oxo-4-androstene-3,17-dione 10 as the third oxidation product of aromatase, a result of the oxidation at C-2 of 19-oxo-androstenedione.



**Scheme 1.4.2: Fishman et al.**

The postulate is interesting since 10 aromatizes non-enzymatically in aqueous solutions at pH 7, with release of the 19-carbon as formate and elimination of the  $1\beta$ -hydrogen. Immunological probes<sup>23a</sup> for  $2\beta$ -hydroxylated-19-oxygenated androgen (exclusive of androgens oxygenated at C-19 only), indicate the presence of such intermediates in microsomal fractions during aromatization. Unfortunately the extent of participation of this mechanism in the estrogen biosynthesis is not clear:

a) human placental microsomes may contain  $2\beta$ -hydroxylating enzymes<sup>23b</sup>,

b) recent work by Caspi<sup>24</sup> indicates a different chemical and biochemical regiochemistry of deformylation: unlabeled formate is released when  $2\beta$ -[ $^{18}O$ ]-marked 10 deformylates whereas biochemical aromatization of the same substrate releases labeled formate.

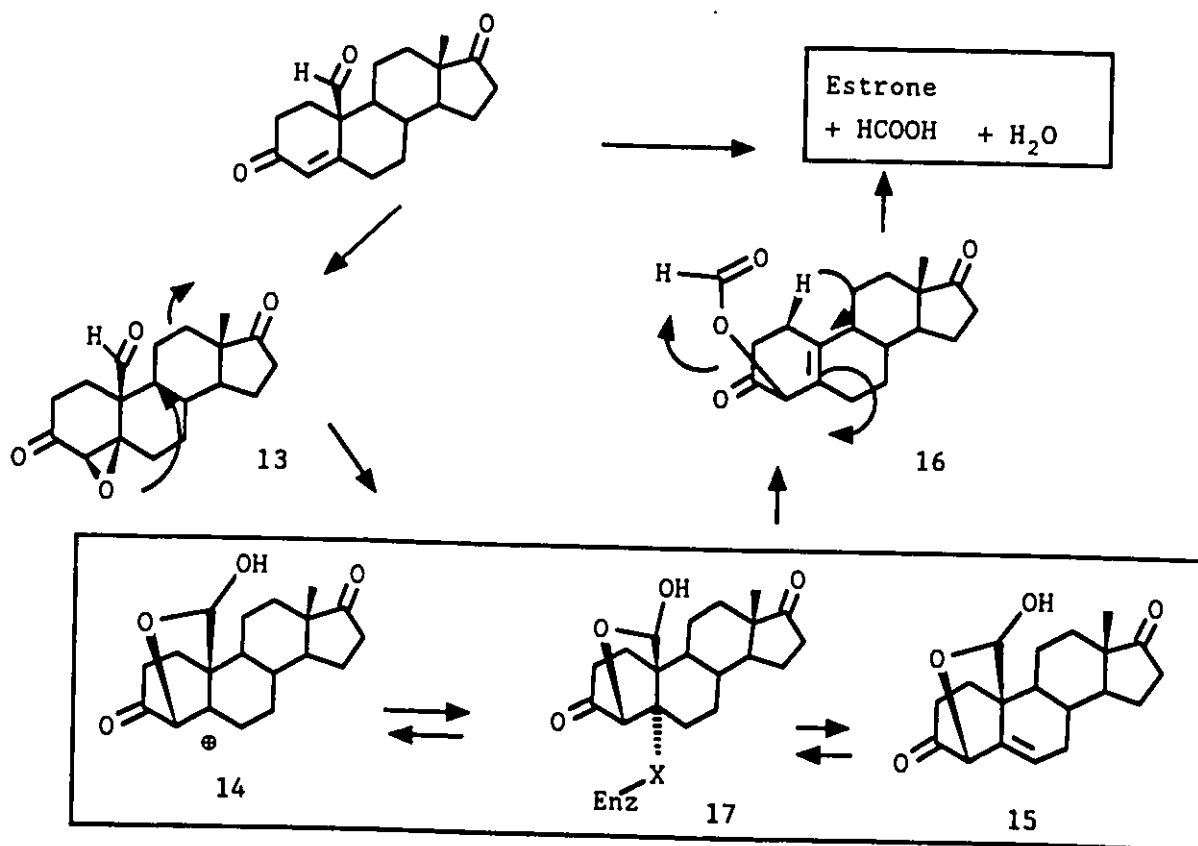
This shows that the last step of enzymatic aromatization does not release free 2 $\beta$ -hydroxy-19-oxo-4-androsten-3,17-dione in the medium; incubation show that aromatase cannot aromatize exogenous sources of 10. Fishman<sup>22d</sup> recently published experiments similar to Caspi's, with diverging results. At present this issue is unclear.

Thus catalysis may still involve such an intermediate, as it is possible that an enzyme-bound intermediate, resembling 10, aromatizes in the enzyme active site under such steric and electronic restrictions to direct 2 $\beta$ -hydroxyl elimination with retention of the third oxygen in the formate; from this perspective, Caspi's labeling work shows that there may be competition between the neighboring 2 $\beta$ -hydroxy and aqueous nucleophiles for the C-19 assisted elimination. Molecular models of 10 show that the 2 $\beta$ -hydroxy axial conformation 11 required for elimination of the C-19 aldehyde is strained: the activation barrier may be too high to achieve this conformation in aqueous solution at room temperature.

### 1.4.3 Oxidation at C-4,5 double bond

An alkene can be converted to an epoxide<sup>25</sup> by treatment with nucleophilic or electrophilic sources of oxygen. Epoxides are known intermediates of the final ring closure in terpene biosynthesis<sup>26</sup> setting all ring junctions of C-30 steroids in one concerted step.

In 1975, Morand<sup>27</sup> postulated that the third oxidation in the biosynthesis of estrone proceeds via 19-oxo-4,5-epoxy-androstane-3,17-dione **13**. The following mechanism would account for the stereochemistry and regiochemistry observed in aromatase.



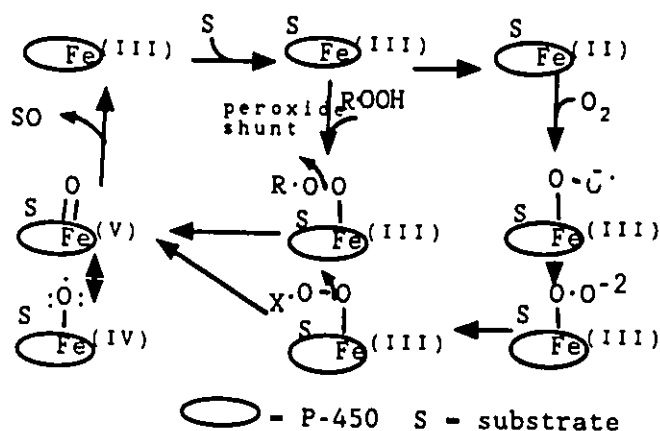
Scheme 1.4.3: Morand et al.

Epoxide **13**, its 4,5 $\alpha$ -isomer, and C-19 oxidized analogues such as 19-carboxy-4,5- $\epsilon$ -epoxy-androstane-3,17-dione can chemically rearrange to estrone under various conditions<sup>28</sup>. It is conceivable that enzymatically, a stabilized carbocation or an enzyme assisted epoxide cleavage will rearrange to **15**. To further investigate this mechanism,

epoxide analogues of 13 were synthesized during the course of this thesis. These and analogues of 15 and 17 were found to interact with aromatase. They will be discussed in chapter 2.

#### 1.4.4 Oxidation at C-19: Formation of Iron hydroperoxide.

The general nature of the catalytic cycle for cytochrome P<sub>450</sub><sup>29b</sup> is shown below.

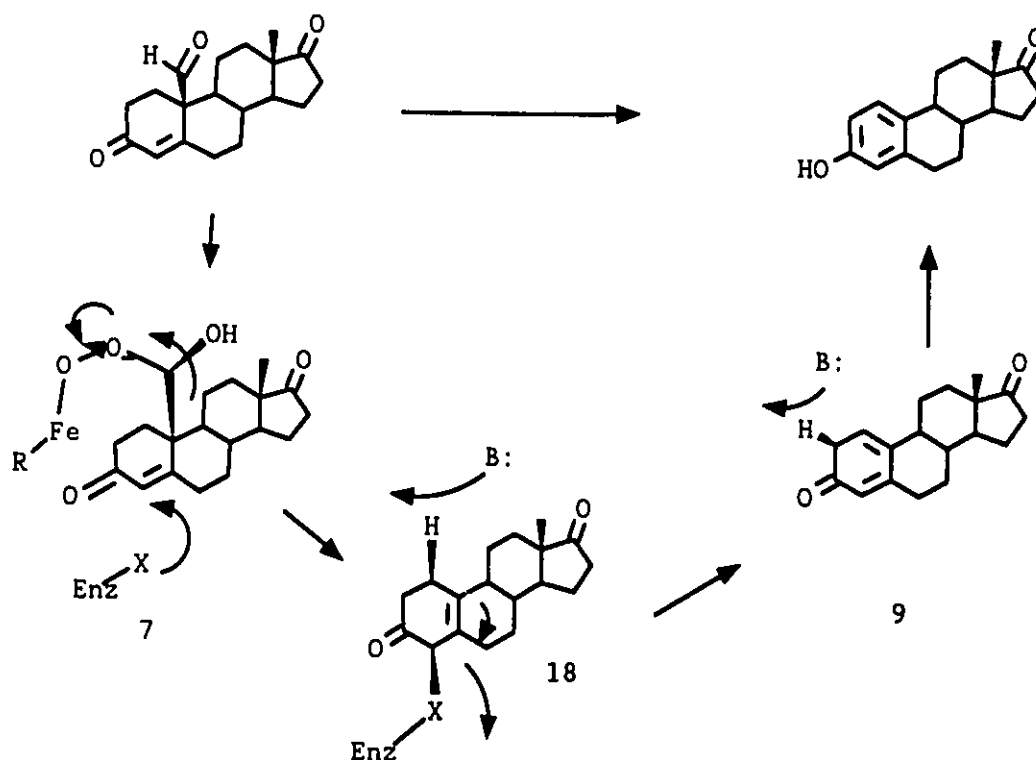


Scheme 1.4.4.1: P<sub>450</sub> catalytic cycle.

The exact species of heme-Fe-O<sub>2</sub> which oxidizes the substrate has not been resolved. There is direct evidence for the generation of a superoxide radical anion<sup>29a</sup> and a peroxide<sup>29b</sup> in models. These have led researchers<sup>29c</sup> to propose that the active intermediate iron species in the third oxidation of the cytochrome P-450<sup>arom</sup> could be equivalent to a nucleophilic mixed hydroperoxide Fe-OOX. Of course, the C-H oxygen insertions of the first and second oxidation require a more active form of oxygen and it is generally agreed to proceed by reaction with either the Fe<sup>V</sup>=O, a Fe<sup>IV</sup>-O<sup>•</sup> radical, or a Fe<sup>III</sup>-OH<sup>29d</sup> obtained from O-O bond cleavage. This dual mechanism of operation of the iron-oxide in aromatase is commonly accepted<sup>31,32</sup> though not substantiated by direct observation.

From these understandings of the  $P_{450}$  mechanism it is reasonable that the third oxidation of aromatase yield a species resembling the steroid gem-19-hydroxy-iron peroxy androstenedione, the product of nucleophilic addition of iron-peroxide to the 19 aldehyde.

By combining this with Covey's idea<sup>30,15</sup> that an enzyme covalent bond may be involved at C-4 18, the following mechanism is suggested.

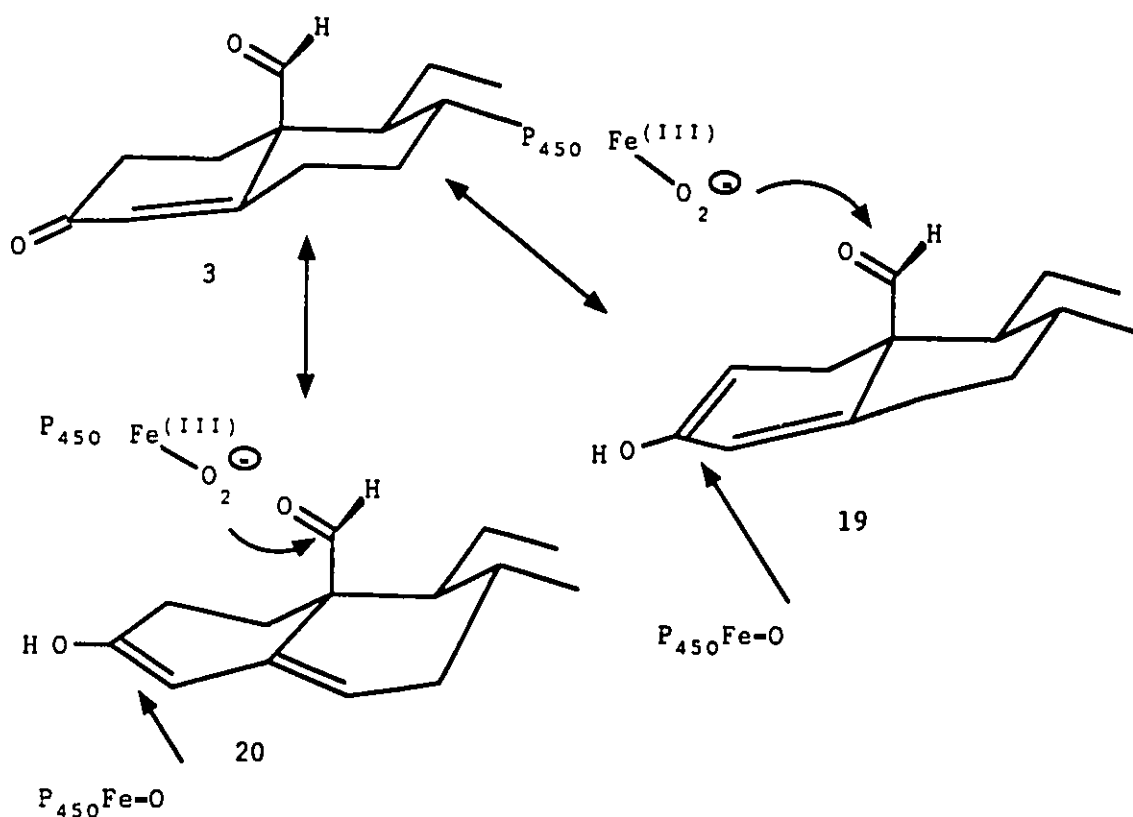


**Scheme 1.4.4.2: Covey et al.**

This scheme helps explain some irreversible inactivation by groups at C-4, C-6 and at C-7. It will be further investigated in chapter 2 where the result of incubations with inhibitors bearing epoxides as nucleophile traps are discussed.

#### 1.4.5 Proposal of New Potential Reactivities

It is established that the two enzyme "intermediates" 19-oxo- and 19-hydroxy-androstenedione are not exchangeable with exogenous pools because they are sequestered on the aromatase. We consider here that the 4-ene-3-one does not remain intact throughout the enzymatic modifications and propose that aromatase actually oxidizes **tautomers** of the androgens. Two reasonable tautomers of the 4-ene-3-one moiety exist. Possible sites of oxidation based on their known chemical reactivity are shown (Scheme 1.4.5). A fundamental difference in this proposal is the possibility of relying on iron-superoxide, a nucleophilic species, instead of perferryl oxide, which is more electrophilic in nature.



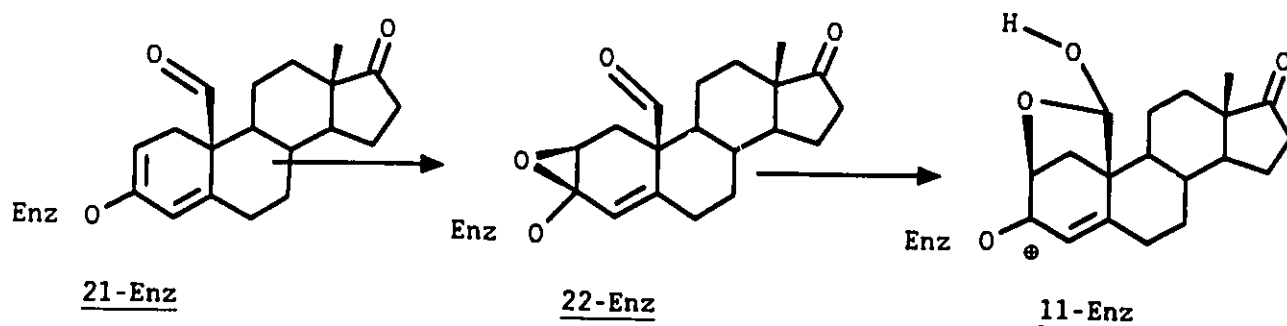
**Scheme 1.4.5:** Oxidation sites of  $\Delta^{2,4}$  and  $\Delta^{3,5}$  diene tautomers

## 1.5 Reactions of androst-3(4),5(6)-diene derivatives

The 4-ene-3-one unveils potential reaction sites by tautomerization. Oxidation of the 2,4-diene tautomer **20** can occur at the C-19 carbonyl and at the newly formed double bonds.

### 1.5.1 C-2,3 Reactivity:

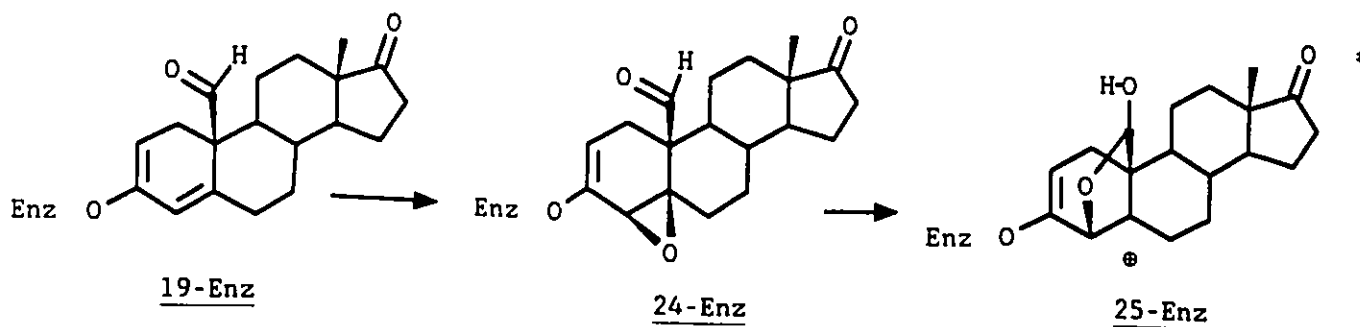
An electron poor  $P_{450}$  species would prefer to react with the C-2,3 double bond. Chemically, oxidation of the silyl enol ether **21** (Enz=tBuMe<sub>2</sub>Si) occurs with cold buffered H<sub>2</sub>O<sub>2</sub>, presumably<sup>31</sup> generating a transient 2,3-epoxide **22** which quickly deformylates to estrogen. The presence of the neighboring C-19-aldehyde is essential to assist in this reaction. Previous investigations with 2-hydroxy-19-aldehyde have shown that the 19-aldehyde **10** does not deformylate<sup>24</sup> via lactol **11** in an aqueous solution. This is probably due to the high strain required in reaching proper orbital overlap. However, an intermediate epoxide such as **22** may lie closer to the reaction pathway and allow formation to this 19-lactol **11**, favoring aromatization. This is further investigated in chapter 3.



**Scheme 1.5.1:** Oxidation of a  $\Delta^{2,4}$  diene; reaction at the C-2,3 position

### 1.5.2 C-4,5 Reactivity:

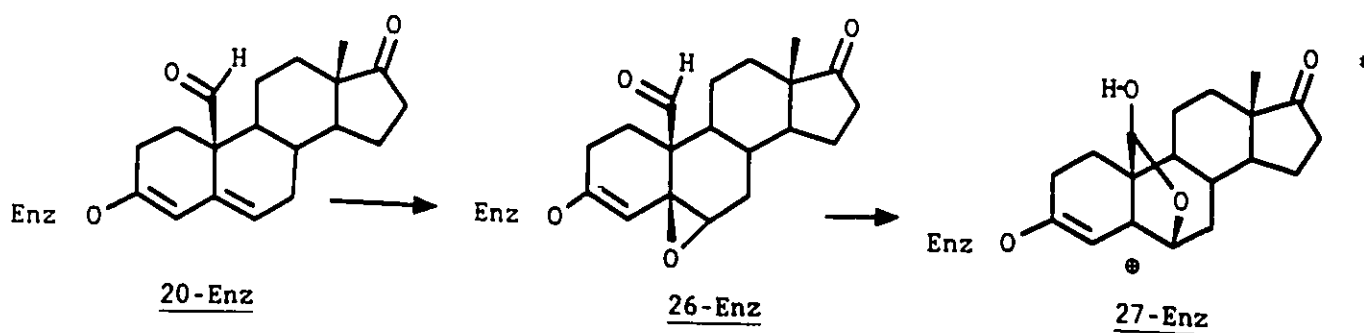
The C-4,5 alkene can undergo oxidation of the double bond to the epoxide **24-Enz** capable of rearranging to the bridged lactol cation **25-Enz**. Analogous compounds readily deformylate<sup>17</sup>.



**Scheme 1.5.2:** Oxidation of a  $\Delta^{2,4}$  diene; reaction at the C-4,5 position

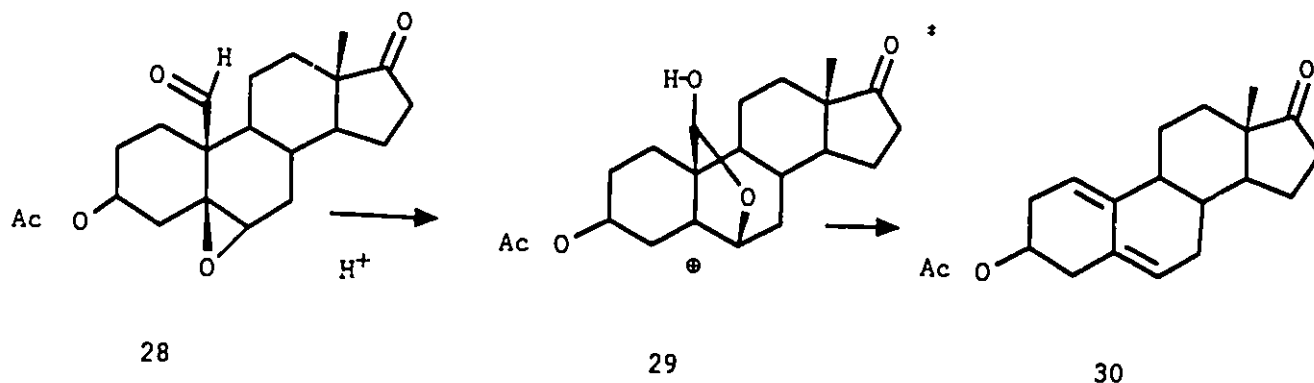
### 1.5.3 C-5,6 Reactivity:

Alternatively, the 5,6 $\beta$ -epoxide **26-Enz** could rearrange to the 19,6-oxo-5-carbonium cation **27-Enz**<sup>27b</sup>.



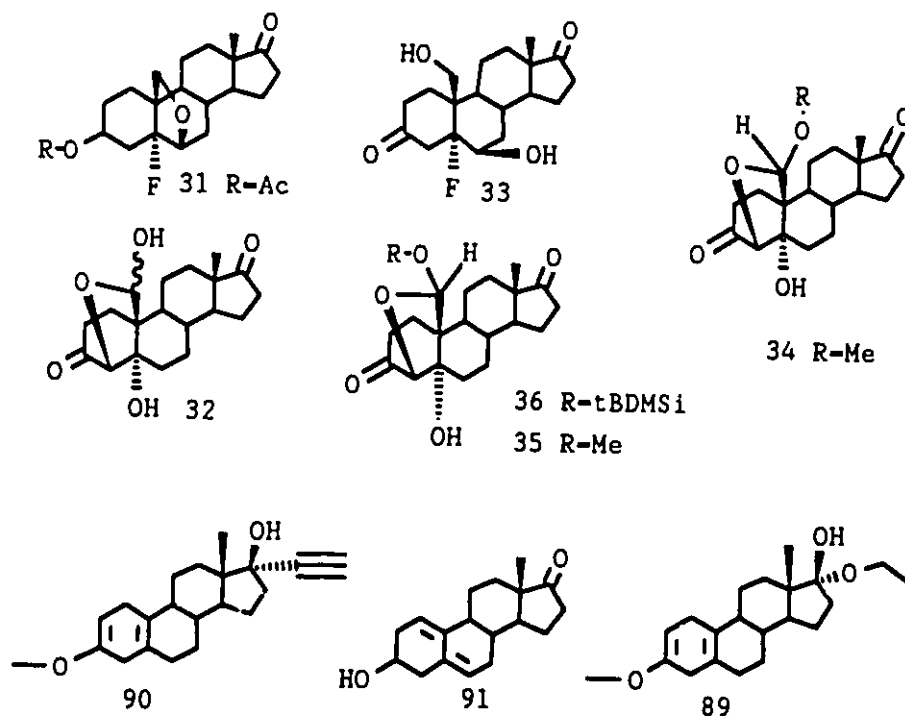
**Scheme 1.5.3.1:** Oxidation of a  $\Delta^{3,5}$  diene; reaction at the C-5,6 position

Under  $\text{CF}_3\text{COOH}$  catalysis, the analogous **28** eliminates to the trans-diene **30**, probably via **29**.



**Scheme 1.5.3.2:** Chemical formation of a trans diene from a 5,6 epoxide.

The approach followed in this thesis to assess the validity of different postulates relied on the incubation of intermediate analogues such as the following.



**Scheme 1.5.3.3:** Steroid analogues incubated with aromatase

**BIBLIOGRAPHY**  
**CHAPTER 1**

1. (a) Brodie A.M.H. (1982) *Cancer Research (suppl)* 42, 3312s  
(b) Brodie A.M.H., Wing L.Y., Goss, P., Dowsett M., Coombes R.C. (1982) *J. Steroid Biochem.* 25 859-865 (c) MacDonald P., Rombaut R., Siiteri P.K. (1967) *J. Clin. Endocr. Metab* 27, 1103
2. (a) Ludwig Breast cancer Study Group (1989) *New England J. Med.* 320, 491-496 (b) Fisher B. *New England J. Med.* 320, 473-484 (c) Mansour E.G., *New England J. Med.* 320, 485-490 (d) Kuhnle R., Delemarre J.F.M., Rao B.R., Stolk J.G., (1986) *Anticancer Research*, 6, 889-892
3. (a) Ryan K.J. (1959) *J. Biol. Chem* 234, 268-272  
(b) E.A. Thompson, P.K. Siiteri, (1974) *J. Biol. Chem* 249, 5373
4. Osawa Y., Higashiyama T., (1980) in "Microsomes, Drug Oxidation and Chemical Carcinogenesis" (Eds: Coon M.J., Conney A.H., Estabrook R.W.) Vol. I, pp. 115-248, Academic press, Orlando, Fl.
5. (a) Kellis J.T., Jr., Vickery L.E., (1987) *J. Biol. Chem*, 262, 4413-4420 (b) Mendelson C.R., Wright E.E., Evans C.T., Porter J.C., Simpson E.R., (1985) *Arch. Biochem. Biophys.*, 243, 480-491 (c) Nakajin S., Shinoda M., Hall P.F., (1986) *Biochem. Biophys. Res. Commun.* 134, 704-710 (d) Tan L., Muto N. (1986) *Eur. J. Biochem.* 156, 243-250 (e) Chen S., Shively J.E., Najakin S., Shinoda M. Hall P.F. (1986) *Biochem. Biophys. Res. Commun.* 135, 713-719 (f) Hagerman D.D. (1987) *J. Biol. Chem*, 262, 2398-2400 (g) Simpson E.R., Evans C.T., Corbin C. Jo, Powell F.E., Ledesma D.B., Mendelson C.R. (1987) *Mol. Cell. Endocrinol.*, 52, 267-272
6. Akhtar M., Calder M.R., Corina D.L., Wright J.N. (1982) *Biochem. J.*, 201, 569
7. Stevenson D.E., Neville Wright J., Akhtar M. (1985) *J. Chem. Soc., Chem. Commun.* 1078
8. Braselton E., Engel L.L., Orr J.C. (1974) *Eur. J. Biochem.*, 48, 35
9. Thompson E.A., Siiteri P.K. (1974) *J. Biol. Chem* 249, 5364
10. (a) Engel L.L. in "Handbook of Physiology, Endocrinology II, Part 1" (1973) American Physiological Society, 474 (Ed: R.O. Greep) (b) Covey D.F., McMullan P., Wixler L., Cabell M. (1988) *Biochem. Biophys. Res. Commun.*, 157, No. 1, 81-86
11. Akhtar M., Skinner S.J.N., (1969) *Biochem. J.* 75, 75
12. Holland H.L., Taylor G., (1981) *Can. J. Chem.*, 59, 2809
13. Hollander N., (1962) *Endocrinology*, 71, 723-728
14. Kelly W.G., Judd D., Stolee A., (1977) *Biochemistry*, 16, 140-146
15. (a) Beusen D., Ph.D. Thesis, (1985), Washington University, 21  
(b) Beusen D.D., Covey D.F. (1984) *J. Steroid Biochem.* 4A, 931-934
16. Holland H.L., Taylor G.J., (1980) *Can. J. Chem.*, 58, 2326

**BIBLIOGRAPHY**  
**CHAPTER 1**

17. Mastalerz H., Ph.D thesis, Univ. of Ottawa, 1981
18. (a)Murray R.I., Dus K.M.(1982) in "Microsomes, drug oxidations and drug toxicity", p.45-52, (Eds: R.Sato and Kato), Wiley Interscience, New York (b) Simons S.S., Thompson E.B.,Johnson D.F.(1979) *Biochemistry* 18, 4915-4922
19. March J.,(1985) in "Advanced Organic Chemistry" p.991, Wiley Interscience, Toronto and refs.therein
20. March J.,(1985) in "Advanced Organic Chemistry" p.272, Wiley Interscience, Toronto
21. March J.,(1985) in "Advanced Organic Chemistry", p.627, Wiley Interscience, Toronto
22. (a)Fishman J.,Hosoda H.(1974) *J.Am.Chem.Soc.* 96, 7325-7329  
(b)Fishman J., Goto J. (1977), *Science*, 195, 80-81  
(c)Fishman J., Raju M.S. (1980) *J.Biol.Chem.* 255,4472-4477  
(d)Fishman J.,(1986) *Steroids* 47, 50
23. (a)Hahn H.F., Fishman J. (1984) *J.Biol.Chem.* 259, 1689-1693  
(b)Fishman J., Dixon D. (1967) *Biochemistry* 6, 1683-1687  
(c)Reed K.C.,Ohno S. (1976) *J.Biol.Chem.* 251, 1625-1631
24. Caspi E.,Wicha J.,Arunalacham T.,Nelson P.,Spiteller G.(1984)*J.Am.Chem.Soc.*,106,7289
25. Gorzinsky-Smith J. (1984) *Synthesis*,1, 629-656
26. (a)Willet J.D.,Sharpless K.B.,Lord K.E.,VanTamelen E.E.,Clayton,R. " (1967)*J.Biol.Chem* 242,4182 (b)Barton D.H.R., Jarman T.R., Watson K.C., Widdowson D., Boar R.B., Damps K.J. (1975) *J.Chem.Soc.Perkin Trans.I*,1134  
(c)Eschenmoser A.,Ruzicka L.,Jeger O.,Arigoni D. (1955) *Helv.Chem.Acta* 38,1890
27. (a)Morand P., Williamson D.G., Layne D.S., Lompa-Krzymien L., Salvador J. (1975) *Biochemistry* 14,635 (b) Morand P., Kalapurackal M., Lompa-Krzymien L., Van-Tongerloo A. (1976) *J.Theor.Biol.* 56,503 (c)Morand P., Lompa-Krzymien L., Williamson D.G., Layne D.S., Labow R.,Salvador J.(1975) *Steroids*, 26,387
28. (a)Mastalerz H.,Morand P.(1981)*J.Org.Chem.*,46,1206-1208  
(b)Mastalerz H.,Morand P.(1982) *J. Chem. Soc. Perkin Trans. I*, 2611-2615
29. (a)Sakjurai H.,Ishizu K.,Okada K. (1984) *Inorg.Chim.Acta* L9-L11. (b) Groves, J.T (1979) *Adv.Inorg.Bioch.* 119  
(c) Bruice T.C. (1988) *Aldrichimica Acta* 21, p.87 also Guengerich F.,Macdonald T.,(1984),*Acc.Chem.Res.*,17,p.9 and refs.therein (d) Traylor T.G.,Lee W.A.,Jones S.E. Srivatsa G.S. Mincey T.C. (1983) *Inorg.Chem.*, 22,3903
30. Covey D.F., Hood W.F. (1982) *Cancer research (suppl)*,42,3327s
31. Cole P.A., Robinson C.H., (1988) *J.Am.Chem.Soc.*,110,1284
32. Stevenson D.,Wright J.,Akhtar M.(1985) *J.Chem. Soc. Chem. Commun.* p. 1078

## Chapter 2 Section 1

### 2 Objectives and strategy of this thesis.

The main thrust of this thesis is directed at finding new lead compounds capable of inhibiting the enzyme aromatase. In order to achieve this, over 80 different steroids have been incubated with aromatase. Of these, 29 steroids were synthesized here. The intermediates leading to these inhibitors are described in chapters 2 and 3. They are characterized in chapter 4. The inhibition kinetics were determined in double substrate experiments with the crude microsomal fraction of human placental tissue. The results are reported in this chapter.

An additional goal of this thesis resides in establishing bioorganic parallels between the hypothesized mechanisms of operation of aromatase, and the chemical degradation of similar steroids. This bioorganic approach assists in generating ideas and tools towards the better understanding of aromatase and its mechanism of action. The bioorganic chemistry of selected steroids are reported in chapter 3.

The strategic approach to these goals was established as follows.

- The analysis of known aromatase pharmacophores (usually reported on crude microsomal fractions) was reviewed here. The kinetic data, when available, were normalized and collated here. The published postulates on the different modes of inhibition were also reviewed.

- Computer simulation and molecular overlap of steroidal and non-steroidal inhibitors led to postulate three aromatase steric and electronic active center considerations. These were useful towards the elaboration of new pharmacophores,

and led to the synthesis of many hitherto unreported steroids.

## **2.1 Postulates on the aromatase active center.**

The three main postulates resulting from this strategy are briefly described here and will be elaborated individually later:

### **1) The C-19 hydrophobic cavity postulate: (C-19-Cav postulate):**

■ An enzyme cavity may exist near the substrate C-19 position. This may accommodate large reactive groups on potential inhibitors. Steroidal C-19 acids, esters and ethers were synthesized to test this hypothesis.

---

### **2) The C-3 chelation postulate: (C-3-Chel postulate):**

■ Chelation to the aromatase heme-iron could be accomplished in the active center via inhibitor-bound moieties. Steroidal C-3 ethylene glycol, thiane and thiolane derivatives were synthesized in order to test this hypothesis. A number of C-19 derivatives were made in an attempt to trap active species of the heme-iron during catalysis.

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### **3) The C-5 nucleophile postulate: (C-5-Nu postulate):**

■ An active site nucleophile may exist near the C-4,5,6 position of the substrate. Two distinct categories of traps were synthesized to address this possibility: 4,5 and 5,6 steroidal epoxides. As seen in chapter 1 there exists postulates of aromatase catalysis which operate either via an

amino acid  $\alpha$ -directed nucleophile or a  $\beta$ -directed nucleophile (such as a heme-iron-hydroperoxide species). Thus, inhibitors bearing both  $\alpha$ - and  $\beta$ -epoxides were synthesized with various C-19 moieties.

■ Although interpretation of the data obtained from microsomal fractions requires more flexibility than kinetics established with pure enzymes, most literature references have concerned themselves with the crude microsomal fraction. These are points of comparison for pharmacophore Structure Activity Relationships. The fact that aromatase is a large multistage membrane protein may account for the difficulty in reconstituting a functional and representative protein. An additional support for the pharmacological screening of potential inhibitors with a crude fraction comes from the additional enzyme systems normally present in the microsomal fraction; the presence of esterases and dehydrogenases during the incubation can be a crucial one. A crude microsomal fraction thus mimics more closely the natural processes which would occur in the body upon treatment with a steroid drug.

In order to provide a strong platform for Structure Activity Relationships, a number of steroids, in addition to those synthesized in this thesis, were incubated and correlated here. These compounds were chosen to complete the investigations of the three main postulates cited above. All counted, this thesis reports the incubation of over 80 steroids with the microsomal fraction of human placental aromatase.

■ The characteristics of some potential enzyme-intermediates were also examined both from a chemical (nucleophilic attack, degradation) and from a computer simulated perspective.

### **2.1.1 Inhibitors of aromatase: main categories.**

Steroidal inhibitors<sup>8,16</sup> of aromatase are often substituted at the C-1 $\alpha$ , C-4, C-6, C-7 $\alpha$  and the C-19 position. Major inhibitor classes seem to contain a 1,4-diene or a 4,6-diene, a heteroatom at C-4, or a C-19 label. Affinity labels comprise common alkylating agent such as halogens,  $\alpha$ -bromo-acetamides, nitrogen mustards and photoaffinity labels (such as thiophenylazide).

Most of the reported inhibition data reported in the literature is of the fixed dose screening type. These are correlated here and some deductions are presented in this thesis; this correlation assisted in establishing some parameters of the active site topology.

A comprehensive analysis of the following aromatase classes of inhibitors was undertaken. When possible, difference in binding free energy ( $\Delta\Delta G^\circ$ ) were normalized and calculated. This constitutes the first comprehensive published attempt at quantifying the parameters affecting aromatase inhibitor activity.

#### **Published androgenic pharmacophores known to inactivate or inhibit aromatase.**

---

- C-1 1,4 dienes (class, cause inactivation)
  - C-3 methylene (class, inhibit only)
  - C-4 4-ene-4-heteroatom (esters, ethers;  
aliphatic, aromatic)
  - C-5 reduced (class)
  - C-6 6 oxo compounds (cause inactivation)
  - C-6 4,6 diene (class, cause inactivation)
  - C-6 alkylating agents (cause inactivation)
  - C-7 phenylthio ethers, 7 $\alpha$  only (class)
  - C-10 nor compounds (class)
  - C-17 keto/ol,  $\alpha$ -methyl, acetylene (modifies alignment)
  - C-16 bromo
  - C-19 heteroatoms
  - C-19 allene, propargyl, thiirane, oxirane
-

The term (class) indicates that a large number of derivatives bearing the indicated groups are reported in the literature.

**2.1.2 Novel androgenic inhibitors incubated and reported in this thesis.**

Analysis of these, and comparison with series of non-steroidal inhibitor lead us to postulate that the following pharmacophores would constitute interesting leads towards new inhibitor classes:

bulky C-19 ethers and esters,  
sulfur containing chelating groups,  
oxygen containing chelating groups,  
epoxide inhibitors.

**Novel androgenic inhibitors incubated and reported in this thesis.**

---

|       |                                               |
|-------|-----------------------------------------------|
| C-2   | bromide, 5 $\alpha$ -H                        |
| C-3   | thio- and oxy- ketals (class)                 |
| C-4,5 | epoxide (19 oxygenated)                       |
| C-5   | fluoride                                      |
| C-5,6 | epoxide (bearing 19 derivatives and 3 ethers) |
| C-16  | bromide                                       |
| C-19  | ethers (class)                                |
| C-19  | esters (class)                                |

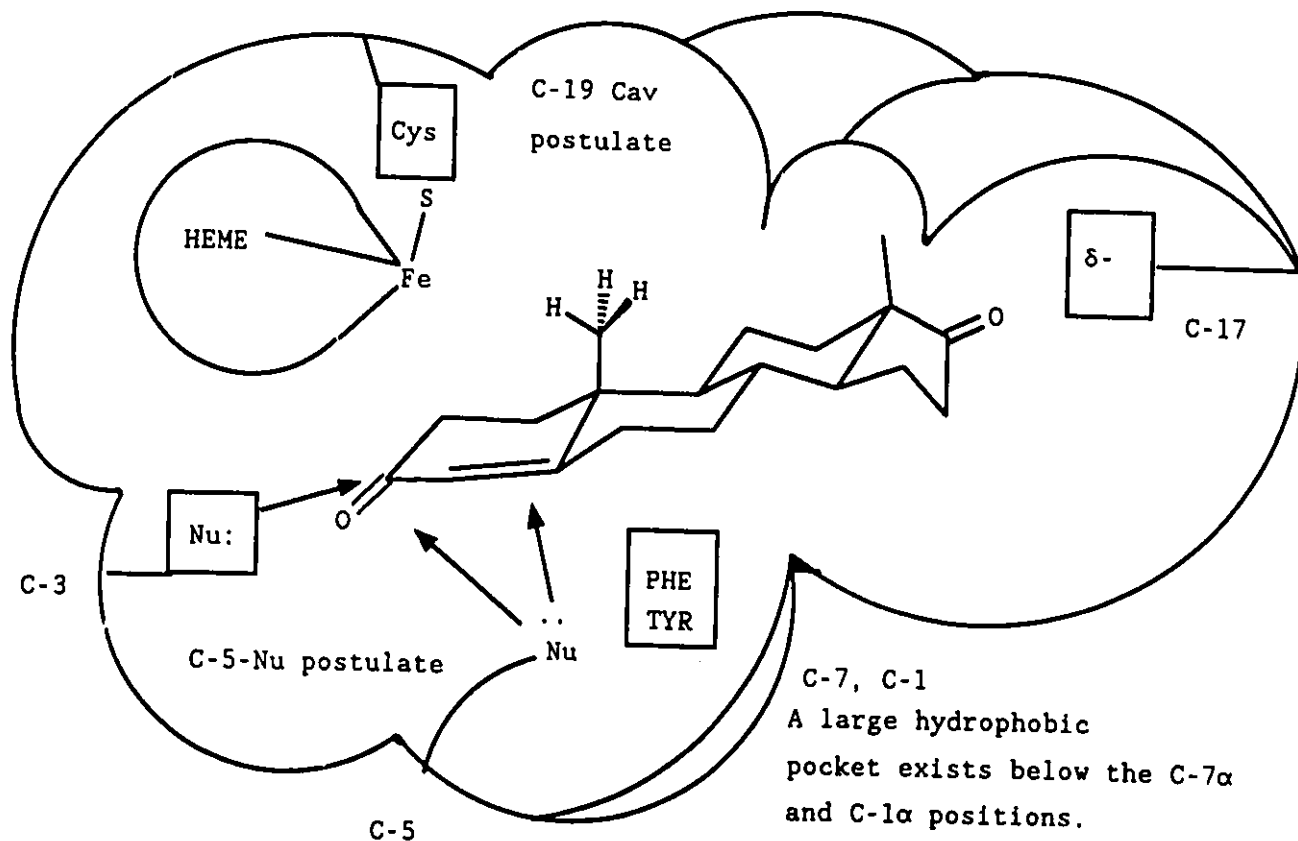
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The term (class) indicates that a large number of derivatives bearing the indicated groups were incubated and reported in this thesis.

The bioorganic chemistry of selected intermediates is described later. The spectral values for  $^{13}\text{C}$ ,  $^1\text{H}$ , ADEPT NMR, MS and IR are listed in the experimental section.

### 2.1.3 Graphic representation of the aromatase active center.

Inspection of the known pharmacophores have led to the following graphic representation of the plausible active center of aromatase.



This working hypothesis of the active center was deduced from literature data. The reasoning is summarized below:

1. The oxidation of the C-19 position by the heme-iron occurs from the  $\beta$ -face above the steroid A,B-ring. Computer simulations (vide infra) can locate only two energy minima

for the position of a heme bound to the C-19. A number of steroids were synthesized and incubated in this thesis, to investigate the potential trapping or chelation by C-3 ethyleneglycol, thiane and thiolane derivatives. (C-3-Chel postulate).

2. C-17-OH steroids exhibit lower affinity than the C-17-ketone for the aromatase active center. The  $\Delta\Delta G^\circ$  calculated for a number of OH/keto pairs seem to indicate a hydrogen bond. This would locate electronegative groups ( $\delta^-$ ) near the C-17 position.

3. There is a large hydrophobic pocket below the C-7 $\alpha$  position. A phenyl spacer seems obligatory for inhibition, possibly indicative of a nearby tyrosine or phenylalanine amino acid to allow for  $\pi$ -stacking.

4. Electrophilic pharmacophores at the C-4 and C-6 position argue favorably for a nearby amino acid nucleophile, possibly a tyrosine or a cysteine. This hypothesis constitutes the basis for the C-5-Nu postulate tested in this thesis with the help of epoxy-steroids.

5. There may be an additional nucleophile near the C-3 ketone. Interpretation of labelling studies and precedents for a hemi-thioketal involvement in  $P_{450}$  catalysis lend credence to this.

6. There is a hydrophobic pocket below the C-1 $\alpha$  position. This may be the same pocket as the C-7 $\alpha$  pocket.

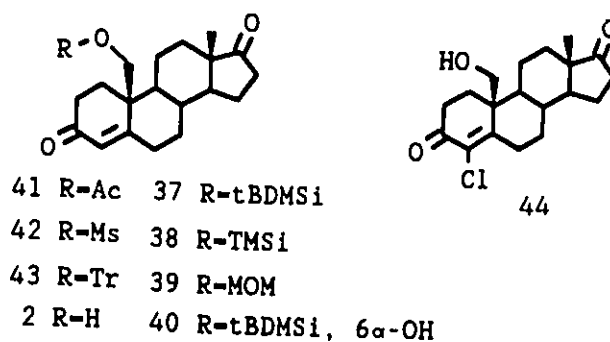
7. There must be a cysteine amino acid to bind the  $P_{450}$  heme.

8. There may be a hydrophobic pocket near the C-19 position. This postulate (C-19-Cav) was the object of computer simulation and extensive steroid incubations in this thesis.

Graphically, the three main postulates investigated in this thesis have origins in the possible mechanisms of aromatase presented in chapter 1. The three main postulates of interest in this thesis are described schematically along with the steroids synthesized to test these and the resulting biochemical conclusions.

#### 2.1.4 The C-19-Cav. postulate: inhibitors synthesized and biochemical results.

■ An enzyme cavity may exist near the substrate C-19 position. This could accommodate large groups and reactive groups on potential inhibitors. The investigation was initiated with steroidal C-19 acids, esters and ethers. The following were synthesized and incubated to test this hypothesis.



Incubations resulted in considerable inhibition for this series of C-19 derivatives. Even the large C-19-triphenylmethyl ether and C-19-tert-butyldimethylsilyl ether derivatives of androstenedione are inhibitors of aromatase. A number of these C-19 modified steroids interact with aromatase and open interesting new classes of inhibitors.

The bulky 19-tBDMS 37 occupies a nearby hydrophobic cavity yet the steroid remains a competitive inhibitor. The

larger 19-trityl **43** also shows competitive inhibition, and higher affinity but no enzyme inactivation. This series confirmed the possibility of a large pocket which accommodates substituents that extend 6.3 Å from the C-19.

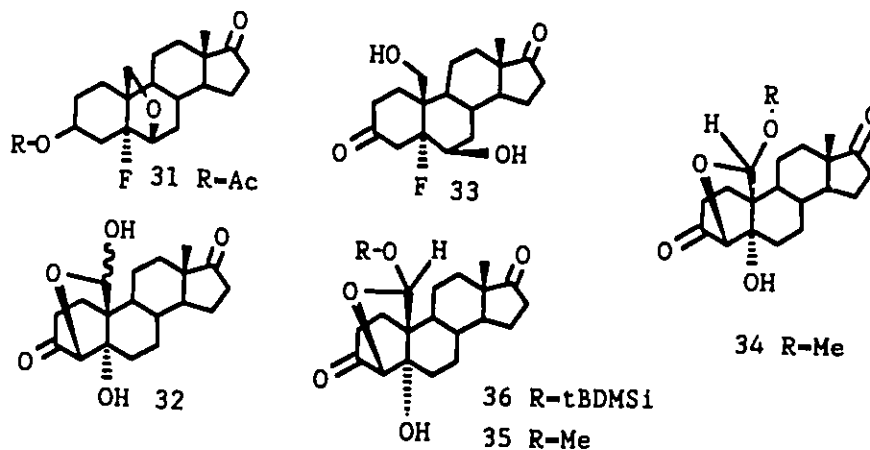
The novel tBDMSi- and Ph<sub>3</sub>C- moieties can be used to realign potential pharmacophores in the androgen binding site, or to protect other steroidal enzymes from non-specific inactivation, thus exhibiting properties of affinity labels and reporter groups in future research.

The 4-chloro-19-ol derivative **44** was synthesized and incubated here for the first time. It is one of the most potent inhibitors of aromatase known. Similarly, the smaller and more reactive 19-TMSi **38** and 19-MOM **39** derivatives were shown here to be potent inhibitors capable of inactivating aromatase, perhaps operating by stabilization of a C-19 radical to trap enzyme active center amino acids.

The mesylate **42** exhibits uncompetitive-like kinetics. The acetate on **41** causes increased affinity for the active center but no inactivating capacity. The inhibitory effect of the 19-acetate group is clearly shown on a number of steroids presented throughout this thesis.

Overall, the C-19 derivatives incubated in this thesis have confirmed that a large pocket exists near the C-19 position. In addition, extremely powerful inhibitors have been discovered in this series and represent a significant contribution towards the elaboration of future aromatase inhibitors.

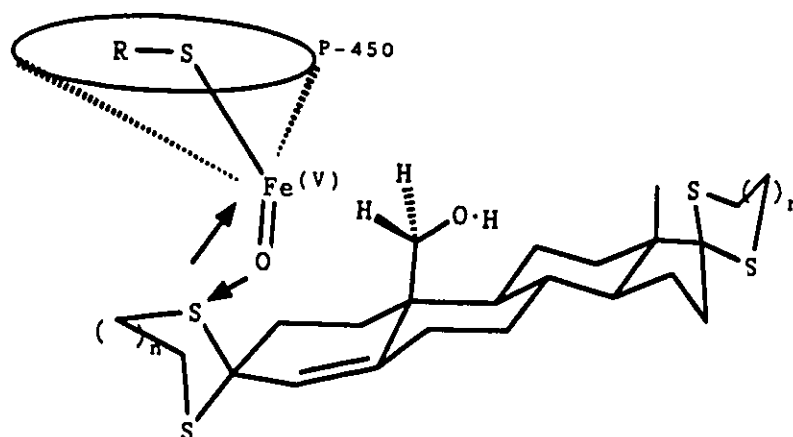
To refine the active site geometry, more rigid steroids were incubated. The following had been synthesized by co-workers.



In the "locked" 19- $\alpha$ -19,4 oxo-cyclic configuration, the 19S methoxy 35 and 19S-tBDMS 36 demonstrate no inactivation, illustrating the dangers of relying on minimized conformations of exocyclic groups to establish enzyme topology. Indeed, an energy window better reflects Koshland's induced fit active center enzyme hypothesis. Although those tested represent a series of poor inhibitors, the possibility of chelation by 19-oxygen bearing steroids was not abandoned, and different approaches were attempted.

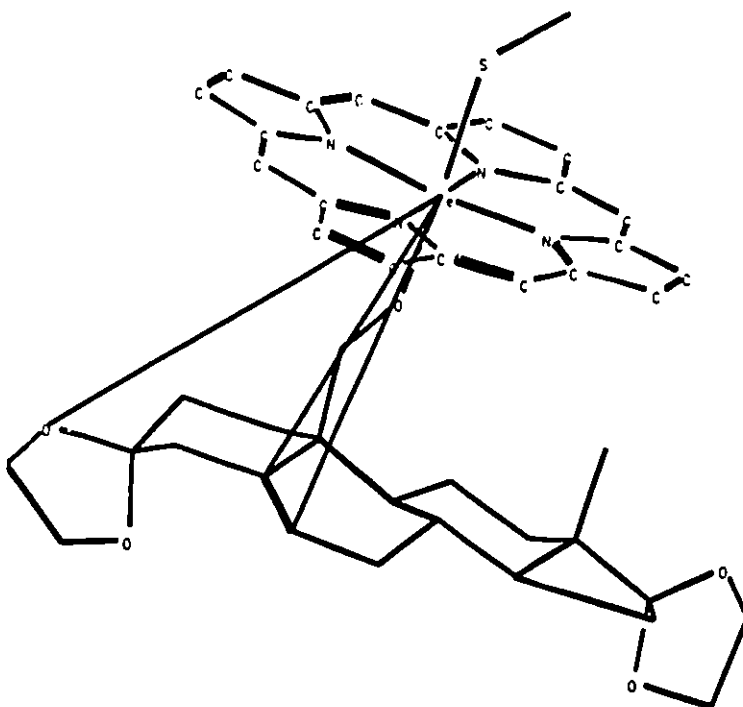
#### 2.1.5 The C-3 Chelation postulate. (C-3 Chel)

■ Inhibitor-bound moieties may cause chelation or undergo oxidation by the heme-iron in the active center. Steroidal C-3 ethyleneketal, dithiane and dithiolane derivatives were synthesized in order to test the hypothesis of this proximity.



In an attempt to rationalize and predict steroid-heme interactions, computer modeling of the aromatase active center were developed during the course of the work. The minimized model, the CH<sub>3</sub>-Porphyrin-Fe (CPF-model), is comprised of a heme-iron group bound to the 19-alcohol, and chelated to C-3 derivatives. This models the potential interaction in the active center, and was used to compare the backbone deformations of a minimized CPF-bound inhibitor with the backbone of the parent inhibitor. One example is presented here in the minimized form. The chelation postulate is shown below, with the desired interaction of the C-3 $\beta$ -derivative interacting with the heme iron. Unfortunately, this model proved difficult to refine based on the incubation data obtained with C-3-derivatives that exhibit weak inhibition and little backbone alterations when chelated to the heme iron.

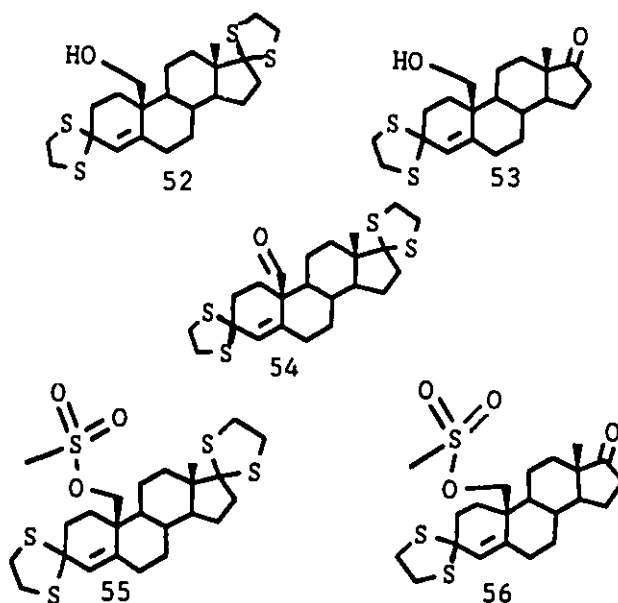
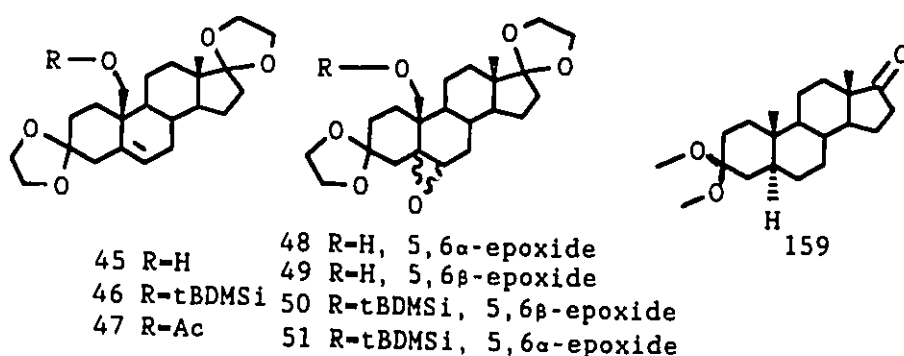
Some pertinent information obtained from the CPF-model came from simulated bond rotation with the heme-iron chelated to various steroids: there are preferred states of energy minima, defining positions where derivatives are expected to interfere with the chelation and therefore lower the affinity for the active center.

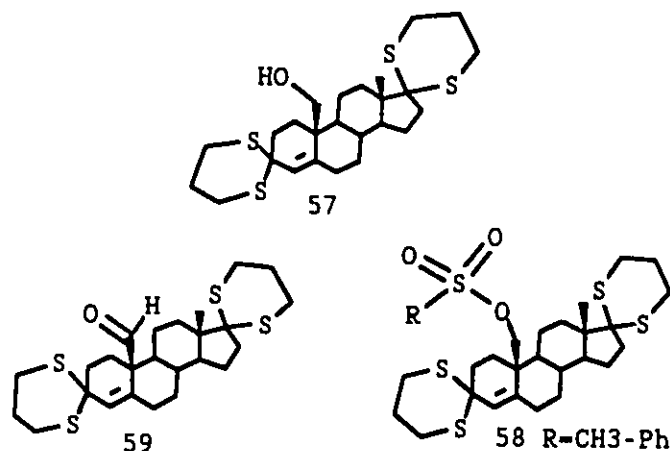


This model may become more useful to simulate the interactions of many moieties and allow predicting the required steric constraints for the oxidations and activations of suicide inhibitors.

#### 2.1.5.1 The C-3 Chel postulate: inhibitors synthesized and biochemical results.

The following steroids were synthesized to test for the possibility of heme-iron chelation to the C-3 derivatives. These exhibited the additional advantage of doubling as potential reducing agents and free radical trapping agents.





Overall, these derivatives exhibited only poor inhibition. Although there are interesting conclusions within the series as to the potentiating effect of different groups, these steroids would not, *a priori*, make good clinical candidates and are not suitable active center trapping agents. The information obtained from these incubations are resumed here.

- There is no significant difference in affinity between the 3,17-bisdithiane- $\Delta^4$ , the smaller 3,17-bis-dithiolane- $\Delta^4$ , and the 3,17-diethyleneketal- $\Delta^5$  derivatives tested.
- A C-17 ketone elicits higher affinity than the corresponding thioketal. (53 affinity >>54. 42>>56)
- A C-17 dithiolane or dithiane substituent is tolerated in the enzyme cavity. These groups do not increase the affinity for the active center, nor do they trap active species there. They may, however, modify the alignment of the C-19 group.
- The difference in inhibition mode between the competitive free alcohols 57, 45 and 52 and the aldehydes 59 and 54 suggests that the 19-alcohols of this series are not oxidized.

■ The 19-aldehyde 59 elicits inactivation, perhaps by causing a re-alignment suitable for inactivation by the C-3 thioketal. The aldehyde 54 does not demonstrate affinity for the active site.

■ Modifications of the 19 position with more reactive mesylate and tosylate caused inactivation. This was also found true in the 4-ene-3-one series. The C-17 dithiolane must allow alignments in the active center such that the 19-mesyl is able to inactivate aromatase.

■ A C-19 mesylate potentiates inactivation and is sufficiently small to retain affinity for the active center. (compare 53,52 vs 56,55)

■ The 17-dithiolane modifies the alignment to cause inactivation by the 19-mesylate 55. (compare 55>56=0)

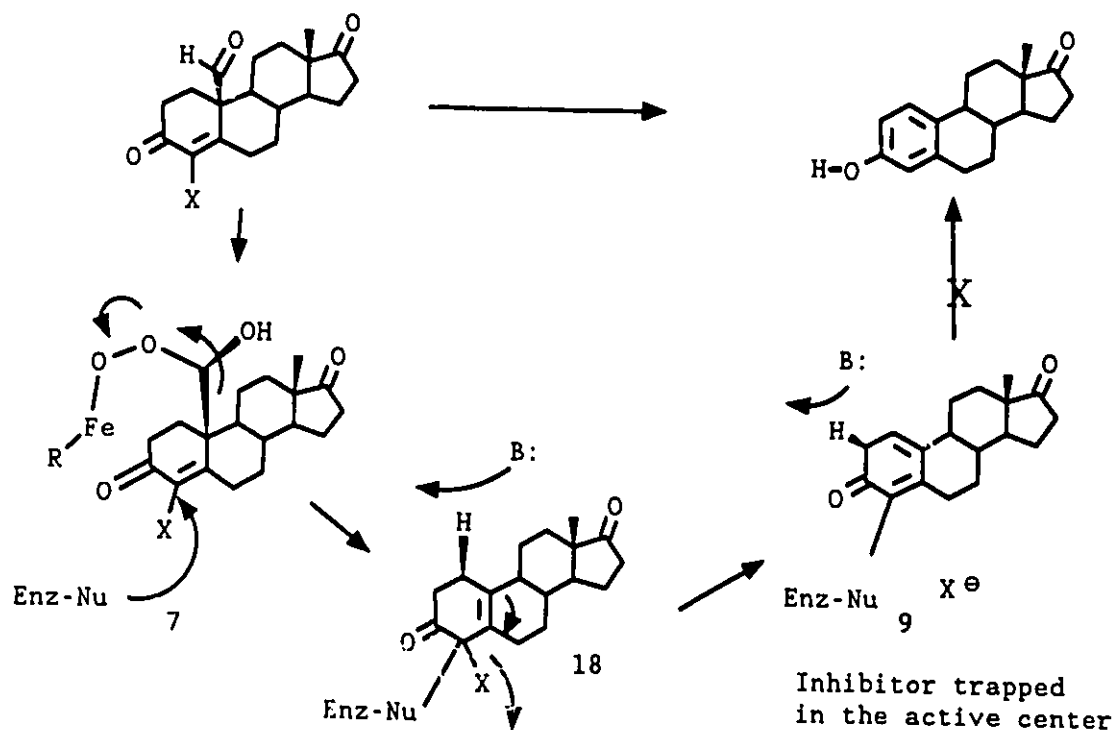
■ A C-19 tosylate does not retain significant affinity and compound 58 is regarded as defining an active site excluded volume.

Surprisingly, some dimethyl derivatives tested here proved to be extremely potent inhibitors of aromatase. The ethyleneketal, dithiane or dithiolanes derivatives did not live up to expectations, despite their theoretical dual ability to chelate the iron-heme and to trap active center free radicals or active oxygen species.

#### 2.1.6 The C-4,5,6 active center nucleophile postulate. (C-5-Nu)

■ An active site nucleophile has been invoked in the literature to explain the suicide-inhibition by C-4 and C-6-derivatives. A reasonable mode of inactivation seen in

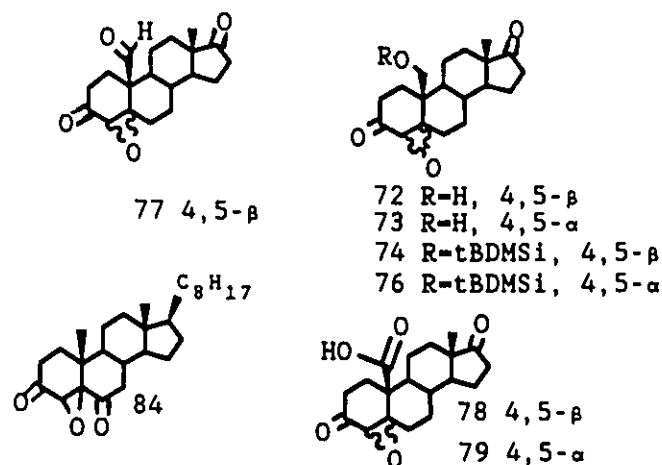
chapter 1 illustrated their involvement via enzyme-bound intermediates such as 18.



#### 2.1.6.1 The C-5-Nu postulate: inhibitors synthesized and biochemical results.

Two distinct categories of traps were synthesized to address this possibility: 4,5 and 5,6 steroidal epoxides. As there exists a rationalization for either an amino acid  $\alpha$ -directed nucleophile or a  $\beta$ -directed nucleophile (such as an heme-iron-hydroperoxide species) both epoxide isomers were synthesized with various C-19 moieties.

These steroid classes demonstrated competitive kinetics only, indicating affinity for the active center, but no inactivation. The data collected thus disfavors inhibitor theories requiring a nearby nucleophile. Although our series of epoxides may not orient properly in the active center it is notable that neither the 4,5- nor the 5,6-epoxide derivatives caused inactivation.

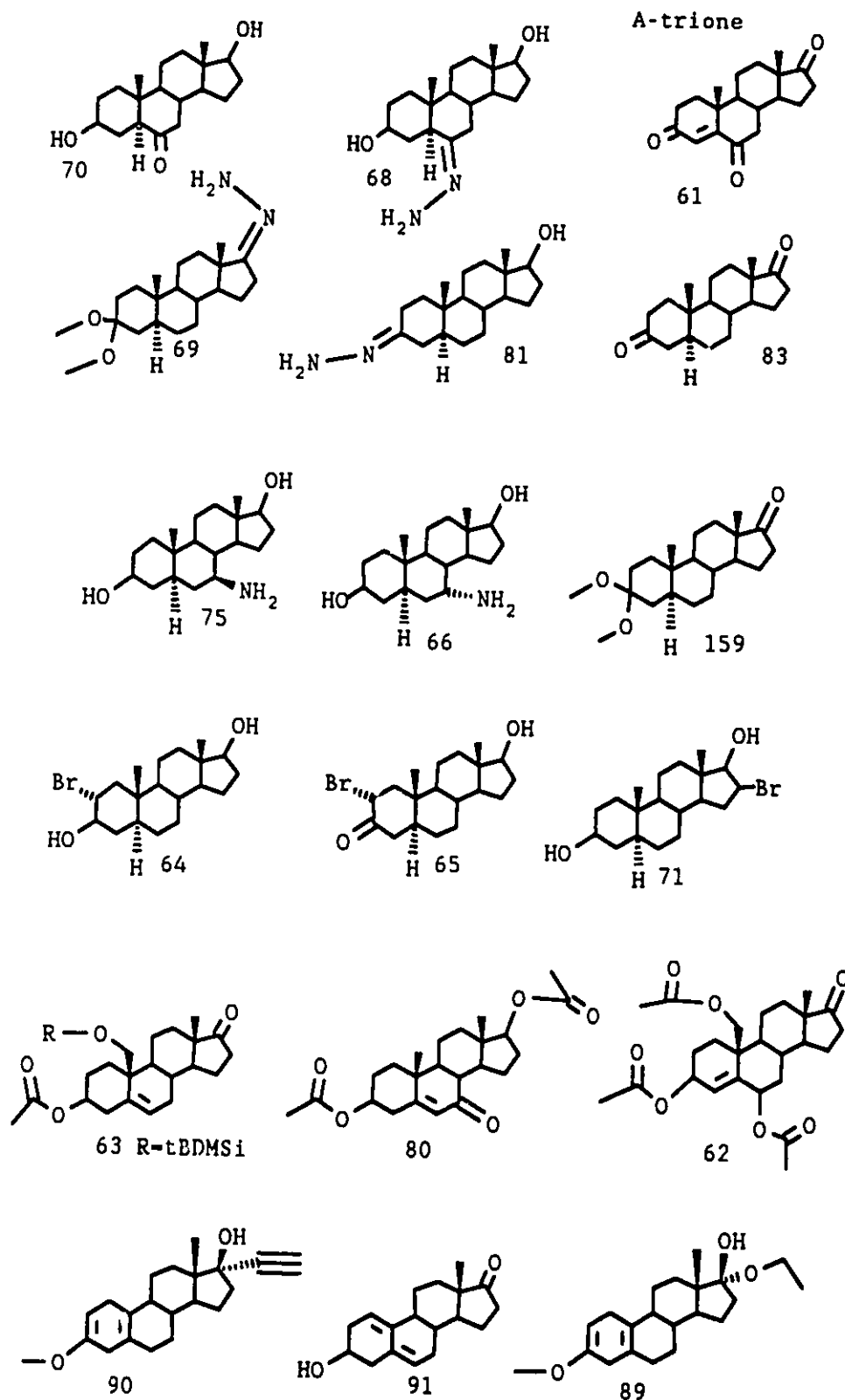


In addition, despite promising computer simulations, no improvement in affinity via chelation was observed. Both series exhibited competitive behavior only and no inactivation via active site nucleophile. The one epoxide causing inactivation was compound 84, a cholesterol derivative with the well known pharmacophore 6-ketone probably responsible for the desired inactivation.

By inspection, the loss of affinity between the parent 19-hydroxy-androstenedione and the epoxides can be ascribed to the presence of a polar environment about the A ring, and realignment caused by the geometry of the C-3 ketone-4,5-epoxides.

#### 2.1.7 Complementary steroids incubated: biochemical results.

In addition to the synthesized steroids above, a number of compounds purchased or synthesized in this laboratory by co-workers were incubated. To assess their homogeneity, chromatography and high resolution mass spectra (with inspection of the ion chromatograms) are reported in chapter 5.



A number of these compounds were found to be very potent aromatase inhibitors. Some conclusions can be drawn from these series of derivatives:

C-3 acetoxy, ethylenedioxy, hydroxy and methoxy are tolerated but do not seem to cause inactivation by themselves.

C-3 hydroxy is tolerated but seems to lower the affinity.

C-3 hydrazone causes weak inactivation.

C-5 $\alpha$ -H androstanes lower the affinity.

C-6 ketones causes inactivation only in conjunction with the 4-en-3-one or 4,5-epoxy-3-one moieties.

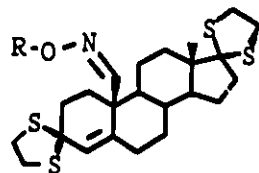
C-10 flat A ring derivatives interact with aromatase, presumably because they are within the Minimum Steric Requirements of the active center.

C-17 can tolerate very large substituents.

The detailed basis for these conclusions are presented later with the pertinent kinetic data.

#### 2.1.8 Bio-organic simulations.

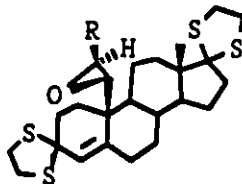
Bio-organic simulations of iron-hydroperoxide were attempted by synthesizing the following steroids and observing the effect of nucleophile addition. Unfortunately, even the  $\alpha$ -effect nucleophiles were not able to add at the C-4 position or elsewhere.



85 R-H

86 R-Me

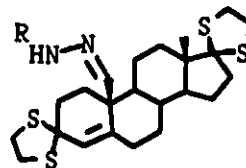
87 R-Bn



93 R-Me

92 R-COOH

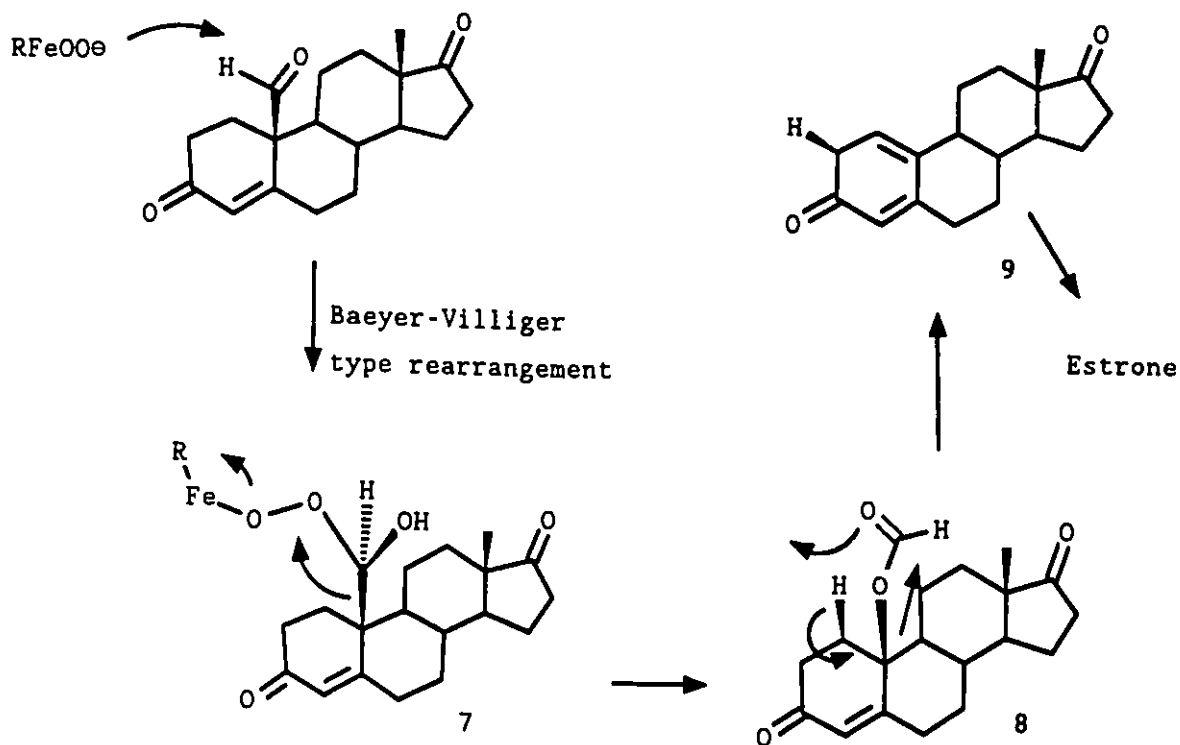
88 R-H



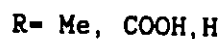
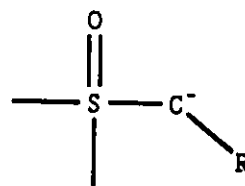
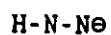
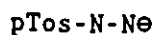
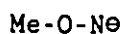
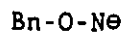
94 R-Tos

82 R-H

The mechanism investigated were rearrangements at the C-10 position and nucleophilic addition at the C-4 position.



Unfortunately, none of the models were successful, either because the models are not applicable or the enzyme active center generates more potent nucleophiles than the chemically generated species listed below:



#### 2.1.9 Section conclusion.

This section summarized the steroids synthesized and incubated with aromatase. The guiding postulates for the synthesis and investigations are presented, along with the interpretations of the different pharmacophores investigated. The subsequent sections will review enzyme kinetics as applied to inhibitors, the literature on a postulate by postulate basis and interpret the biochemical data obtained from the various steroids incubated during this work.

Chapter 3 investigates bio-organic models of aromatase operation. Some novel rearrangements will be detailed there.

Chapters 4 and 5 describe the chemistry experimental and the biochemistry experimental respectively.

## Chapter 2 Section 2

It was shown in chapter 1 that aromatase oxidizes the C-19 methyl of an androgen twice, then by further oxidation produces an estrogen with concurrent loss of the C-19 carbon as formic acid. The nature of the first two oxidized intermediates has been established as the 19-ol and 19-diol (or aldehyde). The process by which the C-19 aldehyde intermediate is then oxidized and expelled, with aromatization of the A ring, still requires clarification.

Mechanisms for this last oxidation by aromatase are proposed to occur in various carbon positions; C-1, C-2, C-19 and C-4, with corresponding mechanisms by which the steroid will rearrange and aromatize.

### **2.2 Use of radiolabel to understand enzyme substrate interactions: affinity, kinetics and mechanism.**

Many enzyme mechanisms can be elucidated with the use of labelled substrates. Such techniques have shown that aromatase removes two of the C-19 methyl hydrogens<sup>1</sup>, releasing them in the aqueous medium. By analyzing the kinetic behavior derived from the incubation of labelled substrates, it is possible to obtain information on the mechanism.

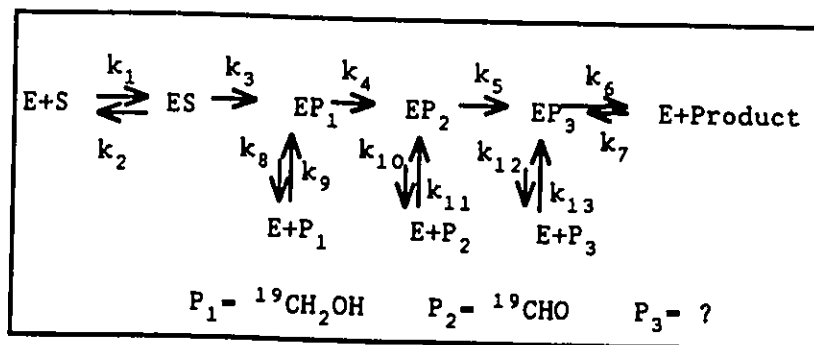
Kinetic isotope<sup>2</sup> effect  $K_H/K_D$  of 3.2 have been observed for 19-d<sub>3</sub>-androstenedione, and 2.3 for 19-d<sub>1</sub>-androstenedione, illustrating that modification of the C-19 position is at least partially rate limiting<sup>3,5</sup>. Deuterium isotope effects<sup>2</sup> of 2 to 4 have been reported for carbon-monoxide insensitive cytochrome P<sub>450</sub>. The isotope effect is presumably associated with the introduction of the oxygen in the 19 carbon-deuterium bonds. It has been suggested that hydroxylation at non activated positions proceed by the direct introduction of an oxene in the C-H bond. This is consistent with the retention of configuration observed. Similarly, the third oxidation of aromatase may involve oxidation of the C-2 position. If this mechanism is operating, a similar kinetic

isotope effect would be expected from the incubation of a 2-deuterated substrate.

Previous investigations with deuterio-testosterone relied on the re-isolation of the starting androgen and the product estrogen. This procedure requires large scale and long incubation times, with the eventual reduction of isotope effects by equilibrium phenomena. Although these probably do not apply to the C-19 oxygenation due to the irreversibility of these reactions, an equilibrium effect at the C-2 position may cancel the isotope effect. Indeed deuteration of the C-2- $\beta$  position is accomplished by base equilibrium of the unsaturated ketone in  $D_2O$ . Therefore, long incubations times may exhibit complex equilibrium phenomena, and non-linear kinetics which would occur undetected during the period required to obtain enough material for isolation.

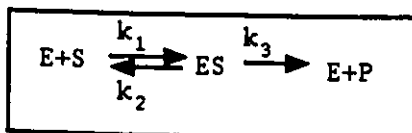
For example, incubation of 4- $^{14}C$ - and 7 $\beta$ - $^2H$ - labelled<sup>6</sup> androstenedione showed that the intermediate 19-ol-Androstenedione (19-OH-A) appears before the 19-oxo-A, though the 19-oxo-A level peaks at 5 minutes, while the 19-ol peaks about 10 minutes. The kinetics involved in the first few minutes thus reflect the first and second oxidations with an initial 19-hydroxylation<sup>6,7</sup> bottleneck.

The kinetics of aromatase in the presence of testosterone can be described by a treatment of the following equation;



Where  $k_8$ ,  $k_{10}$  and  $k_{12}$  are negligible,  
 $k_1 > k_9 > k_{11}$  (by factors of 10 and 100, *vide infra*)  
 and  $k_7 = 0$ . Although no information on  $k_{13}$  has been obtained from re-isolation experiments, it is not unreasonable to assume that  $k_{13} = 0$ .

Michaelis-Menten kinetics will be used throughout this thesis as a first approximation of the aromatase kinetics. Although patently incorrect for a multistage enzyme, this simplification will serve for illustrative purposes and will be used to screen the kinetic data obtained during inhibitor incubations.



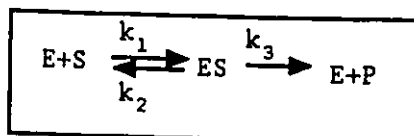
Eq.1

Experimentally, it is possible to follow the enzyme kinetics with tritium and compute enzymatic activity at different testosterone concentrations. Aliquots are taken during the incubation of deuterated testosterone with aromatase at 37 °C. The level of aromatization is determined by co-incubation with 1.7nM 1,2- $\beta$ -tritiated testosterone, known to release the tritium in water during aromatization. Counting the released tritium in the aqueous phase precisely quantifies the extent of aromatization on a very small scale. During linear kinetics, then, the apparent  $K_m$  and the  $V_{max}$  can be extrapolated with this approach.

Compared to the common methodology of isolating the substrate and product to determine deuterium contents, this experiment monitors the enzyme response faster, more accurately and during the first few minutes. Within this time frame, an isotope effect at the third oxidation of aromatase could become evident. This can be detected before effects are cancelled out, before substrate and product can be isolated to determine a deuterium to hydrogen ratio.

#### 2.2.1 Kinetic analysis of an isotope effect at C-2: attempt to elucidate the mechanism with 2-d-testosterone.

By co-incubation with 1,2 $\beta$ -tritiated testosterone, a kinetic isotope effect appears as a variation in the tritium output during aromatization of deuterated and undeuterated substrates.



Eq. 1

Equation 1 describes a typical one-substrate one-enzyme Michaelis-Menten system where

$$K_m = (k_3 + k_2) / k_1 \text{ and } K_{cat} = k_3 ;$$

An isotope effect stems from a different set of equilibrium constants ( $^Dk_*$ ) for the deuterated substrate. Since the deuterated testosterone is sterically indistinguishable from testosterone,  $k_1$  and  $k_2$  are unchanged in Eq. 1 ( $^Dk_1 = k_1$  and  $^Dk_2 = k_2$ ). The  $K_m$  is a reflection of the affinity for the substrate and is mostly unaltered by deuterium since it is usually reasonable to assume that

$$k_3 \ll k_2$$

and  $K_m \approx k_2 / k_1 \approx K_s$ , the dissociation constant.

The enzyme would, however, require more time to oxidize a C-2 deuterium and release the substrate. This decreases  $^Dk_3$ , the turnover number (the catalytic capacity) of the enzyme for deuterated substrate.

$$\begin{aligned} \text{Since} \quad & ^D V_{max} = ^D K_{cat} [E]_0, \\ \text{where} \quad & ^D K_{cat} = ^D k_3 \text{ for an irreversible reaction,} \\ \text{then} \quad & ^D V_{max} = ^D k_3 [E]_0. \end{aligned}$$

The kinetic isotope effect is then due to a difference in the catalytic efficiency, affected by the ratio  $^Dk_3 / ^Hk_3$ . It often results in a lower enzyme velocity.

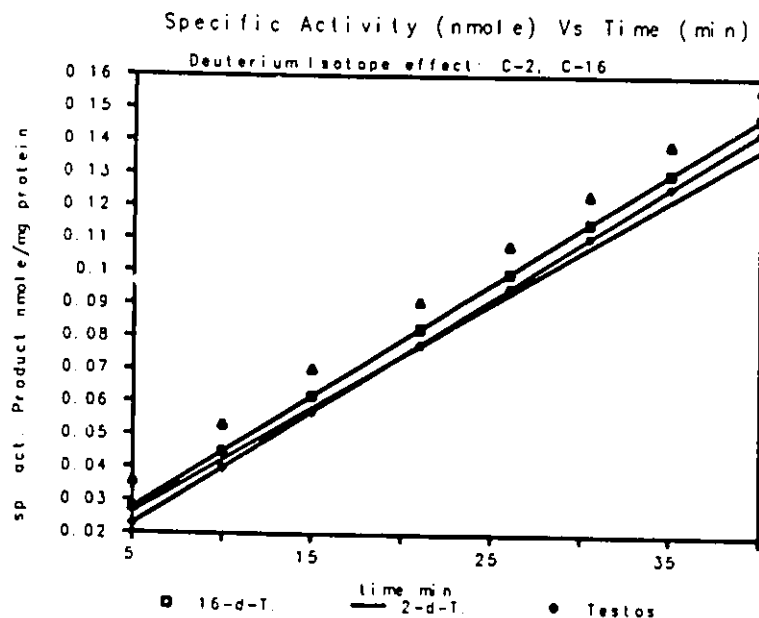


**Figure 2.2.1: Produced tritium vs Time of incubation.**  
**[T] = 1068 nM.**

The results of this experiment are graphed in figure 2.2.1. Triangles represent the value of the control incubation plus one standard deviation. Incubations starting with either deuterated or unlabelled testosterone fall within this standard deviation; the identical kinetics demonstrate no kinetic isotope effect larger than 14 percent. This data affects current postulates discussed in chapter 1. There the loss of a  $2\beta$ -hydrogen is postulated as a result of either;

- A. enolization prior to modification of ring A,
- B. enolization as a final step to aromatization, or
- C. insertion of oxygen in the 2-C-H bond.

If mechanism C is operating, incubation of  $2\beta$ -deuterio-testosterone should result in a kinetic isotope effect similar to that occurring with C-19-deuterated substrate. Experimentally the absence of a measurable isotope effect indicates that loss of the  $2\beta$ -hydrogen is not involved in the rate limiting step, and probably not lost prior to modification of the A ring: there appears a kinetic bottleneck between the second and third oxidation such that enolization prior to this would probably cause a  $^3\text{H}/^2\text{H}$  effect of at least 2.

Absence of effect then disfavors mechanism C. The kinetics can be described by a treatment equation 2 (vide supra). The experimental result suggest that  $K_6$  is not limiting at any time during the incubation.

Unpublished experiments of Fishman *et al.* and Robinson *et al.* indicate that a more complex series of events may occur, with the fate of the 2-deuterium varying under thermal dependence for testosterone but not androstenedione: the importance of this on the mechanism is not clear. (personal communications). From these, the known thermal dependence of  $\Delta\Delta G^\circ$  on substrate-protein hydrogen bonds immediately suggests itself, and warrant further investigation.

#### **2.2.2 Use of inhibitor and co-incubation kinetics in the study of enzyme-substrate interaction.**

The use of inhibitors allows a better understanding of the enzyme mechanism and active site constraints. Pharmaceutically, the control of aromatase via inhibitors would find clinical use in the investigation and control of estrogen-mediated events, such as ovulation, and in the management of estrogen dependent disease states in men and women. These states include hormone-dependent mammary carcinoma (affecting up to 13% of women), endometrioid cancer, gynecomastia and benign prostatic hyperplasia, affecting 70% of the men over 65 years old<sup>8</sup>.

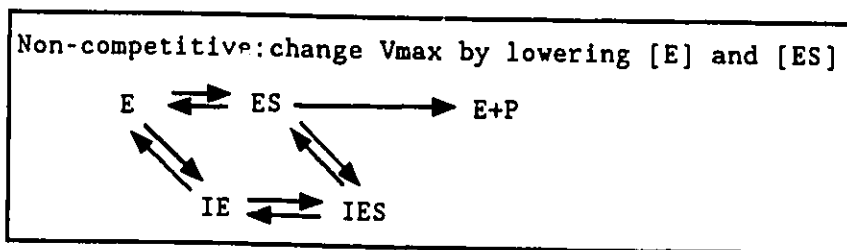
#### **2.2.3 Enzyme inhibitors effects on enzyme kinetics.**

Enzyme inhibitors are commonly sub-divided in four general classes:

- Competitive
- Uncompetitive
- Non-competitive
- Mixed

These are differentiated by their mode of action.

#### 2.2.3.1 NON-COMPETITIVE: Inhibitors lower apparent $V_{max}$ .

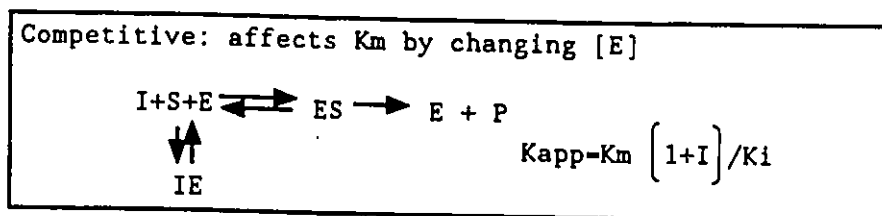


Eq. 3

An inhibitor is non-competitive when affecting enzyme sites in a manner independent of the substrate. The inhibitor affects only the  $V_{max}$  of the enzyme, and the apparent Michaelis-Menten constant ( $K_{app}$ ) equals the  $K_m$  of the enzyme alone. The affinity constant  $K_m$  is unaffected since the inhibitor affects sites other than the active site and natural substrates display the same affinity for the free active site. The inhibitor lowers the  $V_{max}$ , the maximum enzyme turnover, by lowering the concentration of active enzyme  $E$ . The inhibited enzyme species  $IE$  or  $IES$  do not have any significant activity, hence do not participate in the catalytic cycle. Common type of non-competitive inhibition are heavy metal ions reacting with  $-SH$  on the enzyme, distorting the required three dimensional structure.

A test steroid which elicits this type of response can not fit in the active center and yields no steric or electronic information. Alternatively, a similar kinetic profile is obtained from a very reactive steroid as it alters the enzyme concentration before there is competitive effect with the natural substrate. As such, a diagnostic of "uncompetitive" inhibitor is of interest in the screening of the type performed throughout this work. These potent irreversible inhibitors must be differentiated from strict uncompetitive inhibitors by a set kinetic experiments to show time dependent inactivation.

2.2.3.2 COMPETITIVE: Inhibitor increases the apparent  $K_m$ .



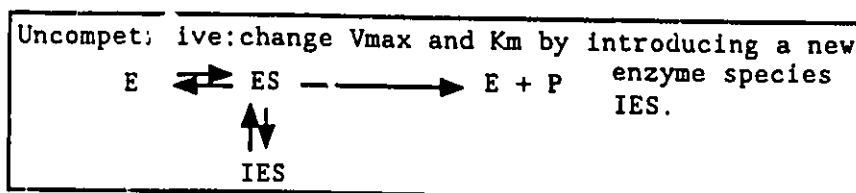
Eq. 4

An inhibitor which increases the apparent  $K_m$  of the enzyme either forces an enzyme conformation unfavorable for a substrate, or the inhibitor has the shape required for interactions in the active site and competes with compounds which also fit the active site.

Recognition of competitive inhibition by synthetic compounds is useful in deducing the nature of the active site<sup>10</sup>. The extent of their respective affinity depends on the relative affinity of the competing compounds (the ratio  $ES/IE$ ), hence depends on the dissociation rates for the substrate and the inhibitor. Increasing the concentration of the substrate  $S$  in the presence of a competitive inhibitor  $I$  will bring the  $K_{app}$  closer to the  $K_m$  by favoring the formation of  $ES$ . Given a fixed concentration of inhibitor, adding enough natural substrate will cancel the effect of the competitive inhibitor entirely. Thus, the maximum enzyme turnover, the  $V_{max}$ , is unchanged: it requires a high concentration of substrate to wash out the inhibitor effect, but it exhibits the same maximum turnover.

The magnitude of the effect on the  $K_m$  relates to the affinity of the steroid for the active site. Comparing series of similar structures will yield series of binding energy differences, reflecting relative affinity for the site: these types of compound are useful in determining Structure Activity Relationships, active site amino acids and active center steric requirements.

**2.2.3.3 UNCOMPETITIVE: Inhibitor changes  
both Vmax and Km.**



**Eq.5**

Uncompetitive inhibitors reduce Vmax and the apparent affinity (increase the Kapp) in the same ratio. The effector combines with the enzyme substrate complex ES. Then  $K_i = [ES][I]/[ESI]$

Uncompetitive inhibition is extremely rare for reactions having a single substrate, but is common for bisubstrate reactions with ping-pong mechanisms.

**2.2.3.4 MIXED COMPETITIVE: Inhibitor changes  
both Vmax and Km.**

In more complex inhibition kinetics, MIXED kinetics are observed; inhibitors can compete for the active site modifying the Vmax and the Km.

**NON-Michaelis-Menten kinetic analysis: Convention for  
inhibitor nomenclature.**

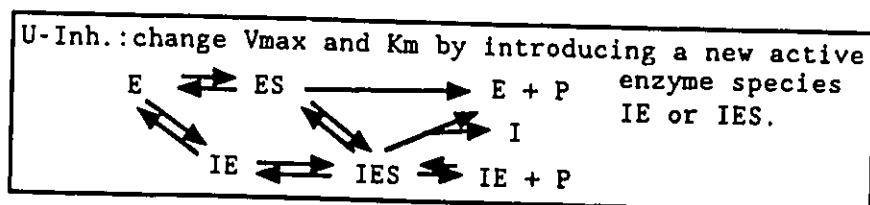
As stated earlier, the Michaelis-Menten formalism applied to the enzyme in this thesis is a simplification of the multistage enzyme aromatase.

The kinetic experiment and screening performed in this chapter did not permit differentiation between mixed-competitive inhibitors and inhibitors (M) which could compete for the active site and irreversibly modify the

enzyme, modifying the  $V_{max}$ , or which could react with the enzyme and remain partially catalytic, affecting both the  $K_m$  and the  $V_{max}$  by introducing new, active, enzymatic species with their own kinetic parameters. Examples include inhibitors that degrade to form other inhibitory species, or inhibitors that bind in and out of the active site.

For the purpose of this screening, these two types of inhibitors are both referred to as mixed-competitive (M), pending more elaborate kinetic experiments aimed at determining irreversibility.

Similarly, the experiments did not differentiate between uncompetitive inhibitors (which under the Michaelis-Menten formalism can exhibit no affinity for the active site) and a most interesting class of inhibitors which would display selectivity for the active site and potent time-dependent inactivation of the enzyme following the following pathways:



For the purpose of this screening, these two types of inhibitors are both referred to as uncompetitive (U), pending more elaborate kinetic experiments aimed at determining the possibility of irreversibility. Very few inhibitors displayed affinity constants or irreversibility constants compatible with eventual clinical applications; the requirement for further kinetic experiments is stated in those cases applicable.

#### 2.2.3.5 KINETIC DIFFERENTIATION.

As shown, considerable information on the mechanism and nature of inhibitor binding is obtained from the enzyme kinetics'. Experimentally, the enzyme is incubated with all required co-factors in the presence of the substrate testosterone and a test steroid. By varying the substrate to inhibitor ratio the following typical graphs are observed:

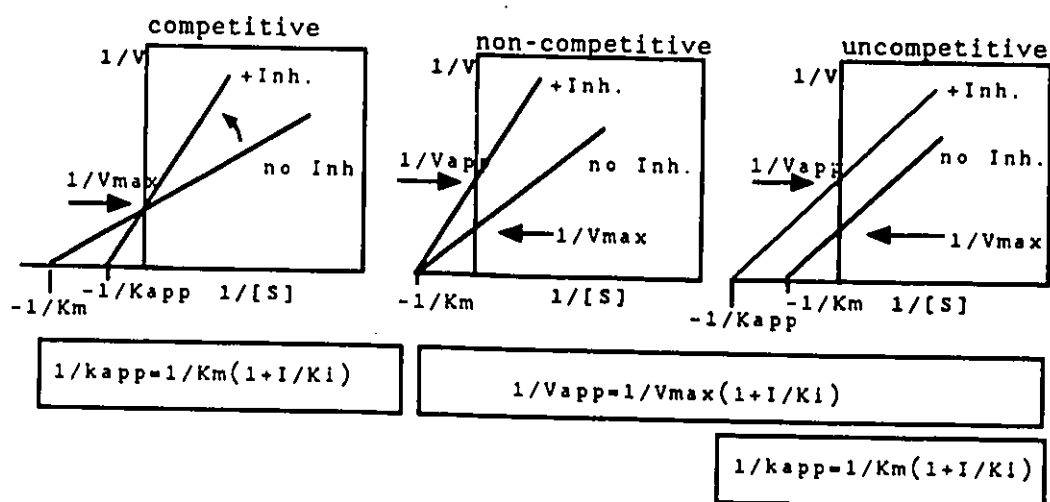


Diagram 2.2.3.5: Lineweaver-Burke plots for classes of inhibitors.

|                 | $K_{app}$               | $V_{app}$                       | $K_i$                               |
|-----------------|-------------------------|---------------------------------|-------------------------------------|
| no inhibitor    | $K_m$                   | $V_{max}$                       |                                     |
| Competitive     | $K_m \cdot (1 + I/K_i)$ | $V_{max}$                       | $I \cdot K_m / (K_{app} - K_m)$     |
| Non-competitive | $K_m$                   | $V_{max} \cdot K_i / (I + K_i)$ | $I \cdot V_{app} / (V_m - V_{app})$ |
| Uncompetitive   | $K_m \cdot (1 + I/K_i)$ | $V_{max} \cdot K_i / (I + K_i)$ | $I \cdot K_{app} / (K_m - K_{app})$ |
| Mixed           | variable                | variable                        |                                     |

By analyzing these plots, a value for  $K_i$  is obtained.

■ For Uncompetitive and Competitive inhibitors,  
 $K_i$  is the concentration of inhibitor that doubles the  
apparent  $K_m$ .

■ For Uncompetitive and Non-competitive inhibitors  
 $K_i$  is the concentration that halves the apparent  $V_{max}$ .  
It is also referred to as  $IC_{50}$  (the median inhibitory  
concentration at which an antagonist exerts half maximal  
effect) .

#### 2.2.3.6 Active site and Chemical selectivity.

It is possible to protect a steroid's inactivating group by means of a latent, unreactive functionality which is uncovered only in the active site. These **suicide inhibitors**<sup>11</sup> react only with the target enzyme that have a high affinity and the proper chemical active center to modify the latent inactivating group. Once uncovered, the reactive group on the inhibitor reacts in the active center and, covalently bound, the enzyme is inactivated. The kinetics<sup>12</sup> of such inactivation represent a special case of irreversible uncompetitive effects, characterized by increasing enzyme protection in the presence of the natural substrate, stoichiometric irreversibility (theoretically each enzyme requires only a few molecules of inhibitor for inactivation) and time dependent loss of activity exhibiting saturation kinetics.

Irreversible inhibitors cause time dependent inactivation. The enzyme activity decreases with time, and can not be recovered after washing the inhibitor from the enzyme preparation. Reversible inhibitors cause time dependent inactivation of the enzyme but can release the enzyme unscathed. The classes of inhibition are determined by the kinetics observed in double substrate co-incubation experiments.

#### 2.2.4 Use of inhibitors to understand the enzyme active site; correlating amino acid sequence to three dimensional folding.

DNA technology and QSAR approaches are complementary in assessing active site topology. Simpson *et al.*<sup>70</sup> sequenced a cDNA insert encoding aromatase cytochrome P<sub>450</sub>. Comparison with other known P<sub>450</sub> sequences yields a putative amino acid sequence for the heme binding region, but no data on the secondary characteristics which define the three dimensional folding<sup>69</sup>. Computer modeling of the amino acid (a.a.) interactions have been carried out by E.R.Simpson (unpublished). They result in a number of possible enzyme conformations, which in turn define the active site topology. The use of inhibitors complement the data required to direct the modeling: a correlated topology of the active site can be obtained by quantitative Structure Activity Relationships (QSAR) of substrate and inhibitors. Cross tabulation yields a hypothesis of the most probable active center topology. The influence of major amino acid to substrate interactions can then be assessed by site specific mutagenesis of the encoding DNA, to generate a slightly altered amino acid sequence. The renatured "isozyme" should display an altered enzyme function illustrating the a.a. substitution.

For example, Simpson has identified a glutamic acid near the C-2 steroidal position which, when mutated to a non polar amino acid, will yield a synthetic isoenzyme with loss of aromatase activity. This can be taken as supportive evidence that glutamic is involved in the mechanism of aromatization, and support postulates which require enolization of the C-2 hydrogen. (*pers.comm.*) The approach followed in the present thesis relied on the more straightforward use of inhibitors: for example, incubation of 2-deuterated testosterone (see p.47) showed no kinetic isotope effects and disfavors a mechanism of aromatization involving C-2-hydrogens in a rate limiting step.

These two approaches can also be applied by approximating the interaction volume of substrate series with the androgen binding site. They can help correlate portion models of the enzyme active site topology and assist in a final working model of the amino acid folding in the active center.

Defining and quantifying the parameters essential for aromatase affinity consequently help the design and generation of lead inhibitors and refine the understanding of the enzyme active center. The synthesis of modified steroid was carried out here for these reasons. The biochemical impact on aromatase are reported in the following sections.

#### **2.2.5.1 Computer modeling, Qualitative Structure Activity Relationship and Comparative Molecular Field Analysis.**

Local entropic phenomena vary with solvent and dielectric constant. These phenomena will affect calculations of a minimized steroidal structures and interaction with the active site surface amino acid side chains. Discussed literature values range from 1 to 5. The dielectric constant to be used for microscopic dimensions (distance dependent) is difficult to calculate.

In modeling, this constitutes a source of error which can not be readily circumvented. Some authors have achieved a high rate of success in QSAR (quantitative structure activity relationship) by setting the dielectric constant at lower values. This is particularly helpful for weak interaction such as  $\pi$ -stacking with phenylalanine, where a  $\Delta\Delta G^\circ$  of transfer from water to ethanol, considered more representative of the protein interior, is of the order of -2.4 Kcal/mol. In this thesis, minimization was normalized using a gross dielectric constant  $\epsilon$  of 1.5.

#### 2.2.5.2 Van der Waals contact radii.

Although the lower normal limits of contact distances between atoms are about 10% smaller than the sum of the corresponding radii, in intermolecular computations the contact distance was set at the Van der Waals radii.

#### 2.2.5.3 Free energy and enthalpy.

In this and the following chapters "free energy" represents the Gibbs free energy. Binding energy is the binding enthalpy.

$$\Delta G = \Delta H - T\Delta S$$

where:

$\Delta H$  = binding energy difference: enthalpy

$\Delta S$  = entropy difference: order

It is possible to apply these to compare two substrate-enzyme interactions by computing first  $\Delta G^*$  for both, then  $\Delta\Delta G^*$ , the difference between the two substrate binding;  
for steroid 1 or 2

$$\Delta G = -RT \ln K_{eq} \quad ; K_{eq} = [\text{Final}]/[\text{Initial}]$$

and

$$\Delta\Delta G^* = -RT \ln [K_{steroid1} / K_{steroid2}]$$

Although  $K_i$  would be a better representation of binding affinity, this constant must be obtained from spectroscopic analysis of  $P_{450}$ -steroid interaction and is seldom reported. Instead, an indexed constant is used.  $K_{steroid} = K_i/K_m$ ; the term  $K_m$  is the normalizing factor, the Michaelis-Menten equilibrium constant for a standard substrate. The term  $K_i$  is the inhibitor constant defined above, representing affinity.

A case in hand is the difference between the binding of androstenedione and testosterone: there is a three fold difference in affinity constants;

ie:  $K_{\text{testos.}}/K_{\text{and.}}=3$ , which represents a  
 $\Delta\Delta G^{\circ} = -0.619\text{Ln}[3] = -0.68 \text{ Kcal at body temperature.}$

Assuming a similar change in energy of transfer from free solution to the enzyme surface,

$$\Delta G = G_{\text{enzyme surface}} - G_{\text{solution}}$$

the difference in free energy is due to entropic or enthalpic phenomena. For similar molecules, a first approximation is that the entropic change  $\Delta S$  is similar, then the free energy difference reflects mostly the difference in enthalpy or the binding energy difference. Factors which contribute to this are numerous, amongst them the surface area, distribution of polar and non polar areas, dipole, ionic and van der Waals interactions.

A major difference between testosterone and androstenedione is the loss of a C-17 ketone. Postulating the involvement of a hydrogen bond to the 17 ketone in the active site, the substitution to a hydroxyl represents a free energy loss of approximately 0.6 Kcal/mol when compared to similar changes. This value, significantly lower than the 3-6 kcal/mol usually assigned a hydrogen bond, reflects the difference ( $\Delta\Delta G^{\circ}$ ) between

(a hydrogen bond for the ketone in water ) and  
(a hydrogen bond for the ketone on the protein surface),

instead of the absolute value  $\Delta G^{\circ}$  of an additional hydrogen bond. Values for such hydrogen bond transfers are typically about 0.6 kcal/mol for glucocorticoid receptors.

If such correlations are confirmed with other pair of substrate exhibiting the C-17 keto/alcohol substitution, it is reasonable to invoke a hydrogen bond to the C-17 keto in the binding site.

#### 2.2.5.4 Surface Area

The water accessible surface of a protein is related to the van der Waals envelopes of surface atoms. Water molecules are considered spheres of 1.4 Å radius. The accessible surface is defined as the area described by the center of the water molecule that rolls over the van der Waals envelope of a side chain. There is a linear relationship between this area and the  $\Delta G_{\text{transfer}}$  for hydrophobic side chains. For polar surfaces this is smaller but still of comparable magnitude. The proportionality constant is approximately 0.025 kcal/mol Å<sup>2</sup>.

#### 2.2.6 Active site topography.

Given the reasonable premise that similar high affinity steroids bind to the enzyme in similar fashion it is possible to approximate a map of aromatase active center by overlapping low energy conformers of the inhibitors. This enzyme-allowed volume is a map representing a functional active site topography, one in which a prospective affinity inhibitor must reside in order to fit the active center

Minimization using the program PCModel (Serina Inc.) yields the most probable conformation of a steroid calculated with MM2 force field, all Allinger options and Hartree-Fock Pi subroutines. In view of Koshland's "induced-fit theory" of enzyme-substrate interactions, Marshall recommends retaining species within 6 kcal of the lowest energy conformer. To reflect this, additional conformations with higher kinetic energies are obtained by simulated bond rotation of angular and pending groups. Dihedral maps are included. Some volume manipulation routines are performed with the programs Sybill and Alchemy. (TRIPOS Associates v.4/1989) Overlapping a judicious choice of inhibitor and conformations, it is then possible to approximate the enzyme allowed volume. The difference in affinity between two

inhibitors is related to the difference in free energy of the enzyme-steroid complex. Adequate comparisons help determine probable sites of interactions and the nature of binding.

Poor affinity inhibitors must interact with fewer binding sites, and steroids with no affinity for the enzyme must not fit in the active center, the outer boundaries of the acceptable cavity determine the disallowed volume. This derives from combining disallowed or unfavored inhibitors, and subtracting these from the enzyme allowed map. This manipulation yields an excluded volume where an inhibitor can not reside without decreasing the affinity for the active site.

Cramer<sup>46</sup> has significantly advanced Comparative Molecular Field Analysis (CoMFA) by integrating similar steric maps with considerations of electrostatic environment and treating the resulting numerical representation with partial least square analysis.

This representation of the electronic parameters affecting inhibitors requires extensive computing power. Although many researchers<sup>71</sup> have obtained excellent result from such statistical analysis on different enzyme systems, the tools available during this thesis precluded such massive computations.

Methods relying on multiple regression analysis using free energy related physical values such as melting point, partition coefficients, hydrophobicity  $\pi$ , polarizability (MR), resonance  $\chi$ , and induction  $F$  were not selected.

Nevertheless, the binding contributions associated with substituent ( $\Delta\Delta G^\circ$ ) presented in this thesis were compiled and normalized from literature data in view of future work by other workers, when more elaborate interpretation can be performed relying on multiple regression analysis of biological response BR (Ki/Km). The constants used will then be:

Biological response: Constants

Ki/Km

Derived binding parameters observed

$\Delta G^{\circ} \propto \log[Ki/Km]$  (normalized to Androstenedione)

Calculated binding parameters: Constants

polar surface area,

non-polar surface area,

hydrophobic surface area

This thesis retains a more graphical approach, relying on computed atomic data, an attempt to determine the principal components affecting aromatase-steroid interactions.

By plotting favorable and unfavorable structure interactions, such maps help understand and predict inhibitor potential, loss of inhibition, loss of inactivation, and eventually help model an inhibitor by modeling the required re-alignment in the active center to expose the right enzyme group to an inhibitor substituent.

**2.2.7 Kinetic strategy to screening aromatase inhibitors.**

Substrate induced inactivation require a high affinity for the aromatase active site. By measuring aromatase activity at different inhibitor concentrations it is possible to select test steroids which will interact with aromatase, modifying or occupying the enzyme active site.

A Structure Activity Relationship (SAR) is obtained by incubating a series of representative test steroids containing key substituent groups we desire to study. Those active then undergo further kinetic analysis to elucidate site interaction. For this purpose, decreasing concentrations ( $10^{-5}$  to  $10^{-9}$  Molar) of steroid are incubated until the smallest useful inhibitor concentration is

determined. For aromatase, a suitable inhibitor will inhibit enzyme activity at concentrations of  $5\mu\text{M}$  to  $0.1\mu\text{M}$ , a very good inhibitor still inhibits at concentrations as low as  $5\text{ nM}$ . The two clinical drugs aminoglutethimide and teslac both have  $K_i$  about  $800\text{ nM}$  against androstenedione ( $K_m \approx 20\text{--}60\text{ nM}$ ). The ratio  $K_i/K_m$  is used to compare data from preparations with different enzyme activity.

Competitive assays are then performed in the presence of excess NADPH and testosterone or androstenedione. We have used testosterone substrate to measure the enzyme activity; in the high NADPH environment of our experimental setup there is less interference from  $17\beta$ -dehydrogenase which would certainly reduce the  $17$ -keto of androstenedione, and complicate interpretation of the affinity constants and aromatase activity.

A promising compound will undergo the sequence of incubations below.

#### **Method 1.**

Determine observed Velocity (Vobs) after a time of 30 minutes. Continue decreasing the inhibitor concentrations until no effect on Vobs is observed. Typically  $[\text{Inh}] = \{100\mu\text{M} \text{ to } 10\mu\text{M}\}$ ;  $[\text{T}] = \{22, 202, \text{ or } 402\text{ nM}\}$ . This reveals whether the steroid interacts with the enzyme. Comparison with standards indicates how potent the inhibitor is.

**Result: Vobs as % of control. Interaction yes/no.**

#### **Method 2.**

Determine two Vobs at two chosen inhibitor concentrations,  $[\text{T}] = \{2000, 401.7, \text{ or } 21.7\text{ nM}\}$ ; and  $[\text{Inh}] =$  fixed. This allows determination of the apparent maximum Velocity (Vapp) and apparent Michaelis-Menten constant (Kapp), both required to compute the mode of interaction and affinity.

**Result: Vobs<sub>1</sub>, Vobs<sub>2</sub>, Vapp, Kapp,  $K_i$ , ( $V_{\text{max}}$ ,  $K_m$ ), %Kapp/ $K_m$  and %Vapp/ $V_{\text{max}}$  indicates type of interaction.**

### Method 3.

Determine six points  $[Inh] = \text{fixed}$ ,  $[T] = \{2400 \text{ to } 22 \text{ nM}\}$ .

Result:  $V_{obs1-6}$ ,  $V_{app}$ ,  $K_{app}$ ,  $(V_{max}, K_m)$ ,  $K_i$ , interaction, kinetics.

The enzyme data is represented with Eadie-Hofstee plots to magnify departure from linearity, and Lineweaver-Burke<sup>13</sup> plots to determine  $V_{max}$  (which can only be approximated as a limiting value at infinite substrate concentration). More precise data analysis can be carried out by direct linear plot according to Eisenthal and Cornish-Bowden<sup>14</sup>. These graphic determinations yield the apparent inhibition constants. In this thesis, a numerical algorithm equivalent to the Eisenthal graphic representation was developed because of the greater accuracy in statistical weight assigned each experimental point. Poor data points are removed according to the student T-distribution when confidence is less than 95%.

### 2.3 The C-19 cavity postulate:

#### Preliminary correlation between literature-based inhibitory activity of non-steroidal inhibitors and C-19-derived androgens.

New leads in pharmacophore development were generated by modeling electronic overlap between glutethimide (or benzoflavone) inhibitors and androgens. These leads could prove useful as affinity labels and would presumably be capable of inducing active site re-alignment in aromatase.

The trends observed in different inhibitor categories will be assessed below to allow information cross over between the available Structure Activity Relationships of non-steroidal inhibitors and androgenic compounds.

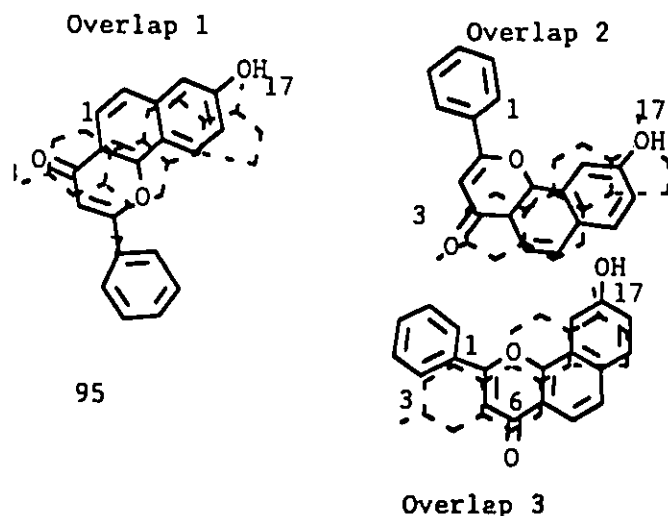
#### 2.3.1 Benzoflavone derivatives.

9-Hydroxy-naphthoflavone<sup>33</sup> is a very potent competitive inhibitor of aromatase with a  $K_i$  in the nanomolar range. It induces a difference spectrum indicative of a shift from high to low spin iron when added to substrate saturated enzyme. The shift in Soret maxima are typical of low spin, oxygen ligated  $P_{450}$ . Vickery proposed that the naphthoflavone occupies the steroid binding site. His data is re-analyzed here for matching pharmacophoric patterns. Scheme 2.3.1 illustrates regions where the naphthoflavone backbone is set to match the steroidal carbon frame. Analysis of the Structure Activity Relationship (SAR) between various hydroxy-derivatives demonstrates a requirement for the hydroxy at the position shown in scheme 2.3.1, with very dramatic loss of inhibition as it is moved away. This allows a more precise modeling with the steroidal backbone. Three

reasonable overlaps account for competition with a steroid backbone in the active site. These were generated using the MMx force field.

### 2.3.1.1 Topological considerations of 9-OH-benzoflavone.

Steric overlap 1 places the phenyl ring near the C-6,7 position, where it is known that phenyl substituents increase inhibition. Overlap 2 places oxygenation in the C-3,4 region and a phenyl in the C-1,2,19 region. Active site mapping of steroids has shown a large hydrophobic pocket in the C-1 region. The preferred overlap#3 matches the C-17 D-ring oxygen, a derived C-7 position and a match is found for the C-6 keto pharmacophore (a well known enhancer of aromatase inhibition). The C-1,2,19 region again possess hydrophobicity, unsaturation and bulk. This lead was investigated with published aminoglutethimide series of inhibitors.



**Scheme 2.3.1:** Overlap of naphthoflavone inhibitors with the androgen backbone (Testosterone is dashed and slightly offset for clarity).

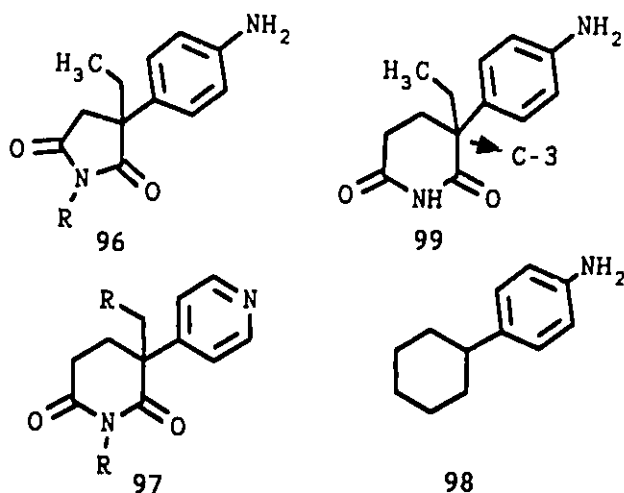
### 2.3.2 Aminoglutethimide derivatives.

Many derivatives of aminoglutethimide **99** (AG) are inhibitors of aromatase. It was of interest to model the work done with these derivatives in the hope that there exist some overlap between their active center and the steroid-recognition sites on the enzyme.

Although the backbone of aminoglutethimide can be freely overlapped with the basic androgen skeleton, and has much rotational freedom about the glutethimide to aromatic ring bond, it is flat and rigid in what corresponds approximately to rings A and C in scheme 2.3.2.1. AG derivatives are type II inhibitors, indicating arylamine coordination to the heme-iron of the cytochrome P<sub>450</sub>.

The aromatic aniline can be substituted for a pyridyl group. Either a pending piperidinedione (glutarimide), cyclohexyl<sup>31</sup>, or pyrrolidinedione<sup>32</sup> are required for activity.

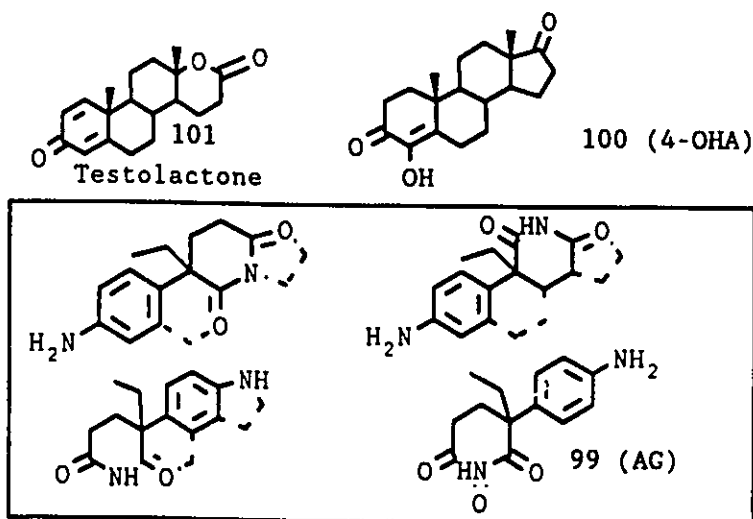
In the AG series, the lipophilicity is shown to play an important role in the inhibitory activity of C-1 or C-3 pyridyl homologues<sup>30</sup>. There is evidence of a relationship between inhibition, the length of the alkyl chain at position C-3 and the lipophilicity. Activity increases until R=octyl<sup>30</sup>.



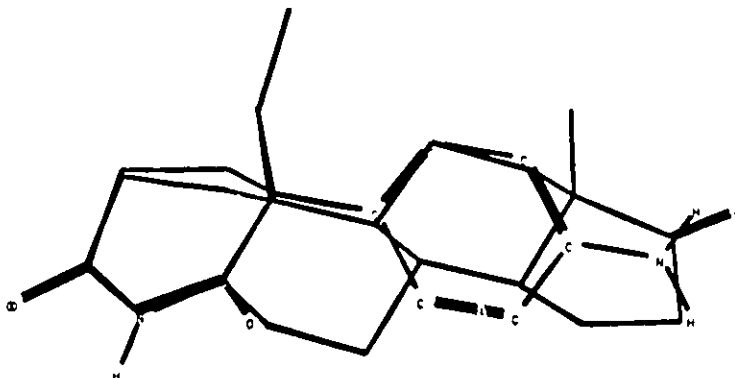
**Scheme 2.3.2:** Aminoglutethimide and derivatives.

### 2.3.2.1 Topological considerations: Aminoglutethimide.

By inspection, steric overlap of the glutarimide or cyclohexyl rings seems consistent with a portion of the steroidal binding site. The possible "overlap" between known inhibitors and AG 99 is shown below.



Aminoglutethimide overlap with steroid backbone.



**Scheme 2.3.2.1:** Aminoglutethimide overlap with androstenedione. Best overlap possible generated using molecular mechanics based on MMx approach.

Considering the amide bond to be 40 % double bond character there may be considerable electronic overlap between the glutarimide portion of aminoglutethimide and the A-ring of known aromatase inhibitors such as testolactone 101

or 4-OHA 100 derivatives. The overlap of the homologous pyridyl-glutathimide C-3 alkyl series with the androgenic C-19 methyl then becomes intriguing.

From these we infer that the aminoglutethimide and androgen backbone will overlap sterically, and the AG-3-ethyl reasonably overlaps with the androgenic C-19 substituent. The SAR performed on non-steroidal inhibitors can then be carried over to steroidal inhibitors to better understand the activity of some androgen structural features, and vice-versa.

The addition of bulky substituents to a drug molecule often results in the emergence of antagonists since it permits the utilization of auxiliary binding sites in the receptor<sup>6 8</sup>.

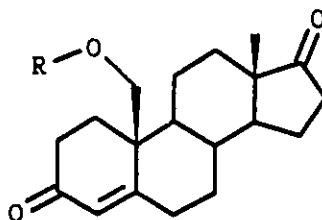
It is reasonable that an increasing binding affinity to aromatase may arise from placing suitably hydrophobic groups at the C-19 position of androstenedione-19-ol, corresponding approximately to the region of steric overlap on these non-steroidal series. This hypothesis is based on the tentative correspondence between the C-1, C-19, C-11 region (on the  $\beta$  face of the steroid A-ring) with:

- 1) the determinant C-3 alkyl bulky moiety of the glutethimide derivatives
- 2) the benzoflavone derivatives with phenyl ring in the same region increasing inhibitory activity,
- 3) the C-4 and C-7 $\alpha$ - phenyl thioether derived androgen demonstrating inhibitory activity.

This postulate indeed proved fortunate.

The inhibitor data generated in this thesis from the compounds listed below, and with more complex C-19 derived androgens (see C-3-chel postulate section) confirms that bulk up to approximately 6Å is acceptable at the angular C-19 position.

### 2.3.3 C-19 derived androgens postulated as potential inhibitors.



2 R-H

|                               |                             |
|-------------------------------|-----------------------------|
| 43 R-Tr                       | 37 R-tBDMSi                 |
| 102 R-Bn                      | 38 R-TMSi                   |
| 103 R-Tf                      | 39 R-MOM                    |
| 104 R-tBu(Ph) <sub>2</sub> Si | 40 R-tBDMSi, 6 $\alpha$ -OH |
| 105 R-p-TOS                   | 41 R-Ac                     |
|                               | 42 R-Ms                     |

**Scheme 2.3.3:** C-19 androgens synthesized to test the C-19-cavity postulate. Some proved too labile to be tested.

The steroids in scheme 2.3.3 were synthesized to test the C-19 cavity postulate. The starting material for these 19-derived test compounds is the 19-oxygenated androstenedione 2. It is a base sensitive compound which is difficult to handle, on a large scale, under conditions required to make the more reactive C-19 alcoholates; deformylation seems to occur, generating a powder with broad spectral bands.

As would be expected of a neopentyl alcohol, the C-19 position exhibits slow reaction times. In order to synthesize the desired bulky derivatives at the neopentyl alcohol, it is best to use S<sub>N</sub>1-type reaction conditions, preferably with carbocationic reagents. The steroid 19-trityl androst-4-ene-3,17-dione-19-ol 43 was synthesized in 41% yield by reaction of the free alcohol 19-OH-A 2 with Ph<sub>3</sub>C<sup>+</sup> BF<sub>4</sub><sup>-</sup> in refluxing CH<sub>2</sub>Cl<sub>2</sub> for 5 days.

In order to enhance the reactivity of the 19 hydroxyl towards reagents, reaction with sodium hydride followed by quenching with bromo reagents can be used. Unfortunately this procedure is difficult to scale up and yields are

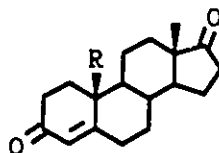
unreliable; the 19-alkoxide presumably deformylates. Instead, we found that reaction of bromo reagents in benzene in the presence of AgNO<sub>3</sub> and Et<sub>3</sub>N is of general use for the hindered C-19 neopentylic alcohol, despite low yield (≈30%) and typically long reaction time (1 week).

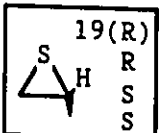
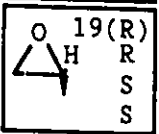
Sn<sub>2</sub> reaction conditions can be productive with hindered alcohols when silane<sup>62</sup> reagents are used. These react via hexacoordinated silicon, and are therefore less sensitive to steric constraints. The general procedure of Corey<sup>61</sup> was modified to synthesize 19-tertbutyldimethylsilyl androst-4-ene-3,17-dione-19-ol **37** in 71% yield by reacting the free 19-alcohol in DMF with tBDMSi-Cl/Imidazole at 77 °C under N<sub>2</sub> for 13 hours.

#### **2.3.3.1 Review of the literature on aromatase inhibitors bearing a C-19 group.**

It was essential to normalize the literature data available for these types of derivatives. Below is the first comprehensive collation of C-19 derived aromatase inhibitors.

TABLE 2.3.3.1: C-19 androgens reported as inhibitors.



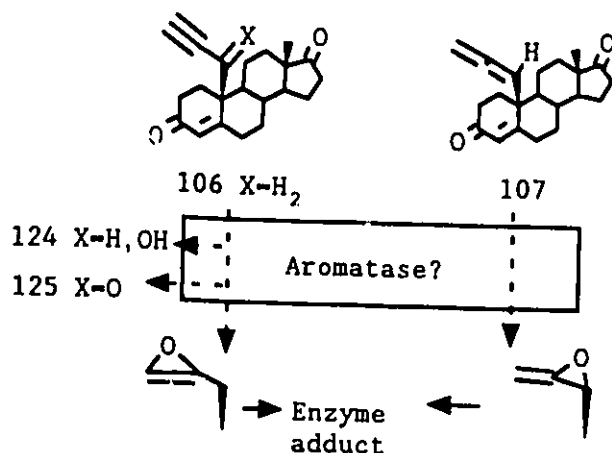
| ref.                 | Code   | R                                                                                 | T/A<br>incubation | K <sub>i</sub> /K <sub>m</sub><br>X100 | K <sub>i</sub><br>μM   | Mode                             | kcal/MAG <sup>*</sup><br>/mol |
|----------------------|--------|-----------------------------------------------------------------------------------|-------------------|----------------------------------------|------------------------|----------------------------------|-------------------------------|
| 37                   | 106    | -C-C≡CH (PED)←                                                                    | T                 | 10/45                                  | 22                     | TDI                              | -0.9                          |
| 39                   | 107    | -C-C=CH <sub>2</sub>                                                              | T                 | 14/45                                  | 19                     | TDI                              | -0.2                          |
| 38                   | 106    | -C-C≡CH                                                                           | A                 | 23/40 <sup>a</sup>                     | 57                     | TDI                              | +0.3                          |
| 45<br>74<br>45<br>74 | 108    |  | A<br>A<br>A<br>A  | 2/20<br>1/39<br>120/20<br>75/39        | 10<br>3<br>600<br>192  | Comp<br>Comp.<br>Comp.<br>Comp.  | +1.4<br>+2.3<br>-1.1<br>-0.4  |
| 45<br>74<br>45<br>74 | 109    |  | A<br>A<br>A<br>A  | 10/20<br>7/39<br>180/20<br>75/39       | 50<br>18<br>900<br>192 | Comp.<br>Comp.<br>Comp.<br>Comp. | +0.4<br>+1.1<br>-1.4<br>-0.4  |
| 49                   | 110    | -C≡N                                                                              | A                 | IC <sub>50</sub> = 2500                |                        |                                  |                               |
| 56                   | 111    | -CN <sub>3</sub>                                                                  | A                 | 5/25                                   | 20                     | Comp.                            | +1.0                          |
| 56                   | 112    | -CSSO <sub>2</sub> Me                                                             | A                 | 1/25                                   | 4                      | TDI                              | +2.0                          |
| 73                   | 113    | -CSH                                                                              | A                 | 47/4400                                | 1                      | ---                              | -2.8 *                        |
| 37                   | 114    | -H <sub>2</sub> CSMe                                                              | A                 | 1                                      | --                     | ---                              | ----                          |
| 27                   | 115    | -HC(OH)CCSiMe <sub>3</sub>                                                        | T                 | 529                                    | --                     | Comp.                            | ----                          |
| 27                   | 116    | -H <sub>2</sub> COSO <sub>2</sub> Me                                              | T                 | ---                                    | --                     | Mixed                            | ----                          |
| 37                   | 117    | -HCF <sub>2</sub>                                                                 | A                 | 1000/40 <sup>b</sup>                   | 2500                   | TDI                              | -2.0                          |
| 37                   | 118    | -H <sub>2</sub> CF                                                                | A                 | IC <sub>50</sub> =                     | 2.9                    | Comp                             | ----                          |
| 37                   | 119    | -C=O                                                                              | A                 | IC <sub>50</sub> =                     | 4                      | Comp                             | ----                          |
| 48                   | 120    | H,17αC≡CH                                                                         | A                 | 4800/90                                | 533                    | Comp                             | -2.5 *                        |
| 21                   | 120    | H,17αC≡CH                                                                         | A                 | ---                                    | --                     | TDI                              | *                             |
| 59                   | 121    | -CH <sub>2</sub> CH <sub>3</sub>                                                  | A                 | 81/91                                  | 89                     | Comp                             | +0.4 to -0.1                  |
| 59                   | 122(S) | -HC(OH)CH <sub>3</sub>                                                            | A                 | 1100/91                                | 1208                   | Comp                             | -2.6 to -3.2                  |
| 59                   | 122(R) | -HC(OH)CH <sub>3</sub>                                                            | A                 | 990/91                                 | 1088                   | Comp                             | -2.7 to -3.0                  |
| 59                   | 123    | -C(=O)CH <sub>3</sub>                                                             | A                 | 150/91                                 | 164                    | Comp                             | -0.2 to -0.4                  |

personal communications: a: J.O.Johnson b. C.Robinson

TDI: Time dependent inhibition. Comp: Competitive inhibition. Mixed: mixed mode inhibition. T/A incubation: specifies the reference substrate against which the inhibitor was incubated. T: testosterone. A: androstenedione. IC<sub>50</sub>: inhibitor concentration required to cause 50% inhibition. \*: This data has been disputed or is inconsistent. The incubation temperature was 30° in ref.73.

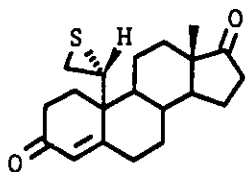
Derivatives with heteroatoms at C-19 are obvious substrate analogues of aromatase and many of these compete for the active site. Some are modified, presumably undergoing enzyme mediated oxidation at the C-19 carbon followed by deformylation. This has led to suicide inhibitors which require the oxidation to uncover the alkylating group.

Extensive work <sup>38,49</sup> has been reported with triple bonds at this position. The propargyl **106**<sup>37</sup> (PED) and allene **107**<sup>37</sup> are extremely potent inactivators of aromatase. The extent of the inhibition by PED has been disputed due to inconsistent  $K_i$  reported, though it remains a most potent inhibitor. PED is undergoing clinical trials for breast cancer in England. These inhibitors exhibit time-dependent, NADPH-mediated loss of activity. As they are not chemically reactive, they must undergo metabolic transformation before inactivation of the enzyme can occur. The tentative intermediates **124** or **125** could be enzyme generated, but fail to explain enzyme inactivation. Instead, the mechanism of inactivation conceivably involves oxidation<sup>39</sup> in the active center to generate an oxirene or allene oxide species; reactive epoxides which can alkylate the enzyme.

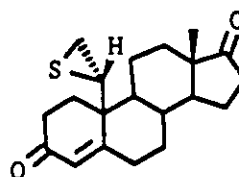


**Scheme 2.3.3.1:** postulated mechanism of action by PED.

The less reactive C-19 thiirane and oxirane **108** (R,S) and **109** (R,S) do not appear to be sufficiently reactive to cause inactivation without the presence of the putative double bond. Nevertheless, the C-19 thiirane and the C-19



108 19(S)

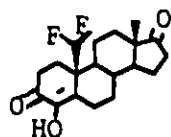


108 19(R)

oxiranes are very potent competitive reversible inhibitors. The isomers have shown differential inhibitory activity, with 36-80 fold higher activity found for the (19R)-isomers<sup>45</sup>. The published data is not consistent and calculates to a large difference in  $\Delta\Delta G^\circ$ . The preference corresponds to a difference in free energy of the substrate-enzyme complex of 2.2 to 2.7 times in favor of the R isomer.

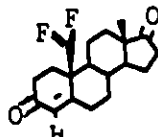
Published spectral analysis confirms that these inhibitors bind to the heme iron. The tighter coordination of the 19R derivatives has been interpreted to favor a heme iron above carbon positions C-1 and C-2 in the A ring. A number of inhibitors synthesized in this thesis were designed in the hope of similarly binding to the heme-iron from a pending C-19 and a C-3 moiety. (*vide infra*)

Marcotte and Robinson<sup>37</sup> synthesized and tested 19-fluorinated androstenedione. While the mono fluorinated 118 is aromatized, the difluorinated 117 acts as a mechanism-based inhibitor, possibly via an acyl fluoride. Time dependent release of tritium from C-19 labelled <sup>3</sup>H-CF<sub>2</sub> indicates the mechanism<sup>73</sup> probably involves HO-CF<sub>2</sub>, which quickly rearranges to the reactive acyl fluoride in solution. Mann<sup>62</sup> combined the 19-F<sub>2</sub> and the 4-OH pharmacophores on one inhibitor. This combination leads to slight loss of activity.



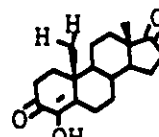
code 126

IC<sub>50</sub>  $\mu$ M 3.3



117

1.3



100

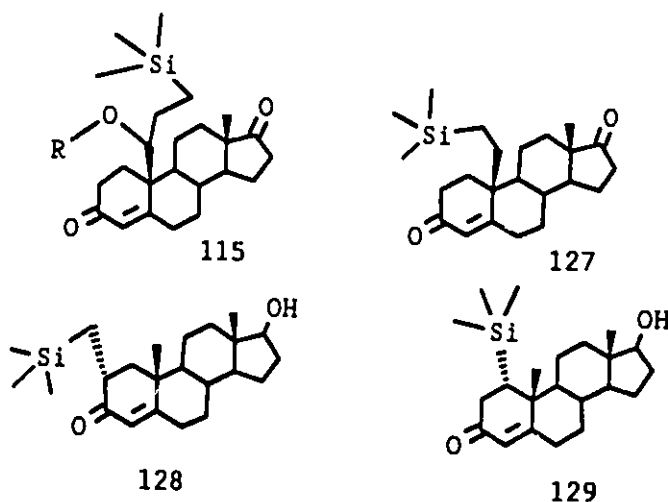
0.2

### 2.3.3.2 Heteroatom based derivatives: reasoning behind preparing 19-sulfonic derivatives.

In 1985, Akhtar<sup>56</sup> reported the synthesis and incubation of a number of C-19 heteroatom inhibitors. The 19-azido **111** and 19-methylthiomesylate-androstenedione **112** were shown to be potent inhibitors. The thiomesylate derivative causes time dependent inactivation of the enzyme. Akhtar entirely disregarded the leaving group capacity of this C-19 difunctional molecule.

The thiomethyl ether **114** causes type II spectral shift typical of sulfur coordination to the heme iron. Counsell has shown that C-19 amino<sup>52</sup> bioisosteres are poor competitive inhibitors. He has investigated 19-sulfur<sup>51</sup> derivatives with considerably more success. As methanesulfonate derivatives are known affinity labels<sup>64</sup>, we synthesized and incubated for the first time a number of 19-mesyl and tosyl derivatives, the simplest being the 19-mesyl-androst4-ene-3,17-dione **42**. It displayed mixed inhibitor kinetics and seemed a lead compound worthy of further investigation.

Weintraub<sup>27</sup> designed inhibitors based on the documented silylation of proximal nucleophiles with enzyme generated  $\beta$ -silyl-stabilized carbonium or radical. (Scheme 2.3.3.2) The most potent of these is the 19-hydroxy-19-ethyl-(2'-tri-methylsilyl) derivative **115**. It exhibits competitive kinetics.



**Scheme 2.3.3.2:** Silylated steroids screened in the literature on aromatase inhibition.

The objective of the C-19 cavity postulate was to confirm and exploit a cavity in the active center of aromatase situated in the vicinity of the C-19 steroidal position. Inspection of the steroid inhibitor literature (table 2.3.3.1) confirmed that minor steric bulk was permissible. Inspection of naphthoflavone and aminoglutethimide inhibition data suggested a greater cavity. Available 19-silyl ethers were convenient starting points for this investigation. Some were synthesized in the hope that their bulky silyl groups would perhaps cause mechanism-based inactivation, in addition to answering the steric question of the C-19 cav. postulate.

#### 2.3.4: Inhibition by derivatives with locked conformations.

Incubation of very few 19-pharmacophores with 19-chirality have been published. They might offer a key to a preferred positioning of the C-19 pending groups in the active center of aromatase. An important differential activity has been observed with the isomers of the C-19 thiirane and oxirane three membered rings of 108 and 109. The R isomers were shown to exhibit 26 to 80 fold higher affinity for the active center of aromatase. The C-19 thiirane is free to rotate, with an energy minima over the A ring and one away from the A/B plane. The X-ray crystal data of the R isomers indicates the solid state preference of the three membered ring resides with the heteroatom over the A ring. To assess whether such conformations might illustrate a favorable active site conformation, steroids with conformations locked via 19 ether bridges were incubated. In addition, those chosen for this incubation set were intermediate analogues of reactivity postulates presented in chapter 1.

A first series of steroids was screened. These exhibit a locked conformation via C-19,4 and C-19,6 ethers. The 19-methyl ethers 34 and 35, the 19-silyl ether 36 and the alcohol epimers of 32 were tested against testosterone.

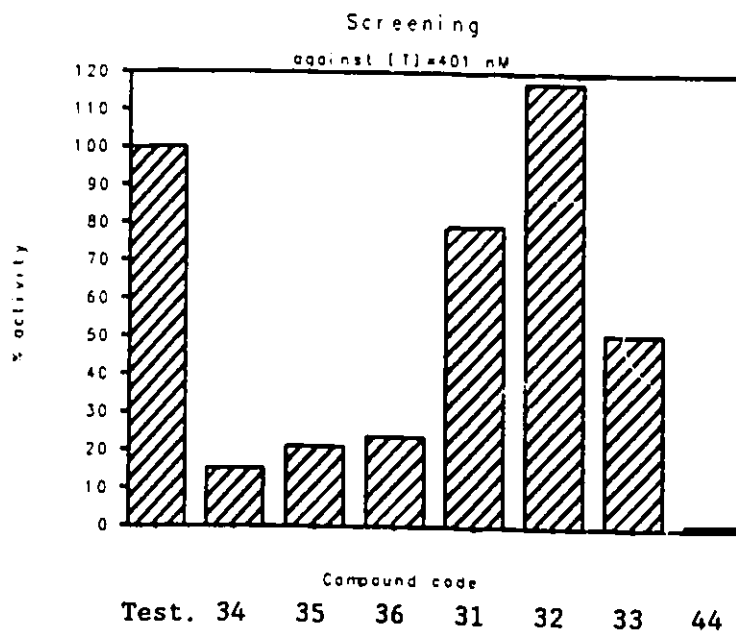
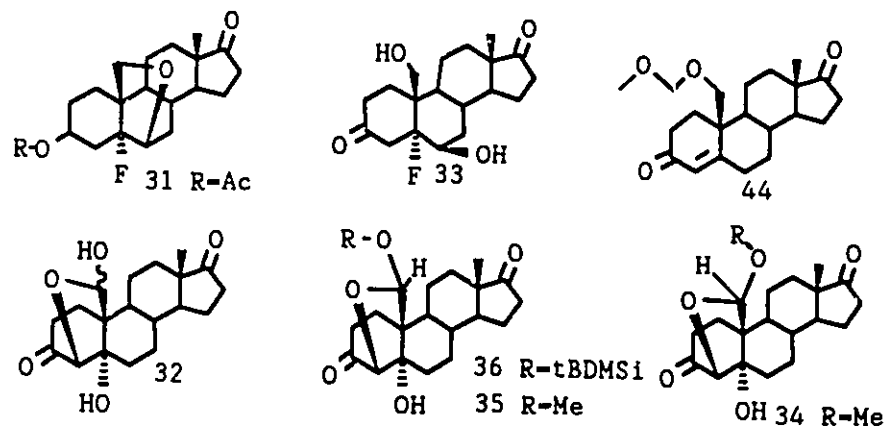


**Figure 2.3.4:** Screening of 19-bridged ethers against testosterone at 401 nM. The inhibitor concentrations [Inh.] range from 130 to 12  $\mu$ M. Incubations as follows: [ $1\beta, 2\beta$ - $^3$ H] Testosterone (0.1  $\mu$ Ci, 1.7 nM) and cold testosterone ([T]=401.7 nM in 40  $\mu$ L ethanol) is co-incubated with inhibitor (in 40  $\mu$ L ethanol) and human placental aromatase (70  $\mu$ g protein) at 37  $^{\circ}$ C for 30 min. in the presence of excess NADPH.

**Table 2.3.4:** Screening of 19-Bridged ethers: 19,4-oxa and 19,6-oxa androgen

| Code   | [Inh.] | Mode | Ki      | %V      |       | Description          |
|--------|--------|------|---------|---------|-------|----------------------|
| Method | M      | %Km  | $\mu$ M | T@401nM | %Vmax | (Androst-3,17-dion-) |

**Enzyme allowed: included in the active center:**

|    |         |         |       |     |     |     |                                   |
|----|---------|---------|-------|-----|-----|-----|-----------------------------------|
| 31 | 1       | 1.19E-5 | - --- | --- | 79  | --- | 5 $\alpha$ -F-6,19-oxa-3-OAc      |
| 33 | 1       | 1.17E-5 | - --- | --- | 51  | --- | 5 $\alpha$ -F-6 $\beta$ ,19-diol  |
| 35 | 2,3     | 1.29E-4 | C 156 | 229 | 21  | 99% | 5 $\alpha$ -ol-19,4-oxa-(19S)MeO  |
| 36 | 2       | 1.00E-4 | C 165 | 156 | 24  | 116 | 5 $\alpha$ -ol-19,4-oxa-(19S)tBDM |
| 39 | 1       | 1.20E-4 | - --- | --- | 0   | --- | 4-ene-19-MOM                      |
| T  | control | none    | - --- | --- | 100 | 100 | Testosterone control              |

**Enzyme excluded: Excluded from the active center:**

|    |   |         |       |     |     |     |                                  |
|----|---|---------|-------|-----|-----|-----|----------------------------------|
| 32 | 1 | 1.16E-5 | I --- | --- | 117 | 100 | 5 $\alpha$ -ol-19,4-oxa-19-ol    |
| 34 | 2 | 1.31E-4 | N? 90 | 126 | 15  | 49  | 5 $\alpha$ -ol-19,4-oxa-(19R)MeO |

Abbreviations: Inhibitor Mode: C competitive; M mixed; N noncompetitive; I insignificant effect; A- Androstenedione; MeO- Methoxy; AcO- acetoxy; T- testosterone; DMO- Dimethoxy; tBDMS- tertButyldimethylsilyloxy ether. Incubations were as follows: [1 $\beta$ ,2 $\beta$ -<sup>3</sup>H] Testosterone (0.1  $\mu$ Ci, 1.7nM) and cold testosterone (in 40 $\mu$ L ethanol) were co-incubated with inhibitor (in 40 $\mu$ L ethanol) and human placental aromatase (70  $\mu$ g protein) at 37 °C for 30 minutes in the presence of excess NADPH.

Methods: 1: Cold testosterone [T]= 401.7nM only,  
 1a: Cold testosterone [T]= 21.7nM only,  
 2: Cold testosterone [T]= 21.7 and 401.7nM,  
 3: Cold testosterone [T]= 21.7 to 2400 nM.

■ %V expresses the percent ratio of the observed enzyme activity Vobs with inhibitor divided by the control enzyme activity with 40 $\mu$ L of ethanol only, with no inhibitor, in the presence of the testosterone concentration specified.

■ %Vmax is a special case of %V where the Vobs is extrapolated to determine the maximum apparent activity Vapp of the enzyme in the presence of the inhibitor. The effect %V is considered negligible when the value observed is within 10% of control. (110% to 90%)

■ %Kapp is the percent ratio of the extrapolated apparent constant Kapp divided by the Michaelis-Menten constant Km.

■ The Ki is the inhibitor concentration producing half maximal aromatization of [1 $\beta$ ,2 $\beta$ -<sup>3</sup>H] Testosterone (0.1  $\mu$ Ci, 1.7nM) under the conditions of incubation used. When both Kapp and Vapp were affected in a different ratio, approximations of the mixed inhibitor's Kik and Kiv represent the affinity constants Ki calculated when only the effect on the Vapp (Kiv) or the effect on the Kapp (Kik) is taken into consideration, independently of the other effects.

The selection of which steroid is enzyme-allowed in the active center is based on competition with testosterone for the active site; it will then exhibit competitive kinetics and affect the Kapp. The volume of the compound then defines an **enzyme allowed volume**. If a compound has no effect on the kinetics of testosterone, it does not fit in the active center and it defines an **enzyme disallowed, or excluded volume**.

The 19,4 oxa alcohol epimers 32 showed no inhibition, while the methyl and tBDMSi ethers were competitive inhibitors. It would not have been unreasonable to expect a differential inhibition in the presence of the epimers 34, 35 or 36.

Inactivation kinetics of (19S)-5-hydroxy-19-methoxy-4 $\beta$ ,19-oxido-5 $\alpha$ -androstane-3,17-dione **35** determined at 129  $\mu$ M indicates a  $K_i$  of 167  $\mu$ M and induces no enzyme inactivation. It exhibited clear effect on the apparent affinity, with  $K_{app}$ =177%  $K_m$ , promoting a fair enzyme inhibition but no inactivation at this concentration. It is a fair competitive inhibitor possessing fair affinity for the active center.

**Table 2.3.4.1:** Aromatase inhibition by **35**, (19S)-5-hydroxy-19-methoxy-4 $\beta$ ,19-oxido-5 $\alpha$ -androstane-3,17-dione.

| Compound             | Vmax<br>nm/min/mg     | Kapp<br>nM        | Ki<br>$\mu$ M | Mode | Inhibitor<br>$\mu$ M |
|----------------------|-----------------------|-------------------|---------------|------|----------------------|
| Testos.<br>$\pm$ STD | 0.0055<br>$\pm$ .0001 | 767<br>$\pm$ 23   | -             |      | none                 |
| <b>35</b>            | 0.0075<br>$\pm$ .0002 | 1360<br>$\pm$ 130 | 167           | C    | 129                  |

Values are given  $\pm$  one standard deviation. Incubation method 3: [1 $\beta$ ,2 $\beta$ -<sup>3</sup>H] Testosterone (0.1  $\mu$ Cl, 1.7nM) and cold testosterone ([T]-21.7 to 2400 nM in 40 $\mu$ L ethanol) were co-incubated with inhibitor (in 40 $\mu$ L ethanol) and human placental aromatase (70  $\mu$ g protein) at 37 °C for 30 min. in the presence of excess NADPH. Data analysis according to Eadie-Hofstee.

Incubations of the bridged ether series showed no marked difference in affinity between the (R) and the (S) diastereomers. There is a very small % $K_m$  decrease observed for the R-methyl ether **34**. Since this falls within the  $\pm$ 10% error margin, the steroid was categorized as a non-competitive inhibitor. Although this could be a special case of time dependent inhibition, no further work was performed to clarify the situation as the affinity was deemed too low for clinical applications.

Rigid rotor rotation of the C-10,19 bond allows computer simulations of the enzyme intermediate androstenedione-19-ol; the 19-alcohol can rotate 300° within an energy window of 6 kcal/mol. An energy-well occurs when the 19-ol is situated over the 4-alkene, at a dihedral angle  $\angle$  C-5,10,19,O-19 of -30°. This favorable conformation corresponds closely to the position of the oxygen ether in 19,4 bridged ethers. If the 19-OHA intermediate is found in the conformation illustrated, the locked conformation of the 19,4-bridged ethers would improve binding and catalysis, under the assumption that no other interactions require space above the A ring.

This hypothesis unfortunately fails as all bridged steroids tested here exhibited poor affinity. The best overlap of the carbon backbones is illustrated below and shows clearly that the major deviation arise at the C-4 position. The Root Mean Square (RMS) deviation on all matching non hydrogen atoms is 0.28Å and the average deviation 0.22Å. C-4 and C-5 cause most of the deviation, with the C-4 almost 1Å above the ring.

The low affinity of these steroids may then be due to such large backbone distortions, or may be due to the presence of the additional C-5 $\alpha$ -OH substituent. 19,4 and 19,6 bridged ethers were abandoned as potential intermediate analogues.

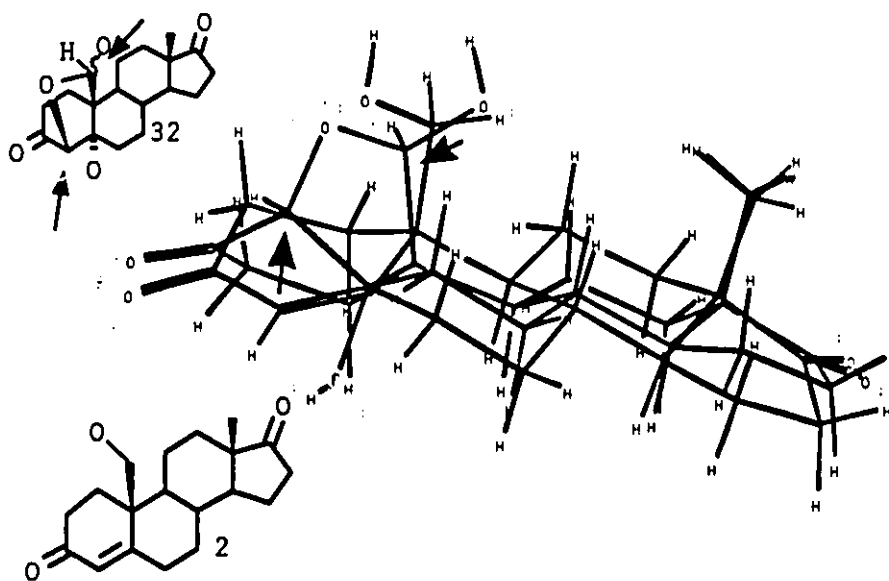


**Figure 2.3.4.1:** Overlap of 19-OHA 2 and compound 32.

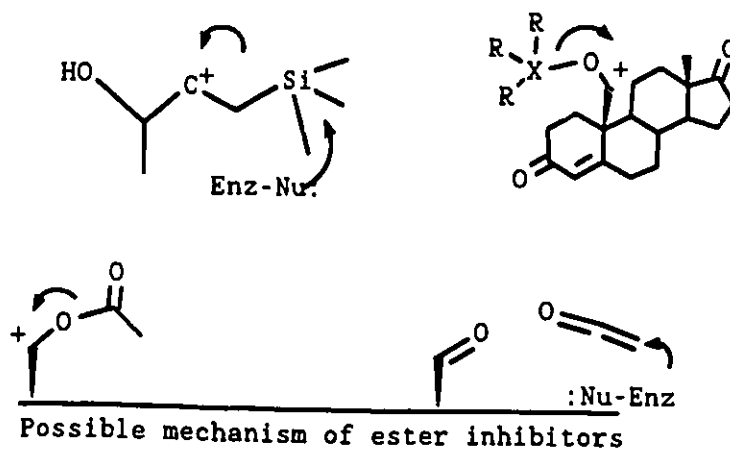
The "locked" 19-ol-19,4 oxo-cyclic 19S methoxy 35 and 19S-tBDMS 36 steroids bear bulky C-19 derivatives and must fit in or near the active center. This series of bridged compounds are intermediate analogues invoked in chapter 1 as potential aromatase intermediates involved in the C-4,5 and C-5,6 reactivity postulates. The poor affinity of the intermediates tested seem to disfavor the postulates.

The C-19 cav. postulate, however, seemed a promising one as the compounds tested do fit, to some extent, in the active center, elucidating competitive kinetics in the presence of testosterone, but no inactivation. This clearly demonstrates the dangers of relying on minimized conformations of exocyclic groups (from the thiirane X-ray) to establish parallel enzyme topology; an energy window better reflects Koshland's induced fit hypothesis.

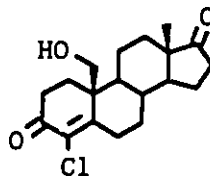
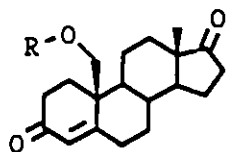
### 2.3.5 Inhibition by C-19 derivatives which can potentially stabilize a C-19 radical or cation.

We proceeded to synthesize and incubate potential mechanism-based aromatase inhibitors with  $\beta$ -silane,  $\beta$ -methoxyl, and  $\beta$ -triphenyl ethers at the C-19 position.

Silylated substrates are not well known enzyme inhibitors. They may operate by the mechanism illustrated in scheme 2.3.5. It was hoped that generation of an enzyme generated  $\beta$ -silyl stabilized radical (carbonium) would lead to silylation of a proximal enzyme nucleophile.



**Scheme 2.3.5:** Stabilization of a C-19 cation.



- 41 R-Ac 37 R-tBDMSi  
42 R-Ms 38 R-TMSi  
43 R-Tr 39 R-MOM  
2 R-H

**Table 2.3.5:** 19-derivatives and analogues.

| Code | [Inh.]<br>Method | [Inh.]  |           | Ki<br>μM | %V      |       | Description  |
|------|------------------|---------|-----------|----------|---------|-------|--------------|
|      |                  | M       | %Km, Mode |          | T@401nM | %Vmax |              |
| 37   | 2                | 1.07E-4 | ---, C    | 270 μM   |         | 102%  | 19-TBDMS-A   |
| 38   | 2                | 9.70E-5 | 90, M     | mixed    |         | 29    | 19-TMS-A     |
| 39   | 1                | 1.20E-4 | ---, -    | ---      | 0       | ---   | 19-MOM-A     |
| 41   | 2                | 1.39E-4 | 542, -    | 31       |         | 139   | 19-AcO-A     |
| 42   | 1                | 1.25E-4 | ---, -    | ---      | 44      | ---   | 19-Ms-A      |
| 43   | 2                | 1.02E-5 | ---, C    | 10       |         | 115   | 19-Tr-A      |
| 44   | 2                | 1.10E-4 | 36, M     | mixed    |         | 2     | 19-OH-4-Cl-A |
| 2    | 2                | 1.16E-4 | 1, -      | low      |         | 0     | 19-OH-A      |
| T    | -                | -----   | ---, -    | ---      | 100     | 100   | Testosterone |

Abbreviations and method as for table 2.3.4.; C competitive inhibition; M mixed inhibition; A- Androstenedione; MeO- Methoxy; AcO- acetoxy; Ms- Mesylate; TMS- Trimethylsilyl ether; tBDMS- tertButyldimethylsilyloxy ether; Tr- Trityloxy ether; MOM- Methoxymethyloxy ether.

Results shown in table 2.3.5 demonstrate considerable inhibition is in this series of C-19 groups. Even the large C-19-triphenylmethyl ether and C-19-tert-butyldimethylsilyl ether derivatives of androstenedione are inhibitors of aromatase, confirming the original C-19 cav. postulate.

The novel methoxy methyl (MOM) ether 39 is a potent inhibitor derived from the C-19 cavity postulate and the postulated mechanism of aromatase. It commanded further investigations, as it causes 100% inhibition at 120 μM in a competitive assay against testosterone at 0.4 μM.

Kinetics of androst-4-en-19-ol-3,17-dione, 19-methoxymethyl ether **39** determined at 40  $\mu\text{M}$  yield an apparent maximum velocity  $V_{\text{app}} = 56\% V_{\text{max}}$  and a  $K_{\text{app}} = 165\% K_{\text{m}}$ . From these the inhibitor concentration required to get  $V_{\text{app}} = 50\% V_{\text{max}}$  is approximated at  $\text{IC}_{50} = 51\ \mu\text{M}$ , and the concentration required to get  $K_{\text{app}} = 50\% K_{\text{m}}$  at  $K_{\text{ik}} = 61\ \mu\text{M}$ . This indicated that steroid **39** is a good mixed inhibitor with good affinity for the active site and induces enzyme inactivation.

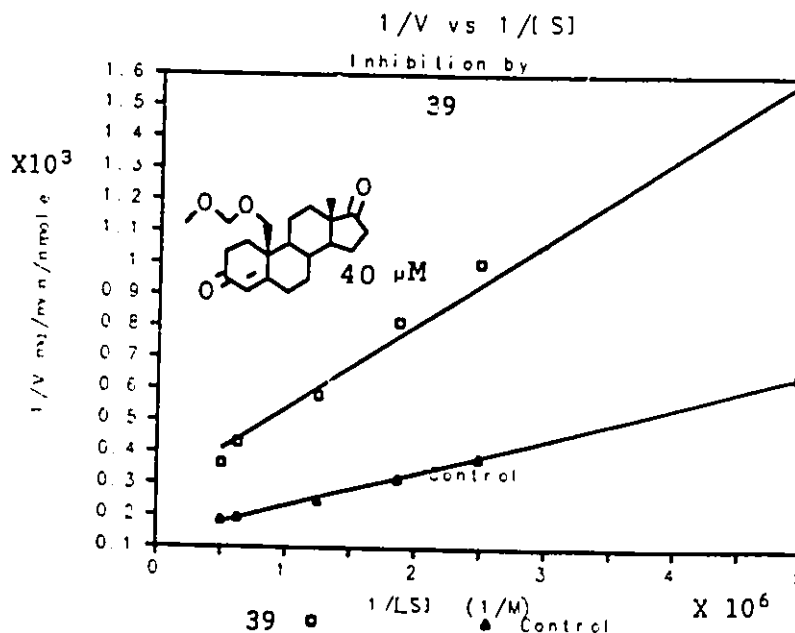


**Figure 2.3.5.1:** Aromatase inhibition by **39**, androst-4-en-19-ol-3,17-dione, 19-methoxymethyl ether: 19-MOM-A.

| Compound    | $V_{\text{max}}$<br>nm/min/mg | $K_{\text{app}}$<br>nM | $K_{\text{i}}$<br>$\mu\text{M}$ | Mode | Inhibitor<br>$\mu\text{M}$ |
|-------------|-------------------------------|------------------------|---------------------------------|------|----------------------------|
| Testos.     | 0.0078                        | 803                    |                                 | -    | none                       |
| $\pm 1$ STD | $\pm 0.0006$                  | $\pm 108$              |                                 |      |                            |
| <b>39</b>   | 0.0044                        | 1330                   | 61 $K_{\text{ik}}$              | M    | 40                         |
|             | $\pm 0.0015$                  | $\pm 676$              | 51 $K_{\text{iv}}$              |      |                            |

Values are given  $\pm$  one standard deviation. Incubation method 3 is described in table 2.3.4. Testosterone concentration ranging from 201 to 2000 nM. Data analysis according to Eisenthal.

Inhibition kinetics of androst-4-en-19-ol-3,17-dione, 19-tertbutyldimethylsilyl ether **37** determined at 107  $\mu\text{M}$  showed no effect on the apparent velocity and a  $K_{app} = 138\% K_m$ . From these the inhibitor concentration required to lower the  $K_{app}$  at 50%  $K_m$  was calculated as  $K_i = 279 \mu\text{M}$ . This indicated that steroid **37** is a fair competitive inhibitor with fair affinity for the active site and induces no enzyme inactivation.

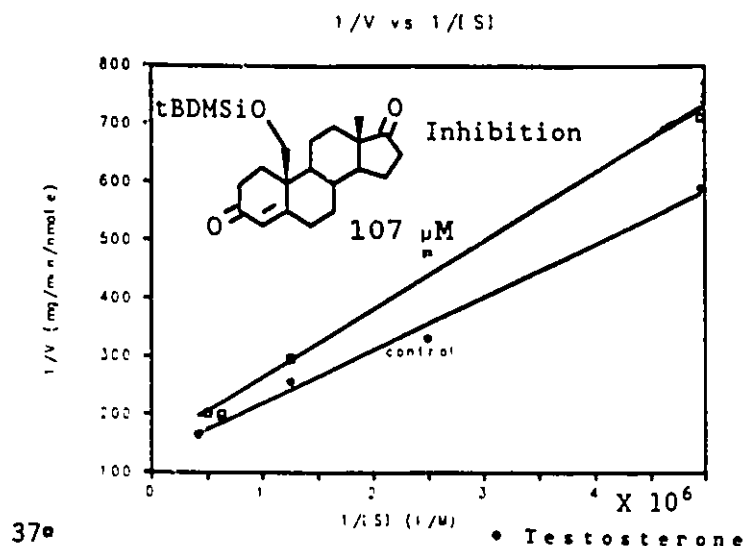


**Figure 2.3.5.2** Aromatase inhibition by **37**, androst-4-en-19-ol-3,17-dione, 19-tertbutyldimethylsilyl ether: 19tBDMS-A.

| Compound               | Vmax<br>nm/min/mg      | $K_{app}$<br>nM  | $K_i$<br>$\mu\text{M}$ | Mode | Inhibitor<br>$\mu\text{M}$ |
|------------------------|------------------------|------------------|------------------------|------|----------------------------|
| Testos.<br>$\pm 1$ STD | 0.0075<br>$\pm 0.001$  | 717<br>$\pm 242$ |                        | -    | none                       |
| <b>37</b>              | 0.0075<br>$\pm 0.0016$ | 991<br>$\pm 331$ | 279                    | C    | 107                        |

Values are given  $\pm$  one standard deviation. Incubation method 3 is described in table 2.3.4. Testosterone concentration ranging from 201 to 2402 nM. Data analysis according to Eisenthal.

Inhibition kinetics of androst-4-en-19-ol-3,17-dione, 19-trimethylsilyl ether **38** determined at 107  $\mu\text{M}$  exhibited an apparent maximum velocity  $V_{app} = 13\% V_{max}$  and an affinity  $K_{app} = 181\% K_m$ . From these the inhibitor concentration computed to lower the apparent  $K_{app}$  to 50%  $K_m$  was  $K_{ik} = 132\ \mu\text{M}$  and  $K_{iv} = 16\ \mu\text{M}$  for  $V_{app} = 50\% V_{max}$ . This indicated that steroid **38** is a good mixed inhibitor with good affinity for the active site and induces enzyme inactivation.

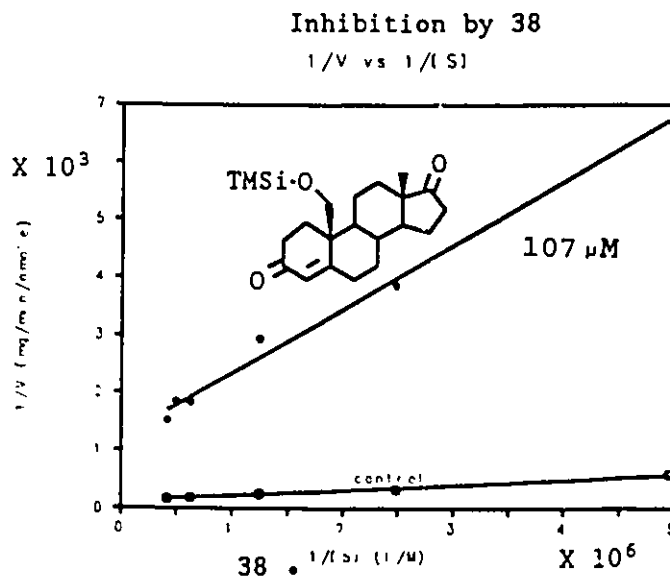


**Figure 2.3.5.3** Aromatase inhibition by **38**, androst-4-en-19-ol-3,17-dione, 19-trimethylsilyl ether: 19-TMS-A.

| Compound               | $V_{max}$<br>nm/min/mg | $K_{app}$<br>nM   | $K_i$<br>$\mu\text{M}$      | Mode | Inhibitor<br>$\mu\text{M}$ |
|------------------------|------------------------|-------------------|-----------------------------|------|----------------------------|
| Testos.<br>$\pm 1$ STD | 0.0075<br>$\pm .0010$  | 717<br>$\pm 242$  |                             | -    | none                       |
| <b>38</b>              | 0.0009<br>$\pm .0002$  | 1300<br>$\pm 455$ | $K_{ik}=132$<br>$K_{iv}=16$ | M    | 107                        |

Values are given  $\pm$  one standard deviation. Incubation method 3 is described in table 2.3.4. Testosterone concentration ranging from 201 to 2402 nM. Data analysis according to Eisenthal.

Inhibition kinetics of androst-4-en-19-ol-3,17-dione, 19-triphenylmethyl ether **43** determined at 10  $\mu\text{M}$  gave an apparent maximum velocity  $V_{\text{app}} = 90\% V_{\text{max}}$  and a  $K_{\text{app}} = 144\% K_{\text{m}}$ . From these the inhibitor concentration required to lower the  $K_{\text{app}}$  to 50%  $K_{\text{m}}$  is computed as  $K_{\text{ik}} = 23 \mu\text{M}$ . This indicated that steroid **43** is a good competitive inhibitor with good affinity for the active site and induces no enzyme inactivation. This compound has potential as a fluorescent probe.

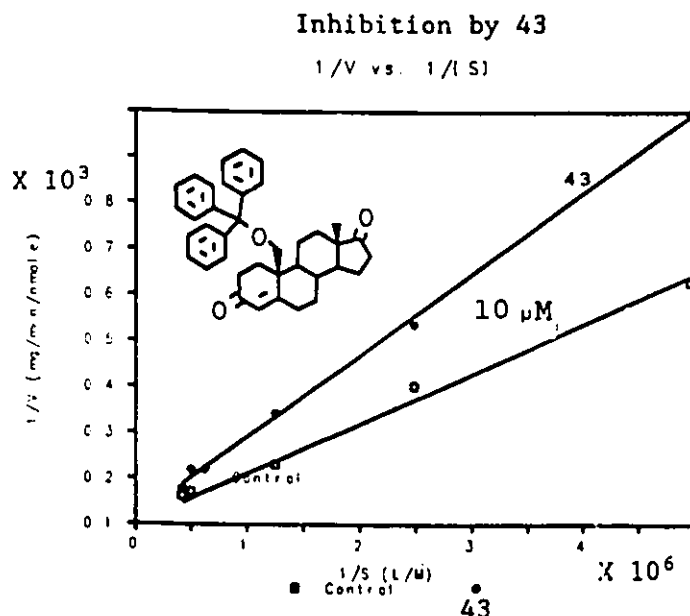


**Figure 2.3.5.4** Aromatase inhibition by **43**, androst-4-en-19-ol-3,17-dione, 19-triphenylmethyl ether: 19-Tr-A.

| Compound    | $V_{\text{max}}$<br>nm/min/mg | $K_{\text{app}}$<br>nM | $K_{\text{i}}$<br>$\mu\text{M}$ | Mode | Inhibitor<br>$\mu\text{M}$ |
|-------------|-------------------------------|------------------------|---------------------------------|------|----------------------------|
| Testos.     | 0.0098                        | 1080                   |                                 | -    | none                       |
| $\pm 1$ STD | $\pm 0.0009$                  | $\pm 214$              |                                 |      |                            |
| <b>43</b>   | 0.0089                        | 1540                   | 23                              | C    | 10                         |
|             | $\pm 0.0004$                  | $\pm 162$              |                                 |      |                            |

Values are given  $\pm$  one standard deviation. Incubation method 3 is described in table 2.3.4. Testosterone concentration ranging from 201 to 2402 nM. Data analysis according to Eadie-Hofstee.

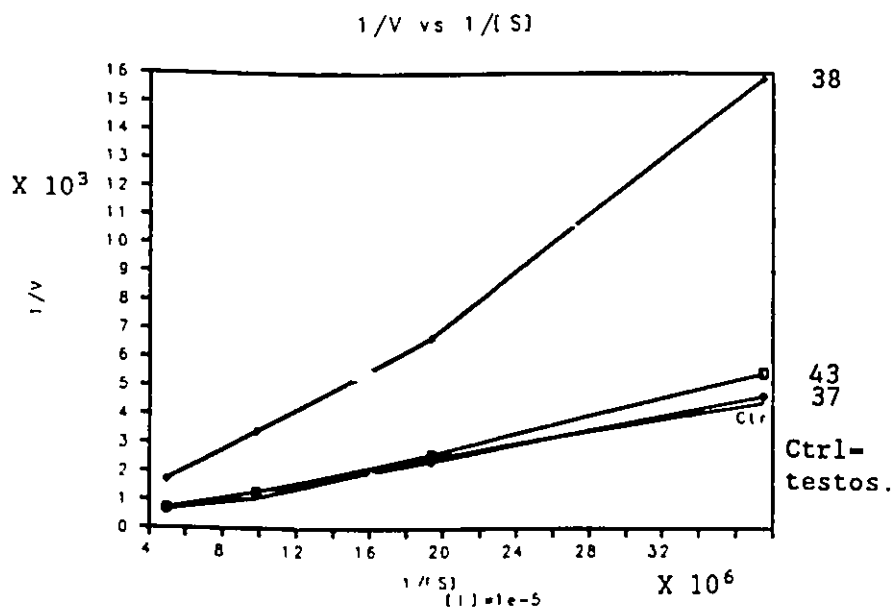


**Figure 2.3.5.5:** Lineweaver-Burke plots of 37, 38, 43 against a control testosterone (Ctrl).

Comparison of 37, 38 and 43 at  $10 \mu\text{M}$  demonstrates the good inhibitory capacity of 38. Compounds 43 and 37 have little effect at such low concentrations.

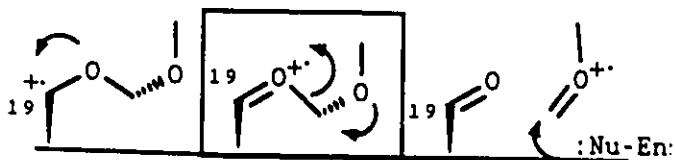
#### 2.3.6 Chemical reactivity of the tBDMSi, TMSi, Trityl and MOM groups. (compounds 37, 38, 39, and 43)

Chemically, the 19-trityl 43, 19-tBDMSi 37 and the 19-MOM 39 are stable and are not expected to deprotect readily in the presence of acetate or thiolate ions under mild aqueous reactions (between pH 4-8.5).

It is also improbable that the inhibitors 19-MOM 39 and the 19-TMSi 38 are deprotected to the 19-alcohol in the medium, since no  $V_{\text{max}}$  effects would then be observed from the free 19 alcohol. Their inactivation of aromatase may, however, go through a deprotection in the active center, where high local concentrations prevail; this could explain why the two which exhibit inactivation, both MOM and TMSi, are more reactive than the strictly competitive and chemically more stable trityl and tBDMSi ethers.

### 2.3.6 Postulated mechanism of action.

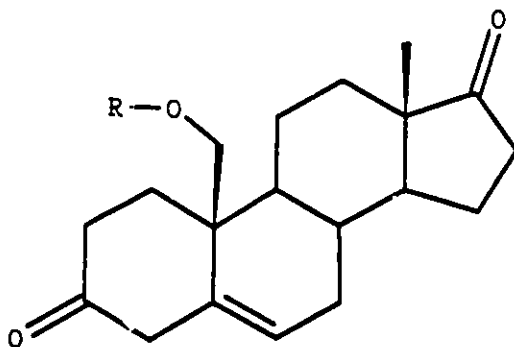
The possible mechanism of silyl derived inhibitors was reviewed earlier. Similarly, the MOM group may cause inactivation via C-19 stabilized carbocation or radical, to cause decomposition of the 19-methoxymethyl ether and generation of oxonium species in the active center, where it would be available to react with an enzyme nucleophile and inactivate the enzyme.



Possible mechanism of MOM ether inhibitor

**Scheme 2.3.6:** Postulated mechanism of inactivation by MOM.

### 2.3.7 Future inhibitors: synthesis of $\Delta^{5,6}$ derivatives



130 R- tBDMSi

The  $\Delta^4$  and  $\Delta^5$ -androgens would provide valuable comparison between the biological impact of the deconjugated 3-on-5,6-ene and the 3-on-4,5-ene. The synthesis is described here. Biochemical analysis will be presented under a different cover.

#### 2.3.7.1 Reaction of 19-OH-A with tBUOK

Treatment of 19-OH-A with two equivalents of base yields an intractable tar. As the C-19 position seems sensitive to deformylation, the 19tBDMSi derivative was used instead.

#### 2.3.7.2 Preparation of 5-androsten-19-ol-3,17-dione, 19-tert-butyldimethylsilyl ether (130).

The 4-carbanion was generated by deconjugation with 10 equivalents of potassium metal dissolved in degassed (with N<sub>2</sub>\*) tBuOH, at RT for 2½ hrs. Quenching the 4-carbanion in glacial acetic acid, followed by neutralization resulted in 45% yield of the 5-ene 130. TLC indicated that the deconjugated steroid is less polar than the enone, a property<sup>64</sup> found in  $\beta,\gamma$ -unsaturated ketones. The mass spectra showed a weak parent ion at m/z 416, the expected M-57 peak at 359 (100%) and silane at 73. <sup>1</sup>H NMR showed a broad absorption at  $\delta$  5.56 ppm corresponding to the 6-CH alkene proton (shifted from 5.85 in the starting material). A broad doublet at  $\delta$  3.25 and a sharp doublet at  $\delta$  2.86 (J=6Hz) were assigned to the allylic 4-CH<sub>2</sub> hydrogens. The axial 4 $\beta$ -CH probably couples with the broad C-6 vinyl proton whereas the equatorial 4 $\alpha$ -CH only shows geminal coupling of 6 Hz. Higher resolution clarified the broad doublet to a fine split doublet with J=1.5 and 16 Hz.

These assignments are consistent with the interpretation proposed for the NMR spectrum of  $\Delta^5$ -testosterone<sup>63</sup>, with a broad doublet at  $\delta$  3.15 and a sharp doublet at  $\delta$  2.66.

---

\* Rigorous exclusion of O<sub>2</sub> is required to prevent oxidation to three more polar reaction products (TLC); presumably the result of oxidation at the C-4 anion.

## Chapter 2 Section 4

### 2.4.1 C-19 esters as affinity labels.

Although the neopentylic C-19 position is known to react poorly with nucleophiles, enzyme oxidation to a 19-radical, a 19-hydroxy radical or a 19-cation, would activate an intermediate then able to react with the enzyme as a transfer affinity label. A number of C-19 esters were synthesized and incubated.

Inhibition kinetics of 19-acetoxyandrostenedione **41** determined at 139  $\mu\text{M}$  yield an apparent affinity  $K_{app}$  three times the  $K_m$ , and insignificant effect on the velocity  $V_{app} = 92\% V_{max}$ . From these the inhibitor concentration required to lower the  $K_{app}$  to 50%  $K_m$  is calculated to be  $K_i = 62 \mu\text{M}$ . This indicates that steroid **41** is a good competitive inhibitor with good affinity for the active site, inducing no enzyme inactivation.

**Table 2.4.1** Aromatase inhibition by 19-acetoxyandrostenedione **41**, and 19-mesyloxyandrostenedione **42**.

| Compound               | $V_{max}$<br>nm/min/mg | $K_{app}$<br>nM | $K_i$<br>$\mu\text{M}$      | Mode       | Inhibitor<br>$\mu\text{M}$ |
|------------------------|------------------------|-----------------|-----------------------------|------------|----------------------------|
| Testos.<br>$\pm 1$ STD | 0.0055<br>$\pm .0001$  | 767<br>$\pm 23$ |                             | -          | none                       |
| <b>41</b>              | 0.0050<br>$\pm .0011$  | 2480            | 62                          | C          | 139                        |
| <b>42</b>              | 0.0043<br>$\pm .0013$  | 519<br>$\pm 97$ | 87 $K_{ik}$<br>63 $IC_{50}$ | non linear | 42                         |

Values are given  $\pm$  one standard deviation. Incubation method 3 is described in table 2.3.4. Testosterone concentration ranging from 201 to 2000 nM. Data analysis according to Eadie-Hofstee.

Simons and Thompson reported promising results with C-21 cortisol-mesylate as affinity label for glucocorticoid receptors. These react readily with thiol anions while reactions with other biological nucleophiles are at least three orders of magnitude slower.

Our mesylate 19-mesyloxyandrostenedione **42** is the oxygen analogue of the well known type II inhibitor 19-thiomesyl-androstenedione **112** (with a 19-sulfur instead of oxygen). Compound **42** was synthesized and tested at 42  $\mu$ M. It exhibits non linear inactivation kinetics. At a 77/1 ratio of inhibitor to testosterone it exhibits a 63% effect on aromatase activity. Extrapolating the data to a straight line yields an approximation for comparative purposes. The steroid induced a 68% effect on  $K_m$  and a 79 % effect on the  $V_{max}$ . It would then be a weak uncompetitive inhibitor with an approximate  $IC_{50}$  of 63  $\mu$ M inducing a 50% reduction in the  $V_{max}$ .

#### 2.4.2 Inhibition by 4-chloroandrostenedione-19-ol (**44**)

4-Chloroandrostenedione was briefly reported as a competitive inhibitor. By placing groups on the steroid it is possible to disturb the alignment in the active site and improve inhibition characteristics. Thus, the related 4-androsten-4-chloro-19-ol-3,17-dione **44** was synthesized and found to exhibit inhibition in the same order of magnitude as 5 $\alpha$ -androstanedione **83** with a residual activity of 47% at 1.2  $\mu$ M. A 4-chloro pharmacophore thus seems a reasonable substituent to modify the alignment of other aromatase pharmacophores. The synthesis of this compound is discussed in chapter 3.

**Table 2.4.2 Aromatase inhibition by clinical candidate inhibitors**

| Cmpnd %V |    | [Inh.] $\mu$ M | Inhibitor                          |
|----------|----|----------------|------------------------------------|
| 44       | 12 | 3.6            | 4-chloro 19-hydroxyandrostenedione |
|          | 47 | 1.2            |                                    |
| 2        | 32 | 1.3            | 19-hydroxyandrostenedione          |
|          | 55 | 0.13           |                                    |
| 83       | 53 | 1.3            | 5 $\alpha$ -androstane-3,17-dione  |
|          | 67 | 0.13           |                                    |

Values are given  $\pm$  one standard deviation. Incubation method; 20  $\mu$ L [ $1\beta$ ,  $2\beta$ - $^3$ H] Testosterone (0.1  $\mu$ Cl, 1.7nM), 40  $\mu$ L testosterone is co-incubated with 40 $\mu$ L inhibitor (in ethanol) and human placental aromatase (70  $\mu$ g protein) at 37 °C for 30 min with excess NADPH.

**2.4.3 Conclusion: Simple C-19 derivatives confirm the C-19 cavity postulate and uncover novel inhibitors capable of aromatase inactivation.**

The original postulate that there may be a cavity in the active center, above the A/B rings of the substrate androgens, was established by analysis and computer simulations of published steroidal and non-steroidal inhibitors of aromatase. This was a fortunate hypothesis. A number of C-19 derived androgens were synthesized and tested for competitiveness in the presence of testosterone substrate. These C-19 modified steroids interact with aromatase and offer interesting alternative to new classes of inhibitors.

- The bulky 19-tBDMSi ether of 19-hydroxyandrostenedione (19-tBDMSiO-A, 37) occupies a nearby hydrophobic cavity by remaining a competitive inhibitor.

■ The larger 19-trityl ether 43 (19-TrO-A) shows higher affinity but no enzyme inactivation.

■ The acetate 41 (19-AcO-A) causes increased affinity for the active center but exhibits no inactivating capacity. The inhibitory effect of acetates was clearly shown on a number of tested steroids (vide infra). Covey has postulated that the presence of a non-specific esterase in a biological medium may be responsible for the inhibition caused by other esters. No attempts at elucidating the mechanism of operation was made during this thesis.

These uncovered a large hydrophobic volume which accommodates substituents that extend 6.3 Å from the C-19, confirming the C-19 cavity postulate.

These novel derivatives can be used to realign potential pharmacophores in the androgen binding site and to protect other steroidal enzymes from non-specific inactivation, as affinity labels and as reporter groups.

Other test compounds exhibited hitherto unpublished pharmacophores capable of aromatase inactivation.

■ The smaller and more reactive 19-TMS 38 and 19-MOM 39 derivatives may be inactivating aromatase by stabilization of a C-19 radical to cause enzyme inactivation.

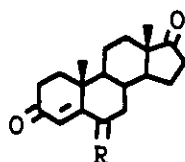
■ The mesylate 42 exhibits uncompetitive-like kinetics.

#### 2.4.4 Inhibitors combining C-19 groups and C-6 pharmacophores.

These C-19 groups were then used to advantage in enhancing other potentially C-6 inactivating pharmacophores on the steroidal backbone.

#### 2.4.5 Derivatives at C-6: Oxo, Fluoro, Amine.

Holland<sup>2</sup> has shown that aromatization of deuterated 6 $\beta$ -d-androstenedione or 4-d-androstenedione occurs with retention of the label in the recovered estrogen: this indicates that the enzyme mechanism probably does not involve a C-5,6 alkene. Yet the 6-keto derivative **A-trione** 131 is one of the most common clinical inhibitors in use. It exhibits relatively poor affinity, competitive kinetics and pseudo first order enzyme inactivation caused by an enzyme generated intermediate<sup>2,4</sup>.



|                                                     | Code | R                                    | Ki<br>$\mu$ M | Mode    | ref. |
|-----------------------------------------------------|------|--------------------------------------|---------------|---------|------|
| A-trione                                            | 131  | =O                                   | 1.1           | Comp.   | 75   |
|                                                     | 132  | $\beta$ -OOH                         |               | Irr.    | 29   |
|                                                     | 133  | $\beta$ -OH                          | 11            | Comp.   | 75   |
|                                                     | 134  | $\alpha$ -OH                         | 15            | Comp.   | 75   |
|                                                     | 135  | $\alpha$ -Br                         | 0.0034        | Comp.   | 36   |
|                                                     | 136  | $\beta$ -Br                          | 0.8           | Irr.    | 36   |
|                                                     | 137  | $\alpha$ -F                          |               |         |      |
|                                                     | 138  | $\beta$ -F                           | "excellent"   |         | 76   |
|                                                     | 139  | $\beta$ -OCOCH <sub>3</sub>          | 20            | Comp.   | 75   |
|                                                     | 140  | $\alpha$ -OCOCH <sub>3</sub>         | 4             | Comp.   | 75   |
|                                                     | 141  | $\beta$ -OCOCH <sub>2</sub> Br       | 45            | Comp.   | 75   |
|                                                     | 142  | $\alpha$ -OCOCH <sub>2</sub> Br      | 2.8           | Irr.    | 75   |
| Derivatives with 17 $\beta$ -OCOCH <sub>2</sub> Br: |      |                                      |               |         |      |
|                                                     | 143  | $\beta$ -Br                          | ND            | TDI     | 75   |
|                                                     | 144  | =O                                   | 42            | TDI     | 75   |
|                                                     | 145  | $\beta$ -CMe                         | ND            | TDI     | 75   |
|                                                     | 2    | 19-OH-A                              | 13            | Control | 75   |
|                                                     | 1b   | Androst.                             | 50            | Control | 75   |
|                                                     | 146  | 16 $\alpha$ -Br-6-one                | "weak"        |         | 35   |
|                                                     | 147  | 2 $\beta$ ,6 $\beta$ -F <sub>2</sub> | "weak"        |         | 76   |

**Table 2.4.5:** Known C-6 pharmacophores.

TDI is time dependent inactivation. Irr. is irreversible. Comp. is competitive.

#### 2.4.6 Interpretation of literature data on C-6 pharmacophores.

Other proposed mechanisms of inactivation have invoked delocalization of the charge towards the C-6 instead of the C-4 moiety. This induction would lower the olefinic character at C-4, a hypothesis<sup>28</sup> retained for the two isosteres 6 $\alpha$ -fluoro 137 and 6 $\beta$ -fluoro 138. Alternatively, stronger reactive groups at C-6 can operate directly on the enzyme active site, without going first through enzyme-linked activation. It is known<sup>16d</sup> that both 6-epimers of androstenedione-6-ol 133, 134 are aromatized. Tan modified these competitive inhibitors to make the more reactive epimers of 6-hydroperoxy-androstenedione 132; these<sup>29</sup> are NADPH-independent irreversible inhibitors and do not require enzyme activation. It is not unreasonable to postulate that inactivation occurs as a result of oxidation of the active center cysteine by the C-6 hydroperoxide.

Bellino and Osawa<sup>35</sup> found that both epimers of 6-bromo-androstenedione 135 and 136 are potent inhibitors of aromatase. By more careful kinetics, Osawa<sup>36</sup> determined that the 6 $\alpha$ -epimer 135 exhibits a  $K_i$  of 3.4 nM, gives a type-I P-450 binding spectrum<sup>35</sup> and is one of the most potent competitive inhibitor known, whereas the 6 $\beta$ -epimer 136 is a mechanism based irreversible inhibitor with a  $K_i$  of 800 nM.

Inspection of table 2.4.5 confirms that a number of the C-6 oxygenated androgens published are time dependent inactivators or irreversible inactivators. These are of great clinical interest for their permanent effect on aromatase activity.

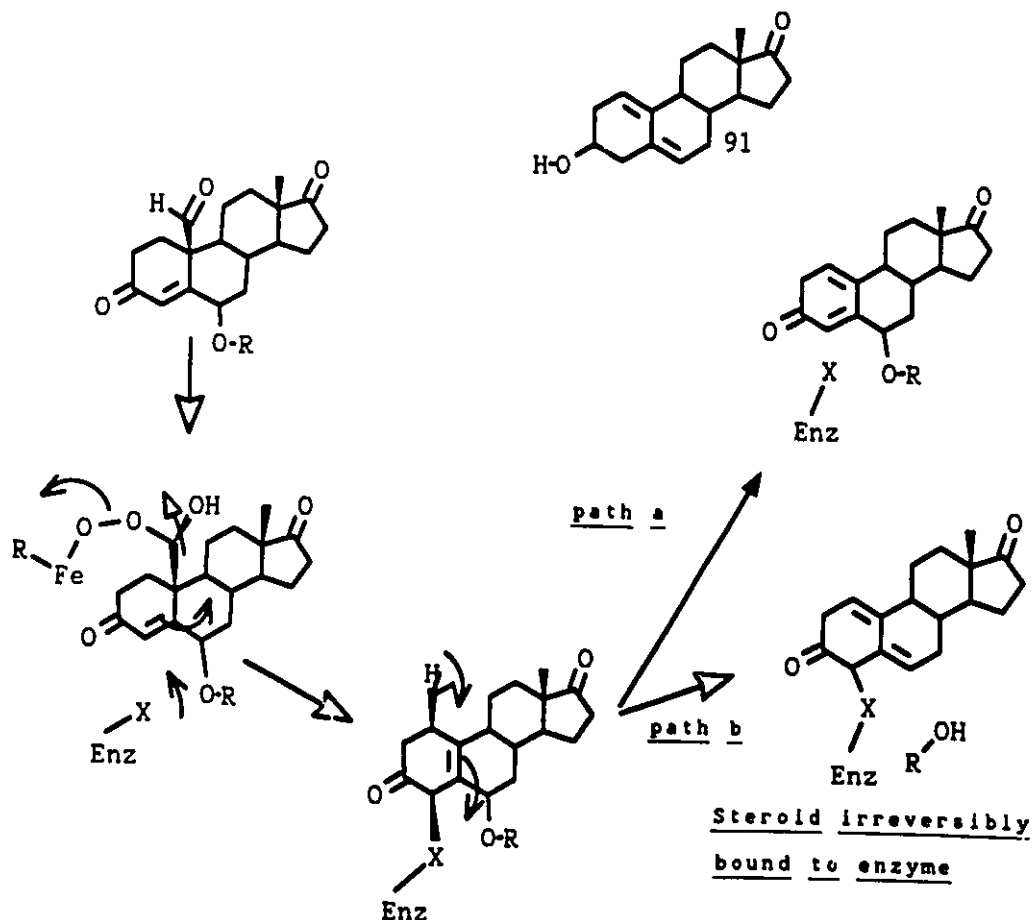
##### 2.4.6.1 Mechanism of inactivation by oxygenated species at C-6.

Useful C-6 modified inhibitors which successfully combines known pharmacophores include androsta-1,4,6-triene-3,17-dione 148 ATD, and androst-4,6-dien-4-ol-3,17-dione 149.

|                          | ATD  | 4-OHA | A-trione |
|--------------------------|------|-------|----------|
|                          |      |       |          |
| code                     | 148  | 100   | 131      |
| IC <sub>50</sub> $\mu$ M | 0.18 | 0.25  | 0.43     |
| ref.                     | 24   | 20    | 24       |

**Table 2.4.6:** Common pharmacophores.

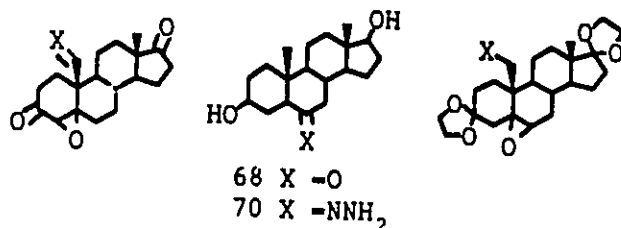
The successful clinical pharmacophores represented are 1,4-diene, 4-enol and 6-ketone. Combinations of these groups often yield time dependent inhibitors. A C-6 leaving group could cause inactivation by a mechanism similar to the addition-elimination proposed for C-4 oxygenated inhibitors; if path **B** competes with the normal path **A**, subsequent elimination of the C-6 leaving group would result in a covalently bound steroid.



**Scheme 2.4.6:** Postulated nucleophile addition-elimination

#### 2.4.7 Incubations designed to question the C-6 addition-elimination postulate.

In order to test these mechanisms, the intermediate analogue estra-1(10),5(6)-diene-3-ol-17-one **91** was incubated here and found to interact at the active center with good affinity. To test the requirement for the 4-ene in this postulate compound **68** and **70** were incubated. The reduced **70** can not undergo conjugated addition; the kinetics displayed indicate a poor competitive inhibitor. The 6-hydrazone **70** was thought to trap a nearby nucleophile; preliminary screening categorized it as a poor uncompetitive inhibitor, yet it affects both the observed enzyme affinity and the enzyme maximal catalysis. These may indicate a nearby nucleophile that can operate independently of the 4-ene, or there may be enough reactivity to react directly. To trap such a potential nucleophile near carbon positions 4, 5 or 6, synthesis and incubation of a number of 4,5 and 5,6 - $\alpha$  and - $\beta$  epoxides were carried out. (*vide infra*)



These steroid classes demonstrated competitive kinetics, interacting with the active center, but not causing inactivation, which disfavor inhibitor theories requiring a nearby nucleophile. Although this series of epoxides may not orient adequately in the active center it is notable that neither the 4,5 nor 5,6-epoxide derivatives caused inactivation.

A number of published inhibitors bear C-4, C-5 or C-6 pharmacophores. A first effort at discovering aromatase inhibitors concentrated on screening C-6 modified androgens. Incubation methods 1 and 2 were selected for a rapid screening of steroids designed to increase understanding of the C-6 pharmacophores. Full kinetics (method 3) was determined on promising test compounds.

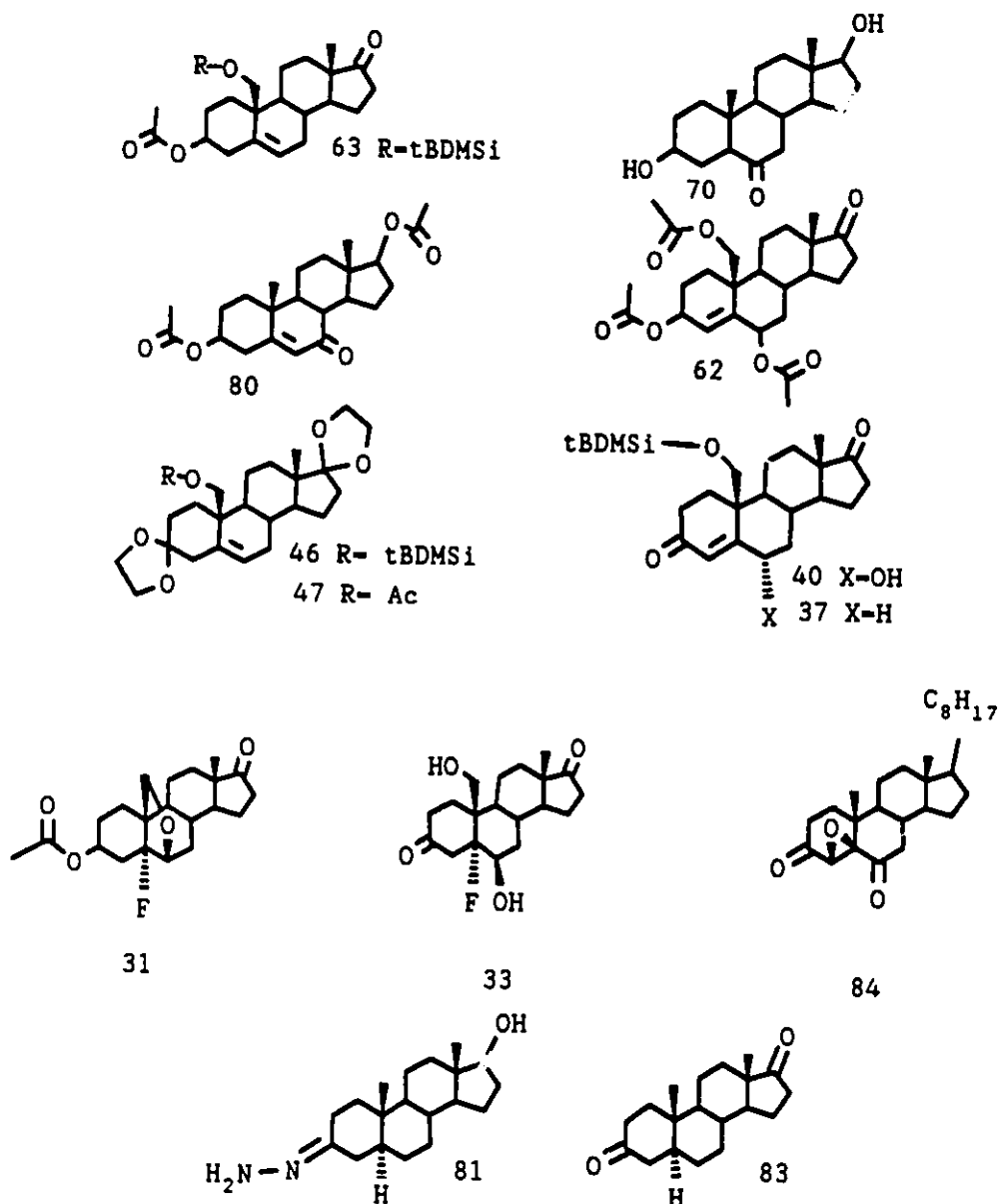


Table 2.4.7.1: Screening 19-derivatives with 6-pharmacophores.

| Code | Method | [Inh.]<br>μM | Mode | Ki<br>μM | AV<br>T=401nM%Vmax |     | Description                   |
|------|--------|--------------|------|----------|--------------------|-----|-------------------------------|
| T    |        | none         |      | 0.8      | 100                | 100 | Testosterone                  |
| 31   | 1      | 12           | -    | ---      | 79                 |     | A-5αF-3-OAc-6,19-ether-17-one |
| 33   | 1      | 12           | -    | ---      | 51                 |     | A-5αF-3,17-dione-6β,19-diol   |
| 40   | 1a     | 143          | -    | ---      | 55*                |     | A-19-tBDMSi-6α-ol             |
| 46   | 1a     | 113          | -    | ---      | 77*                |     | EG-5,6-ene-19-tBDMSi          |
| 47   | 2      | 101          | M    | 163      | 67                 |     | EG-5,6-ene-19-OAc             |
| 62   | 2      | 116          | C    | 73       | 98                 |     | A-3,6,19-OAc                  |
| 63   | 1      | 119          | -    | ---      | 38                 |     | A-3-OAc-5,6-ene-19-tBDMSi     |
| 70   | 3      | 128          | C    | 105      | 64                 | 114 | A-3-ol-5α-H-6-one             |
| 80   | 2      | 116          | C    | 127      | 85                 | 138 | A-6-ene-7-keto                |
| 83   | 1      | 124          | -    | ---      | 11                 |     | A-5α-H-3,17-dione             |
| 84   | 1      | 127          | -    | ---      | 11                 |     | Chol-4,5α-epoxy-6-oxo-3-one   |

Abbr. as for table 2.3.4. EG-androsta-3,17-diethyleneglycol = [T] = 21 nM.

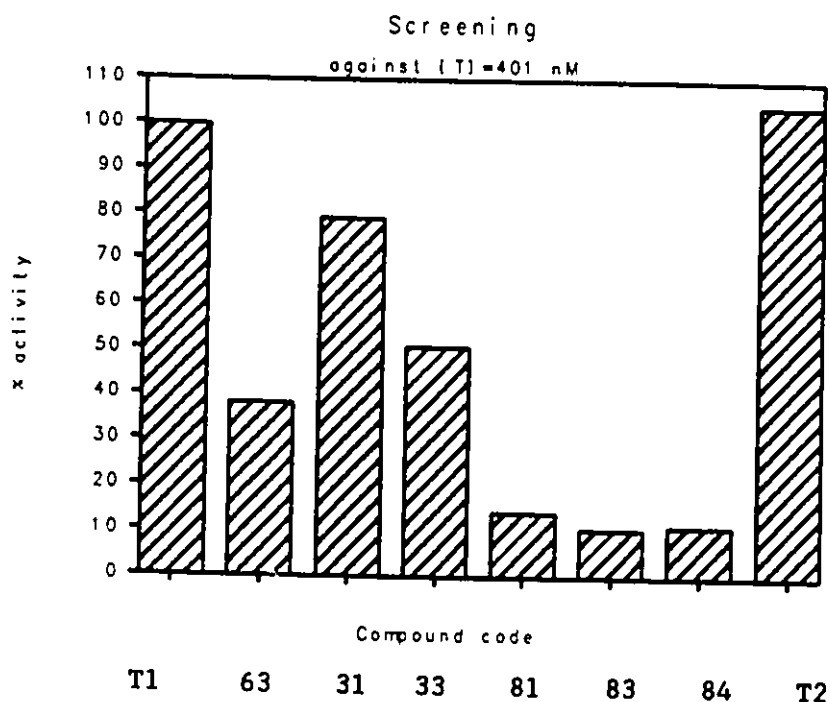


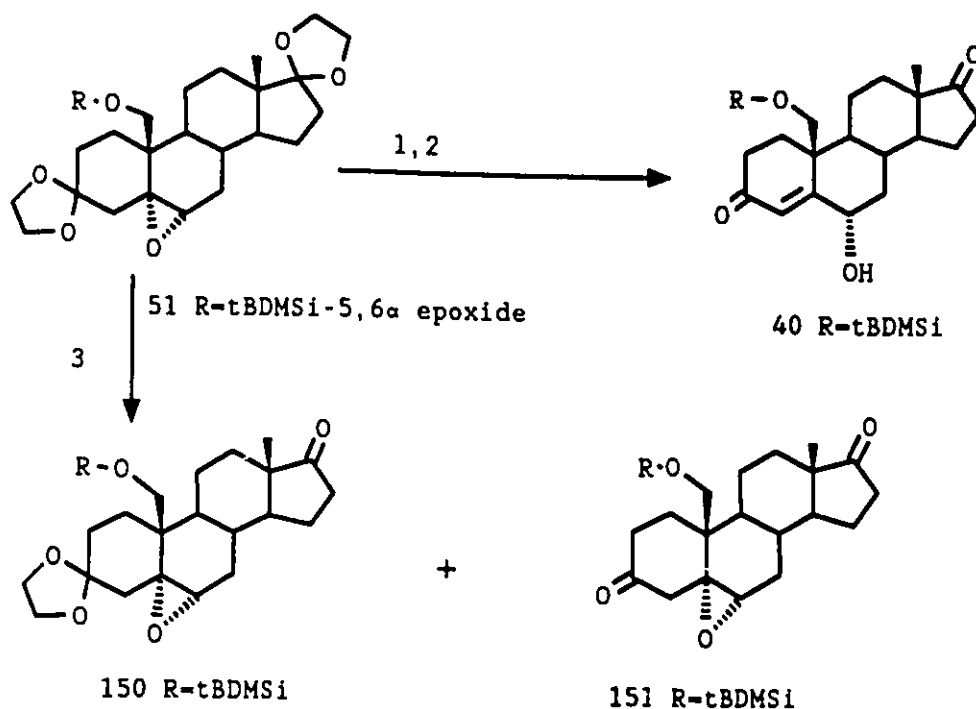
Figure 2.4.7.1: Screening of 19 and 6 derived steroids. T1 is Testosterone at the beginning and T2 is the testosterone at the end of incubations.

The 3-acetate **31** combines a 3-acetate, 5 $\alpha$ -fluorine and a cyclic 19,6 ether. It has poor activity against aromatase. The 3,6-diacetate **62**, exhibits increased affinity ( $K_i$  of 73  $\mu$ M) and competitive inhibition in the same order as **70**, the 3,17-diol-6-one analyzed earlier, but no inactivation. The C-3-acetate 5,6-en-6-one **80** shows fair competitive inhibition but also loses the inactivating potential of **63**, since none of these 3-acetates affect the  $V_{max}$ . This seems to rule out transesterification as a mechanism of inactivation for **63**.

Compound **40**, the 19-terbutyldimethylsilyl ether of 4-androsten-6 $\alpha$ ,19-diol-3,17-dione, as well as **84** and the key lead compound **63** deserved further investigation.

A ready synthesis of compound **40** was arrived at during the attempted preparation of 5,6-epoxy-androgens. It was discovered that the ethyleneglycol derivatives were acid sensitive and could not be readily deprotected to yield the desirable 5,6-epoxides. By adjusting the approach to less

aggressive reaction conditions, it was found that conditions for transketalization of a diethyleneketal provide a ready avenue to the epoxide ring-opened **40**, instead of the intact B-ring epoxides.



**Scheme 2.4.7.1:** Synthesis of compound **40**

- 
- 1) 8% HCl/Acetone (1:1), rt., 1 hrs.    2) add 10% NaOH  
 3) 3.5% aqueous HClO<sub>4</sub>/acetone (1:18), rt., 2 hrs.
- 

As shown, vigorous reaction conditions (HCl/Acetone) lead to transketalization. The desired epoxide underwent further reaction by acid promoted opening to generate compound **40**, 4-androsten-6α,19-diol-3,17-dione, 19-tertbutyl-dimethylsilyl ether. The structure of this UV positive compound was confirmed by a correct parent ion and expected fragmentation pattern in the mass spectra. The IR spectra shows the presence of the two ketones, one of them unsaturated. The <sup>1</sup>H NMR exhibits loss of the ketal multiplet to uncover the C-19 methylene AB quartet, a broad doublet assigned to the C-6α-H-C-O, a fine split doublet in the alkene region, along with the presence of the tert-butyl and dimethylsilyl peaks. Further data is given in the experimental.

Inhibition kinetics of 4-androsten-6 $\alpha$ ,19-diol-3,17-dione, tertbutyldimethylsilyl ether **40** determined at 121  $\mu$ M computes to a  $K_i$  of 121  $\mu$ M and no enzyme inactivation. Compound **40** doubled the  $K_{app}$  at this concentration but had no marked effect on the  $V_{app}$ . It is therefore a fair competitive inhibitor possessing no inactivating capacity. This compound is a component of enzyme allowed topological maps.

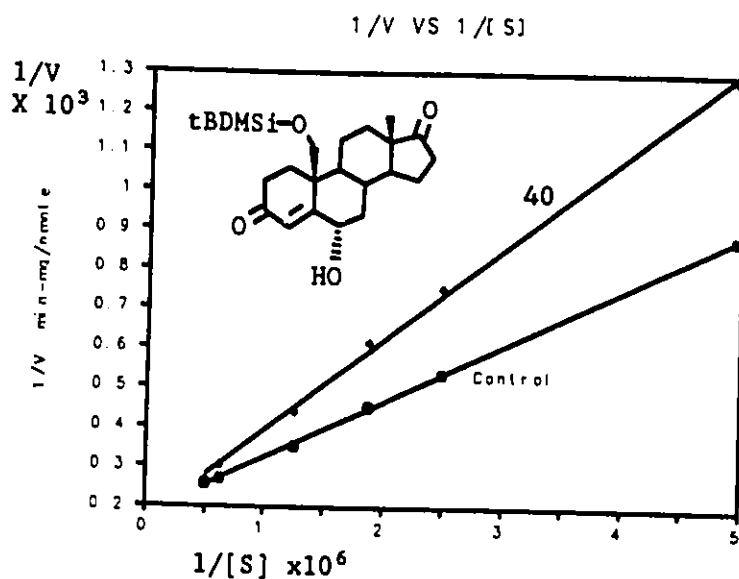


**Figure 2.4.7.2:** Aromatase inhibition by **40**, 4-androsten-6 $\alpha$ ,19-diol-3,17-dione, 19-tertbutyldimethylsilyl ether.

| Compound  | Vmax<br>nm/min/mg | K <sub>app</sub><br>nM | K <sub>i</sub><br>$\mu$ M | Mode | Inhibitor<br>$\mu$ M |
|-----------|-------------------|------------------------|---------------------------|------|----------------------|
| Testos.   | 0.0055            | 767                    |                           | -    | none                 |
| $\pm$ STD | $\pm$ .0001       | $\pm$ 23               |                           |      |                      |
| <b>40</b> | 0.0065            | 1530                   | 121                       | C    | 120                  |
|           | $\pm$ .0002       | $\pm$ 104              |                           |      |                      |

Values are given  $\pm$  one standard deviation. Incubation method 3; 20  $\mu$ L [ $1\beta$ ,2 $\beta$ -<sup>3</sup>H] Testosterone (0.1  $\mu$ Cl, 1.7nM), 40  $\mu$ L testosterone (final concentration ranging from 201 to 2000 nM) is co-incubated with 40 $\mu$ L inhibitor in ethanol and human placental aromatase (70  $\mu$ g protein) at 37 °C for 30 minutes in the presence of excess NADPH. Data analysis according to Eadie-Hofstee.

Inactivation kinetics of 5-androsten-3 $\beta$ ,19-diol-17-one, 3-acetate,19-tertbutyldimethylsilyl ether **63** determined at 39  $\mu$ M computed to a  $K_i$  212  $\mu$ M and some enzyme inactivation. Compound **63** exhibited  $V_{app}$ =84%  $V_{max}$  and very little effect on the apparent  $K_{app}$ . It is therefore a weak non competitive inhibitor possessing poor affinity for the active center. Since the nature of this protocol did not differentiate between competitive and time-dependent inhibitors, precursors of more potent inhibitors would appear as non-competitive if they have poor affinity for the active site.

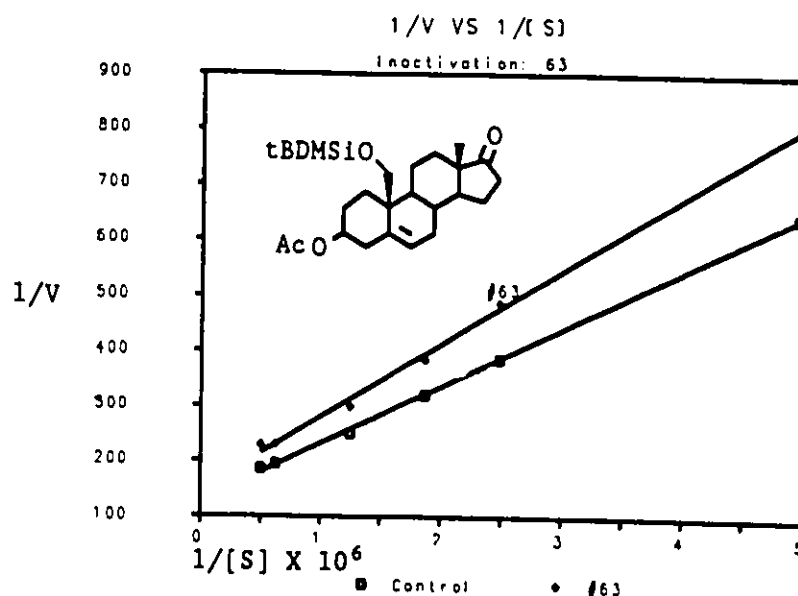
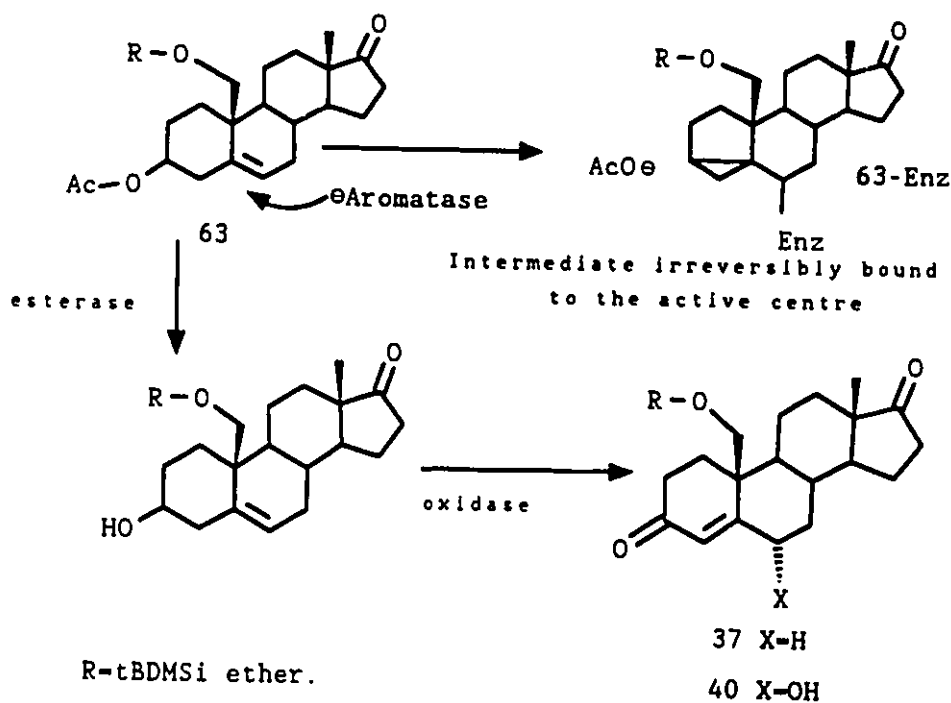


**Figure 2.4.7.3** Aromatase inhibition by **63**, 5-androsten-3 $\beta$ ,19-diol-17-one, 3-acetate,19-tertbutyldimethylsilyl ether.

| Compound  | $V_{max}$<br>nm/min/mg | $K_{app}$<br>nM | $K_i$<br>$\mu$ M | Mode | Inhibitor<br>$\mu$ M |
|-----------|------------------------|-----------------|------------------|------|----------------------|
| Testos.   | 0.0078                 | 803             |                  | -    | none                 |
| $\pm$ STD | $\pm$ .0006            | $\pm$ 108       |                  |      |                      |
| <b>63</b> | 0.0066                 | 817             | 212              | N    | 39                   |
|           | $\pm$ .0011            | $\pm$ 283       | $\pm$ 51         |      |                      |

Values are given  $\pm$  one standard deviation. Incubation method 3 as for previous compound. Data analysis according to Eisenthal.

The intriguing inhibitor **63** displays poor affinity and inactivates aromatase in an apparently "non-competitive" manner. This may be caused by the irreversible addition of an enzyme nucleophile at C-6 of the 5-ene-3-acetate, with loss of the C-3 acetate. There is chemical precedent for C-6 nucleophilic additions with displacement of a C-3 methoxy to generate an A-ring cyclopropane. The kinetics of inhibition shown in figure 2.4.7.3. were determined in a strict time-independent competitive assay; no pre-incubation was performed. It is important to note that a similar kinetic profile is obtained from a very reactive steroid as it alters the enzyme concentration before there can be competitive effects with the natural substrate.



**Scheme 2.4.7.4:** Possible mode of inactivation by compound **63**.

An other plausible mode of action of **63** would occur via conversion by other enzymes commonly present in the crude microsomal preparation. The most obvious sequence of events would be de-esterification via non-specific esterases found in the crude microsomal preparations, followed by conversion of the 5-en-3-ol to the 4-en-3-one androgen **37**. Although quite a reasonable sequence, it is not a probable one for **63**, as both 4-en-3-one-19-derivatives **37** and **40** were reported in this thesis as strictly competitive inhibitors, exhibiting no

inactivation potential. The mode of inactivation by compound 63 remains to be investigated.

Kinetic analysis of cholesta-3,6-dione-4,5 $\beta$ -epoxide indicated that 84 produces aromatase inactivation with a  $K_i$  of 224  $\mu$ M. The inhibitor exhibits a  $V_{app}=83\%V_{max}$  and  $K_{app}=84\%K_m$  at a concentration of 43  $\mu$ M. It is therefore a weak inhibitor possessing poor affinity for the active center. No further investigation was done on this compound.

SAR: This compound indicates tolerance at C-17. The poor affinity may be caused by such a large group disturbing the androgen orientation in the binding site. "Inactivation" could be the result of interactions with C-4,5-epoxide, or the known pharmacophore 4-en-3,6-dione.

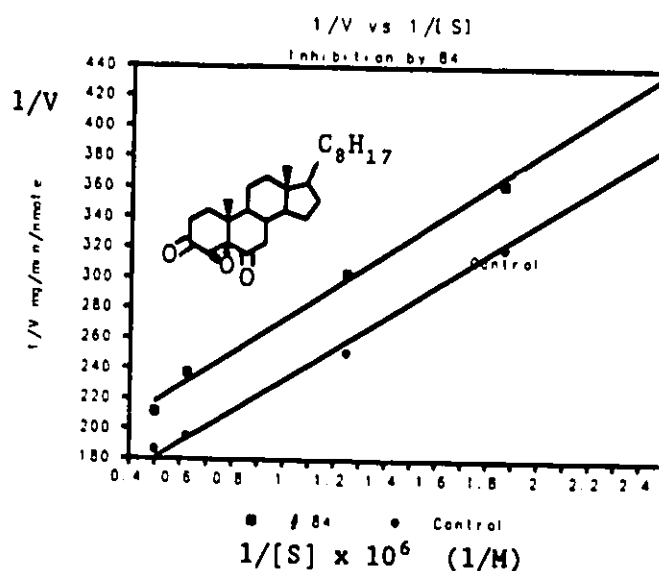


**Figure 2.4.7.5.** Aromatase inhibition by 84, cholesta-3,6-dione-4,5 $\beta$ -epoxide.

| Compound  | $V_{max}$<br>nm/min/mg | $K_{app}$<br>nM | $K_i$<br>$\mu$ M | Mode | Inhibitor<br>$\mu$ M |
|-----------|------------------------|-----------------|------------------|------|----------------------|
| Testos.   | 0.0078                 | 803             |                  | -    | none                 |
| $\pm$ STD | $\pm$ .0006            | $\pm$ 108       |                  |      |                      |
| 84        | 0.0065                 | 676             | 224              | U    | 43                   |
|           | $\pm$ .0010            | $\pm$ 104       | $\pm$ 60         |      |                      |

#### 2.4.7 Conclusion for combined C-19 and C-6 derivatives:

This series of analogues did not increase the affinity of the important lead compound 63. To test the hypothesis that 63 inactivates aromatase via nucleophilic displacement at C-6 with loss of the 5-ene-3-leaving group, the 3-acetate was replaced with the more stable ethyleneglycol. The ethyleneglycol derivative (which would require a Lewis acid to activate) increases the affinity, but loses the ability to inactivate. Similarly, the 19-methyl-7-one derivative 80 demonstrates little inhibition.

The previous screens reveal test steroids which failed to demonstrate inhibition and inactivation. These yield information used in plotting the enzyme disallowed or enzyme disfavored topological maps.

Some preliminary conclusions to date:

C-3 acetoxy, ethylenedioxy, hydroxy and methoxy are tolerated but do not seem to cause inactivation by themselves.

C-3 hydroxy is tolerated but seems to lower the affinity.

C-3 hydrazone causes weak inactivation.

C-5 $\alpha$ -H lowers the affinity.

C-4,5 epoxide may cause inactivation when oriented on a cholesterol backbone.

C-6 ketone causes inactivation only in conjunction with the 4-en-3-one.

C-10 flat A ring derivatives interact with aromatase, presumably because they are within the Minimum Steric Requirements of the active center.

C-17 can tolerate a cholesterol side chain, though this lowers the affinity.

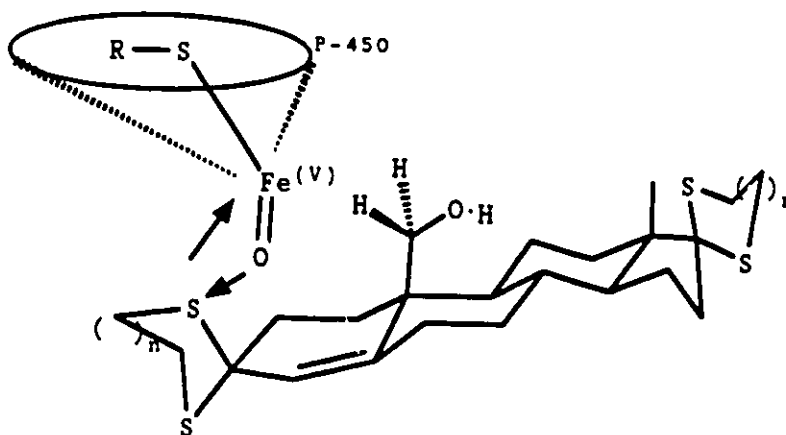
C-19 tBDMSi can fit in the active site.

### 2.5.1 THE C-3 CHELATION POSTULATE.

The second main active site postulate investigated in this thesis is the possibility of improving inhibitor kinetics via chelation to the aromatase heme-iron, with the potential of blocking the active site by reduction of a heme-iron species with a steroid-bound heteroatom.

This objective could be accomplished in the active center via inhibitor-bound moieties bearing oxygen or sulfur atoms such as steroidal C-3-ethylene glycol, dithiane and dithiolane derivatives. Before these could be synthesized, however, three essential questions needed answers:

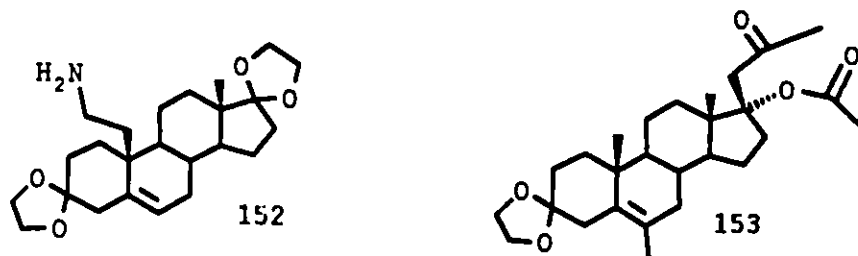
- 1) Are there any precedents for this type of group in the pharmacopeia?
- 2) Will the addition of the C-3 groups cause backbone distortions that are not permissible for the enzyme cavity?
- 3) Is the heme-iron close enough to the steroid C-3 position to allow chelation by a neighboring heteroatom?



**Scheme 2.5.1:** Potential chelation and reduction of a heme-iron intermediate by a neighboring heteroatom.

Since Edifolone and Edogestrone have been used as cardiac depressants, it was reasonable to assume other ketals could be formulated for administration. (US Pharmacopeia).

**Scheme 2.5.1.1:** Edifolone and Edogestrone, cardiac depressants bearing the ketal groups.



Edifolone Acetate  
1987 Searle, SC-35135  
Cardiac depressant  
(antiarrhythmic)

Edogestrone  
PH-218

**2.5.2 Will the addition of the C-3 groups cause backbone distortions that are not permissible for the enzyme cavity ?**

Schematically, the objective of this postulate was to position a heteroatom sufficiently close to the putative heme-iron position to either chelate or to intercept an active oxygen. An obvious advantage of relying on dithianes and glycols is the presence of a heteroatom on both sides of the steroid A-ring. An essential steric consideration was the bulk and backbone distortions permissible about the A-ring.

A preliminary solution to this question was to model the minimized structures of known inhibitors exhibiting A/B ring distortions. Comparing these with a natural substrate with great affinity for the active site would give a measure of the steric latitude near the C-3 position.

**2.5.2.1 Derivatives bearing the 1,4-diene or 4,6-diene moieties: literature and conformational analysis of the backbone distortions about the C-3,4 positions.**

The analysis of conformational differences between known inhibitors and the usual substrate androstenedione can yield useful information on the active site geometry. As the C-3-Chel. postulate describes, there are reasons to place a heteroatom near the heme-iron in the active center. In order to determine the steric latitude about this position, a reasonable approach consists in overlapping computer generated minimized structures of aromatase inhibitors with distorted A-rings.

The  $\Delta^{1,4}$  pharmacophore is found on many potent aromatase inhibitors. It flattens the A ring, with a RMS deviation of 0.2 Å and major effects at the C-1 and C-2 carbon positions. The O-3 is pushed down by 0.3 Å.

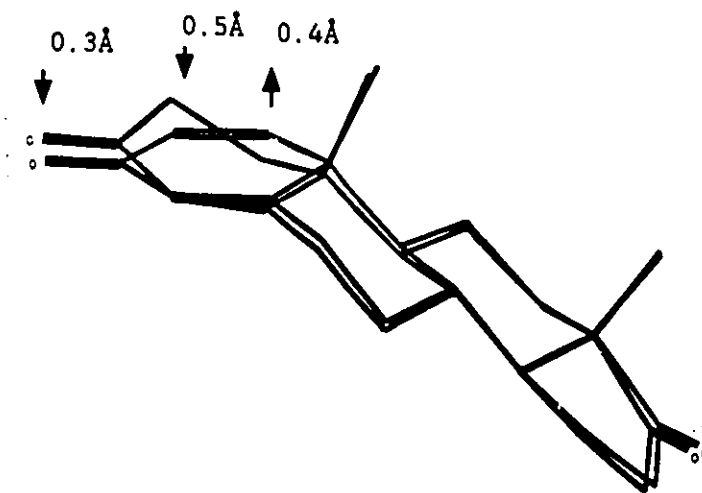
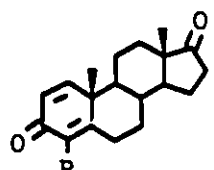


**Figure 2.5.2.1:** Overlap of the  $\Delta^4$  and  $\Delta^{1,4}$  backbone.

Important classes of such inhibitors owe their inactivating character to the presence of a 1,4-dien-3-one or a 4,6-dien-3-one pharmacophore. These pharmacophoric patterns form the basis of inhibition in the clinically useful  $\Delta^{1,4,6}$  compound **148** (ATD) and the  $\Delta^1$ -testolactone (**Teslac**) **101**.

**Table 2.5.2:** Reported inhibitors bearing the  $\Delta^{1,4}$  and  $\Delta^{1,4,6}$  pharmacophores.

|  |                   |                                                                                   |               |               |                       |              |
|-----------------------------------------------------------------------------------|-------------------|-----------------------------------------------------------------------------------|---------------|---------------|-----------------------|--------------|
| Code                                                                              | R                 | R                                                                                 | Ki<br>$\mu$ M | Km<br>$\mu$ M | Ki/Km<br>$\times 100$ | Mode ref.    |
| 154                                                                               | NH <sub>2</sub>   |                                                                                   | 0.068         | ---           | ---                   | Irr. 25      |
| 155                                                                               | H,                | 7 $\alpha$ -SPHNH <sub>2</sub>                                                    | 0.01          | 0.053         | 19                    | Irr. 42      |
| 156                                                                               | H,                | 1-CH <sub>3</sub>                                                                 | 0.250         | ---           | ---                   | TDI 54,20    |
| 156                                                                               |                   |                                                                                   | 0.082         | 0.03          | 273                   | Irr. 8b,24 * |
| 157                                                                               | NH <sub>2</sub> , | $\Delta^{1,4,6}$                                                                  | 0.059         | ---           | ---                   | TDI 25       |
| 101                                                                               | H,                | D ring                                                                            | 20            | 0.03          | 67000                 | ND 8b        |
|                                                                                   | Teslac            |  | 35            | ---           | ---                   | TD 18        |
| 148                                                                               | H,                | $\Delta^{1,4,6}$                                                                  | 0.500         | ---           | ---                   | ND 20        |
|                                                                                   | ATD               |                                                                                   | 0.18          | ---           | ---                   | TDI 24       |

TDI: Time Dependent Inhibition, Irr: Irreversible  
ND: Not Determined, \* Inconsistent data published.

Originally reported as a time dependent competitive inhibitor, **148** (ATD) was more recently shown to elicit a time dependent pseudo first order enzyme inactivation profile typical of an intermediate derived from the parent steroid<sup>24</sup>, a so-called mechanism based inhibitor. Henderson reports metandroden<sup>54,20</sup> (SH489) 1-methyl-androsta-1,4-diene-3,17-dione **156** to be an effective irreversible inhibitor of aromatase with a Ki similar to 4-OH-A 100 ( $\approx 250$ nM). Since it is slightly aromatized (1%), Henderson synthesized potential metabolites and established their affinity: none seem as efficient as the parent compound **156**. Other modifications on the ATD backbone include the C-6-methylene<sup>19</sup> and the amine **157**.

It is clear that it is the 1,4-diene pharmacophore which imparts the quality of irreversibility to these inhibitors. The high affinity suggests it as having a "desirable" shape in the active center.

The best overlap of androstenedione and these derivatives was computed by performing a root mean square on all matching atoms, neglecting hydrogens. Very large conformational differences exist at the C-3 ketone oxygens. The overlap of the  $\Delta^{1,4}$ -diene showed the 3-oxygen pushed down 0.3 Å.

Overlap of  $\Delta^{4,6}$  and  $\Delta^4$  derivatives shows distortion in the B ring, a RMS deviation of 0.2 Å with major movement of carbons C-7 and C-6. The 3-oxygen is shifted up by 0.4 Å.

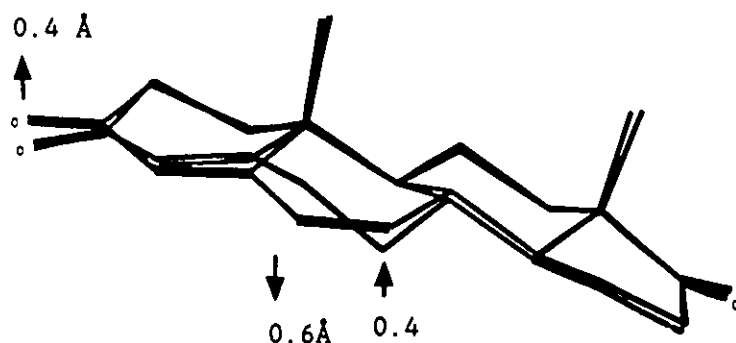


Figure 2.5.2.2: Overlap of the  $\Delta^4$  and  $\Delta^{4,6}$  backbone

#### Conclusion:

The considerable conformational differences between these two parent inhibitors demonstrates a permissible combined movement of 0.7 Å for the 3-oxygen. Similar analysis of the inhibitor 3-methylene-androst-4-ene-17-one 158 lead to the conclusion that the enzyme cavity can also accommodate a C-3 alkene which extends 0.57 Å further along the alkene long axis.

These data answered the first question conducive to investigation of the C-3-chelation postulate via chelation through electronegative C-3 sulfur and oxygen containing moieties.

These derivatives presented the incentive of ready availability through synthesis, and the potential of trapping active species which must exist in the active center during catalysis. Based on the reported 19-sulfur bearing steroids, at the very least this avenue was expected to uncover some potent type-II inhibitors capable of binding tightly to the heme-iron, if the proximity between the sulfur and the heme-iron was adequate.

### 2.5.3 Is the heme-iron close enough to the steroid C-3 position to allow chelation?

Once the steric latitude was established, the question of proximity became crucial: will the heteroatoms introduced on the inhibitors come close enough to the heme-iron, to allow for chelation.

The  $P_{450}$  bound iron in the active center of aromatase responds to chelation by inhibitors bearing a C-19 sulfur, a C-10 $\beta$ -oxygen, and the aminogluthethimide nitrogen. To account for spectral shifts induced by androgen inhibitors, it is implicitly accepted that the heme-iron is situated near the A ring and within a few Å of the C-19 carbon.

#### 2.5.3.1 Iron docking experiments

To better understand these interactions a series of computer simulations were performed. Porphin was used as a basic steric group which represents the heme moiety. Although there can be additional strain due to amino acids or water intercalated between the substrate and the heme, these could not be predicted without more information on the protein itself.

Thus modeling the  $P_{450}$  interactions was accomplished by the "extrapolated" group  $CH_3S$ -Porphin-Fe chelated to an androgen. This "CPF" model was developed as it demonstrated the major steric interactions we desired to study. This model would have other predictive uses in all of  $P_{450}$ -enzyme simulations.

### 2.5.3.2 Effect of heme approach on the steroid backbone.

This model was used to investigate the conformational effects due to heme-substrate interactions. Since it is known that 19-SH-A causes type II spectral shift, the complex was constructed using bond values from the literature. The Fe-S-steroid bond is fixed at 2.5 Å, a maximum value within the published 2.2-2.5 Å length typical<sup>79</sup> of tetracoordinate iron-sulfide or cysteinate reported from X-ray data of other protein. The low spin form of  $P_{450}$  is represented, with a bond to the iron. The tetradentate N-Fe radius was set at 2 Å, leaving the porphin and the square pyramidal iron coplanar. Energy minimization revealed the complex below.

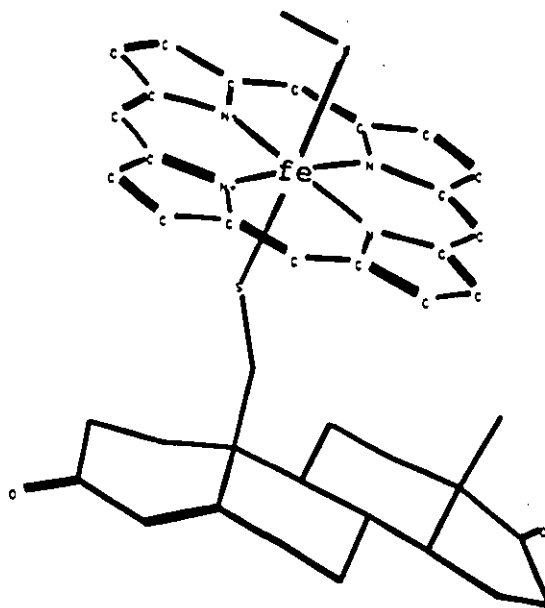
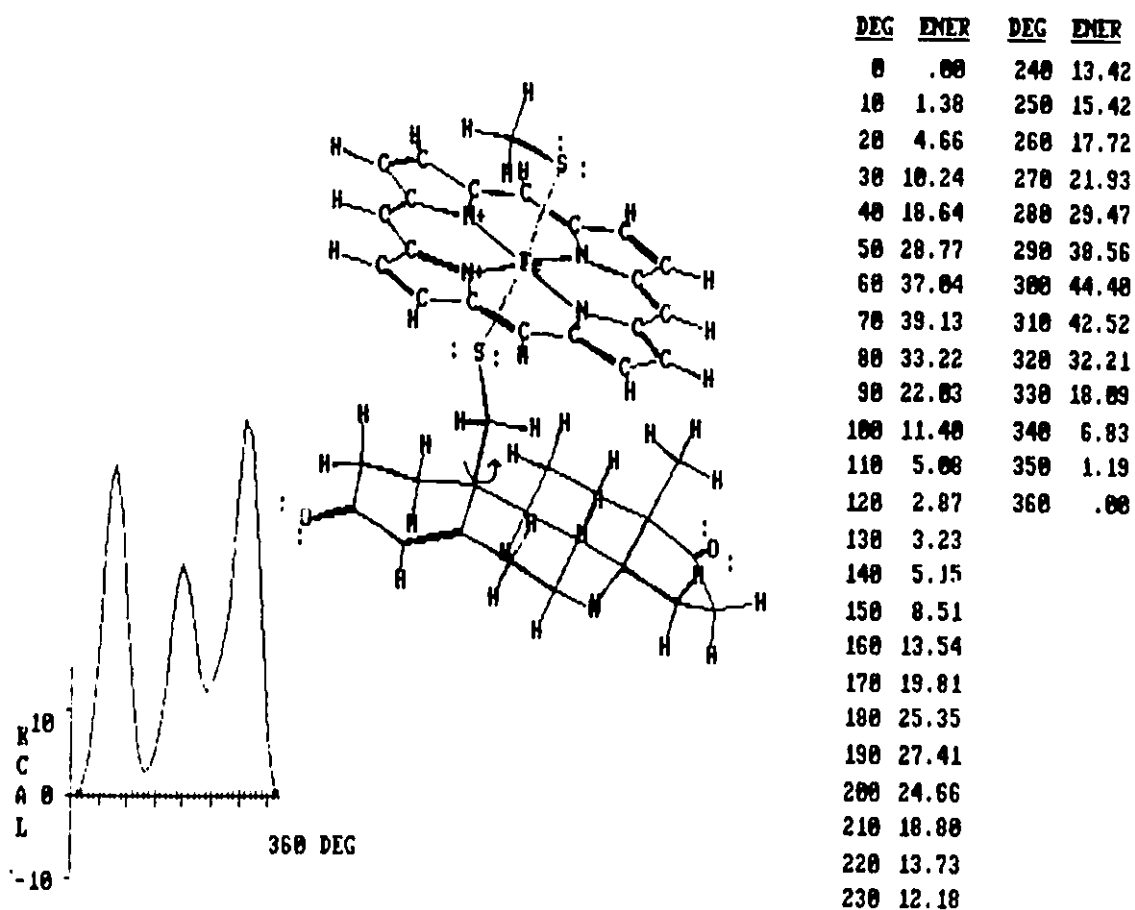


**Figure 2.5.3.1:** docking of porphyrin-Fe and 19-SH-A: CPF-19SHA

Although hydrogens are included in the steric calculations, they are omitted in the drawing for clarity.

**Figure 2.5.3.2:** Rigid rotor approximations at this bond distance were simulated to determine local energy minima.

From a rotation of the C-10,19 bond a rigid rotor approximation of the CPF-19-SHA model presents three local minima, two within a 6 kcal/mol window.



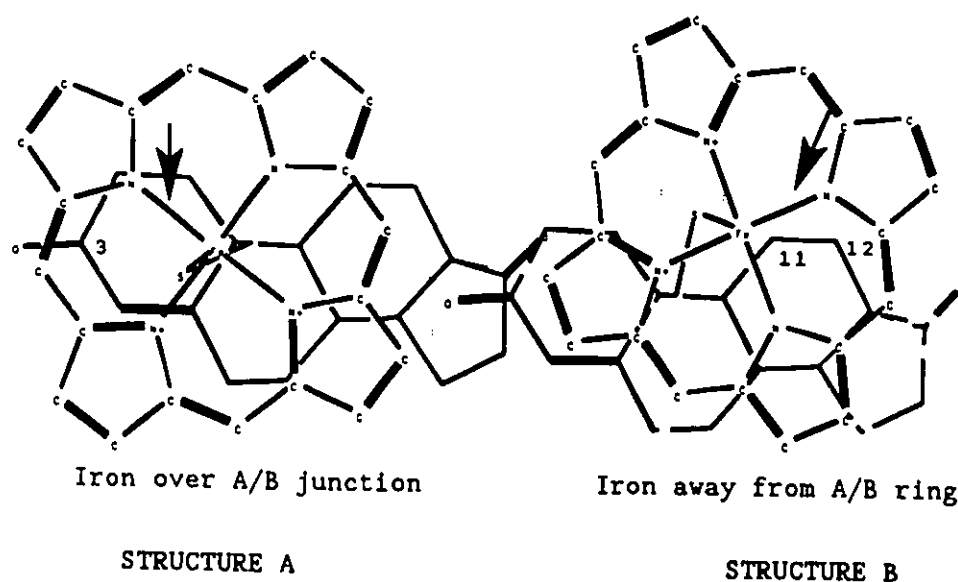


**Figure 2.5.3.3:** Top view of the two energy minima, along the C-10,19 bond.

Structure **A** situates the iron directly above the C-19, an energy window about 30° wide. Structure **B** places the iron away from the A/B ring, in an energy well about 40° wide.

The simulated bond-rotation in **Structure A** indicates that the heme-iron will reside directly above the 19 position and there probably be amenable to chelation by C-3 groups.

**Structure B**, however, demonstrates an other local energy minima locating the heme-iron away from the A/B ring, closer to C-ring carbons C-11 and C-12.

Conformational analysis of the minimized parent steroid before and after binding in the CPF-model indicates that some backbone distortions are caused by the proximity of the heme group. Computations of the best overlap of the minimized structures showed a RMS backbone deviation of 0.1 Å, with ring A being pushed down about 0.2 Å at the C-1 position, an effect of 0.37 Å at the O-3 and a 0.2 Å movement of the C-19 carbon (calculated with a Fe-S bond length of 2.5 Å). From this computation, the steric interactions may cause a loss of affinity for the 3-ethyleneketal derivatives planned.

These computer simulations were conclusive that C-3 derivatives would be within binding distance of the heme-iron, and at the outer acceptable limit of the enzyme active-site allowed volume.

#### 2.5.4 Derivatives bearing C-17 substituents: literature review.

The target synthesis chosen also made the C-17 derivatives available. These seemed acceptable as a literature review, and screening tests performed in previous sections indicated that considerable tolerance exists in the aromatase active center for 17-derivatives.

Pharmaceutically, methylation at C-17<sub>a</sub> is commonly used to prolong the biological half life of a steroid drug.

In steroid biochemistry, modulation is often linked to the oxidation level of C-17 and C-5. For example, aromatase has a threefold preference for androstenedione over testosterone; this corresponds approximately to a net energy difference for the two enzyme-substrate complexes of 0.6 kcal/mol. This value is confirmed by other 17 C-OH/C=O steroid pairs, the substitution causing a three-fold higher affinity for the ketone. The difference may develop from the disruption of a hydrogen bond to the ketone, since distortion of hydrogen bond in other protein environment typically cause a  $\Delta\Delta G^\circ \approx 0.6$  kcal/mol. Covey<sup>72</sup> has reported a SAR on  $\Delta^{1,4}$ -androstadienedione D-ring derivatives and compared the pair 17 C-OH/C-H<sub>2</sub>; the dihydrogen causes a free energy change of only +0.1 kcal/mol, binding almost as tightly as the C-17 alcohol. From his limited set of data available it may be concluded that the oxygen atom at C-17 is not required for tight binding affinity to aromatase as long as the D-ring is intact. Our own compilations indicate a consistently tighter binding in the presence of a 17-ketone.

The considerable latitude found for C-17 inhibitors, and the possibility of binding the heme-iron while in the second local energy minima (Structure B, with the iron above the C-ring) argued favorably for the synthesis and incubation of both C-3 and C-3,17 derivatives.

#### **2.5.5 Inhibition by oxy and thio derivatives at C-3 and C-17.**

Judicious positioning of heteroatoms on substrate<sup>45, 52</sup> increase the affinity for aromatase; these additional interactions with the heme iron are characteristic of many substrates bearing thiol substituents. We tested derivatives with potentially chelating groups at C-3, C-4, C-5, C-6 and C-19. Because of the presumed proximity to the heme a derivatized C-3 group could improve affinity by coordination with the P<sub>450</sub> iron. A 3-ethyleneketal constitutes a suitable choice, harbors an oxygen above and below the A ring and a large polar surface over the A ring.

##### **2.5.5.1 Topological considerations on CPF-Oxygen models.**

A CPF model of the possible interactions can be constructed with the following requirement: due the nature of the program used, docking procedures are best treated by first approximating the best macroscopic fit between moieties, then setting distances for key atoms in this approximation and minimizing. After this, rotamers can be manipulated. Since all parameters of the Fe-O relationship (length, charge, geometry and strength) will change continuously during catalysis, even an X-ray of the Fe-O complex, (if it were available) would only reflect a "stopped" moment in the catalysis.

The same is not true of the CPF-sulfur and nitrogen models as they exhibit type II interactions, actually coordinated and therefore measurable.

##### **2.5.5.2 Determining the Fe-O distance.**

Reported spectral studies of 19-oxygenated substrate and P<sub>450</sub> interactions all indicate a type I spectral shift in absorption; indicating that there is no chelation with the 19-oxygen, but rather displacement of the heme sixth ligand, usually water or dioxygen. For this reason it would be ideal to set an exact distance between the iron and the 19-oxygen; a distance long enough to cause displacement of a ligand and yet not within coordination distance.

A rough approximation for the maximal distance between the iron and the steroidal oxygen is obtained in a linear displacement of the water molecule, and can be set at 5.8 Å. This is calculated as the sum of the minimal distance at which elastic penetration of the ligand molecule's accessible surface (1.4 Å) comes in contact with the steroidal 19-hydroxyl van der Waals envelope ( radius  $\approx 1.4$  Å) with respect to the iron-ligand radius (2.5-3Å).

More realistically, the possibility of displacement at an angle can be modeled by minimizing the hexacoordinated CPF, with water in the sixth ligand position. These calculations result in distances of coordinated water (with hydrogen bonding) Fe-OH<sub>2</sub> about 1.9Å, and a Fe-tBuOH distance of 2.8, before displacement occurs. This agrees well with recent x-ray data<sup>79</sup> of P<sub>450</sub><sup>camphor</sup> collected in the presence of three substrates:

- camphor, which displaces the sixth aqua ligand,
- nor-camphor which does not and is found 3Å away from the water, and
- adamantone which elicits reactions similar to camphor.

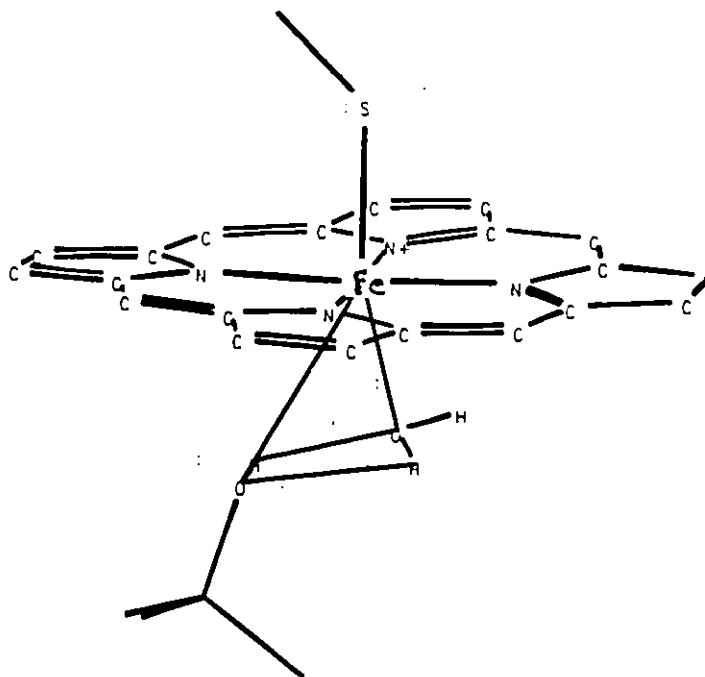


**Figure 2.5.5.2:** Docking of tBuOH onto CPF-OH<sub>2</sub>

#### 2.5.5.3 Docking experiment.

Hydrogen bonding with the steroidal hydroxyl would then be the first disturbance of the ligand van der Waals cloud, and no angular restrictions need be imposed. With this procedure the distance at which the steroidal-hydroxyl-iron distance may cause spectral disturbance by affecting the water ligand was set at 3Å. Although not entirely satisfactory from an electronic point of view, there is some basis for the steric interpretation of such models. The model is illustrated below with the parent compound 45 and CPF, a Fe-O bond length of 3 Å. Possible coordination to the ketal oxygens and the 5,6 alkene is shown.

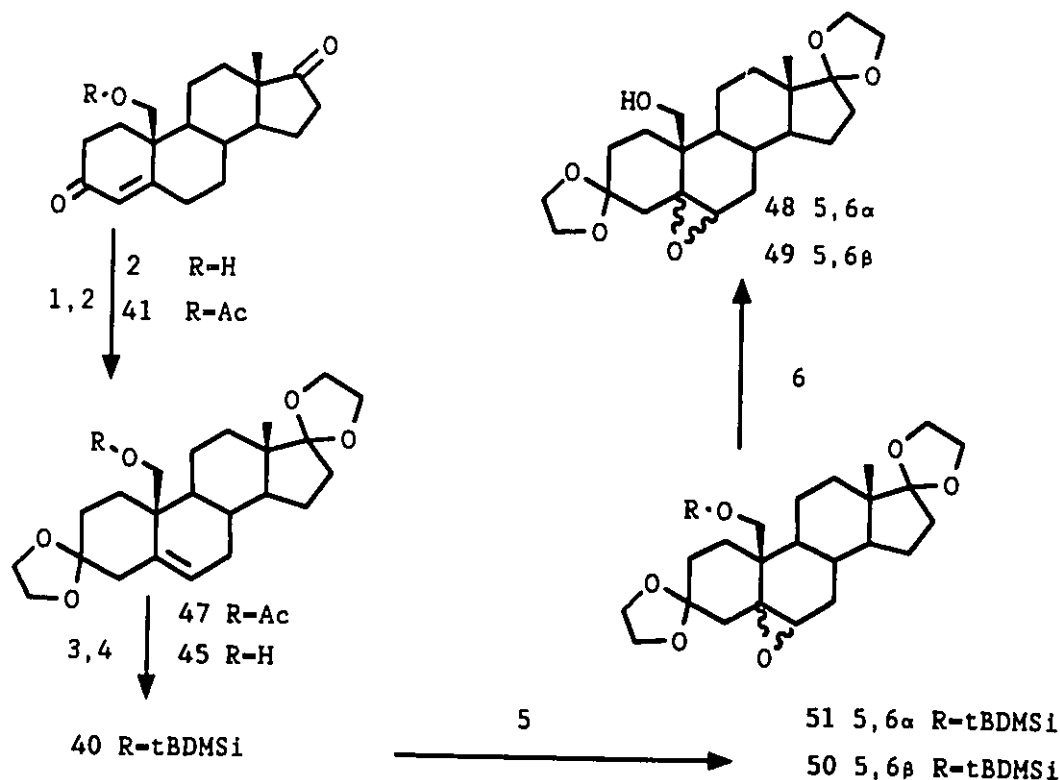


Figure 2.5.5.3: CPF-3,17-EG-19-ol compound 45.

## 2.5.6 KETALS AT C-3 AND C-17.

### 2.5.6.1 Preparation of 5-androsten-19-ol-3,17-dione 3,17-diethyleneketal 19-tertbutyldimethylsilyl ether (40).

Synthesis of the required ketals was undertaken. We found that synthesis of 5-androsten-19-ol-3,17-dione 3,17-diethyleneketal **45** could not proceed directly by reaction of 19-OH-A (**2**) because the 19-hydroxyl deforms. Instead, the 19-alcohol was protected with acetyl chloride in pyridine (icebath). The resulting 19-acetate oil **41** was immediately reacted, without further purification, with ethyleneglycol in a Dean-Stark apparatus, in refluxing benzene, under para-toluenesulfonic acid catalysis (**47**). Deprotection afforded the free 19-alcohol **45**.



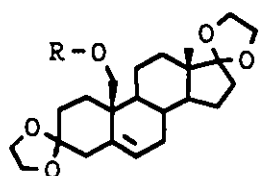
**Scheme 2.5.6.1:**

- 1) AcCl/Pyr, 0°C, 1 hr.
- 2) PhH, HOCH<sub>2</sub>CH<sub>2</sub>OH, TsOH, 24 hr.
- 3) KOH/MeOH
- 4) tBDMSi/ImH/DMF
- 5) mCPBA/CH<sub>2</sub>Cl<sub>2</sub>
- 6) Bu<sub>4</sub>N<sup>+</sup>F<sup>-</sup>/THF

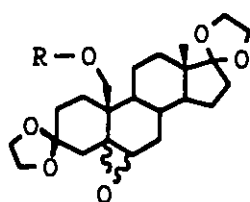
In order to obtain both isomers of the desired epoxides, the  $\beta$ -directing effect of the 19-hydroxyl and 19-acetoxyl groups had to be avoided during epoxidation.

Silylation resulted in a compound which can be epoxidized by mCPEA in  $\text{CH}_2\text{Cl}_2$ . Chromatography (HPLC) afforded both desired 19-silyl-epoxides. These were deprotected with  $\text{Bu}_4\text{N}^+\text{F}^-$  in THF to yield the free alcohols **48**, 19-hydroxy-5 $\alpha$ ,6-epoxy-5 $\alpha$ -androstand-3,17-dien-2-one and **49**, 19-hydroxy-5 $\beta$ ,6-epoxy-5 $\beta$ -androstand-3,17-dien-2-one

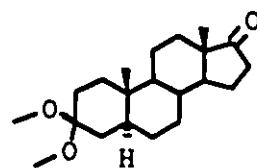
These compounds were incubated with aromatase.



45 R=H  
46 R=tBDMSi  
47 R=Ac



48 R=H, 5,6 $\alpha$ -epoxide  
49 R=H, 5,6 $\beta$ -epoxide  
50 R=tBDMSi, 5,6 $\beta$ -epoxide  
51 R=tBDMSi, 5,6 $\alpha$ -epoxide



159

The simple glycol **45** displays competitive kinetics but a poor affinity index of 15,000 ( $100 \times K_i/K_m$ ). Modifying the binding with tBDMSi leads to **48**, a steroid causing no detectable inhibitory effect at  $10^{-4}$  M concentration. The 19-acetate **47** exhibits mixed inhibitions and an affinity similar to the starting 19-alcohol **45**.

**Table 2.5.6.1:** Ketals: C-3 Ethyleneglycol (EG).

| Cmpd         | %Kapp<br>Method | %Vapp | Ki<br>( $\mu$ M) | Mode    | Inhibitor<br>( $\mu$ M) |                                    |
|--------------|-----------------|-------|------------------|---------|-------------------------|------------------------------------|
| 45           | 3               | 211   | 119              | 105     | C                       | (117) EG-19-OH-5,6-ene             |
| 46           | 1b              |       | 77               |         | I                       | (113) EG-19-tBDMSiO-5,6-ene        |
| 47           | 2               | 162   | 67               | 162 Kik | M                       | (101) EG-19-OAc-5,6-ene            |
| 48           | 1b              |       | 91               |         | I                       | (132) EG-5,6 $\alpha$ -epoxy-19-OH |
| 49           | 1b              |       | 103              |         | I                       | (125) EG-5,6 $\beta$ -epoxy-19-OH  |
| 50           | 3               | 190   | 113              | 88      | C                       | (80) EG-5,6 $\beta$ -epoxy-19-SiO  |
| 51           | 3               | 155   | 102              | 83      | C                       | (99) EG-5,6 $\alpha$ -epoxy-19-SiO |
| 159          | 1a              |       | 0                |         | C                       | (101) 5 $\alpha$ -A-3-DMO-17-one   |
| Testosterone |                 | 100   | 0.906            | -       |                         | Testosterone                       |

Abbreviations and method as for table 2.3.4. Incubation 1a against 400nM and 1b is against 21.7 nM. EG- Androsta-3,17-diethyleneketal, DMO- Dimethoxy; tBDMSi- tertbutyldimethylsilyl ether; AcO- Acetate.

Kinetics of 5-androsten-19-ol-3,17-dione 3,17-diethyleneketal **45** determined at 117  $\mu$ M display no effect on the apparent maximum velocity and a  $K_{app}=211 \pm K_m$ . From these the inhibitor concentration required to get  $V_{app}=50\% V_{max}$  is computed at  $K_i= 105\mu$ M. This indicates that steroid **45** is a competitive inhibitor with fair affinity for the active site but does not induce enzyme inactivation.

**Table 2.5.6.3:** Aromatase inhibition by **45**;  
5-androsten-19-ol-3,17-dione, 3,17-diethyleneketal

| Compound               | Vmax<br>nm/min/mg     | Kapp<br>nM       | Ki<br>$\mu$ M | Mode | Inhibitor<br>$\mu$ M |
|------------------------|-----------------------|------------------|---------------|------|----------------------|
| Testos.<br>$\pm 1$ STD | 0.0078<br>$\pm .0006$ | 803<br>$\pm 108$ |               | -    | none                 |
| <b>45</b>              |                       |                  | 105           | C    | 117                  |

Values are given  $\pm$  one standard deviation. Incubation method 3 is described in table 2.3.4. Testosterone concentration ranging from 201 to 2000 nM. Data analysis according to Eisenthal.

#### 2.5.6.4 Epoxides as affinity labels.

The potential affinity labels **48**, **49** and **46** exhibited insignificant (I) effect during screening conditions of high inhibitor to low testosterone concentration. They did not interact with the active center adequately. Modifying these with a bulky silyl tBDMSi group (**50**, **51**) improved the affinity slightly. Further studies on compound **51** indicated that it retained little effect under more clinically acceptable concentrations of 2 to 10  $\mu$ M.

#### 2.5.6.5 Topological conclusions to C-3,17 diethyleneketal:

C-3,C-17 diethyleneketal derivatives are not valuable inhibitors of aromatase. They do not increase affinity over parent ketones. Whether this is due to the displaced 5,6 alkene or to the bulky group at C-3 and C-17 is unclear. C-5,6 epoxides alter the ring A conformation. The  $\alpha$  epimer "lifts" the C-3 glycol closer to the postulated CPF-iron. Despite the additional polar volume, no significant increase in affinity is observed. Chelation to the heme iron is not probable, therefore, and indicates either that the substrate do not fit near the heme iron to reflect the CPF model used here, or the heme iron is not available near the C-3, nor C-5,6 $\beta$  face.

The synthesized epoxides had the additional task of trapping a nucleophile near the 4,6 carbons. These did not deviate from competitive effects, did not trap nucleophiles in the active site, and argue against the  $\alpha$  proximal active site nucleophile. Simpler epoxides with no ethyleneglycol groups were investigated and are reported in section 6.

#### 2.5.7 THIOKETALS AT C-3 AND C-17

Thio-androgens are amongst the most potent inhibitors of aromatase. 19-SH-A 113 exhibits type II spectral shifts typical of direct binding to the P<sub>450</sub> iron, and C-10 thiols inactivate aromatase, presumably via 10-sulfenyl mediated rearrangements. Nelson has recently shown 2 $\alpha$ -SH-A 160 to be a competitive (but weak) inhibitor.

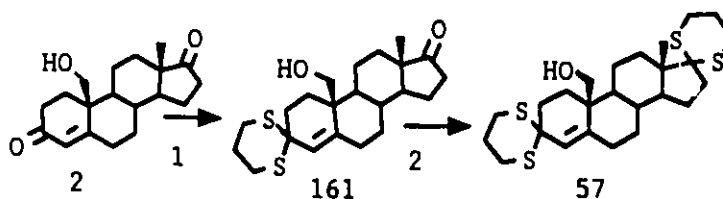
Thioketals were chosen as an alternative to the unsuccessful ethylene glycol. The longer carbon-sulfur bond would move the thioether closer to the putative  $\beta$ -heme and might demonstrate better coordination. In addition to retaining the backbone  $\Delta^4$  alkene, these are potential mechanism-based reducing agents which can trap active-site iron-perferryl species or C-2 radical (an intermediate required for Fishman's postulated C-2 oxidation).

When overlapped with the substrate 19-OH-A 2, the minimized conformation of the synthesized bisdithiane 57 shows little backbone distortions except from the additional rings at C-3 and C-17, and more closely resembles the 19-hydroxy-androstenedione substrate.

#### 2.5.7.1: Synthesis of 3,17-bisdithiane androgens as potential inhibitors.

##### 57) Preparation of 19-hydroxy-4-androstene-3,17-dione, bispropylenethioketal. (57)

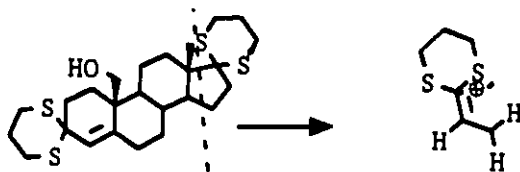
Reaction of the starting material with propanedithiol in  $\text{CHCl}_3$  with  $\text{BF}_3$  catalyst gave excellent yields of the bis-dithiane 57 after 4 days. Since  $\alpha, \beta$ -unsaturated ketones are more reactive towards ketalization than C-17 ketones, it is possible to obtain high yields of 3-dithiane-17-one by stopping the reaction after a few hours.



1) propanedithiol,  $\text{BF}_3$ ,  $\text{CHCl}_3$  4 hours 2) 4 days

**Scheme 2.5.7.57:** Preparation of 19-hydroxy-4-androstene-3,17-dione, bispropylenethioketal. (57)

The monothioketal 161 can be obtained by stopping the derivatization procedure after a few hours and chromatography. The bistihioketal 57 requires longer reaction times and was characterized by an acceptable high resolution MS. A peak at  $m/z$  145 was assigned to the degradation of a C-17 propyldithiane  $[\text{C}_6\text{H}_9\text{S}_2]$ , since we observed similar degradation with 3,17-bisdithiolane but not 17-keto-derivatives. 17-dithiolane protective groups are similarly characterized by a peak at  $m/z$  131  $[\text{C}_5\text{H}_7\text{S}_2]$ . (*vide infra*)



145 peak

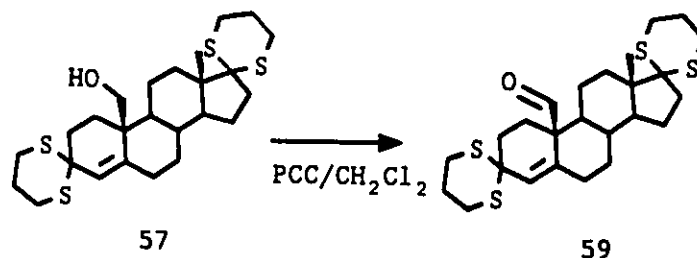
The dithiane is much less polar than the starting diketone and has a purple Kober chromogen when heated with H<sub>2</sub>SO<sub>4</sub> on silica. <sup>1</sup>H-NMR displays a shifted 4-CH ( $\delta$  5.76) and a shifted 18-methyl ( $\delta$  0.967) in addition to the expected multiplet of thiomethylene found at  $\delta$  2.57-2.94 (CH<sub>2</sub>S) and  $\delta$  2.46-2.58 (CH<sub>2</sub>-C-S). ADEPT <sup>13</sup>C NMR indicates loss of the ketone quaternary carbons and replacement with higher field absorptions at  $\delta$  50.14 (17-C=S<sub>2</sub>) and  $\delta$  63 (3-C=S<sub>2</sub>).

#### 2.5.7.2: Synthesis of bithioketals derived at C-19:

##### 58) Preparation of 19-hydroxy-4-androstene-3,17-dione, bispropylenethioketal, 19-toluenesulfonic ester. (58)

Tosylation was carried out in the presence of p-toluenesulfonyl chloride at 60 degrees for 2 hours. This afforded a white foam in 76% yield. Care must be taken not to overheat the mixture, as a rearrangement product occurs (see chapter 3). The mass spectra of the tosylate thus exhibits no high end, even under milder chemical ionization MS. The high mass shows a M-172 peak, which corresponds to loss of the C-19 tosylate [M-CH<sub>3</sub>Ph-SO<sub>3</sub>H]. This is a stable fragment seen in the lower end, along with the characteristic m/z 145 peak. TLC shows a typical green Kober chromogen slightly less polar than the starting alcohol. The para substituted phenyl appears in the <sup>1</sup>H NMR, along with the extra methyl at  $\delta$  2.43. ADEPT shows no major change except a 6 ppm deshielding for the 19-CH<sub>2</sub>, alkene carbons shielded by 1 ppm and the C-10 shielded by 2 ppm. The extra peak at 21.7 ppm corresponds to a tosyl-methane, and the tosyl aromatic carbons are found at low field.

**59) Preparation of 4-androsten-3,17,19-trione, 3,17-bispropylenethioketal. (59)**

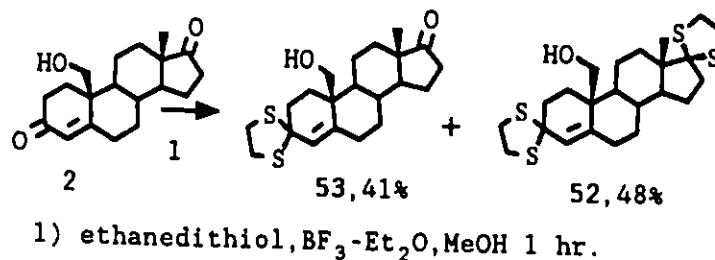


**Scheme 2.5.7.59:** Preparation of 4-androstene-3,17,19-trione 3,17-bispropylenethioketal 59

The desired 19-aldehyde **59** was obtained in 48% yield from the PCC oxidation of **57** in buffered  $\text{CH}_2\text{Cl}_2$ , followed by chromatography. The mass spectra displays a parent ion at  $m/z$  480  $[\text{P}^+]$  and a  $[\text{M}-\text{CO}]$  peak at 452, in addition to the characteristic 145  $[\text{C}_6\text{S}_2\text{H}_9]$  peak. IR contains a carbonyl absorption at  $1710\text{ cm}^{-1}$  assigned to the 19- $\text{C}=\text{O}$  aldehyde. NMR confirms the presence of the aldehyde,  $\delta$  9.79 ( $^1\text{H}$ ) and  $^{13}\text{C}$  202.6 (19-CHO). A 4 ppm deshielding of the quaternary 5- $\text{C}=\text{C}$  to 139.3 ppm reflects the presence of the aldehyde.

**52) Preparation of 19-hydroxy-4-androstene-3,17-dione 3-ethylenethioketal and 19-hydroxy-4-androstene-3,17-dione bisethylenethioketal.**

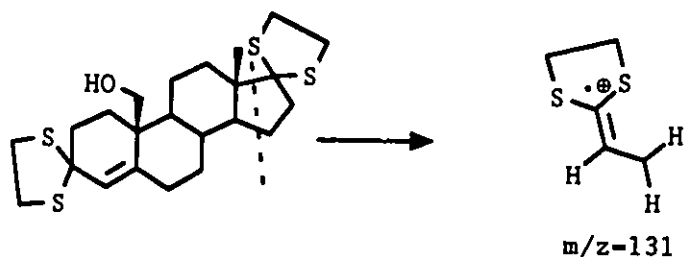
The dithiolane derivatives were obtained in good yield by reaction of the 3,17-ketones with ethanedithiol in methanol, under  $\text{BF}_3$ -etherate catalysis.



**Scheme 2.5.7.52:** Synthesis of 19-hydroxy-4-androstene-ethylenethioketals 52 and 53.

## 52) Spectral analysis of bisethylenethioketal 52.

The bisdithiolane is characterized by a parent ion at  $m/z$  454 ( $P^+$ ) and a peak at  $m/z$  131 attributed to  $[C_5H_7S_2]$ , obtained from the degradation of the D-ring ethylenethioketal in 3,17-bisdithiolanes.

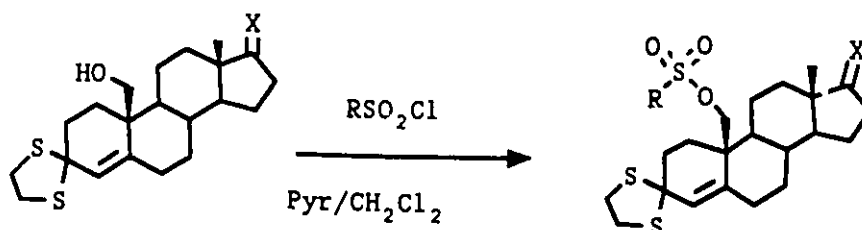


The  $^1H$  NMR displays a shifted 4-CH (5.82p) a shifted 18-methyl (0.905p), a shifted 19-methylene (3.61-3.93) in addition to the expected multiplet thiomethylene found at 3.03-3.4 integrating for eight protons ( $CH_2S, 8H, m$ ). ADEPT  $^{13}C$  NMR indicates loss of the ketone quaternary carbons and replacement with higher field absorptions at  $\delta$  80.02 (17- $C=S_2$ ) and 65.1 (3- $C=S_2$ ). The shift from  $\delta$  50.14 for dithiane to 80.02 for the dithiolane 17- $C=S_2$  is noteworthy.

## 53) Spectral analysis of the 3-ethylenethioketal 53.

The more polar monothioketal derivative 53 is characterized by a parent ion at  $m/z$  378 [ $P^+$ ], and no peak at  $m/z$  131. The  $^1H$  NMR displays a 4-CH at a value of  $\delta$  5.83 ppm, similar to the bisdithiolane, an 18-methyl similar to the starting material (0.859 ppm) and a shifted 19-methylene complex at  $\delta$  3.66 (19- $CH_2O$ , 2H, d of ABq.), in addition to the multiplet of four thiomethylene protons found at 3.18-3.58 ( $CH_2S, 4H, m$ ). ADEPT  $^{13}C$  NMR indicates substitution of the C-3 ketone carbon for a higher field absorptions at  $\delta$  65.1 (3- $C=S_2$ ). The 17-keto is retained at 220.3 (17- $C=O$ ), close to the starting material ( $\delta$  220.1).

**Scheme 2.5.7.56:** Synthesis of 19-ester derivatives



53 X-O

52 X-SCH<sub>2</sub>CH<sub>2</sub>S

56 R-CH<sub>3</sub> ; X-O

162 R-CH<sub>3</sub>Ph ; X-SCH<sub>2</sub>CH<sub>2</sub>S

55 R-CH<sub>3</sub> ; X-SCH<sub>2</sub>CH<sub>2</sub>S

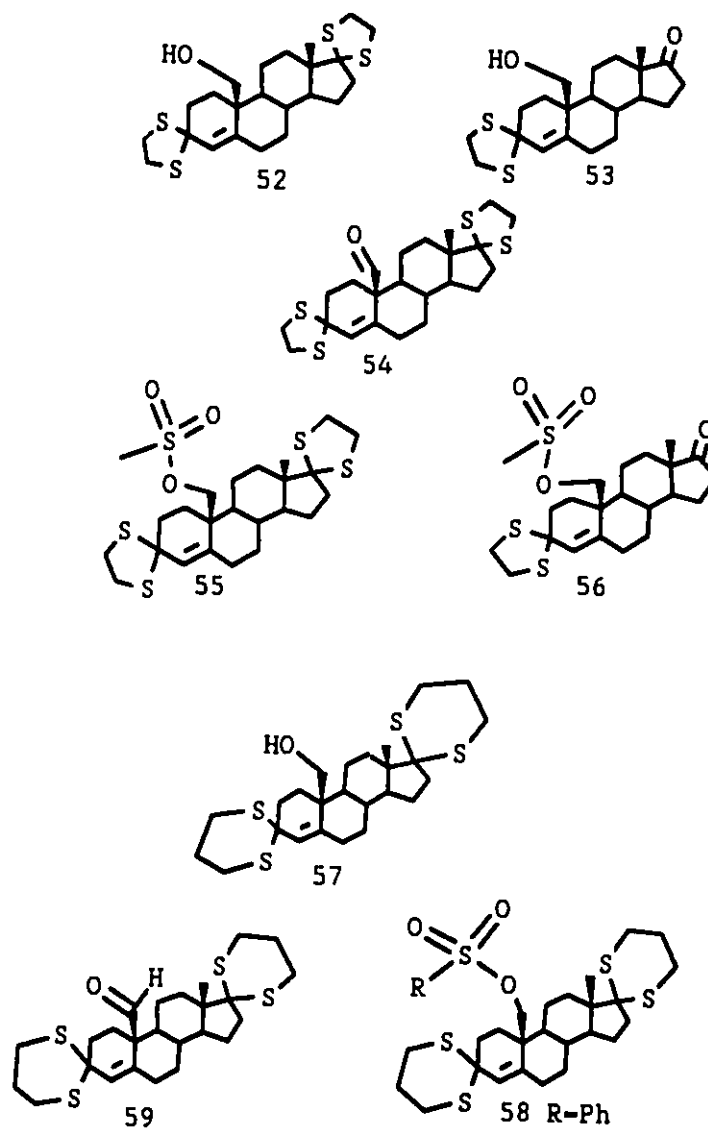
**56) Preparation of 19-hydroxy-4-androstene-3,17-dione 3-ethylene-thioketal, 19-methanesulfonic ester. (56)**

The 19-alcohol **53** was derivatized in 2 hours at 42°C in the presence of mesyl chloride. Chromatography afforded the mesylate in 68% yield. The mass spectra shows a weak parent ion at  $m/z$  456 [ $P^+$ ] and an [ $M-CH_3SO_3H$ ] peak at 360 (100%). IR shows bands at 1360 ( $SO_2$ ), 1180 ( $S=O$ ) and 960, 840, 530, along with the expected 17-C=O carbonyl at 1740  $cm^{-1}$ . The  $^1H$  NMR has a shifted 19-CH<sub>2</sub>O AB quartet (4.35 ppm) and a new peak at  $\delta$  3.01 due to the  $CH_3SO_2$ . The ADEPT  $^{13}C$  spectra exhibits changes similar to those found for other sulfonates described: a methanesulfonate peak at  $\delta$  37.7, a 19-CH<sub>2</sub>O deshielded by 5 ppm (to 70.5), a 10-C (or 13-C) deshielded by 18 ppm and alkene carbons shielded by 1 ppm.

**55) Preparation of 19-hydroxy-4-androstene-3,17-dione bisethylene-thioketal, 19-methanesulfonic ester. (55)**

The 19-methanesulfonate was obtained from the alcohol **52** by derivatization with mesyl chloride in  $CH_2Cl_2$ /pyridine at 42°C. The bisdithiolane exhibits a weak parent ion at  $m/z$  532 [ $P^+$ ], a [ $M-CH_3SO_3H$ ] peak at 436, as well as the characteristic peak at  $m/z$  131 attributed to D-ring [ $C_5H_7S_2$ ]. IR shows bands at 1350 ( $SO_2$ ), 1170 ( $S=O$ ) and 950, 530  $cm^{-1}$ . The  $^1H$  NMR has a shifted 19-CH<sub>2</sub>O AB quartet (4.45p) and a new peak at  $\delta$  3.0 due to the  $CH_3SO_2$ . The ADEPT  $^{13}C$  spectra exhibits changes similar to those found for other steroid sulfonates.

The following derivatives were incubated.



**Scheme 2.5.7.2: Dithiane and dithiolane inhibitors synthesized.**

**Table 2.5.7.2:** Incubation results of dithianes and dithiolanes.

| Cmpd | %Kapp | %Vmax | Ki $\mu$ M  | Mode | ( $\mu$ M) Inhibitor:androst-4-ene- |
|------|-------|-------|-------------|------|-------------------------------------|
| 52 3 | 129%  | 91%   | 103 $\mu$ M | C    | (104) 19-OH-3,17-bisdithiolane      |
| 53 3 | 303   | 92    | 57          | C    | (116) 19-OH-3-dithiolane-17-one     |
| 57 3 | 218   | 116   | 101         | C    | (120) 19-OH-3,17-bisdithiane        |
| 45 3 | 211   | 119   | 105         | C    | (117) EG-19-OH-5,6-ene              |
| 54 3 | 101   | 111   | ----        | I    | (102) 19-CHO-3,17-bisdithiolane     |
| 59 3 | 77    | 78    | 168         | U    | (51) 19-CHO-3,17-bisdithiane        |
| 55 3 | 77    | 60    | 176 Kiv     | M    | (54) 19-Ms-3,17-bisdithiolane       |
| 56 3 | 173   | 97    | 171         | C    | (125) 19-Ms-3-dithiolane-17-one     |
| 58 2 | 92    | 65    | ----        | N    | (95) 19-Ts-3,17-bisdithiane         |
| 42 3 | 79    | 68    | 87          | M    | (41) 19-Ms-Androstenedione          |
| T    | 100   | 100   | 0.906       | -    | Testosterone control                |

Abbreviations and method as for table 2.3.4 EG: 3,17-diethyleneglycol, CHO- aldehyde; Ms Mesylate; Ts- Tosylate; U is uncompetitive; N is non competitive; M is mixed inhibition.

■ The free alcohols 53, 52, 57 exhibited affinities similar to the control ethyleneglycol 45. They did not cause enzyme inactivation. Conclusion are made with compound codes referred in brackets.

■ A C-17 dithiolane or dithiane substituent is tolerated in the enzyme cavity. These groups do not increase the affinity for the active center, but modify the alignment of the C-19 group such that they can not be oxidized at the C-19 position. (45~52~57, 42 affinity >> 56 ) They are presumed not substrates, nor oxidized at the C-19 position since these steroids are competitive whereas their oxidized 19-oxo derivatives exhibit different kinetics; the 19-oxodithiane 59 is "uncompetitive" (kinetically not differentiated from an irreversible inhibitor in these sets of experiments)

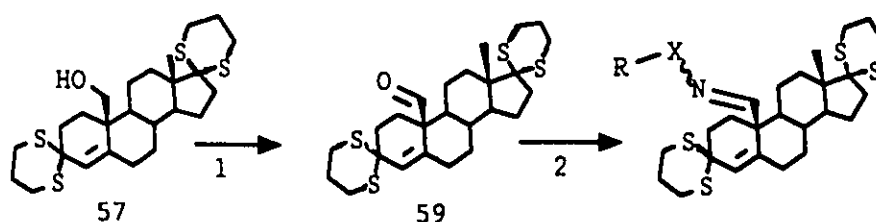
and the 19-oxo-dithiolane 54 does not demonstrate affinity for the active site.

- A C-17 ketone elicits higher affinity than the corresponding thioketal. (53 affinity >>52. a 42>>56≈55.
- There is no significant difference in affinity between the 3,17-bisdithiane- $\Delta^4$ , the smaller 3,17-bisdithiolane- $\Delta^4$ , and the 3,17-diethyleneketal- $\Delta^5$ .
- The best competitive inhibitor was compound 53 (19-OH-3-dithiolane-17-one). It was further investigated at 10  $\mu$ M. It does not exhibit significant inhibition at  $\mu$ 's concentration.
- Modifications of the 19 position with more reactive mesylate and tosylate caused **inactivation**. The C-17 dithiolane must induce a realignment in the active center such that the 19-mesyl is able to inactivate aromatase.
- A C-19 mesylate potentiates inactivation and is sufficiently small to retain affinity. (compare 53,52 vs 56,55)
- The 17 dithiolane modifies the alignment to cause inactivation by 19-mesylate 55. (compare 55>56=0)
- A C-19 tosylate does not retain significant affinity and compound 58 is regarded as defining an active site excluded volume. The electronic clouds about the sulfonate may cause loss of affinity.
- We would postulate that the aldehyde 59 causes alignment suitable for inactivation by the C-3 thioketal in one of the mechanism discussed earlier.
- The C-19 aldehyde seems to potentiate inactivation. Compound 54 is enzyme disallowed. (52,54)

Since deviation from the basic androstenedione-19-ol backbone is minimal, it appears that these dithiolane, ethyleneglycol and dithiane derivatives encounter sufficient steric constraints about the C-3 position to lower the affinity significantly.

The dithiolane 19-mesylate is strictly a competitive inhibitor, and the bisdithiolane is a "mixed mode" inhibitor, possibly causing inactivation. This suggests the possibility of an alternative position in the active center for the inactivation by the bisdithiolane; If they both shared the same position, one would expect the 17-one derivative to be at least as active as the 17-dithiolane derivative, yet this is not observed.

The mode of operation of the 19-aldehyde bisdithiane was not further elucidated. It awaits a more thorough Structure Activity Series. Key members bearing  $sp^2$  C-19 derivatives were synthesized and await biochemical analysis. Some bio-organic chemistry on these is reported in chapter 3.



87  $RX-PhCH_2O-$   
94  $RX-pTOSNH_2-$

1)  $PCC/CH_2Cl_2$  2)  $RONH_2/H^+$  or  $RNNH_2/H^+$

**Scheme 2.5.7.94:** Synthesis of future aromatase inhibitors bearing  $sp^2$  derivatives at the C-19 position.

### 2.5.7.3: The C-3 chelate postulate: conclusions:

The C-3 chelate postulated that a heteroatom at the C-3 position might be able to chelate with the heme-iron in the active center of aromatase, increasing the affinity and, with the choice of sulfur heteroatoms, perhaps also reduce the

active iron-oxygen species which occur during catalysis. The approach led to the synthesis of a number of ethyleneglycol, dithiane and dithiolane derivatives.

Although most of these presented a competitive profile, none of the 19-alcohol derivatives were able to increase affinity or cause inactivation of the enzyme.

Knowledge gained from the C-19 postulate, however, offered the possibility of inactivation through a C-19 handle. Test compounds bearing groups such as aldehydes, mesylate and tosylates were thus synthesized and incubated. The results were of great interest as a number of these proved to be not only competitive inhibitors but exhibited kinetics typical of enzyme inactivation.

The more surprising aldehyde derivatives were intriguing since a commonly accepted intermediate in the aromatization of the natural substrates is a C-19-aldehyde (or diol) androstenedione, and thus an aldehyde pharmacophore would not be expected to cause inactivation. That it does, however, can be explained by invoking an altered alignments of the thioketals, due to the presence of the aldehyde.

That the mesylate-thioketal derivatives caused inactivation was less surprising in view of the potency of the androstenedione-mesylate discovered during the C-19 postulate investigation.

There is thus some merit to the C-3 chelate postulate, though the possibility of causing inactivation must be further evaluated with the help of pharmacophores causing a realignment in the active center. A few novel C-19 derivatives investigated during the C-19 cav. postulate may prove helpful in this regard.

**2.6 The C-4,5,6 active center nucleophile postulate.  
(C-5-Nu)**

An active site nucleophile has been invoked in the literature to explain the suicide-inhibition by C-4 and C-6-derivatives. A reasonable mode of inactivation seen in chapter 1 illustrated their involvement via enzyme-bound intermediates. This postulate requires an enzyme bound nucleophile near the C-4,5,6 position of the substrate. Two distinct categories of traps were targeted to question this possibility: 4,5 and 5,6 steroidal epoxides.

Epoxides are small, chemically reactive moiety which would point to the presence of an active site nucleophile near the C-4 position. As seen in chapter 1 there exists postulates of aromatase catalysis which operate either via an amino acid  $\alpha$ -directed nucleophile or a  $\beta$ -directed nucleophile (such as a heme-iron-hydroperoxide species). Thus, inhibitors bearing both  $\alpha$ - and  $\beta$ -epoxides were synthesized to trap such nucleophiles. Advances explored in the previous sections suggested a number of additional derivatives which were also synthesized.

As discussed in the previous sections, there are two prime concerns in designing inhibitors:

- 1) Will the target compound fit in the active center, there to exhibit competitive kinetics ? How does it compare, structurally, to other known inhibitors ?
- 2) Are the pending pharmacophores capable of further increasing the pharmacological value of the inhibitor, by causing enzyme inactivation or increasing residence time?

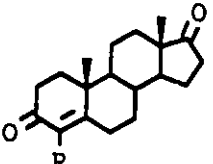
From a chemical point of view the reactivity of epoxides towards nucleophiles is well documented and a series of epoxy steroids would confirm whether or not there exists an active site nucleophile near the steroidal C-4,5,6 positions.

The incubation of selected epoxide inhibitors would then help in confirming or not the probable presence of nucleophilic species in the active center. Judicious selection would also assist in determining the favored position of attack and the stereochemistry of the nucleophilic addition, certainly a major contribution to better understanding the extremely potent inhibitors possessing pharmacophores at the C-4 and C-6 positions.

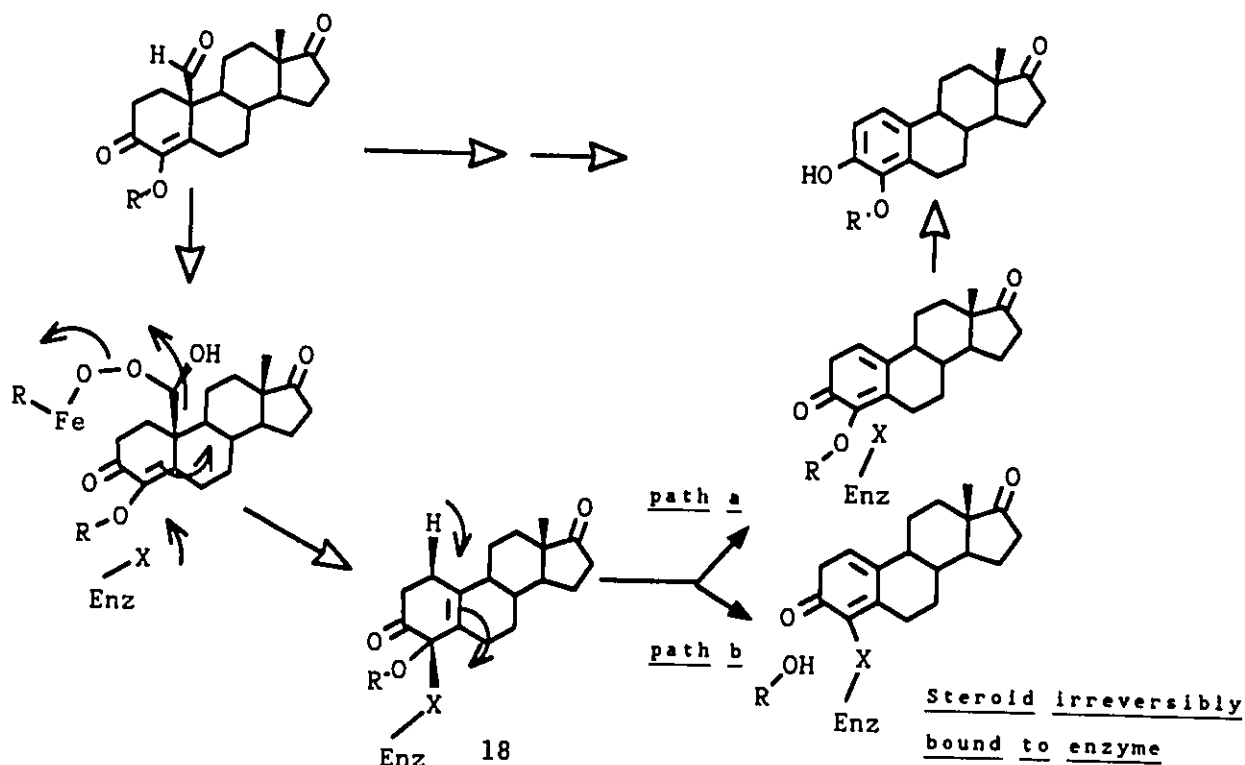
#### **2.6.1 Literature review. Derivatives at C-4: Cl, OH, OAc, OMe, oxirane**

Androstenedione derivatives bearing 4-ene-4-oxygen groups are reported as promising steroidal inhibitors of aromatase. The first clinical study of compound **100** was undertaken in 1984<sup>17</sup>. Groups led by Brodie<sup>16e</sup> have since tested a number of C-4 oxygenated androgens for aromatase inhibition. More recently, Lombardi<sup>25</sup> and collaborators have synthesized 4-amino-androgens that are also mechanism-based irreversible inhibitors. Such modifications on  $\Delta^{1,4}$  or  $\Delta^{4,6}$  androgen backbones also elicit irreversible inhibition.

**Table 2.6.1:** Inhibitors bearing C-4 pharmacophores.

|  |                      | Code                   | R   | K <sub>i</sub><br>μM | K <sub>i</sub> /K <sub>m</sub><br>x100 | Mode | ref. | ΔΔG°<br>kcal/mol |
|-----------------------------------------------------------------------------------|----------------------|------------------------|-----|----------------------|----------------------------------------|------|------|------------------|
| 100                                                                               | OH                   | 0.054                  | 100 | TDI                  | 20, 77, 44                             | 0.0  |      |                  |
| 163                                                                               | OAc                  | ND                     |     | Comp.                | 26                                     |      |      |                  |
| 164                                                                               | OMe                  | ND                     |     | Comp.                | 26                                     |      |      |                  |
| 165                                                                               | SH                   | IC <sub>1.5</sub> -63% |     | Comp.                | 44, 26                                 |      |      |                  |
| 166                                                                               | NH <sub>2</sub>      | 0.037                  |     | Ir.                  | 25                                     |      |      |                  |
| 167                                                                               | Cl                   | ND                     |     | Comp.                | 26                                     |      |      |                  |
| 168                                                                               | SAc                  | 0.073                  | 138 | Comp.                | 44, 26                                 | -0.2 |      |                  |
| 169                                                                               | SMe                  | 0.059                  | 111 | Comp.                | 44                                     | -0.1 |      |                  |
| 170                                                                               | SEt                  | 0.041                  | 77  | Comp.                | 44                                     | +0.2 |      |                  |
| 171                                                                               | SBu                  | 0.065                  | 122 | Comp.                | 44                                     | -0.1 |      |                  |
| 172                                                                               | SPh                  | 0.036                  | 68  | Comp.                | 44                                     | +0.2 |      |                  |
| 173                                                                               | 4,5-epoxy            | ND                     |     | Comp.                | 26                                     |      |      |                  |
| Δ <sup>4,6</sup>                                                                  |                      |                        |     |                      |                                        |      |      |                  |
| 175                                                                               | NH <sub>2</sub>      | 0.059                  |     | TDI                  | 25                                     |      |      |                  |
| 176                                                                               | OH                   | ND                     |     |                      | 26                                     |      |      |                  |
| Δ <sup>1,4</sup>                                                                  |                      |                        |     |                      |                                        |      |      |                  |
| 177                                                                               | OH                   | ND                     |     |                      | 26                                     |      |      |                  |
| 178                                                                               | NH <sub>2</sub>      | 0.068                  |     |                      | 25                                     |      |      |                  |
| 174                                                                               | OH, 19-CHO           | 3.0                    | --  | Comp. TDI            | 78                                     |      |      |                  |
| 126                                                                               | OH, 19F <sub>2</sub> | IC <sub>50</sub> =3.3  | --  | Comp.                | 81, 0.2 μM for 4-OH-A                  |      |      |                  |

Inhibitor 4-hydroxy-androstenedione **100** (4-OHA) shows time-dependent, NADPH-mediated loss of activity. The mechanism possibly involves catalytic conversion to an intermediate which then causes active site inactivation. Covey<sup>18</sup> invoked enzyme assisted addition-elimination at C-4 to explain the irreversible inhibition caused by 4-OHA **100** and 4-acetoxy-androstenedione **163**.

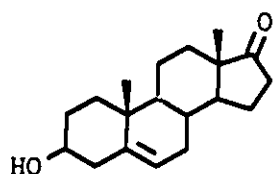


**Scheme 2.6.1:** Possible mechanism of inactivation at C-4.

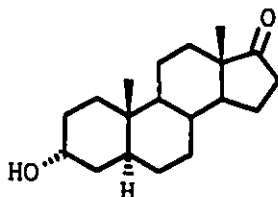
Marsh<sup>26</sup> found that the inhibitory activity of sulfur derivatives 4-SH **165** and 4-SAc **168** is lower than the corresponding oxygen isosteres. Evaluation<sup>44</sup> of a number of 4-thio-ethers by Abul-Hajj indicate that an alkyl chain up to three carbons long fit in the active site. The straight chain derivatives with 4-S-C<sub>4</sub>, 4-S-C<sub>5</sub> and the benzyl 4-S-CH<sub>2</sub>Ph elucidate little inhibition (IC<sub>18%</sub>, IC<sub>8%</sub>, IC<sub>8%</sub> = 1.5  $\mu$ M, respectively). The aromatic 4-S-Ph and 4-S-Ph-p-CH<sub>3</sub> causes inhibition while 4-S-Ph-p-OCH<sub>3</sub>, -p-NH<sub>2</sub>, -p-Br, and -p-NO<sub>2</sub> cause no inhibition at 1.5  $\mu$ M. This report on 4-thio alkyl and numerous 4-thiophenyl derivatives indicate a hydrophobic pocket which accommodates substituents 4.3 Å in width and 5.5 Å in length. These results suggest that electronic factors can not enhance interactions with the active site, though this may be due to steric inaccessibility as there is a sharp drop in activity at a distance of 5.3 Å from the C-4 sulfur atom in both the aromatic and aliphatic series.

### 2.6.2 Derivatives at C-5: 5 $\alpha$ -H.

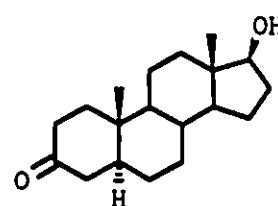
Naturally produced C-5 $\alpha$ -reduced androgens<sup>22</sup> are recognized inhibitors of aromatase, while C-5 $\beta$ -reduced androgens do not display significant affinity. 5 $\alpha$ -dihydro-testosterone (DHT), the 17-ketone 5 $\alpha$ -androstanedione and androsterone bind to aromatase with an affinity similar to testosterone.



dehydroepiandrosterone  
179



androsterone  
180



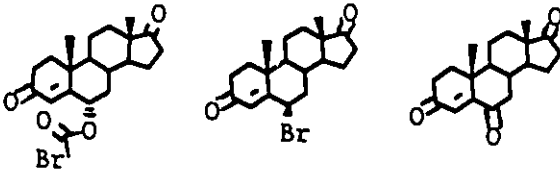
dihydrotestosterone DHT  
181

---

These natural substrates are proposed in the natural control of estrogen synthesis, where the ratio of reductase to aromatase determines the concentration of reduced steroid, hence the feedback on estrogenesis. In vitro data supports the potential role of 5 $\alpha$ -reduced C-19 steroids in a number of estrogen-dependent cancers such as breast and endometrioid<sup>22c</sup> cancer. The 5 $\alpha$ -hydrogen prevents aromatization, and synthetically it may be possible to use this to increase the half life of a test inhibitor. We therefore sought to combine this effect with other substrate modifications during preliminary screenings. Some fluoride analogues were also investigated. Results are presented in section 7 and reflect the trend that 5-reduction lowers affinity for aromatase.

### 2.6.3 Derivatives at C-6.

The published C-6 derivatives aromatase inhibitors have been reviewed in the section concerning the C-19 *cav.* postulate (pages 95-99). The inhibitors of interest are the following:



|                          |     |     |      |
|--------------------------|-----|-----|------|
| code                     | 142 | 136 | 131  |
| IC <sub>50</sub> $\mu$ M | 2.8 | 0.8 | 0.43 |
| ref.                     | 75  | 36  | 24   |

**Scheme 2.6.3:** Potent C-6 derived inhibitors causing irreversible inactivation or pseudo-first order time dependent inactivation. IC<sub>50</sub> are not normalized to the enzyme activity (K<sub>i</sub>/K<sub>m</sub>) and can not be directly compared in these different preparations.

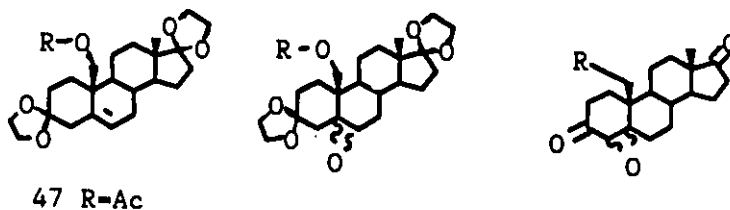
Within the framework of the C-19 Cav. postulate, additional C-6 and C-19 polyfunctional test compounds were incubated. All of those tested in section 4 (page 100, table 2.4.7.1) displayed competitive behavior only, with the exception of 5-androsten-19-ol-3,17-dione diethyleneketal, 19-acetate 47.

The weak competitive inhibition by 70, a 3,17-reduced form of A-trione 131, suggested that the 4-ene-3-one was a requirement for inactivation by the 6-keto pharmacophore.

More reactive groups are sought here to trap the putative 4,5,6-active site nucleophile.

#### 2.6.4 Epoxide inhibitors as nucleophile traps.

To trap the nucleophile invoked for C-4 and C-6 inhibitors, synthesis and incubation of a number of 4,5 and 5,6 $\alpha$ - and  $\beta$ -epoxides was carried out.



**Scheme 2.6.4:** C-4, C-5 and C-6 target inhibitors.

The targeted 4,5 epoxides seemed valuable lines of investigations. The 5,6-epoxide series have been shown to be strictly competitive inhibitors during the C-3-Chol. postulate. (*vide supra*, p.123)

The fundamental question of whether the androgenic 4,5-epoxide derivatives were liable to fit in the active center was answered by determining the molecular overlap between target epoxy-steroids and natural substrate.

#### **2.6.5 Literature precedent: inhibition by androgen derivatives bearing 4,5 epoxide.**

Epimers of 173, androsta-4,5-epoxy-3,17-dione<sup>8a,26</sup> have been briefly reported as inhibitors of aromatase. This would indicate that the targeted steroids would likely fit in the active center of aromatase. Attempts at modifying the realignment would assist in increasing the inhibitory capacity, and hopefully inducing inactivation.

##### **2.6.5.1 Topological considerations on $\alpha$ -epoxide/androgen overlap.**

Overlap of the minimized structures for the 4,5 $\alpha$ -epoxide 73 and the natural substrate androstenedione reveal minimal backbone deviation except for a 0.15 Å movement at C-4 and 0.1 Å at the O-3 position, with an overall RMS deviation of 0.05Å.

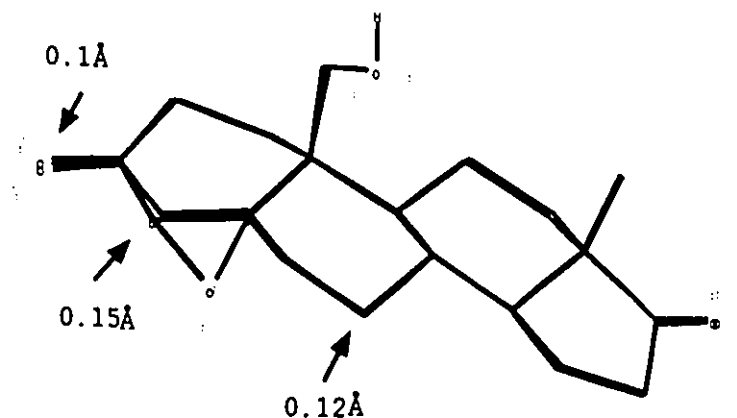


**Figure 2.6.5.1:** Overlap between  $\alpha$ -epoxide 73 and androstenedione.

Similar overlap with the potent inhibitor 4-OH-A 100 confirms that this series may have the potential of binding in the active center without major backbone distortions.

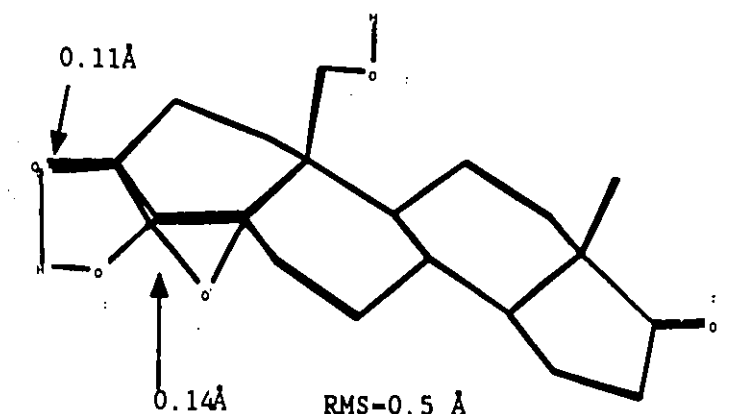


**Figure 2.6.5.2:** Overlap between  $\alpha$ -epoxide 73 and 4-OH-androstenedione.

From these simulations, it would appear that very minor backbone distortions are expected from the  $\alpha$ -epoxide target inhibitors. The major effect of the epoxide group would be the addition of a strong  $\alpha$ -polar cloud caused by the epoxide ether, in addition to a nucleophile trap hopefully aligned to trap active center nucleophiles attacking from the  $\beta$ -face.

#### 2.6.6 Topological considerations on $\beta$ -epoxide/androgen overlap.

The  $\beta$ -epoxide derivatives, on the other hand, offer the possibility of nucleophilic attack from the  $\alpha$ -face, in addition to the potential Lewis acid effects from the overhead heme-iron. However, the arrangement alters the backbone significantly, as demonstrated below with 72, illustrating a RMS deviation of 0.3 Å, and a greatest deviation occurring at the O-3, mostly in the A ring.

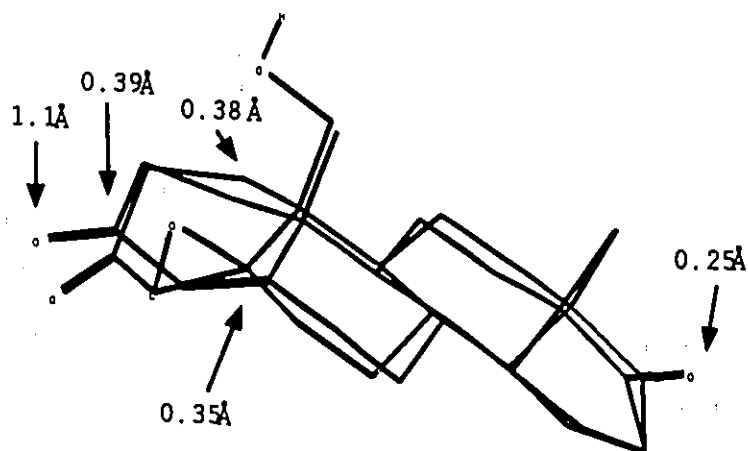


**Figure 2.6.6.1:** Overlap between  $\beta$ -epoxide 72 and androstenedione.

In addition, the high polar surface area due to the epoxide is now **above** the ring, near the putative heme porphyrin and thus capable of contributing to displacement of the aqueous sixth ligand, or coordinating to the iron. Rigid rotor analysis of the 19-OH-A-CPFe ether was modeled and the resulting two low energy rotamers are illustrated.

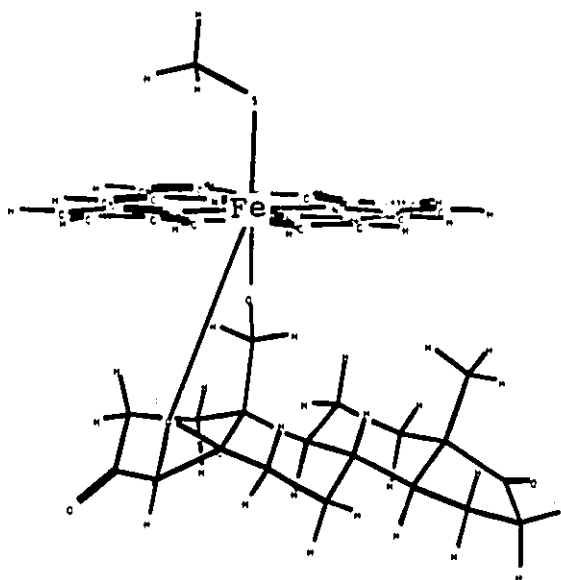
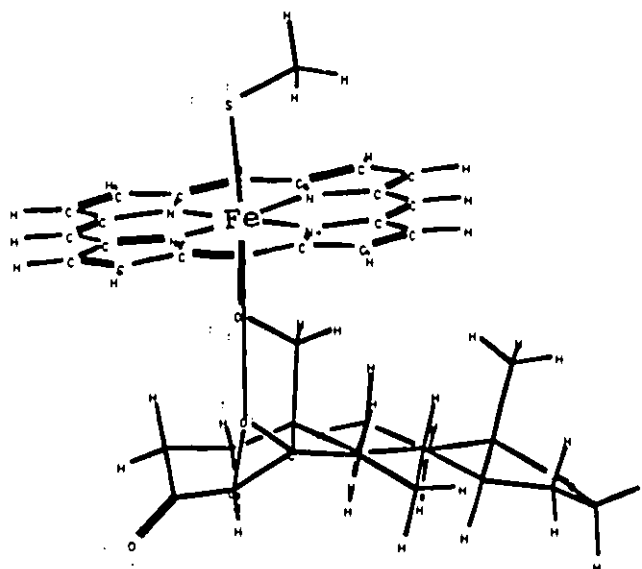


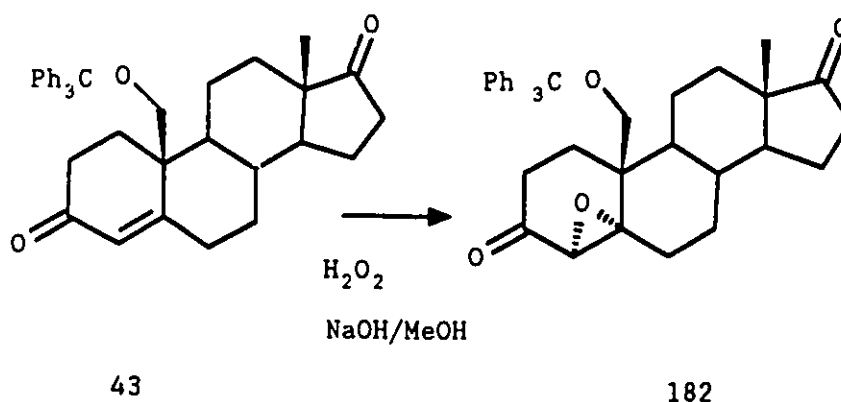
**Figure 2.6.6.2:** Two lowest energy rotamers of CPF bound to androsta-4,5 $\beta$ -epoxy-3,17-dione-19-ol 72.

### 2.6.7 Synthesis of epoxide derived androgens as potential inhibitors of aromatase.

Epoxide inhibitors were synthesized as potential aromatase inhibitors which could lodge in the active center, where they could trap an active enzyme nucleophile such as one postulated near the C-4 position. Since the free 19-alcohol directs epoxidation almost exclusively from the  $\beta$ -face of the steroid<sup>65</sup>, transformation of the steroidal 4-en-3-one **2** to the 3-one-4,5 $\beta$ -epoxide **72** proceeds directly by  $\text{H}_2\text{O}_2/\text{NaOH}$  oxidation. However, to obtain the  $\alpha$ -4,5-epoxides, a bulky 19-oxy-derivative<sup>66</sup> group is necessary; it offsets the  $\beta$ -face directing effect of the 19-alcohol and increases the steric hindrance over the A-ring, favoring oxidation from the  $\alpha$  face.

#### 2.6.7.1 Preparation of 19-trityloxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstane-3,17-dione.

An early attempt thus exploited the epoxidation of 19-trityloxy-androstenedione. The reaction gave low yields of the desired  $\alpha$ -epoxide, and the 19-silyloxy derivative was synthesized instead.

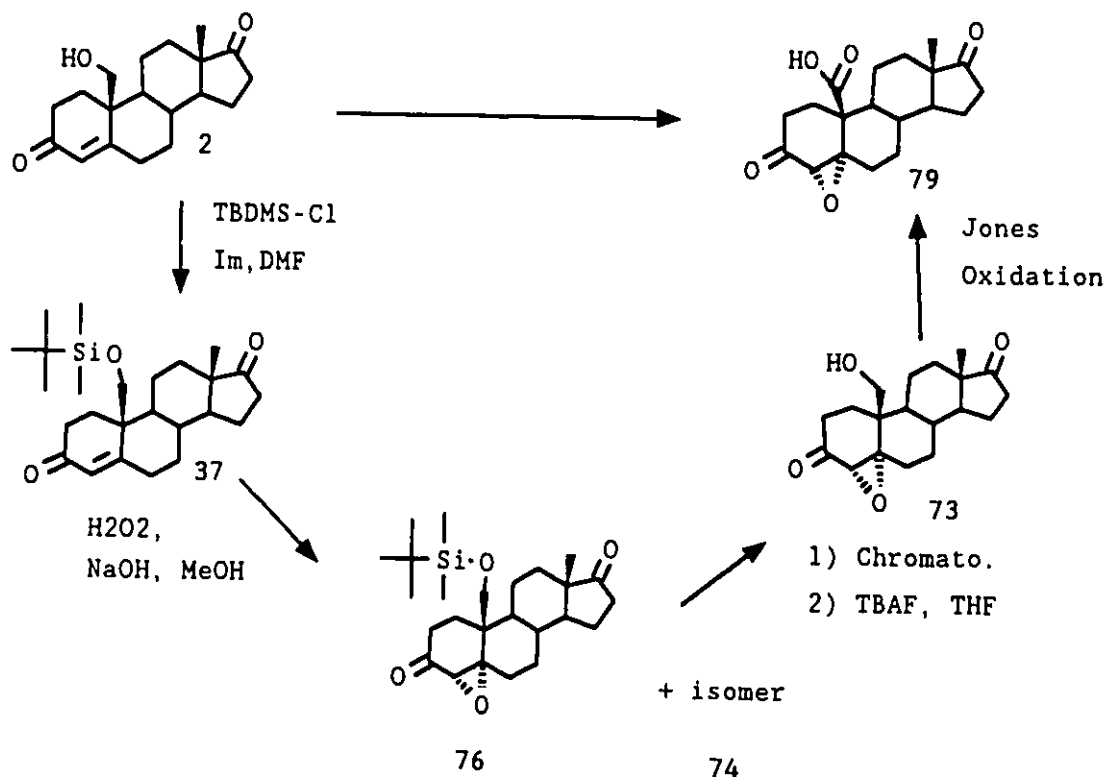


Scheme 2.6.7.182

**2.6.7.2 Preparation of 19-tertbutyldimethylsilyloxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstand-3,17-dione 76.**

Epoxidation of the tBDMS derivative generates both epoxide isomers in a 6/4  $\beta/\alpha$  ratio and this reaction can be conducted on 10 gram scale. Chromatography, deprotection and oxidation of the resulting alcohol with Jones's reagent affords the  $\alpha$ -epoxy-acid.

Spectroscopic data is presented in chapter 3, where the chemistry of these derivatives is investigated.



**Scheme 2.6.7.2:** Synthesis of target epoxide-bearing steroids.

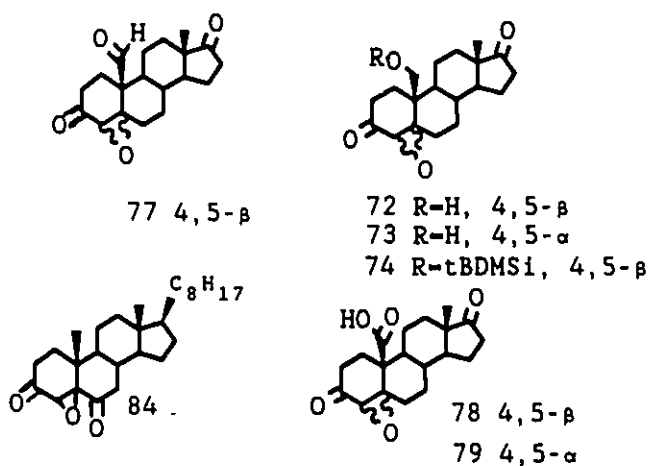


**Table 2.6.7:** Incubation results of androgen epoxide incubations.

| Cmpd   | %Kapp | %Vmax | Ki    | Mode | (μM) Inhibitor |
|--------|-------|-------|-------|------|----------------|
| method |       |       | μM    |      |                |
| T 3    | 100   | 100   | 0.906 | -    | Testosterone   |

**Androsta-5,6-epoxy-3,17-bisethyleneglycol-19-derivatives:**

|    |    |     |     |   |                             |
|----|----|-----|-----|---|-----------------------------|
| 48 | 1b | 91  |     | I | (132) 5,6α-epoxy-19-OH      |
| 49 | 1b | 103 |     | I | (125) 5,6β-epoxy-19-OH      |
| 50 | 3  | 113 | 88  | C | (80) 5,6β-epoxy-19-tBDMSO   |
| 51 | 3  | 102 | 83  | C | (99) 5,6α-epoxy-19-tBDMSO   |
| 84 | 3  | 84  | 224 | U | (43) Chol-4,5-epoxy-3,6-oxo |

**Androsta-4,5-epoxy-3,17-dione-19-derivatives:**

|    |   |     |     |   |                           |
|----|---|-----|-----|---|---------------------------|
| 72 | 3 | 107 | 18  | C | (10) 4,5β-epoxy-19-OH     |
| 73 | 3 | 141 | 48  | C | (144) 4,5α-epoxy-19-OH    |
| 74 | 3 | 105 | 12  | C | (11) 4,5β-epoxy-19-tBDMSO |
| 78 | 3 | 101 | 376 | C | (131) 4,5β-epoxy-10-COOH  |
| 79 | 3 | 97  | 61  | C | (48) 4,5β-epoxy-19-CHO    |

Abbreviations and methods as for table 2.3.4. I is insignificant effect. C is competitive effect. U is uncompetitive. Incubation 1b is against testosterone at 21.7 nM.

Method 3 represents the same kinetic protocol as in previous sections. The preferred method of computations was a mathematical equivalent to the Eisenthal direct plot approach.

#### 2.6.8: Conclusions concerning the epoxide series.

- All steroid-epoxides exhibited competitive kinetics only, with loss of affinity with respect to the natural substrates testosterone and androstenedione. This series of novel aromatase inhibitors cast serious doubts on the involvement of the invoked active-site nucleophile near the C-4,5,6 steroid positions.
- Comparison between the 4,5 $\alpha$ - and the 4,5 $\beta$ -epoxy-androgens indicates a 4-fold greater affinity of aromatase for the  $\beta$ -epoxide configuration, despite the much greater backbone distortions calculated for the  $\beta$ -epoxides. The implications seem that a  $\beta$ -polar group, or the upwards orientation of the C-3 ketone in the  $\beta$ -epoxide series, increase the affinity.
- There is no difference in affinity between the 19-silylated 74 and the corresponding free alcohol 72. This confirms the previous data on the C-19 cav. postulate that there exists a large cavity above the C-19 steroidal position when in the active center.
- There seems to be a decrease of affinity as the level of oxidation increases at the C-19 position. A systematic compilation of the available literature performed after this set of incubations confirmed this.
- There is quite a difference in affinity between the diethyleneketal series of 5,6-epoxides and the androgen 4,5-epoxides.

Although a more thorough comparison between the various 19-derivatives was planned, the other  $\alpha$ -epoxide derivatives synthesized were unfortunately not amenable to solubilization under the incubation conditions, precipitating out of

solution and making the comparison of kinetics unreliable.

#### **2.6.9 Conclusion concerning the C-4,5,6 postulate.**

The epoxides tested here would most certainly react with a nearby nucleophile (or a nearby electropositive center) if this nucleophile was present. The result of this investigation into the C-5 Nu. postulate casts a strong doubt on the current theory explaining inhibitor mechanisms involving an enzyme nucleophile entering at C-4 or C-5.

A number of potent aromatase inhibitors were discovered, strongest among them 19-hydroxy-4,5 $\beta$ -epoxy-5 $\beta$ -androstane-3,17-dione 72 and the tertbutyldimethylsilyl derivative 74.

The impact of this investigation at the C-6 nucleophilic attack is less clear as the lower affinity of the diethylene-ketal series may be representative of an alternative docking site in the active center.

#### **2.6.10 Conclusions concerning the influence of 19-oxygenation on the affinity.**

Following the interpretation of the incubation results in this series, and in view our remark that epoxides in the  $\beta$ -configuration display about 4 times more affinity than  $\alpha$ -epoxides, the following table 2.6.10.1 was compiled from our data and from the literature. It demonstrates that increasing oxidation level at the C-19 seems to decrease the affinity for most reported steroids. The series of  $K_i$  presented can be compared horizontally only. Vertical comparison can not be performed without normalization to a common substrate, since 100 fold differences in  $K_m$  have been reported in the literature. The compounds incubated during this thesis are directly comparable as they all were incubated with the same aromatase preparation which showed very little change of activity during the tests.

Table 2.6.10.1: Comparison of 19-derivatives and biological affinity.

| Ki (μM) for the 19 derivative shown. |                                                                 |                                       |                    |                        |                                                 |
|--------------------------------------|-----------------------------------------------------------------|---------------------------------------|--------------------|------------------------|-------------------------------------------------|
| <sup>19</sup> CH <sub>3</sub>        | C-OH                                                            | HC=O                                  | COOH               | CH <sub>2</sub> OtBDMS | STEROID                                         |
| 0.053                                | 0.65                                                            | 0.65                                  | --                 | 107                    | A-4-ene-3,17-dione <sup>39b</sup>               |
|                                      | 48                                                              | --                                    | 85                 | --                     | A-4,5α-epoxy-3,17-dione                         |
|                                      | 18                                                              | 61                                    | 376                | 12                     | A-4,5β-epoxy-3,17-dione                         |
|                                      | I                                                               | --                                    | --                 | 88                     | EG-5,6β-epoxy                                   |
|                                      | I                                                               | --                                    | --                 | 83                     | EG-5,6α-epoxy                                   |
|                                      | 103                                                             | I                                     | --                 |                        | A-3,17-dithiolane                               |
|                                      | 101                                                             | 168                                   | --                 |                        | A-3,17-dithiane                                 |
| 0.0047                               | 0.013                                                           | 0.024                                 | --                 |                        | A-3-CH <sub>2</sub> -4-ene-17-one <sup>15</sup> |
|                                      | <sup>19</sup> C-C≡CH                                            | HOC-C≡CH                              | O=C-C≡CH           |                        | Androst-4-ene-3,17-dione                        |
|                                      | 0.004                                                           | 0.049 (RS)                            | 0.350              |                        | reference <sup>39b</sup>                        |
|                                      | 0.023                                                           | 27 (S)*                               | 12                 |                        | reference <sup>38</sup>                         |
|                                      |                                                                 | 2.5 (R)*                              |                    |                        |                                                 |
|                                      | <sup>19</sup> C-C≡CH                                            | HOC-C≡CH                              |                    |                        | Δ <sup>4,6</sup> -A-3,17-dione                  |
|                                      | 0.13                                                            | 4.8 (S)                               |                    |                        | reference <sup>58</sup>                         |
|                                      |                                                                 | 32 (R)                                |                    |                        | reference <sup>58</sup>                         |
|                                      | <sup>19</sup> CH <sub>2</sub> CH <sub>3</sub>                   | HOCHCH <sub>3</sub>                   | O=CCH <sub>3</sub> |                        | Androst-4-ene-3,17-dione                        |
|                                      | 0.081                                                           | 9.9 (R)                               | 0.15               |                        | reference <sup>59</sup>                         |
|                                      |                                                                 | 11 (S)                                |                    |                        | reference <sup>59</sup>                         |
|                                      | <sup>19</sup> CH <sub>2</sub> CH <sub>2</sub> SiMe <sub>3</sub> | HOCHCH <sub>2</sub> SiMe <sub>3</sub> |                    |                        | Androst-4-ene-3,17-dione                        |
|                                      | I                                                               |                                       | 0.56               |                        | reference <sup>27</sup>                         |

Ki is a measure of affinity of the steroid against testosterone. The C-19 group is specified. A is androstane. ref.39b uses a different preparation of aromatase. None of the Ki values are normalized. They can only be compared horizontally and internally: 100 fold differences in Km have been reported in the literature. I is insignificant binding.

From a stereochemical point of view, it would be of interest to observe a consistent preference for one isomer at the C-19 position. Unfortunately, the available data<sup>38, 39</sup> is sparse, and not always consistent.

#### 2.6.11 Active site topography:

The receptor allowed volume map 1 represents the allowed volume obtained by combining the volume map of allowed steroids. The map indicates a large hydrophobic cavity above the C-19 position where a C-19 trityl or silyl can reside. From this, considerable steric latitude is expected at this position, making plausible realignment of the steroid in the active site by derivatizing the C-19 position.

The C-19 can be transformed to a chemical trap by conjugation to a reactive moiety residing in the newly identified pocket. In addition, a (usually bulky) reporter group can be placed at C-19 to monitor the mechanism of aromatization.

Refinements of this allowed volume can be achieved by considering poor affinity inhibitors which must be near the outer boundaries of the acceptable cavity. Steroids which do not have any affinity for the enzyme presumably do not fit (or do not reach) the active center.

The sum total of the maps of inactive compounds defines the  $V_I$ . It is possible to determine the favorable volume, by subtracting the two maps to yield an excluded volume. Map 2 is  $V_{dis}$ , the enzyme disallowed volume where an inhibitor decreases affinity for the active site.

$V_{dis} = V_I - V_{all} =$  Volume responsible for negative interactions.

$V_{ess} = V_I \cap V_{all} =$  Receptor essential volume

Table 2.6.11: Mapping of the active center

| Cmpd | method | %Km | %Vmax | Ki $\mu$ M | Mode | ( $\mu$ M) Inhibitor |
|------|--------|-----|-------|------------|------|----------------------|
|------|--------|-----|-------|------------|------|----------------------|

Enzyme Allowed: Included Volume  $V_{all}$ : MAP 1

|    |   |     |     |    |         |                                |
|----|---|-----|-----|----|---------|--------------------------------|
| 74 | 3 | 191 | 105 | 12 | C (11)  | A-4,5 $\beta$ -epoxy-19-tBDMSO |
| 72 | 3 | 157 | 107 | 18 | C (10)  | A-4,5 $\beta$ -epoxy-19-OH     |
| 43 | 3 | 144 | 90  | 23 | C (10)  | A-19-TrO                       |
| 41 | 3 | 323 | 92  | 62 | C (139) | A-19-AcO                       |

Enzyme disallowed: Volume of inactive species  $V_I$ : MAP 2

|    |   |     |     |     |         |                                |
|----|---|-----|-----|-----|---------|--------------------------------|
| 37 | 3 | 138 | 100 | 279 | C (107) | A-19-tBDMSiO                   |
| 78 | 3 | 135 | 101 | 376 | C (131) | A-4,5 $\beta$ -epoxy-10-COOH   |
| 34 | 2 | 90  | 49  | 126 | V (131) | A-5 $\alpha$ -ol-19,4-O-19-MeO |

Enzyme Favorable: Electronic

|    |  |    |     |        |         |             |
|----|--|----|-----|--------|---------|-------------|
| 43 |  | 90 | 144 | 23     | C (10)  | A-19-Trityl |
| 41 |  | 92 | 323 | 62     | C (139) | A-19-Ac     |
| 42 |  | 79 | 68  | 87 Kik | M (41)  | A-19-Ms     |
| 39 |  | 56 | 165 | 61 Kik | M (40)  | A-19-MOM    |

Abbreviations and methods as for table 2.3.4.

**Figure 2.6.11.1: Enzyme Allowed: Included Volume  $V_{i1}$ :  
(two different perspectives)**

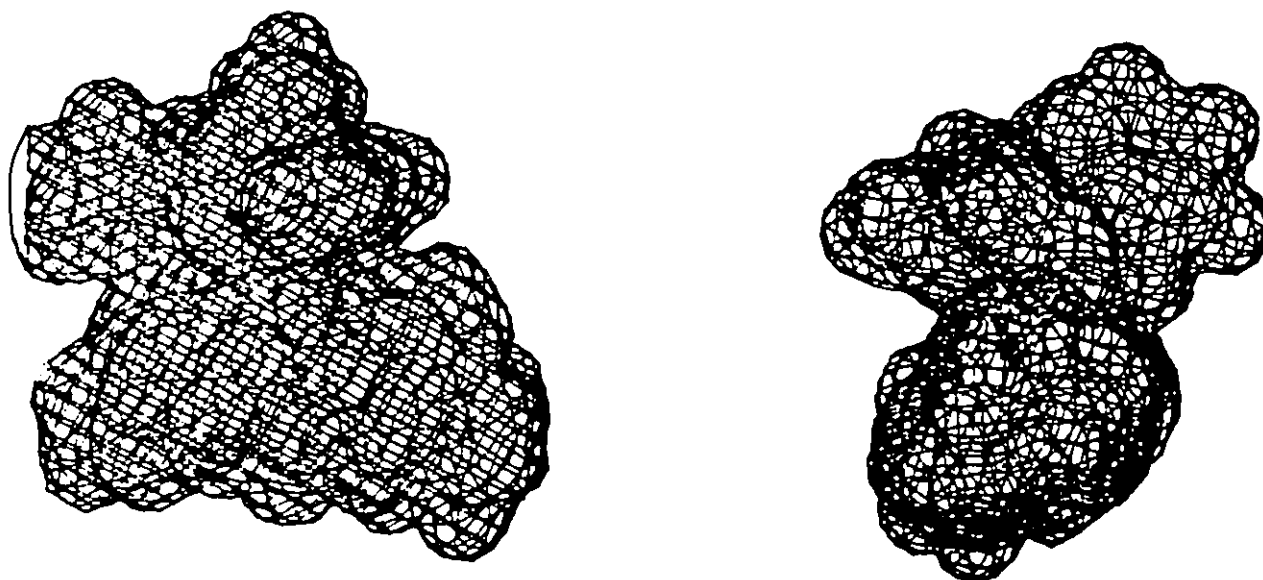
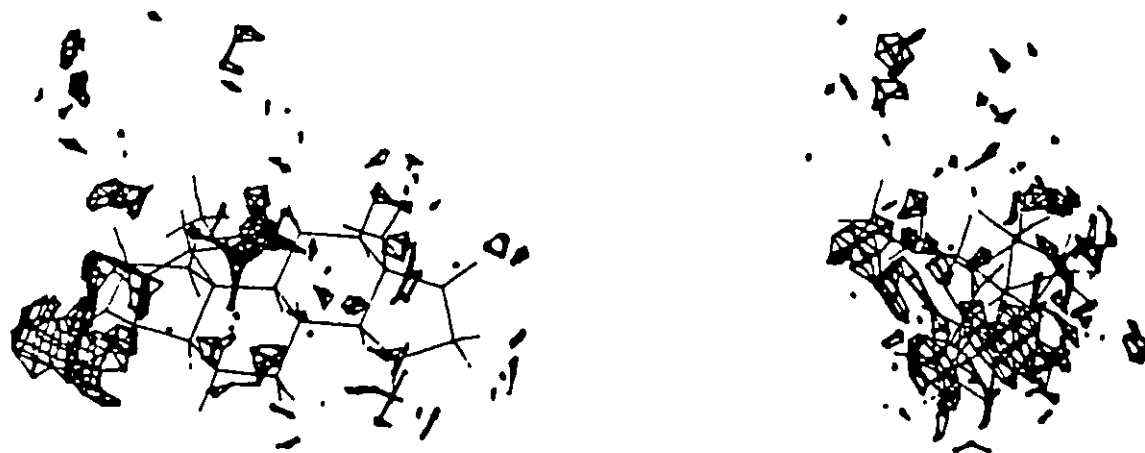


**Figure 2.6.11.2: Enzyme disallowed:  $V_{d1}$ .  
Difference Volume between Allowed and excluded species.  
(two different perspectives)**

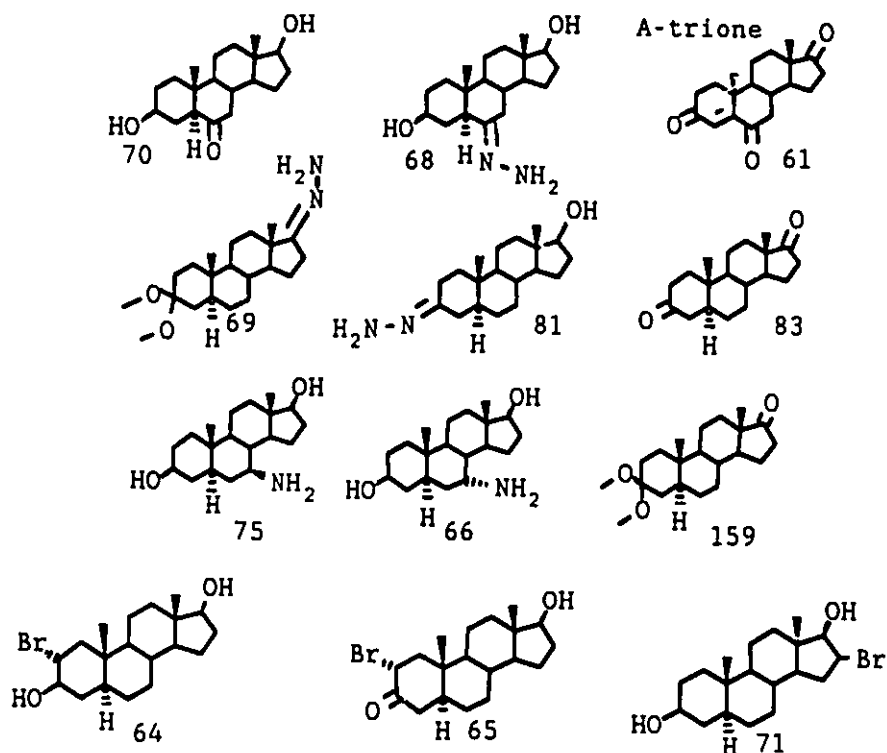


These maps were generated using the Sybill software. Electronic refinements were not included in this analysis. Analysis of these maps reveals that there may be an excluded area over ring-B There also appears to be a large disallowed pocket about the C-3 ketone of androstenedione derivatives. Both disallowed volumes are mostly due to compound 34 and are not conclusive at present. Indeed cross reference with other C-3 derivatives demonstrate that this exclusion is less restrictive on other androgen backbones.

## Chapter 2 Section 7

### 2.7.1 Generation of new lead compounds

Additional substituent screens were performed on various available steroids. These illustrated groups and functionality with a potential to elicit inhibition according to our previous analysis of the active center.



**Scheme 2.7.1:** Reduced steroids incubated with aromatase. Screening

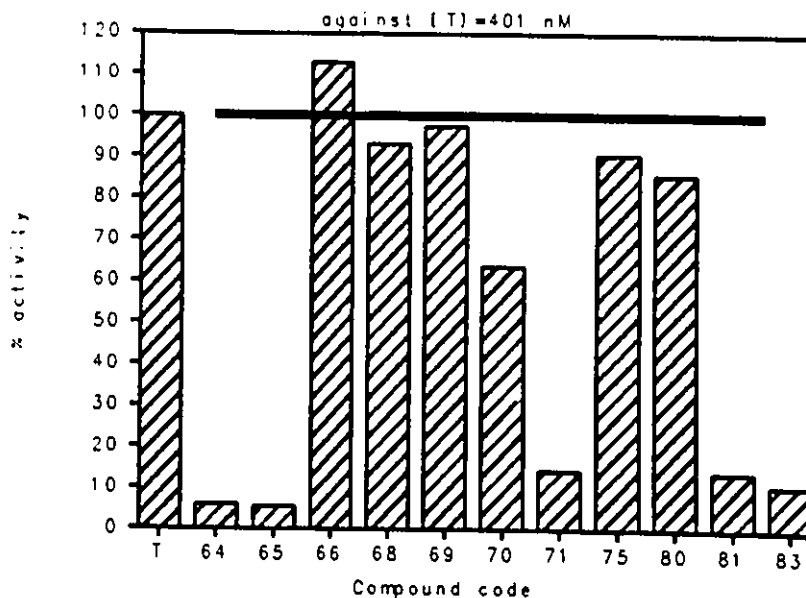


**Figure 2.7.1.1:** Activity of aromatase in the presence of potential inhibitors.

### 2.7.1 Involvement of a 6-keto in inhibition by androgens.

By combining interesting C-6 oxo, C-6 hydrazone or C-3 hydrazone with 5-hydrogen or 5-fluoride, this series possess both a potentially deactivating moiety and a reduced A-ring.

Reduction at the C-5 carbon is expected to lower the affinity for the active site. However, incubation of a modified A-ring steroid may clarify mechanisms involving a specific substituent by removing complicating factors from other substituents. This could for example distinguish the irreversible effect of a 6-ketone in A-trione **61** from the complicating interaction caused by a 6-keto-4-ene-3-one by analyzing compound **70**.

6-keto androgens are postulated to cause inactivation by direct enzyme nucleophile "1,2 addition" at the C-6 keto, or by "1,4 addition" to the enone. The absence of inactivation by epoxide inhibitors presented in the previous sections weaken the theory of a nearby nucleophile.

To assess whether inactivation can occur after removing the 4 double bond, **68** and **70** were incubated. Both resemble A-trione but lack the 4-ene-3-one moiety; if an enzyme linked nucleophile is involved by direct addition to the C-6, it may be trapped by the hydrazone; if the enzyme linked nucleophile is only available to react with the C-4 double bond, no inactivation could occur in the absence of this moiety.

The 6-hydrazone **68** (table 2.7.1) seems a true uncompetitive inhibitor, influencing the apparent Kapp and the apparent Vapp in the same proportion. Since there are no other regions where inactivation could readily occur on this molecule, the data confirms the possibility of intervention on the C-6 hydrazone moiety.

However, to reconcile the data on known C-6 derivatives with the results of the epoxide incubations, a mechanism not involving enzyme-nucleophile intervention must be invoked.

The approximate affinity constant  $K_i$  is expectably low, and the inactivation elucidated at a concentration of 117  $\mu\text{M}$  is 68% against testosterone. Inhibition by the control steroid 6-keto 70 shows some competitive activity. The free energy contribution for a substituent change from 6-C=O to 6-C=NNH<sub>2</sub> is -0.2 kcal/mol on the 5 $\alpha$ H-3,17-diol backbone.

Compound 70 is a reduced form of the potent A-trione. It clearly shows that the 4-en-3-one is required to potentiate the 6-keto inhibitory mechanism. Whether the difference in activity is due to the orientation in the active center would be surprising as the hydrazone displays some inactivation.

The 3-hydrazone 81 inactivates aromatase to the same extent as the natural substrate 83 which serves as a benchmark.

Table 2.7.1: C-5 reduced inhibitors: incubation results.

| Code         | {Inh.]Mode |               |   |       | $K_i$<br>M | %V      |       | Description                            |
|--------------|------------|---------------|---|-------|------------|---------|-------|----------------------------------------|
|              | Method     | $\mu\text{M}$ |   | %Kapp |            | T=401nM | %Vmax |                                        |
| 64           | 2          | 117           | U | 56%   | 1E-4       | 6       | 4     | 2 $\alpha$ -Bromo-3,17diol             |
| 65           | 1          | 117           | - | --    | ----       | 5       | --    | 2 $\alpha$ -Bromo-3-one-17-ol          |
| 66           | 2          | 115           | I | 73    | 3E-4       | 113     | 90    | 7 $\alpha$ -NH <sub>2</sub> -3,17-diol |
| 68           | 2          | 117           | U | 64    | 2E-4       | 93      | 68    | 6-NNH <sub>2</sub> -3,17-diol          |
| 69           | 2          | 117           | I | 100   | 2E-2       | 93      | 94    | 17-NNH <sub>2</sub> -3-DMO             |
| 70           | 2          | 128           | C | 223   | 1E-4       | 64      | 114   | 6-keto-3,17-diol                       |
| 71           | 2          | 145           | M | 303   | 7E-5 Kik   | 14      | 34    | 16-Bromo-3,17-diol                     |
| 75           | 2          | 152           | I | 132   | 5E-4       | 91      | 108   | 7 $\beta$ -NH <sub>2</sub> -3,17diol   |
| 81           | 1          | 129           | - | --    | ----       | 14      | --    | 3-NNH <sub>2</sub> -17-ol              |
| 83           | 1          | 124           | - | --    | ----       | 11      | --    | 3,17-dione                             |
| 159          | 1          | 101           | - | --    | ----       | 0%      | --    | 17-keto-3-DMO                          |
| Testosterone |            | C             |   |       | 8E-7       | 100     | 100   | Testosterone                           |

Abbreviations as in table 2.3.4. inhibitor mode: C competitive, "M" mixed, "N" noncompetitive, I insignificant effect; MeO-Methoxy; AcO-acetoxy; DMO-Dimethoxy.  $K_i$  is in moles/liter in this table.

A dramatic drop of inhibitory activity is observed with all 5 $\alpha$ -androstane derivatives screened here. The overlap with the parent **A-trione** illustrate the distortion of ring A. Differences at C-4 (0.44Å) and the O-3 oxygen are notable. The average deviation is 0.10Å and the RMS deviation 0.15Å. These positions are mobile, however, and can adopt the conformation of the parent enone. On first analysis it is thought that the steric overlap of most backbone atoms would be significant in solution; the major steric difference residing with the C-4 position, and the substitution to a C-3 and C-17 alcohol. These affect the dipole moments significantly, which may be the principal components accounting for the loss of biological response.

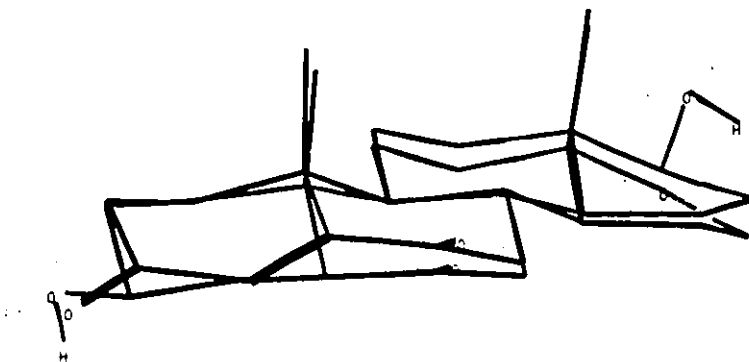


**Figure 2.7.1.2:** Overlap of A-trione and its reduced form, 5 $\alpha$ -androstane-6-one-3,17-diol 70.

5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol-6-one **70**, a C-3,17 reduced form of A-trione, displays competitive inhibition with a  $K_{app}$ =194%  $K_m$ , but loses the characteristic inactivation caused by the parent compound A-trione; reduction of the A ring lowers the affinity and the ability to inactivate the enzyme. The data shows unequivocally that the 4-ene-3-one is required for efficient inactivation by a C-6 ketone.

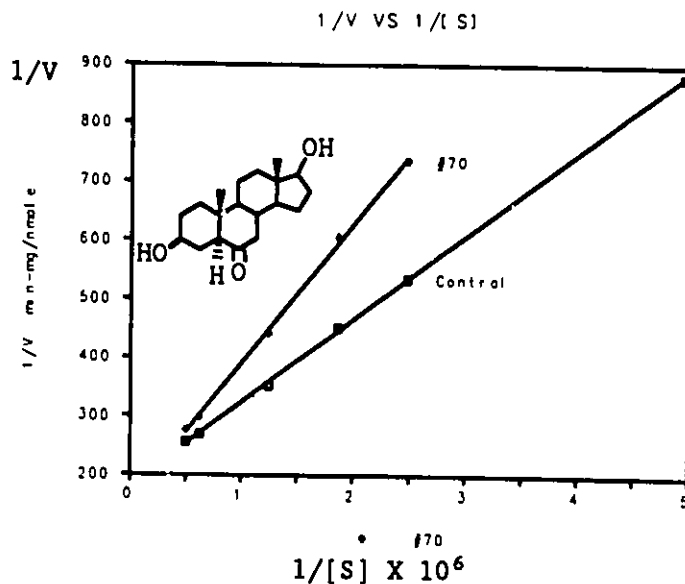


**Figure 2.7.1.3:**

Aromatase inhibition by **70**, 5 $\alpha$ -androstane-3 $\beta$ ,17 $\beta$ -diol-6-one.

| Compound  | V <sub>max</sub><br>nm/min/mg | K <sub>app</sub><br>nM | K <sub>i</sub><br>μM | Mode | Inhibitor<br>μM |
|-----------|-------------------------------|------------------------|----------------------|------|-----------------|
| Testos.   | 0.0055                        | 767                    |                      | —    | none            |
| ±STD      | ±.0001                        | ±23                    |                      |      |                 |
| <b>70</b> | 0.0064                        | 1490                   | 136                  | C    | 128             |
|           | ±.0001                        | ±88                    | ±106                 |      |                 |

Values are given ± one standard deviation. Incubation method 3; 20 μL [1 $\beta$ ,2 $\beta$ -<sup>3</sup>H] testosterone (0.1 μCi, 1.7nM), 40 μL testosterone (final concentration ranging from 201 to 2000 nM) is co-incubated with 40μL inhibitor (in ethanol) and human placental aromatase (70 μg protein) at 37 °C for 30 min in the presence of excess NADPH. Data analysis according to Eadie-Hofstee.

Inactivation kinetics of 3-hydrazido-5 $\alpha$ -androstan-17 $\beta$ -ol 81 was determined at 43  $\mu$ M. It shows a mild inactivation effect  $V_{app}=69\%V_{max}$ , and a clear affinity effect at  $K_{app}=387\%K_m$ . The affinity constant  $K_i$  can not be calculated directly with mixed kinetics. If the effect on  $K_{app}$  is computed independently from the effect on  $V_{app}$ , the  $K_{ik}=15$   $\mu$ M. If the effect on  $V_{app}$  is taken separately the  $K_{iv}=94\mu$ M. These approximations are made to allow some comparison between different categories of inhibitors. Compound 81 is thus a mixed inhibitor possessing a high affinity for the active center and mild inactivating capacity.

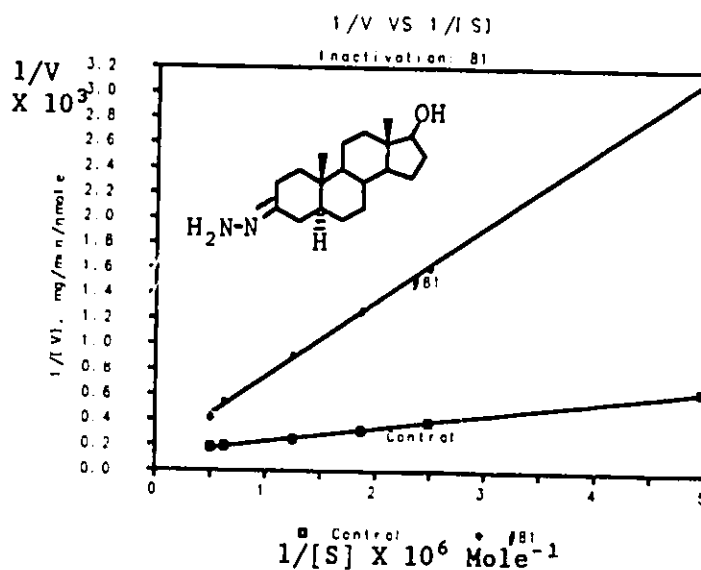


Figure 2.7.1.4: Aromatase inhibition by 81,  
3-hydrazido-5 $\alpha$ -androstan-17 $\beta$ -ol.

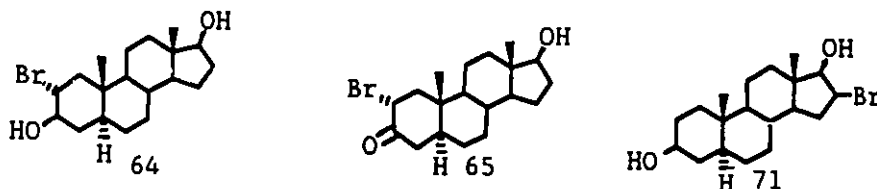
| Compound  | $V_{max}$<br>nm/min/mg | $K_{app}$<br>nM | $K_i$<br>$\mu$ M | Mode | Inhibitor<br>$\mu$ M |
|-----------|------------------------|-----------------|------------------|------|----------------------|
| Testos.   | 0.0078                 | 803             |                  | -    | none                 |
| $\pm$ STD | $\pm$ .0006            | $\pm$ 108       |                  |      |                      |
| 81        | 0.0054                 | 3110            | 15= $K_{ik}$     | "M"  | 43                   |
|           | $\pm$ .0006            | $\pm$ 448       | 94= $K_{iv}$     |      |                      |

Incubation method 3; see table 2.3.4.

Analysis according to Eisenthal.

Incubation for 45 minutes of a low concentration ( $1.3\mu\text{M}$ ) indicates no inhibition, even with a hydrazone to testosterone ratio of 759/1. This confirms that 81 is not a very efficient irreversible mixed inhibitor, since fixing a high ratio of inhibitor should favor inactivation of the enzyme without interference from the natural substrate.

### 2.7.2 Bromo-derived inhibitors.



The chemically more reactive bromo androstenedione might be of interest. In the preliminary screen (table 2.7.1) the 2 $\alpha$ -bromo-androgens 64, 65 and the 16-bromo 71 seemed to exhibit inactivation of aromatase, presumably by reaction at the carbon bearing bromide. To evaluate their potential clinical uses, 71 and 65 were incubated at concentrations of  $1\mu\text{M}$  and  $0.1\mu\text{M}$ . They caused a 40 to 60% inhibition of enzyme activity against  $1.7\text{ nM}$  testosterone.

The results indicate that these bromo inhibitors lower both the  $K_m$  and  $V_{max}$  of aromatase. Their clinical use may be weakened by the non-specific reactivity of alkylating agents.

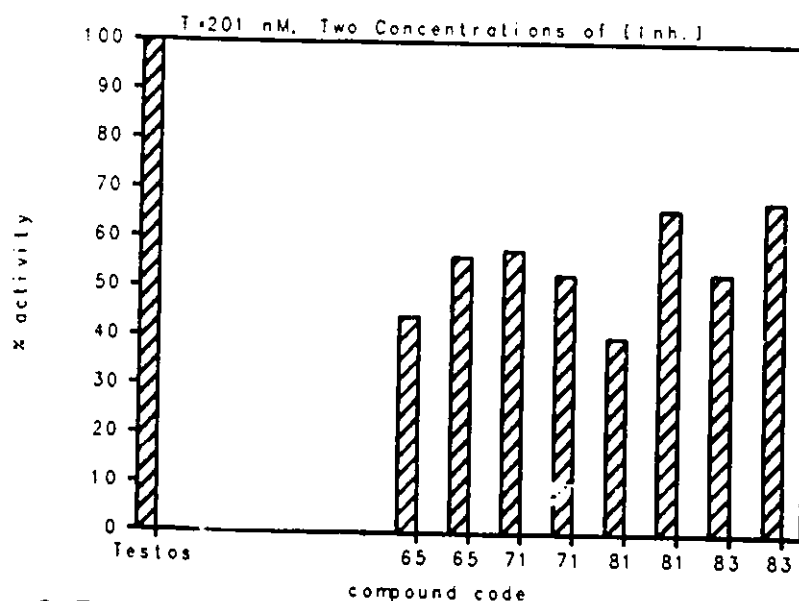


Figure 2.7.2.1: incubation of bromo steroids ; two concentrations of inhibitors.

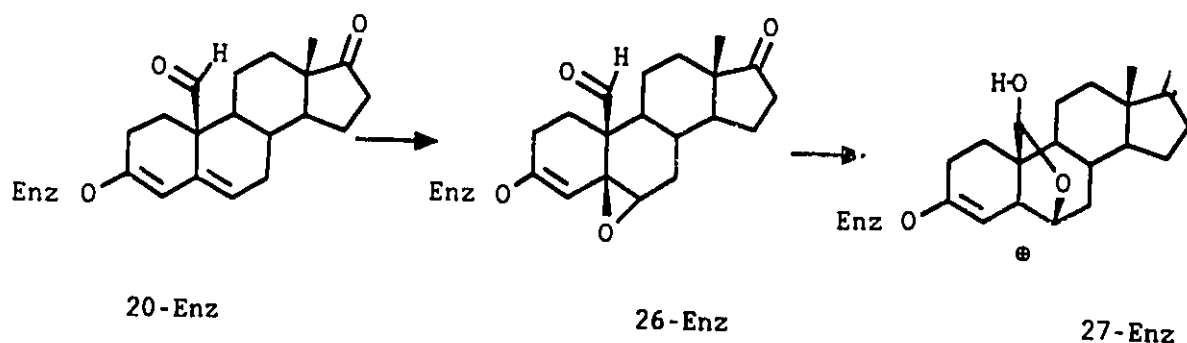
### 2.7.3 $\Delta^{1,4}$ -3-one and $\Delta^{4,6}$ -3-one inhibitors.

The most published aromatase pharmacophores involve a flat A ring with  $\Delta^{1,4}$ -3-one or  $\Delta^{4,6}$ -3-one functionality. Reasonable mechanisms of inactivation will be presented in chapter 3 (bioorganic chemistry). A  $\Delta^1$  releases -0.4 kcal/mol in affinity, and a  $\Delta^6$  releases -0.2 kcal/mol according to our compilation. This suggests that a flat A ring may be an interesting lead for improved binding properties.

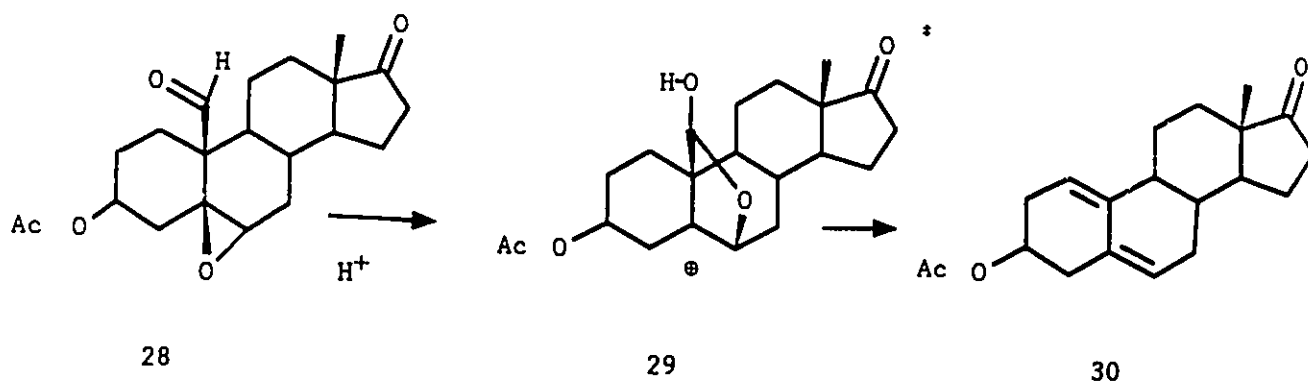
Commercial contraceptives nor-ethisterone, nor-ethisterone acetate, nor-ethynodrel and SCH10015 are inhibitors of aromatase. The  $17\alpha$ -alkyne is thought to be responsible for the prolonged biological activity.

Along such lines, the  $\Delta^{2,5(10)}$ -enol ethers **90** and **89**, analogues of 1,4 diene inhibitors, were tested for aromatase activity. These present the additional incentive that an electron rich 2,3-enol ether would perhaps trap a  $P_{450}$  activated oxygen species in the active center more readily than an 4-en-3-one. Both interact with aromatase, though the  $17\alpha$ -alkyne derivative exhibits much stronger inhibition than the 17-ethyl ether under high inhibitor to testosterone ratio.

Compound **91** was synthesized in our laboratory by Mastalerz as a potential intermediate-analogues. In this thesis it is considered both as a potential intermediate in C-6 keto inhibitor inactivation and as an intermediate in alternative reactivities to consider in aromatase mechanisms.



Under chemical  $\text{CF}_3\text{COOH}$  catalysis, the analogous **28** eliminates to the trans-diene **30**, probably via **29**.



**Scheme 2.7.3.1: Chemical Formation of trans diene from a 5,6 Epoxide**

The small and rigid A-ring of **30** is found to interact at the active center. Kinetics according to method 3 on **91** revealed little effect at  $10\mu\text{M}$  concentrations, but at higher concentrations showed inhibitory effects of the same order of magnitude as commercial contraceptives.

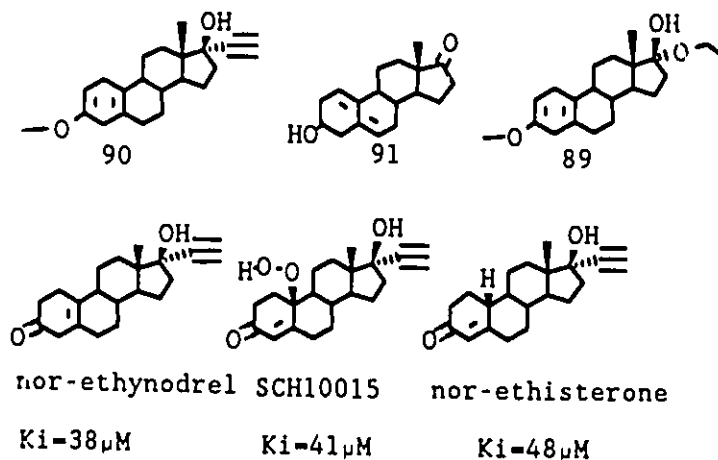
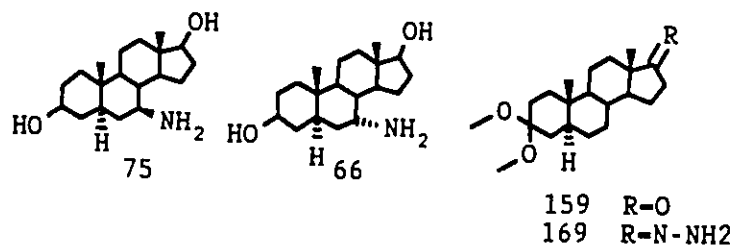


**Table 2.7.3: Inhibitors with A-ring dienes.**

| Cmpnd     | %V | [Inh.] $\mu\text{M}$ | Inhibitor                                                              |
|-----------|----|----------------------|------------------------------------------------------------------------|
| <b>91</b> | 24 | 119                  | estra-1(10),5(6)-diene-3-ol-17-one                                     |
| <b>89</b> | 73 | 116                  | 17 $\alpha$ -EtO-17 $\beta$ -OH-estra-5(10),2(3)-diene-3-MeO           |
| <b>90</b> | 11 | 115                  | 17 $\alpha$ -C $\equiv$ CH-17 $\beta$ -OH-estra-5(10),2(3)-diene-3-MeO |

Method 1: see table 2.3.4: high Inh./Test. ratio



#### 2.7.4 Amine and hydrazone inhibitors.

Surprisingly, the C-17-hydrazone-3-dimethoxyketal **69** causes little inhibition, whereas **159**, the control 17-keto-3-dimethoxyketal is a strong inhibitor under the same conditions.

Since the C-3 dimethoxyl substitution is acceptable in 159, the factor responsible for the lost of affinity must be the 17-hydrazine. It is well known that androgen-17-ol derivatives usually display affinity 3 times lower than 17-ketone derivatives: there may be a repulsion from an enzyme bound hydrogen donor near this position, to explain why the 17-hydrazone is "enzyme-disallowed".

The epimeric 7-amino 5 $\alpha$ -androstanes **75** and **66** elicited little effect. The 7 $\beta$ -amine **75** seems to cause no inactivation and is a very weak competitive inhibitor. The 7 $\alpha$ -amine **66** seems a "mixed" inhibitor displaying little inactivation and low affinity for aromatase.

A number of interesting lead compounds are uncovered in this series. Further work on these will be presented under a different cover.

## Chapter 2 Section 8: SUMMARY

A number of 19-derivatives have been synthesized and tested in this thesis. This has revealed a large cavity of about 6.3 Å near the C-19 position. Novel aromatase inhibitors have been uncovered. Some display extremely potent competitive inhibition, and some display "inactivation" kinetics.

Although crucial for strictly competitive inhibitors, a loss of affinity is largely compensated, from a pharmaceutical point, with mechanism based inhibitors that inactivate aromatase, such as the novel mixed mode inhibitors 19-TMSiO-A 38, 19-MOMO-A 39, 19-Mesylo-A 42, the 4-Cl-19-OH-A 44, the 19-mesyl-3,17-bisdithiolane 55, "uncompetitive" compounds 19-CHO-dithiane 59 and diethylereketal-5-ene-19-acetate 47. Bromo and hydrazone derivatives of androstane were also successful inactivating inhibitors.

### 2.8.1 Conclusions the biochemistry of novel aromatase inhibitors.

The main thrust of this thesis was directed at elucidating the aromatase mechanism of oxidation and finding new lead compounds capable of inhibiting the enzyme aromatase. In order to achieve this, over 80 different steroids were incubated with aromatase. A compilation of all the inhibitor data collected is found in this section.

Computer simulation and molecular overlap of steroidal and non-steroidal inhibitors led to postulate three aromatase steric and electronic considerations.

- 1) The C-3-chelate postulate
- 2) The C-5 Nucleophile postulate
- 3) The C-19-Cav postulate

### 2.8.2 The C-3 chelation postulate: (C-3-Chel postulate):

Chelation to the aromatase heme-iron was sought via inhibitor-bound moieties. A number of steroids were synthesized to test for the possibility of heme-iron chelation to the C-3 derivatives. Thiane and thiolane derivatives offered the additional advantage of doubling as reducing agents and free radical trapping agents.

Some dimethyl derivatives tested here proved to be extremely potent competitive inhibitors of aromatase. The ethyleneglycol series resulted in some competitive inhibitors. They could find use in pharmaceutical developments as they bear large C-19 derivatives. Such pending groups can protect from secondary or adverse reactions due to competing enzyme mobilizations: a large steroid which does fit aromatase may not fit the receptor sites of other enzymes. This is particularly important in many breast cancer patients who need to maintain a low aromatase activity yet require a balanced supplement of estrogenic hormones.

One test compound, 47, exhibited mixed mode inactivation and weak affinity. The mode of operation was not elucidated.

The dithiane or dithiolanes had the theoretical dual ability to chelate the iron-heme and to trap active center free radicals or active oxygen species.

Although there were interesting conclusions within this series as to the potentiating effect of different groups, these steroids do not seem to chelate to the heme-iron and do not exhibit good affinity nor inactivation, with the exception of a most interesting "uncompetitive" inhibitor 19-aldehyde-bisdithiane derivative 59 and the "mixed" mode inhibitor 19-mesylate bisdithiolane 55.

The other competitive inhibitors discovered in these series have served the purpose of disproving the C-3 chelate postulate which aimed at using a C-3 or C-17 handle to access and eventually block the heme-iron in the active center of aromatase. Offshoots of this series have not yet undergone biochemical testing. They are  $sp^2$  derived dithianes bearing heteroatoms extrapolated from the two "inactivating" steroids 59 and 55. These 19- $sp^2$  derivatives would display more latitude above the A/B plane, and perhaps more susceptibility to nucleophilic attack from an incoming enzymic nucleophile.

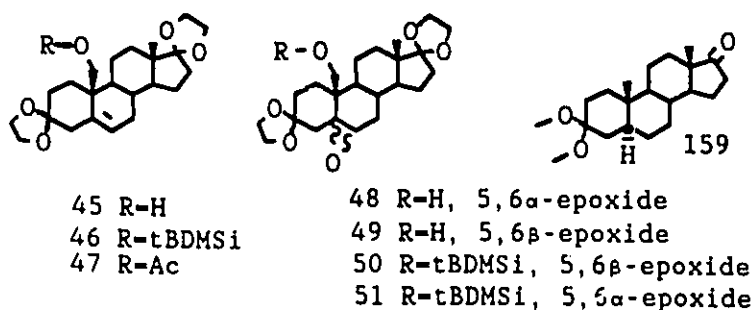


**Table 2.8.2.1:** Ketals: C-3 Ethyleneglycols (EG).

| Cmpd         | %Kapp<br>method | %Vapp | Ki<br>( $\mu$ M) | Mode | Inhibitor<br>( $\mu$ M)              |
|--------------|-----------------|-------|------------------|------|--------------------------------------|
| 45           | 3               | 211   | 105              | C    | (117)EG-19-OH-5,6-ene                |
| 46           | 1b              | 77    |                  | I    | (113)EG-19-tBDMSiO-5,6-ene           |
| 47           | 2               | 162   | 162 Kik          | M    | (101)EG-19-OAc-5,6-ene               |
| 48           | 1b              | 91    |                  | I    | (132)EG-5,6 $\alpha$ -epoxy-19-OH    |
| 49           | 1b              | 103   |                  | I    | (125)EG-5,6 $\beta$ -epoxy-19-OH     |
| 50           | 3               | 190   | 88               | C    | (80)EG-5,6 $\beta$ -epoxy-19-tBDMSO  |
| 51           | 3               | 155   | 83               | C    | (99)EG-5,6 $\alpha$ -epoxy-19-tBDMSO |
| 159          | 1a              | 0     |                  | C    | (101)5 $\alpha$ -A-3-DMO-17-one      |
| Testosterone |                 | 100   | 0.906            | -    | Testosterone control                 |

Abbreviations and method as for table 2.3.4. Incubation 1a against testosterone at 400 nM and 1b is against [T]=21.7 nM. EG- Androsta-3,17-diethyleneketal, DMO- Dimethoxy; tBDMSi- tertbutyldimethylsilyl ether; AcO- Acetate

Dithiane and dithiolane inhibitors synthesized.

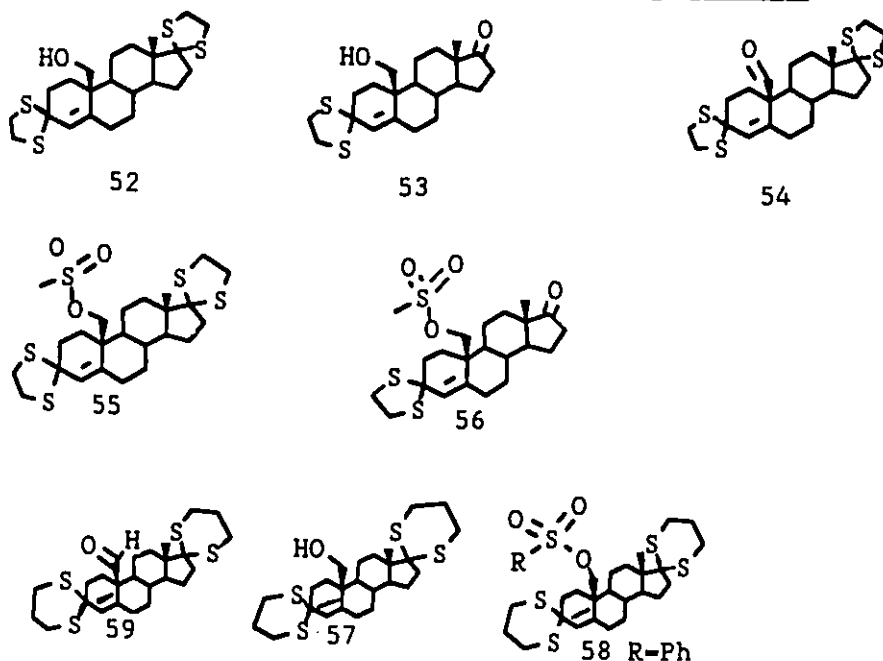


**Table 2.8.2.2:** Incubation results of dithianes and dithiolanes.

| Cmpd   | %Kapp | %Vmax | Ki $\mu$ M | Mode        | Inhibitor:androst-4-ene-<br>[ Inh. ] |
|--------|-------|-------|------------|-------------|--------------------------------------|
| method |       |       |            |             |                                      |
| 52     | 3     | 129%  | 91%        | 103 $\mu$ M | C (104) 19-OH-3,17-bisdithiolane     |
| 53     | 3     | 303   | 92         | 57          | C (116) 19-OH-3-dithiolane-17-one    |
| 57     | 3     | 218   | 116        | 101         | C (120) 19-OH-3,17-bisdithiane       |
| 45     | 3     | 211   | 119        | 105         | C (117) EG-19-OH-5,6-ene             |
| 54     | 3     | 101   | 111        | ----        | I (102) 19-CHO-3,17-bisdithiolane    |
| 59     | 3     | 77    | 78         | 168         | U (51) 19-CHO-3,17-bisdithiane       |
| 55     | 3     | 77    | 60         | 176 Kiv     | M (54) 19-Ms-3,17-bisdithiolane      |
| 56     | 3     | 173   | 97         | 171         | C (125) 19-Ms-3-dithiolane-17-one    |
| 58     | 2     | 92    | 65         | ----        | N (95) 19-Ts-3,17-bisdithiane        |
| 42     | 3     | 79    | 68         | 87          | M (41) 19-Ms-Androstenedione         |
| T      | 100   | 100   | 0.906      | -           | Testosterone control                 |

Abbreviations and method as for table 2.3.4 EG:

3,17-diethyleneketal, CHO- aldehyde; Ms Mesylate; Ts- Tosylate; U is uncompetitive; N is non competitive; M is mixed inhibition.

### 2.8.3 The C-5 nucleophile postulate: (C-5-Nu postulate):

An active site nucleophile has been postulated to exist near the C-4,5,6 position of the substrate. Two distinct categories of traps were synthesized to address this postulate: 4,5 and 5,6 steroidal epoxides.

These steroid classes demonstrated competitive kinetics only, indicating affinity for the active center, but no inactivation. The collected data disfavors inhibitor theories requiring a nearby nucleophile. Some members of this series of epoxides showed considerable affinity for the active site of aromatase, though they unfortunately did not orient in such a way as to cause inactivation. It is notable that neither the 4,5-epoxy-androgens nor the 5,6-epoxide-ethyleneglycol derivatives caused inactivation.

It is interesting to note that the addition of the epoxide groups increased the affinity for the active site by up to 15 times. This suggests a preference for polar groups above the 4,5 plane, away from the C-19,10,1 plane (ie. away from the C-19  $\beta$ -side). A compilation of literature and data gathered here seems to show a definite lowering of affinity with increased polarity above the C-19 position.

The following compounds were synthesized and incubated to reach these conclusions.

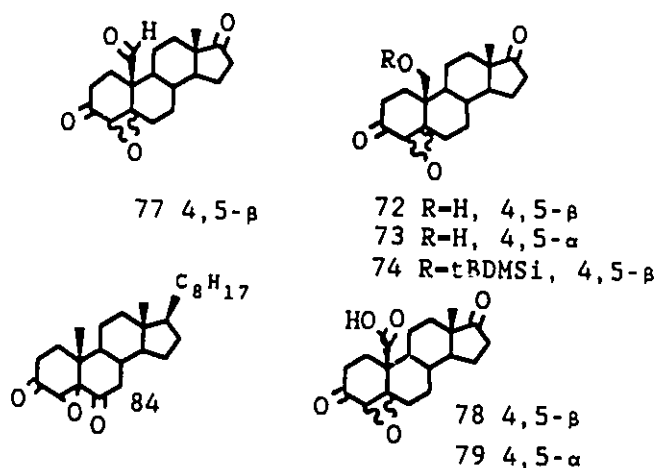


**Table 2.8.3:** Incubation results of androgen epoxide incubations.

| Cmpd   | %Kapp | %Vmax | Ki      | Mode | ( $\mu$ M) Inhibitor |
|--------|-------|-------|---------|------|----------------------|
| method |       |       | $\mu$ M |      |                      |
| T 3    | 100   | 100   | 0.906   | -    | Testosterone         |

**Androsta-5,6-epoxy-3,17-diethyleneketal-19-derivatives:**

|    |    |     |     |     |                                      |
|----|----|-----|-----|-----|--------------------------------------|
| 48 | 1b |     | 91  | I   | (132) 5,6 $\alpha$ -epoxy-19-OH      |
| 49 | 1b |     | 103 | I   | (125) 5,6 $\beta$ -epoxy-19-OH       |
| 50 | 3  | 190 | 113 | 88  | C (80) 5,6 $\beta$ -epoxy-19-tBDMSO  |
| 51 | 3  | 155 | 102 | 83  | C (99) 5,6 $\alpha$ -epoxy-19-tBDMSO |
| 84 | 3  | 83  | 84  | 224 | U (43) Chol-4,5-epoxy-3,6-oxo        |

**Androsta-4,5-epoxy-3,17-dione-19-derivatives:**

|    |   |     |     |     |                                     |
|----|---|-----|-----|-----|-------------------------------------|
| 72 | 3 | 157 | 107 | 18  | C (10) 4,5 $\beta$ -epoxy-19-OH     |
| 73 | 3 | 395 | 141 | 48  | C (144) 4,5 $\alpha$ -epoxy-19-OH   |
| 74 | 3 | 191 | 105 | 12  | C (11) 4,5 $\beta$ -epoxy-19-tBDMSO |
| 78 | 3 | 135 | 101 | 376 | C (131) 4,5 $\beta$ -epoxy-10-COOH  |
| 79 | 3 | 178 | 97  | 61  | C (48) 4,5 $\beta$ -epoxy-19-CHO    |

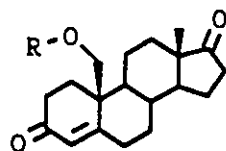
Abbreviations and methods as for table 2.3.4. I is insignificant effect. C is competitive effect. U is uncompetitive. Incubation 1b is against testosterone at 21.7 nM.

**2.8.4 The C-19 hydrophobic cavity postulate:  
(C-19-Cav. postulate):**

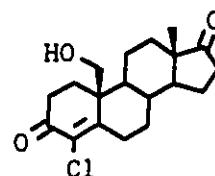
Incubation of steroids bearing large C-19 groups have confirmed our postulate that a large enzyme cavity, 6.3 angstrom deep, resides in the active center near the C-19 position. Steroidal C-19 acids, esters and ethers were synthesized and show considerable competitive inhibition. In addition, a number of test compounds bearing large C-19 groups were incubated.

These C-19 groups can be used to alter alignments of other pharmacophores, to increase the selectivity of an inhibitor for aromatase, lowering the side-effects due to competing enzyme modifications. Compound 43, the 19-trityloxy-derivative of androstenedione, could prove useful as a reporter group in future aromatase investigations.

This series of compounds has uncovered many potent competitive and inactivating pharmacophores. The 4-Cl-19-OH-A 44, 19-TMSiO-A 38 and 19-MOMO-A 39 derivatives may be mechanism based aromatase inactivators. The mesylate 19-MsO-A 42 exhibits uncompetitive-like kinetics, though it may be a straight alkylating agent causing inactivation. We have applied the discovery of a C-19 mesylate inhibitory activity to other series of derivatives with success.



- 41 R-Ac 37 R-tBDMSi  
42 R-Ms 38 R-TMSi  
43 R-Tr 39 R-MOM  
2 R-H



44

Table 2.8.4.1: 19-derivatives and analogues.

| Code | Method | [Inh.]  |           | Ki<br>μM                       | %V      |       | Description  |
|------|--------|---------|-----------|--------------------------------|---------|-------|--------------|
|      |        | M       | %Km, Mode |                                | T@401nM | %Vmax |              |
| 37   | 3      | 1.07E-4 | 138, C    | 279                            |         | 102%  | 19-TBDMS-A   |
| 38   | 3      | 9.70E-5 | 181, M    | 132 KiK<br>16 IC <sub>50</sub> |         | 29    | 19-TMS-A     |
| 39   | 3      | 4.00E-5 | 166, M    | 61 KiK<br>51 IC <sub>50</sub>  |         |       | 19-MOM-A     |
| 41   | 3      | 1.39E-4 | 323, C    | 62                             |         |       | 19-AcO-A     |
| 42   | 3      | 4.20E-5 | 67, M     | 87 KiK<br>63 IC <sub>50</sub>  |         |       | 19-Ms-A      |
| 43   | 3      | 1.02E-5 | 143, C    | 23                             |         |       | 19-Tr-A      |
| 44   | -      | 1.20E-6 | 36, M     | 1.2 IC <sub>50</sub>           |         | 47%   | 19-OH-4-Cl-A |
| 2    | 2      | 1.16E-4 | 1, -      | low                            |         | 0     | 19-OH-A      |
| T    | -      | -----   | ---, -    | ---                            | 100     | 100   | Testosterone |

Abbreviations and method as for table 2.3.4.; C competitive inhibition; M mixed inhibition; A- Androstenedione; MeO- Methoxy; AcO- acetoxy; Ms- Mesylate; TMS- Trimethylsilyl ether; tBDMS- tertButyldimethylsilyloxy ether; Tr- Trityloxy ether; MOM- Methoxymethyloxy ether.

This series of compounds were originally hoped to be intermediate analogues. Their low affinity, however, has made them mostly helpful to assess the active center topology via computer summations of appropriate volumes.

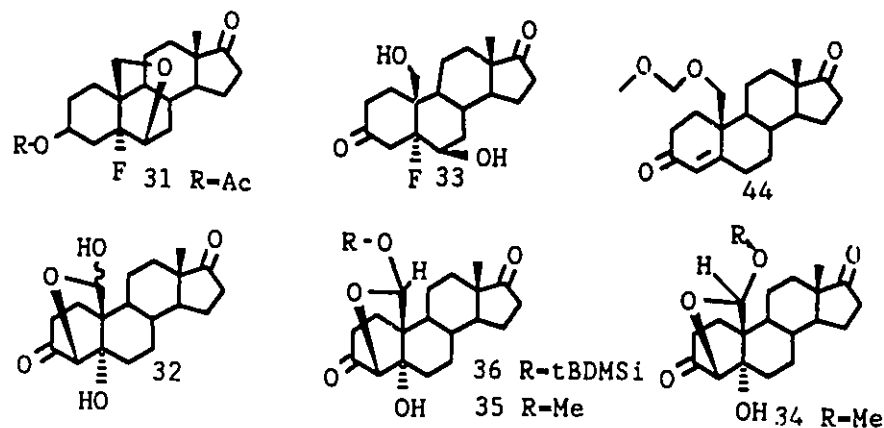


Table 2.8.4.2: Screening of 19,4-oxa and 19,6-oxa androgens.

| Code | [Inh.] | Mode | Ki | %V      |       | Description<br>(Androst-) |
|------|--------|------|----|---------|-------|---------------------------|
|      |        |      |    | T@401nM | %Vmax |                           |

Enzyme allowed: included in the active center:

|    |         |         |       |     |     |     |                                        |
|----|---------|---------|-------|-----|-----|-----|----------------------------------------|
| 31 | 1       | 1.19E-5 | ---   | --- | 79  | --- | 5 $\alpha$ -F-6,19-oxa-3-OAc           |
| 33 | 1       | 1.17E-5 | ---   | --- | 51  | --- | 5 $\alpha$ -F-6 $\beta$ ,19-diol-3-one |
| 35 | 2,3     | 1.29E-4 | C 156 | 229 | 21  | 99% | 5 $\alpha$ -ol-19,4-oxa-(19S)MeO       |
| 36 | 2       | 1.00E-4 | C 165 | 156 | 24  | 116 | 5 $\alpha$ -ol-19,4-oxa-(19S)tBDMS     |
| 39 | 1       | 1.20E-4 | ---   | --- | 0   | --- | 4-ene-3,17-dione-19-MOM                |
| T  | control | none    | ---   | --- | 100 | 100 | Testosterone control                   |

Enzyme excluded: Excluded from the active center:

|    |   |         |       |     |     |     |                                  |
|----|---|---------|-------|-----|-----|-----|----------------------------------|
| 32 | 1 | 1.16E-5 | I --- | --- | 117 | 100 | 5 $\alpha$ -ol-19,4-oxa-19-ol    |
| 34 | 2 | 1.31E-4 | N? 90 | 126 | 15  | 49  | 5 $\alpha$ -ol-19,4-oxa-(19R)MeO |

A screening of test compounds aimed at understanding the C-6-keto pharmacophore and perhaps discovering new lead compounds proved disappointing.

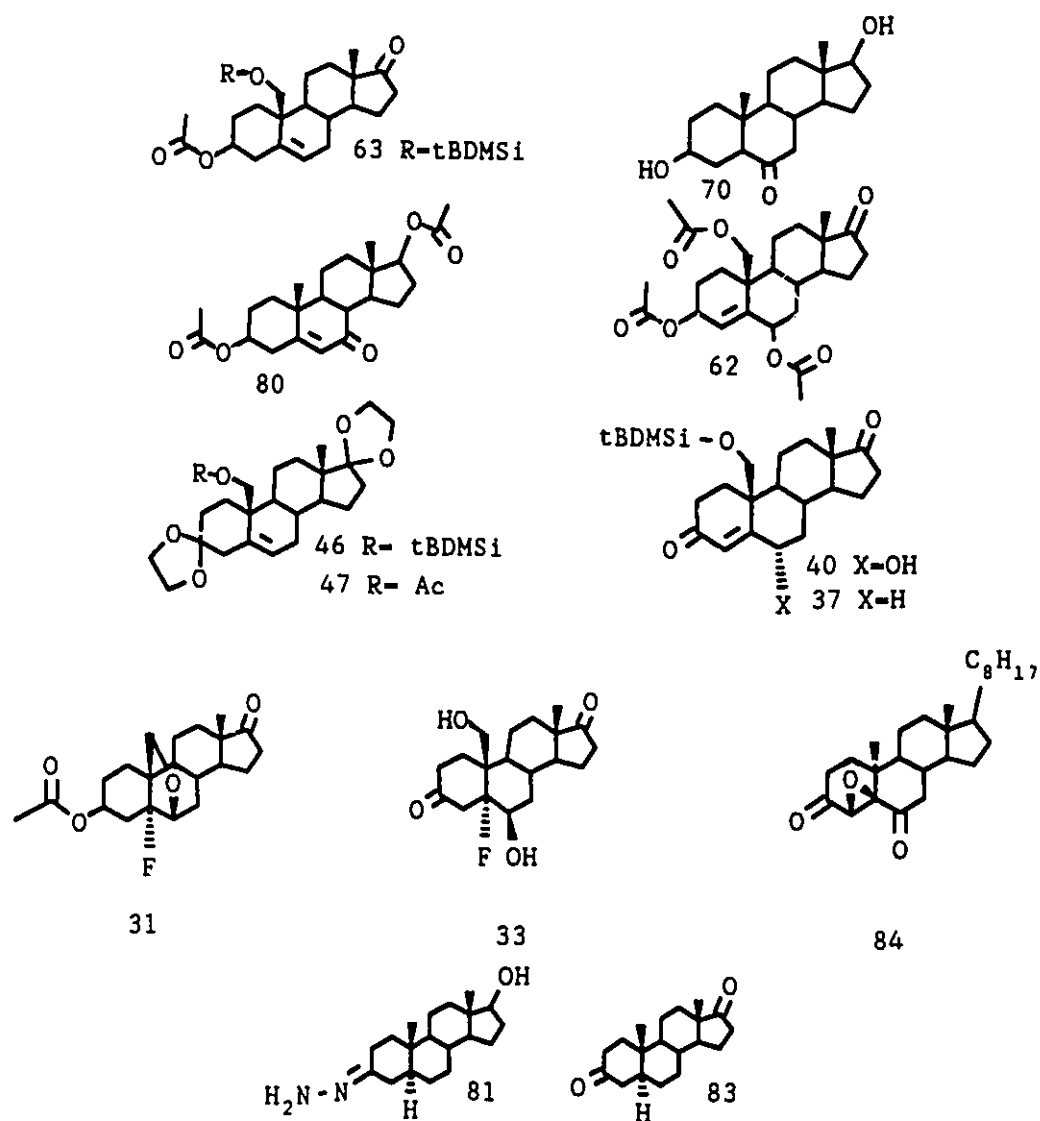


**Table 2.8.4.3:** Screening 19-derivatives with 6-pharmacophores.

| Code |        | [Inh.] |      | Ki  | %V      |       |                               |
|------|--------|--------|------|-----|---------|-------|-------------------------------|
|      | Method | μM     | Mode | μM  | T=401nM | %Vmax | Description                   |
| T    |        | none   | -    | 0.8 | 100     | 100   | Testosterone                  |
| 31   | 1      | 12     | -    | --- | 79      |       | A-5αF-3-OAc-6,19-ether-17-one |
| 33   | 1      | 12     | -    | --- | 51      |       | A-5αF-3,17-dione-6β,19-diol   |
| 40   | 3      | 143    | C    | 121 | 55*     |       | A-19-tBDMSi-6α-ol             |
| 46   | 1a     | 113    | -    | --- | 77*     |       | EG-5,6-ene-19-tBDMSi          |
| 47   | 2      | 101    | M    | 163 | 67      |       | EG-5,6-ene-19-OAc             |
| 62   | 2      | 116    | C    | 73  | 98      |       | A-3,6,19-OAc                  |
| 63   | 3      | 39     | N    | 212 | 38      |       | A-3-OAc-5,6-ene-19-tBDMSiO    |
| 70   | 3      | 128    | C    | 105 | 64      | 114   | A-3-ol-5α-H-6-one             |
| 80   | 2      | 116    | C    | 127 | 85      | 138   | A-6-ene-7-keto                |
| 83   | 1      | 124    | -    | --- | 11      |       | A-5α-H-3,17-dione             |
| 84   | 3      | 43     | U    | 224 | 11      |       | Chol-4,5β-epoxy-3,6-dione     |

### 2.8.5: The search for other lead compounds.

Some dienes were included in the search of lead compounds. An intermediate similar to compound 91 has been invoked in the mechanism of inactivation by C-6-keto pharmacophores. Incubation showed that it exhibits a good affinity for the aromatase active center.

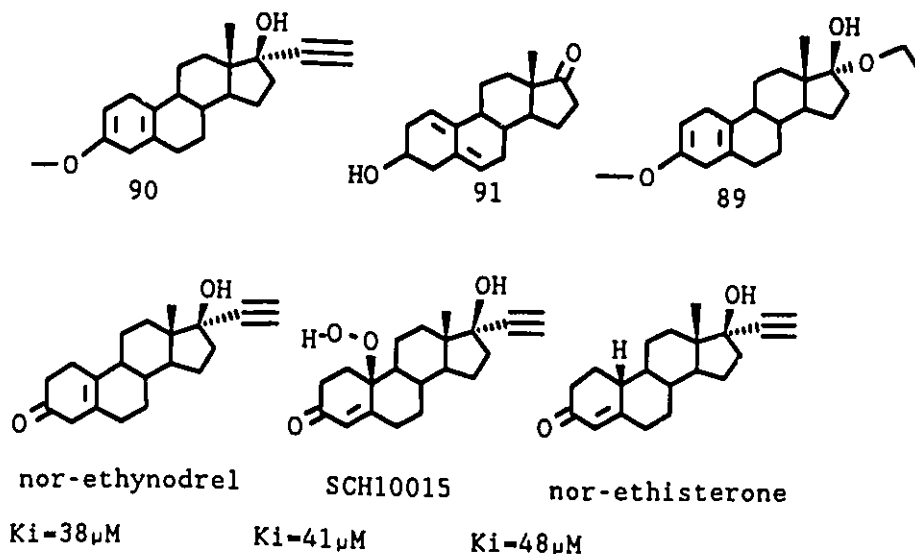


**Table 2.8.5.1:** Inhibitors with A-ring dienes.

| Cmpnd | %V | [Inh.]<br>$\mu$ M | Inhibitor                                                              |
|-------|----|-------------------|------------------------------------------------------------------------|
| 91    | 24 | 119               | estr-1(10),5(6)-diene-3-ol-17-one                                      |
| 89    | 73 | 116               | 17 $\alpha$ -EtO-17 $\beta$ -OH-estra-5(10),2(3)-diene-3-MeO           |
| 90    | 11 | 115               | 17 $\alpha$ -C $\equiv$ CH-17 $\beta$ -OH-estra-5(10),2(3)-diene-3-MeO |

Method 1: see table 2.3.4: high Inh./Test. ratio

Other lead compounds were sought. They proved most interesting in assessing the requirements of the intact conjugated enone for activity of A-trione. Hydrazones at this position, and some bromo derived androgens were also found to be potent aromatase inactivating compounds. Their general alkylating potential, however, may preclude them from having pharmaceutical uses except in research.

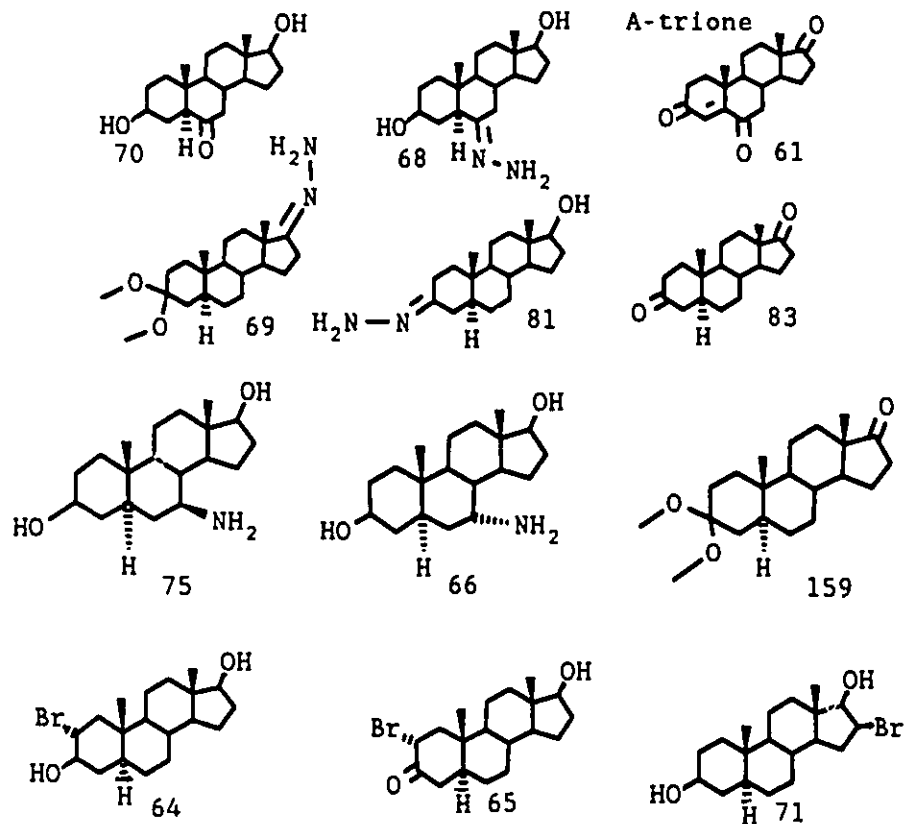


**Table 2.8.5.2: C-5 reduced inhibitors: incubation results.**

| Code         | [Inh.]        | Mode  |   | Ki  | %V                      | Description           |     |                                        |
|--------------|---------------|-------|---|-----|-------------------------|-----------------------|-----|----------------------------------------|
| Method       | $\mu\text{M}$ | %Kapp |   | M   | T=401nM %Vmax           | 5 $\alpha$ -Androsta- |     |                                        |
| 64           | 2             | 117   | U | 56% | 1E-4                    | 6                     | 4   | 2 $\alpha$ -Bromo-3,17-diol            |
| 65           | 1             | 117   | - | --  | ----                    | 5                     | --  | 2 $\alpha$ -Bromo-3-one-17-ol          |
| 66           | 2             | 115   | I | 73  | 3E-4                    | 113                   | 90  | 7 $\alpha$ -NH <sub>2</sub> -3,17-diol |
| 68           | 2             | 117   | U | 64  | 2E-4                    | 93                    | 68  | 6-NNH <sub>2</sub> -3,17-diol          |
| 69           | 2             | 117   | I | 100 | 2E-2                    | 93                    | 94  | 17-NNH <sub>2</sub> -3-DMO             |
| 70           | 3             | 128   | C | 136 | 1.36E-4                 | 64                    | --  | 6-keto-3,17-diol                       |
| 71           | 2             | 145   | M | 303 | 7E-5 KiK                | 14                    | 34  | 16-Bromo-3,17-diol                     |
| 75           | 2             | 152   | I | 132 | 5E-4                    | 91                    | 108 | 7 $\beta$ -NH <sub>2</sub> -3,17diol   |
| 81           | 1             | 129   | M | 387 | 1.5E-5 KiK              |                       | 69  | 3-NNH <sub>2</sub> -17-ol              |
| 81           |               |       |   |     | 9.4E-5 IC <sub>50</sub> |                       |     | 3-NNH <sub>2</sub> -17-ol              |
| 83           | 1             | 124   | - | --  | ----                    | 11                    | -   | 3,17-dione                             |
| 159          | 1             | 101   | - | --  | ----                    | 0%                    | -   | 17-keto-3-DMO                          |
| Testosterone |               |       |   |     | 8E-7                    | 100                   | 100 | Testosterone                           |

Abbreviations as in table 2.3.4. inhibitor mode: C competitive, M mixed, N noncompetitive, I insignificant effect; MeO-Methoxy; AcO-acetoxy; DMO-1-methoxy. Ki is in moles/liter in this table.

**BIBLIOGRAPHY**  
**CHAPTER 2**

1. Caspi E., Arunalacham T., Nelson P., (1986)  
J. Am. Chem. Soc., 108, 1847
2. Holland H.L., Taylor G. (1981) Can.J.Chem., 59, 2809
3. Kelly W.G., Judd D., Stolee A. (1977) Biochemistry, 16, 140
4. Marcotte P.A., Robinson C.H. (1982) Biochemistry 21, 2773 also  
Cancer research (1982) (suppl), 42, 3327s
5. (a) Northrop D.B., (1975), Biochemistry 14, 2644 (b) Northrop  
D.B., Miwa G.T. Garland W.A., Hodshon B.J., Lu A.Y.H.. (1980)  
J. Biol. Chem. 255, 6049
6. Braselton E., Engel L.L., Orr J.C., (1974)  
Eur. J. Biochem., 48, 35
7. Thompson E.A., Siiteri P.K. (1974) J. Biol. Chem 249, 5364
8. (a) Brodie A.M.H., Wing L.Y., Goss, P. Dowsett M., Coombes R.C.  
(1986) J. Steroid Biochem. 25, 859-865 (b) Kaburagi Y., Marino  
M., Kirdani R., Greco J.P., Karr J.P., Sandberg A.A. (1987)  
J. Steroid Biochem. (1987) 26, 739-742
9. Webb, J.L.: (1963) Enzymes and metabolic inhibitors, Academic  
Press also P.D. Boyer, ed.: (1970) The Enzymes, vol. 1.  
Structure and Control, Academic Press, also I.H. Segel  
(1975), Enzyme kinetics, Wiley.
10. R. McGilvery (1970), p. 297 in "Biochemistry, a Functional  
Approach", W.B. Saunders Toronto
11. Walsh C.T. (1984) Ann. Rev. Biochem. 53, 493  
for inactivation kinetics see refs. 9-14 therein
12. Lehninger A.L. (1978) 220, Biochemistry, Worth Publishers, N.Y.
13. Reed K.C., Ohno S. (1976) J. Biol. Chem. 251, 1625-1631 also ref. 7
14. Eisenthal R., Cornish-Bowden A., (1974) Biochem J., 139, 715-720
15. Fishman J., Miyairi S. (1986) J. Biol. Chem. 261, 6772-6777
16. (a) Beusen D., Ph.D. Thesis, (1985), Washington University, USA  
(b) Henderson D., habenicht U.-F., Nishino Y., Kerb U., Etreby  
M.F. El (1986) J. Steroid Biochem. 25, 867-876 (c) Sheean L.A.,  
Meigs R.A. (1983) Steroids, 41 223-241 (d) Engel L.L.,  
Handbook of Physiology, Endocrinology II, Part 1,  
Physiological Society, 474 (1973), Ed.: R.O. Greep (e) Brodie  
A.M.H., Wing L.Y., Goss, P. Dowsett M., Coombes R.C. (1986)  
J. Steroid Biochem 24, 91-96
17. Coombes R.C., Dowsett M., Goss, P., Brodie A.M.H., Gazet J.C.,  
(1984) Lancet, 2, 1237-1239
18. Covey D.F., Hood W.F. (1982) Cancer research (suppl), 42, 3327s
19. Guicidi D., Ornati G., Briatico G., Buzzetti F., Lombardi P.,  
di Salle E. (1988) J. Steroid Biochem. 30, 387-389
20. Henderson D., Norbistrath G., Kerb U., (1986)  
J. Ster. Biochem., 24, 303-306
21. (a) Osawa Y., Yarbrough C., Osawa, Y. (1982) Science, 215,  
1249-1251 (b) Osawa Y., Osawa Y., Yarbrough C., Borzinsky L.  
(1981) Biochem. Soc. Trans. 604 th. meeting Cambridge 11, 656
22. (a) Siiteri P.K., Thompson E.A. (1975) J. Steroid Biochem. 6,  
317-322 (b) Hillier (1980) J. Endocrinology 69, 409 (c) Perel  
B., Stolee K.H., Kharlip L., Blackstein M.E., Kilinger D.W.  
(1984), 58, 467-472 also Kuhnle R., Delemarre J.F.M., Rao  
B.R., Stolk J.G., (1986) Anticancer Research, 6, 889-892
23. (a) Abul-Hajj Y.J. (1986) J. Med. Chem. 29, 582-584  
(b) Abul-Hajj Y.J. (1983) Steroids 41, 783-790;
24. Covey D.F., Hood W.F. (1981) Endocrinology, 108, 1597-1599

# BIBLIOGRAPHY CHAPTER 2

25. Lombardi P., VII Conference on Hormonal Steroids, Spain 1986
26. Marsh D.A., Brodie H.J., Garret W., Tsai-Morris C.-H.,  
Brodie A.M. (1985) *J. Med. Chem.* 28, 788-795
27. Burhart J.P., Weintraub P.M. (1985) *Steroids* 45, 357-374
28. Morato T., Raab K., Brodie H., Hayano M., Dorfman R.,  
*J. Am. Chem. Soc.*, 84, 3764 (1962)
29. Tan L., Petit A. (1985) *Biochem. Biophys. Res. Commun.* 128, p. 613-620
30. Leung C.S., Rowlands C.S., Jarman M., Foster A.B., Griggs L.G.,  
Wilman D.E.V. (1987) *J. Med. Chem.* 30, 1550-1554
31. Kellis J.T., Vickery L.E., (1984) *Endocrinology* 114, 2128
32. Pourgholami M.H., Nicholls P.J., Smith H.J., Daly M.J., Dyas J.  
(1987) *J. Steroid Biochem.* 26, 309 also *J. Med. Chem.* (1986) 29, 520
33. (a) Kellis J.T., Vickery L.E. (1984) *Science* 225, 1032  
(b) Kellis J.T., Vickery L.E. (1987) *J. Biol. Chem.* 262, 4413  
(c) Wakeling, VII conference on hormonal steroids, Spain 1986  
(d) Kellis J.T., Nesnow S., Vickery L.E. (1986) *Bioch. Pharmacol.* 35, 2887-2891
34. Kellis J.T. Jr., Vickery L.E. (1987) *J. Biol. Chem.* 262, 8840-8844
35. Bellino F.L., Gilani S.S.H., Eng S.S., Osawa Y., Duax W.L.  
(1976) *Biochem.* 15, 4730
36. (a) Osawa Y., Osawa Y., Coon M.J. (1987) *Endocrinology* 121, 1010  
(b) Tochigi B., Osawa Y., Takagi S. (1978) 5th Int'l Congress  
on Hormonal Steroids, New Delhi, India p. 820
37. Marcotte P.A., Robinson C.H. (1982) *Biochemistry* 21, 2773 see  
also ref. 18 above.
38. Covey D.F., Hood W.F., Parikh V.D. (1981)  
*J. Biol. Chem.* 256, 1076-1079
39. (a) Metcalf B.W., Wright C.L., Burkhart J.P., Johnston  
J.O. (1981) *J. Am. Chem. Soc.* 103, 3221 (b) Johnston J.O., Wright  
C.L., Metcalf B.W. (1984) *Endocrinology* 115, 776-785
40. Brodie A.M.H. (1981) *Steroids* 38, 693-703
41. (a) Schwarzel W.C., Kruggel W.G., Brodie H.J. (1973)  
*Endocrinology*, 92, 866-880 (b) Abul-Hajj Y.J. (1983)  
*Steroids*, 41, 783-790
42. (a) Bruggemeier R., Floyd E., Counsell R.E. (1978) *J. Med. Chem.*  
21, 1007-1011 (b) Snider C.E., Bruggemeier R.W. (1985)  
*J. Steroid Biochem.* 22, 325-330 (c) Bruggemeier R.W., Snider  
C.E., Kimball J.G. (1982) *Steroids* 40, 679-689 (d) Snider C.,  
Ph.D. thesis, (1986) Ohio State University
43. Solo A.J., Caroli C., Darby M.V., McKay T., Slawnwhite W.D.,  
Hebborn P. (1982) *Steroids* 40, 603-614
44. Abul-Hajj Y.J. (1986) *J. Med. Chem.* 29, 582-584
45. Kellis J.T., Vickery L.E., (1987) *J. Biol. Chem.* 262, 4421-4426
46. Cramer R.D. III (1988) *J. Am. Chem. Soc.* 110, 5959-5962
47. Covey D.F., Hood W.H., Beusen D.D., Carrell H.L. (1984)  
*Biochemistry* 23, 5398-5406
48. Covey D.F., Hood W.H., McMullan P.C. (1986) *Biochem. Pharmacol.* 35, 1671-1674
49. Marcotte P.A., Robinson C.H. (1982) *Steroids* 39, 325-355
50. Bednarski P.J., Porubek D.J., Nelson S.D. (1985)  
*J. Med. Chem.* 28, 775-779
51. Flynn G.A., Johnston J.O., Wright C.L., Metcalf B.W. (1981)  
*Biochem. Biophys. Res. Commun.* 103, 913

**BIBLIOGRAPHY**  
**CHAPTER 2**

52. Lovett J.A., Darby M.V., Counsell R.E. (1984)  
J.Med.Chem. 27, 734-740
53. Yamamoto T., Yoshiji S., Urabe M., Shiroshita K., Fuji M., Honjo H., Okada H. (1987) Acta Obst.Gyneac. Jpn., 39, 849-852
54. Nishino Y., Kerb U., Henderson D.A., Schillinger E., Beier S., Weichert R., Neumann, El Etreyby M.F. (1986) Endocrinology '85, p. 443-446, Eds.; G.M.Molinatti and L. Martini, Elsevier
55. Ayub M., Stinch R., (1986) J.Steroid Biochem. 25, 981-984
56. Akhtar M. (1985) J.Chem.Soc.Chem.Comm., 1733
57. Baldwin J.E., J.Chem.Soc.Chem.Comm. (1976) 734, 736, 738 and J.Chem.Soc.Chem.Comm. (1977), 233
58. Covey D.F., Beusen D.D., Hood W.H., (1982) Fed.Proc. 41, 882 (abstract 3582)
59. Beusen D.D., Carrell H.L., Covey D.F. (1984) Biochemistry 26, 7833-7841
60. F.P.Guengerich, Willard R.J., Shea J.P., Richards L.E., T.L.Macdonald (1984) J.Am.Chem.Soc. 106, 6446-6447.
61. Corey E.J., Venkateswarlu A., (1972) J.Am.Chem.Soc. 94, 6190  
see also H.Mastalerz, Ph.D. Thesis, U. of Ottawa, (1981)
62. Guindon Y., Fortin R., Yoakim C., Gillard J.W. (1984) Tetrahedron Lett. 42, 4717 and refs. therein
63. Williams J.R., Sarkinian G.M. (1974) Synthesis 3332
64. Ringold H.J., Malhotra S.K. (1962) Tetrahedron Lett. 15, 669
65. Kirk D.N., Hartshorn M.P., Steroid Reaction Mechanism, Monograph 7 in Reaction Mechanisms in Organic Chemistry, 1968, Elsevier, New York.
66. (a) H.Mastalerz, Ph.D. Thesis, University of Ottawa, (1981), 59  
(b) Henbest H.B., Jackson W.R. (1967) J.Chem. Soc. C, 2459
67. Kellis J.T., Nesnow S., Vickery L.E. (1986) Bioch. Pharmacol. 35, 2887-2891
68. Nogardy T. Medicinal Chemistry, p.457
69. Schultz G.E., Schirmer R.H. in "Principles of Protein Structure", (1979) Springer Verlag, N.Y.
70. Simpson E.R., Evans C., Corbin C., Powell F., Lesdana D., Mendelson C.R. (1987) Mol. Cell. Endocrinol. 52, 267-272
71. Wolff M.E. in "Quantitative Structure-Activity Relationships of Drugs" (Ed. Topliss J.G.) Chapter 9, pp. 351-388, 1983, Academic Press, New York
72. Covey D.F. in " Sterol Biosynthesis Inhibitors" (Ed; Brodie A.M.H.), Ch. 22, 534-571 (1988) Ellis Horwood Ltd., Cambridge
73. Nelson S.D., Bednarski P.J. (1989) J.Med.Chem. 32, 203
74. (a) Robinson C.H., Childers W. (1987) J.Chem.Soc.Chem.Comm. 320  
(b) Robinson C.H., Furth P.S. (1989) Biochemistry 28, 1254
75. Numazawa N., Tsuji M., Osawa Y., (1986) Steroids 48, V.5-6, p.346
76. Mann J., Pietrzak B. (1983) J.Chem.Soc.Perkin Trans.I, 2681
77. Covey D.F., Wood W.F. (1982) Molecular Pharmacol. 21, 173-180
78. Robinson C.H., Newitt J. (1986) Fed.Proc. 45, 1885 (abstract 2362)
79. Raag R., Poulos T.L. (1989) Biochemistry 28, 917
80. Halpern O., Delfin I., Magana L., Bowers A. (1966) J.Org.Chem. 31, 693-710
81. Mann J., Drew M., Pietrzak B. (1985) J.Chem.Soc.Chem.Comm. 1191
82. Mansuy M. (1984) J.Am.Chem.Soc., 106, 1699

**3.1 The bioorganic chemistry of oxygen bearing steroids.**

In this section, computer analysis of oxygen-bearing aromatase intermediates and inhibitors, along with some investigations into their bioorganic chemistry and reactivity are presented. Sulfur bearing compounds are presented in the next section.

**3.1.1 Reactions of**

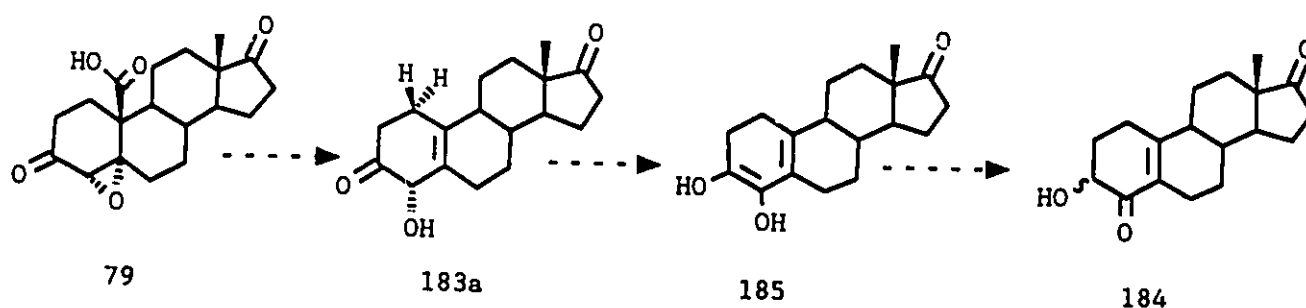
**19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androsterane-3,17-dione 79.**

**Thermal degradation of 79.**

Synthesis and comparison of 79 and 78, the epoxide isomers of 19-carboxy-4,5-epoxy-5 $\alpha$ -androsterane-3,17-dione, confirmed that the novel  $\alpha$ -epoxy-acid 79 undergoes thermal degradation at a temperature 100 degrees lower than the  $\beta$ -epoxy-acid 78.

The higher reactivity of the  $\alpha$ -epoxide 79 can be expected from the possible trans-anti parallel arrangement of the C-10,19 bond and the C-5,Oxygen epoxide bond.

Decarboxylation is followed by epoxide ring opening. When the resulting complex mixture were chromatographed, the minor reaction products seemed converted to the major product estrone. The presumed intermediate 183 and the rearranged 184 were isolated.



**Scheme 3.1.1: Thermal degradation of  
19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstane-3,17-dione. 79**

A suspension of the acid **79** (260 mg) in freshly distilled decalin (b.p. 195°C) was heated at reflux under nitrogen. The reaction was followed by TLC on SiO<sub>2</sub> (CHCl<sub>3</sub>). After one hour, the reaction mixture was chromatographed by Chromatotron using CH<sub>2</sub>Cl<sub>2</sub> and MeOH (0%–5%) as the eluent. In addition to separating estrone, this approach appeared to concentrate one of the desired major compounds.

Past experience with the difficulty in separating the isomers of **184** was confirmed as an apparently clean band again showed two spots by TLC after purification on silica. Chromatography of this fraction with a different eluent system (CHCl<sub>3</sub> 1% / ethylacetate 15% / hexane 84%) allowed concentration of one of the components of the overlapping bands. Re-crystallization in ethylacetate / hexane afforded a major product (69 mg) which was assigned to be **3 $\beta$ -hydroxy-estr-5(10)-ene-4,17-dione 184**.

Although the <sup>1</sup>H NMR appears clean, analysis by ADEPT <sup>13</sup>C showed this to be a mixture of both 3-hydroxy isomers, in a ratio approximately 1:1. Similar attempts with the  $\beta$ -carboxylic acid degradation (below) and by Mastalerz (Ph.D. thesis) resulted in a similar mixture of the 3-isomers. It is tempting to postulate that these isomers are inter-convertible via tautomerization of the 4-keto, and that no amount of chromatography can separate these. NMR experiments were not undertaken to confirm whether this hypothesis is valid.

**Characterization of the pyrolytic degradation of  
19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstand-3,17-dione. 79**

**3 $\xi$ -hydroxy-estr-5(10)-ene-4,17-dione 184:** C<sub>18</sub>H<sub>24</sub>O<sub>3</sub> MS  
m/z: 288(P<sup>+</sup>, 27%),  $\lambda_{max}$ (MeOH) = 247nm. The Infrared spectra  
exhibits two carbonyl absorptions, at 1730(17-C=O) and 1655  
(4-C=O), and alkene peak at 1620(5,10-C=C) cm<sup>-1</sup>. <sup>1</sup>H NMR  
(CDCl<sub>3</sub>):  $\delta$  4.03-4.10 (3-CH<sub>1</sub>-OH, assigned as one doublet for  
each isomer, or an overlap of AB quartet, both fine split by  
the hydroxyl proton. Additional fine splitting is probably  
with a 2-CH<sub>2</sub>. The fine splitting of the 3-CH<sub>1</sub>-OH collapses,  
along with the OH peak, on addition of D<sub>2</sub>O. ADEPT <sup>13</sup>C clearly  
shows 36 carbons, each a doublet, confirming the presence of  
both C<sub>18</sub> isomers. The significant parts of the spectra are  
the presence of 6 quaternary low field carbons assigned as  
follows:  $\delta$  219.84 & 219.78 (17-C=O), 200.50 & 199.69(4-C=O),  
159.21 & 158.18(5-C=C), 129.5 (10-C=C), 71.90 &  
71.78(3-CH<sub>1</sub>-O), 14.04 & 13.09 (18-CH<sub>3</sub>).

**MINOR COMPONENT:** a minor component, a yellow powder was  
tentatively assigned as **4 $\alpha$ -hydroxy-estr-5(10)-ene-3,17-dione  
183a** with some **184** in the ratio 2/1. A mass spectra similar  
to **184** is not surprising. The infrared spectra exhibits two  
unconjugated carbonyls at 1785(3-C=O), 1730(17-C=O) cm<sup>-1</sup>.  
The <sup>1</sup>H NMR shows a sharp singlet for the 4-CH<sub>1</sub>O, a low field  
18-CH<sub>3</sub> and no alkene proton.

There is an additional peak corresponding to a fine  
split doublet in the NMR spectra at 4.09, and the presence of  
an extra methyl peak at 0.893. These additional peaks are  
assigned to the presence of compound **184** in the ratio 2:1  
**183a/184**, by TLC and comparison of the <sup>1</sup>H NMR spectra.

Figure 3.1.1:  $^1\text{H}$ -NMR of 3 $\beta$ -hydroxy-estr-5(10)-ene-4,17-dione  
(184)

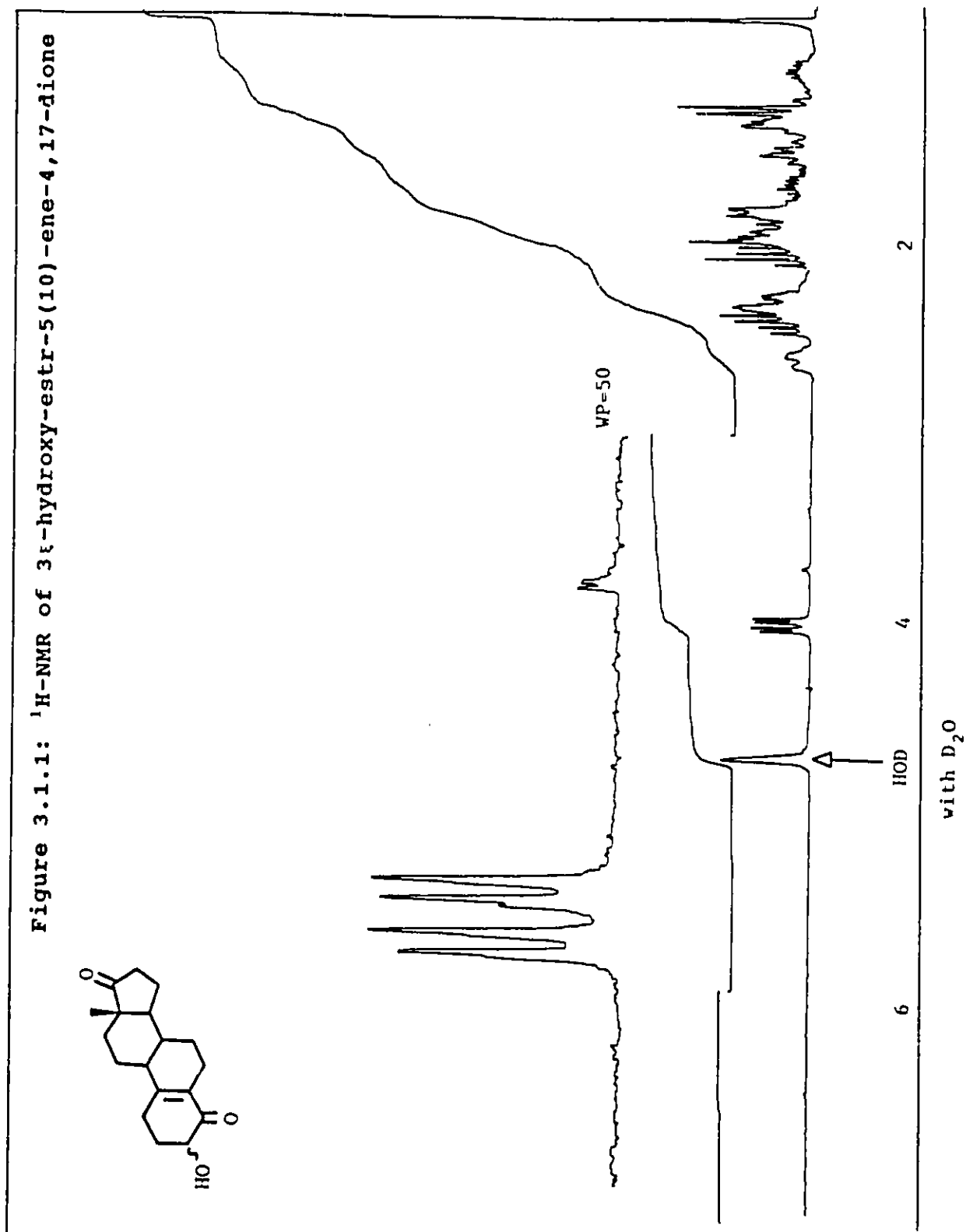
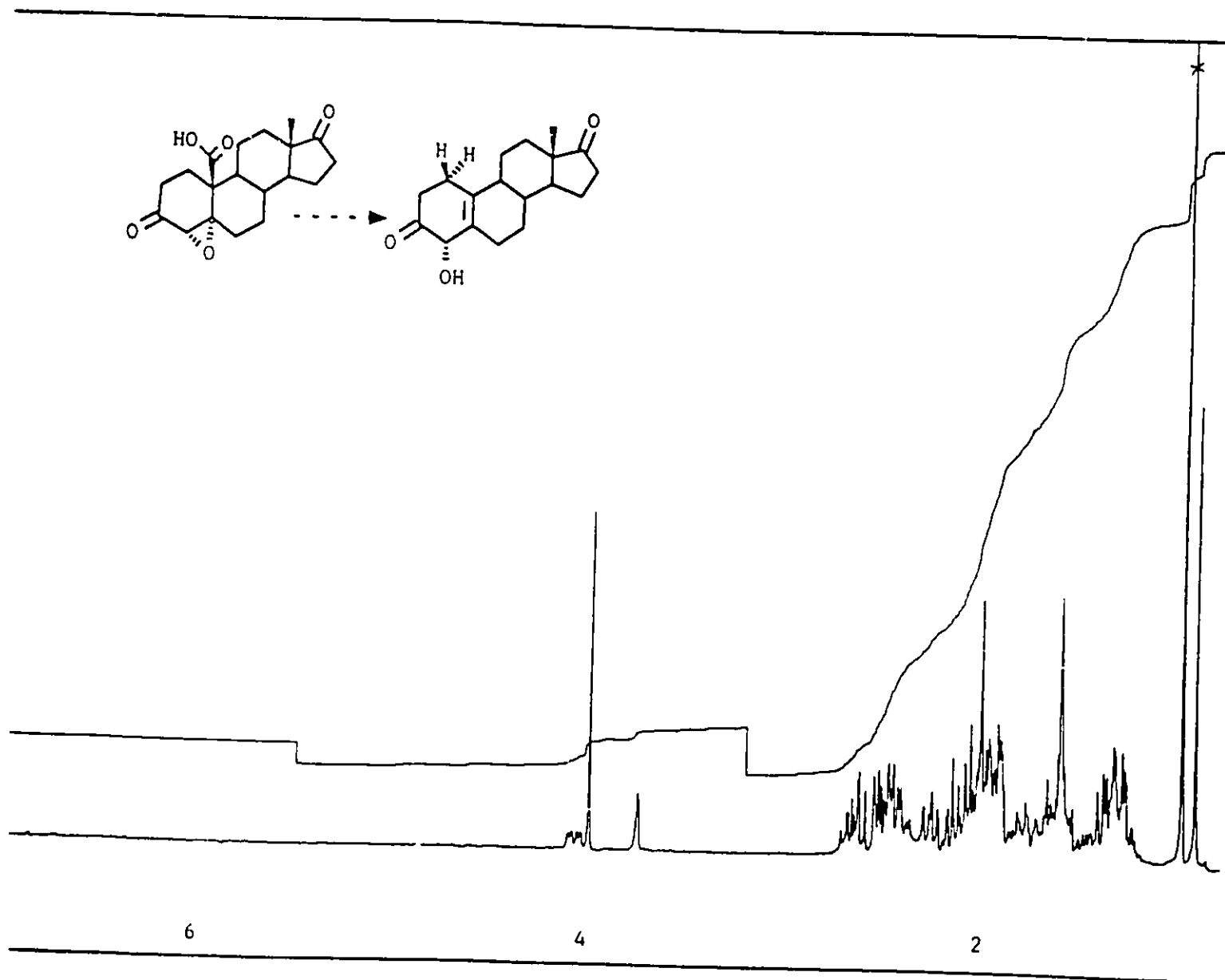
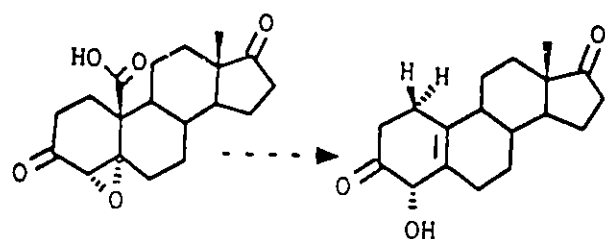


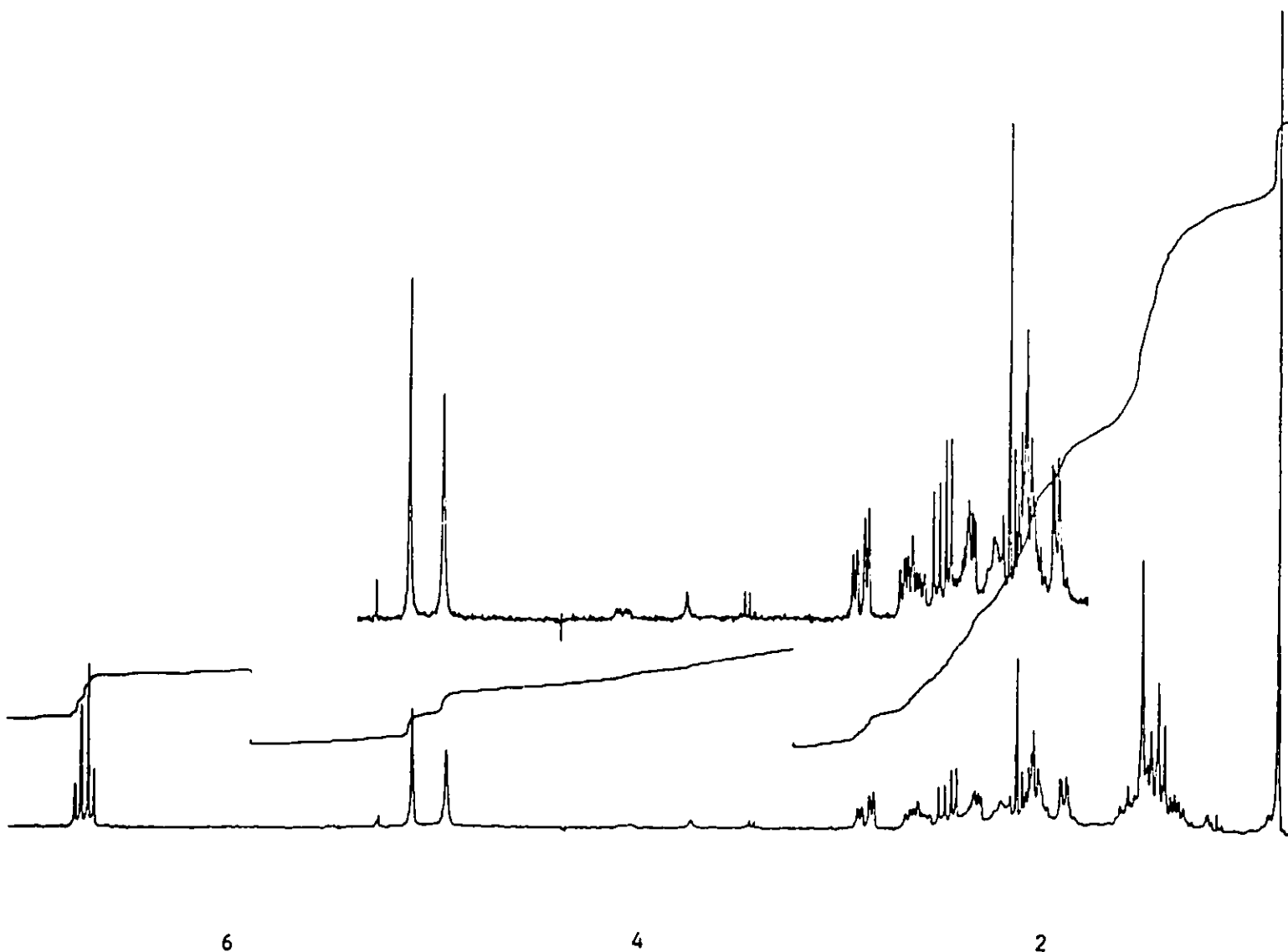
Figure 3.1.2:  $^1\text{H}$  NMR of 4 $\alpha$ -hydroxy-estr-5(10)-ene-3,17-dione (183a) with some 3 $\beta$ -hydroxy-estr-5(10)-ene-4,17-dione (184)



Undefined components of the pyrolysis degradation of  
19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstand-3,17-dione. 79

Additional inseparable polar components correspond to two degradation products (TLC) with two distinct mass spectra (parent ions at 288 and 286), absence of H-C-O in the NMR, two singlets in the alkene region, and an AB quartet about 6.7 reminiscent of the  $\Delta$ -1,4-diene pattern for the 1-CH and 2-CH protons. It is possible that one of these is **estra-1,4-diene-3-hydroxy-17-one**.

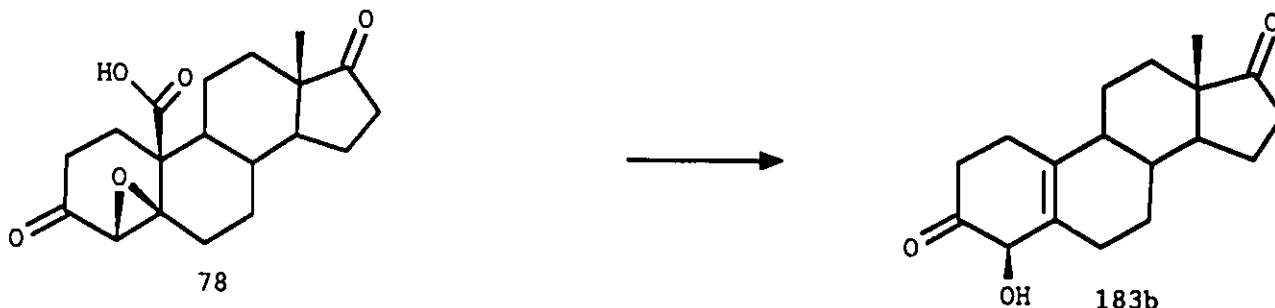
Figure 3.1.3:  $^1\text{H}$  NMR of one unidentified component,  
**estra-1,4-diene-3-hydroxy-17-one**



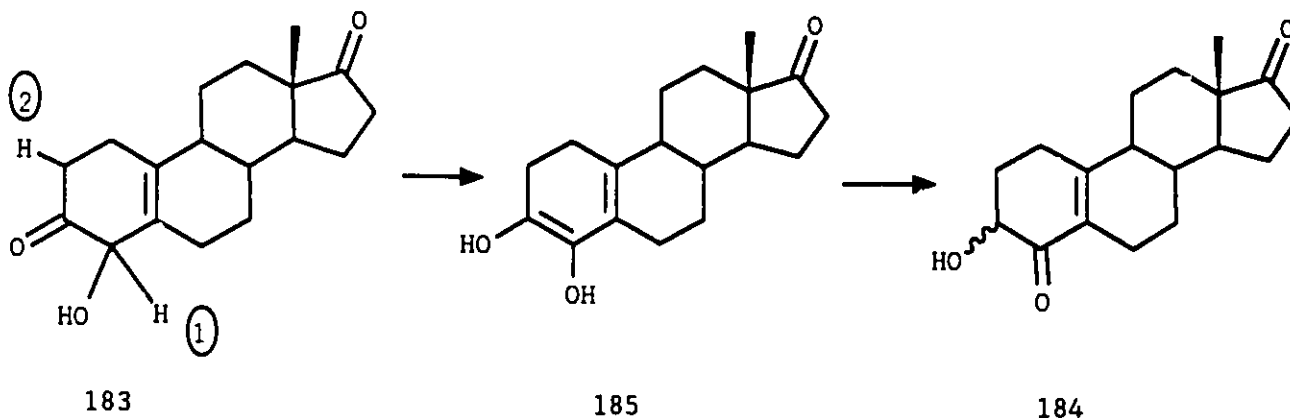
### 3.1.2 Reactions of

#### 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstand-3,17-dione **78**.

The outcome was similar to the thermal decarboxylation<sup>5</sup> of 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstand-3,17-dione **78** which lead to the formation of estrone and 3 $\epsilon$ -hydroxy-estr-5(10)-ene-4,17-dione **184**, with no isolated 4-alcohol **183b**.



**Scheme 3.1.2**



**Scheme 3.1.3**

The instability of the intermediates **183** (4 $\alpha$ -OH and 4 $\beta$ -OH) can be ascribed to enolization of the 4-hydrogen, then ketonization at C-4 obtaining the favorable conjugated product.

**3.1.3 Preparation of 3 $\epsilon$ -hydroxy-estr-5(10)-ene-4,17-dione (184) by thermal degradation of 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstane-3,17-dione. 78**

A suspension of the acid 78 in decalin (b.p. 195°C) was heated at reflux under nitrogen for 2.5 hours. The solvent was evaporated under reduced pressure and the residual semi-solid was chromatographed on basic Woelm alumina grade III. The reaction was followed by TLC on SiO<sub>2</sub> (CHCl<sub>3</sub>) results in 8 compounds, of which estrone and starting material can be identified, in addition to the mixture of 184 alcohol isomers and four other minor components.

**Analysis of the thermal degradation of 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstane-3,17-dione (78) by "flash" pyrolysis in the presence of silylating agents and chromatographic separation.**

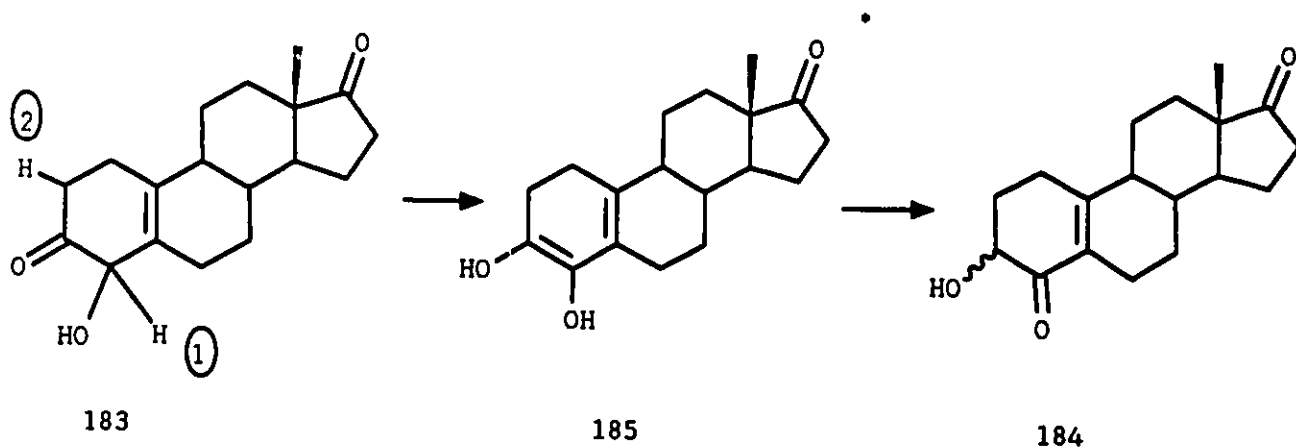
Silylation and direct injection in a pre-heated injection port (200°C) of a gas chromatograph attached to the mass spectrometer afforded a convenient method of analysis of the pyrolysis products of 78. GC separation was effected on a 6% OV-101 column with a temperature ramp of 10°C/min. This experiment afforded three peaks in the GC.

A. The more volatile corresponds to estrone treated in a similar fashion, with appropriate isotopic abundance for C<sub>21</sub>H<sub>30</sub>O<sub>2</sub>Si· and the following ion fragmentation: MS m/z: calc:342; 342(P $\oplus$ ,100%), 257 (26%), 218(24%)

B. The second compound to be separated corresponds to silylated **184**, with appropriate isotopic abundance for the major breakdown product of the parent ion and the following ion fragmentation:  $C_{21}H_{32}O_3Si_1$  calc:360; MS m/z: 360(P $\oplus$ ,2%), 345(M-15, 100%), 244(61%)

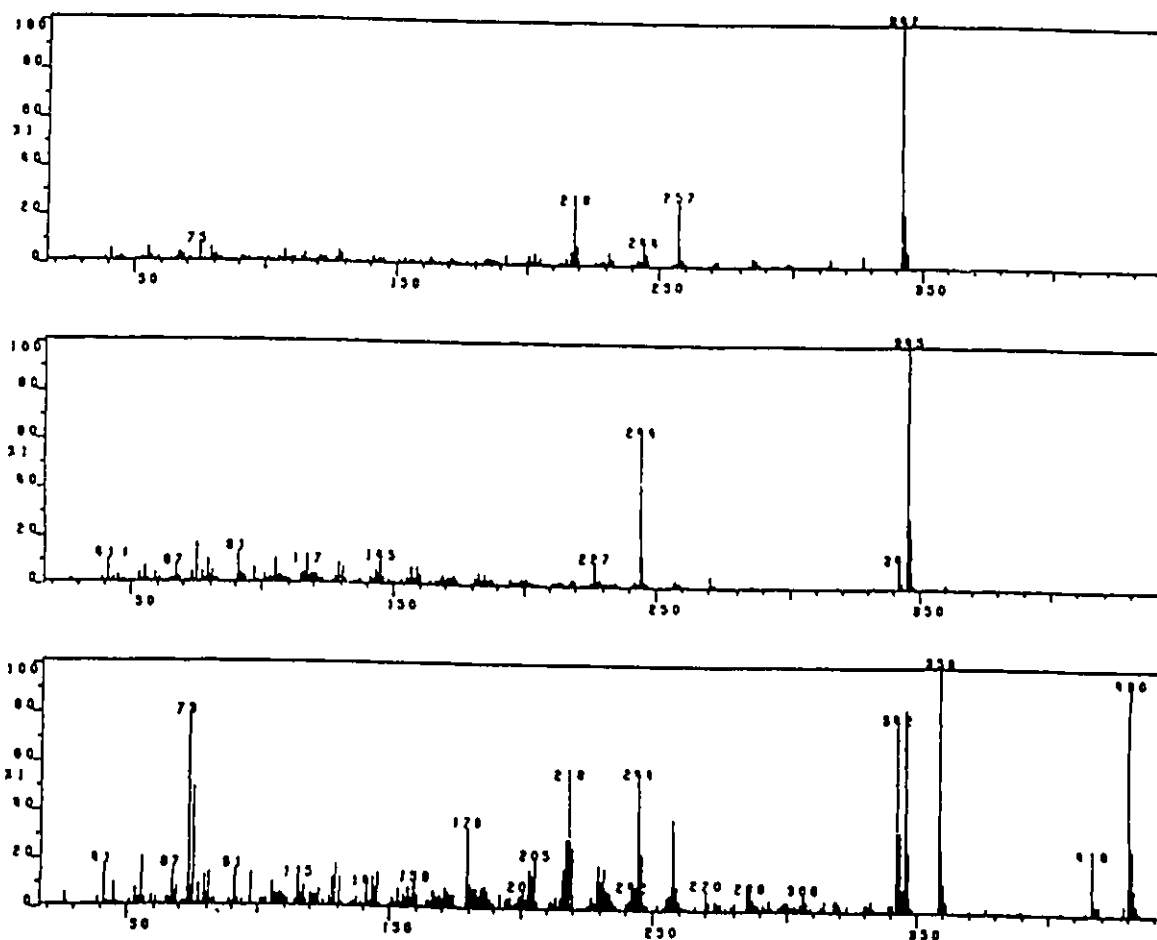
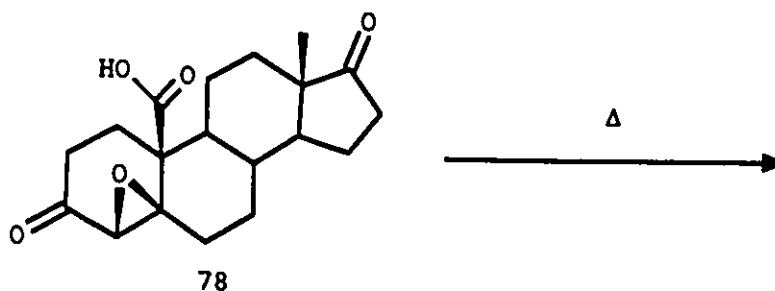
C. The third compound to be separated corresponds, at least in natural abundance and molecular weight, to doubly silylated **estr-3,5(10)-diene-3,4-diol-17-one 185**, with appropriate isotopic abundance for the major breakdown product of the parent ion.

Though the following ion fragmentation is by no means proof of the elusive compound **185**, the data is indicative.  $C_{24}H_{40}O_3Si_2$  calc:432; MS m/z: 432 (P $\oplus$ ,10%), 430 (92%), 416(22%), 360(11%, could be TMSi of compound **184**), 358(100%), 345 (82%, major breakdown of compound **184**), 342(76%, could be TMSi-estrone), 345(M-15, 100%), 244(61%).



**Scheme 3.1.4**

Figure 3.1.4: Mass spectra of the compounds resulting from the flash pyrolysis of 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstand-3,17-dione in the presence of silylating agents, followed by chromatographic separation.



**Reaction of 72 with base: Attempted preparation of 183.**

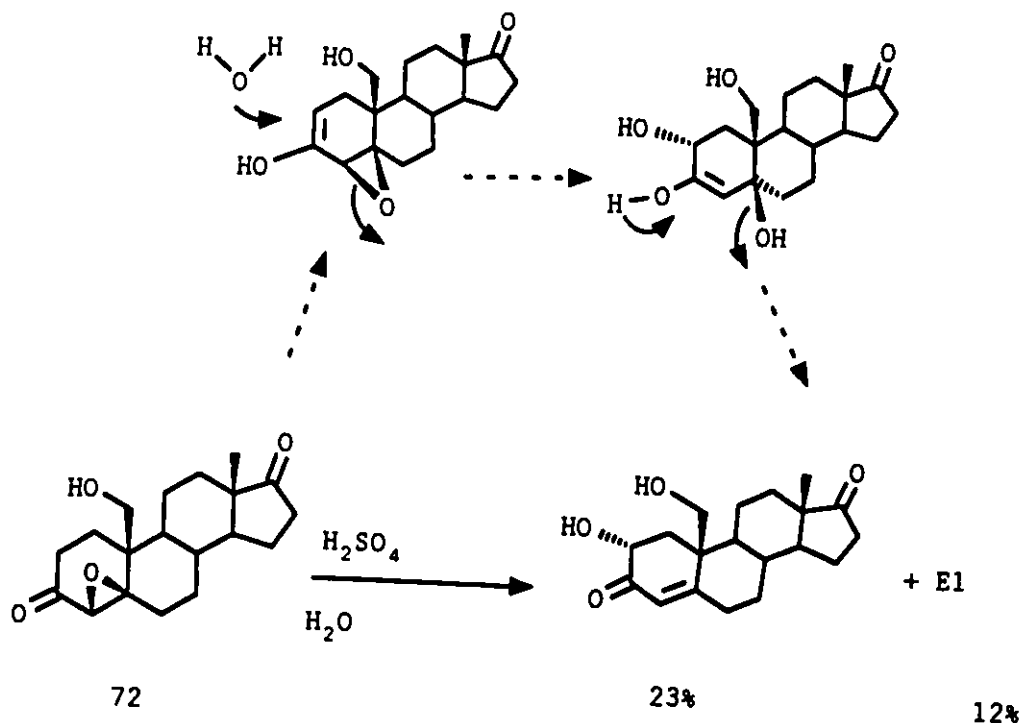
As intermediate 183 is of biological importance we set out to synthesize it. Treatment of  $\beta$ -epoxide 72 with 1.1 equivalent of tBuOK/tBuOH, (or NaOMe/MeOH) gave an insoluble yellowish powder. The large bands found in the  $^{13}\text{C}$  and  $^1\text{H}$  NMR of this substance indicated a probable oligomer.

**Reaction of 72 with acid: Attempted preparation of 183.**

Acid treatment of epoxide 72 resulted in three major rearrangement products, depending on the anion present in solution;  $\alpha$ -Mode and  $\beta$ -mode rearrangement occur as the result of nucleophilic attack at the C-4 or C-5 carbon atoms respectively.  $\alpha'$ -opening of is shown below

**$\alpha'$ -acid promoted epoxide rearrangements:**

Hosoda and Fishman<sup>18</sup> report  $\alpha'$  opening mode of the epoxide 72, resulting in 12% estrone and 23% 2 $\alpha$ -hydroxy compound, presumed to occur by nucleophilic attack of the 2,3-enol with opening of the epoxide.



**Scheme 3.1.5:  $\alpha'$ -epoxide opening.**

**Reaction of 72 with HCl/DMF: formation of 44.**

Reaction of the epoxide 72 with gaseous HCl/DMF generated compound 44 19-OH-4-Cl-Androstenedione. The  $^1\text{H}$  NMR (DMSO- $d_6$ ) spectra showed no alkene proton and a multiplet C-19 methylene: both half of the usual AB quartet ( $J = 5.5$  Hz) was further split twice ( $J_{\text{OH}} = 2.5$  and  $J_{1\alpha} = 0.6$ ). From this observation, it is reasonable to conclude that the C-19 hydroxyl must exist in a preferred conformation, with the O-H interacting with the 4-chloride electron orbitals. The first coupling is thus assigned to the -O-H. There is also fine splitting, possibly a  $^4J$  long range W coupling with the C-1 $\alpha$ -hydrogen. Although addition of 50  $\mu\text{L}$   $\text{D}_2\text{O}$  did not abolish the fine splitting due the O-H in DMSO- $d_6$ , the NMR in acetone- $d_6$  transformed the 19- $\text{CH}_2$  to an AB quartet and the peak for O-H disappeared. ADEPT correlation spectroscopy gave a  $^{13}\text{C}$  spectra with 4 quaternary carbons (190, 219, 162, 127) assigned respectively to C-3, C-17, C-5 and C-4. Absence of a tertiary alkene and presence of a quaternary peak (Cl-C-4), along with a corresponding increased molecular weight in the mass spectra. This confirms that a chloride is indeed found on the C-4 carbon.

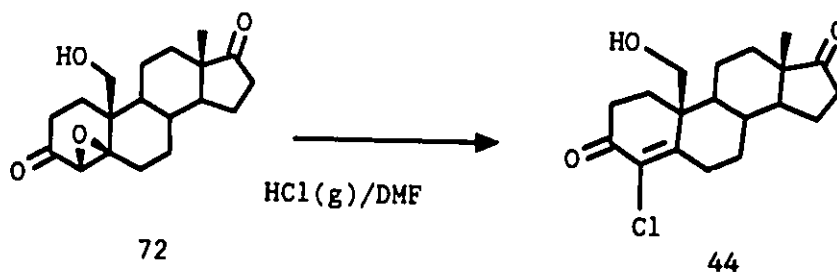
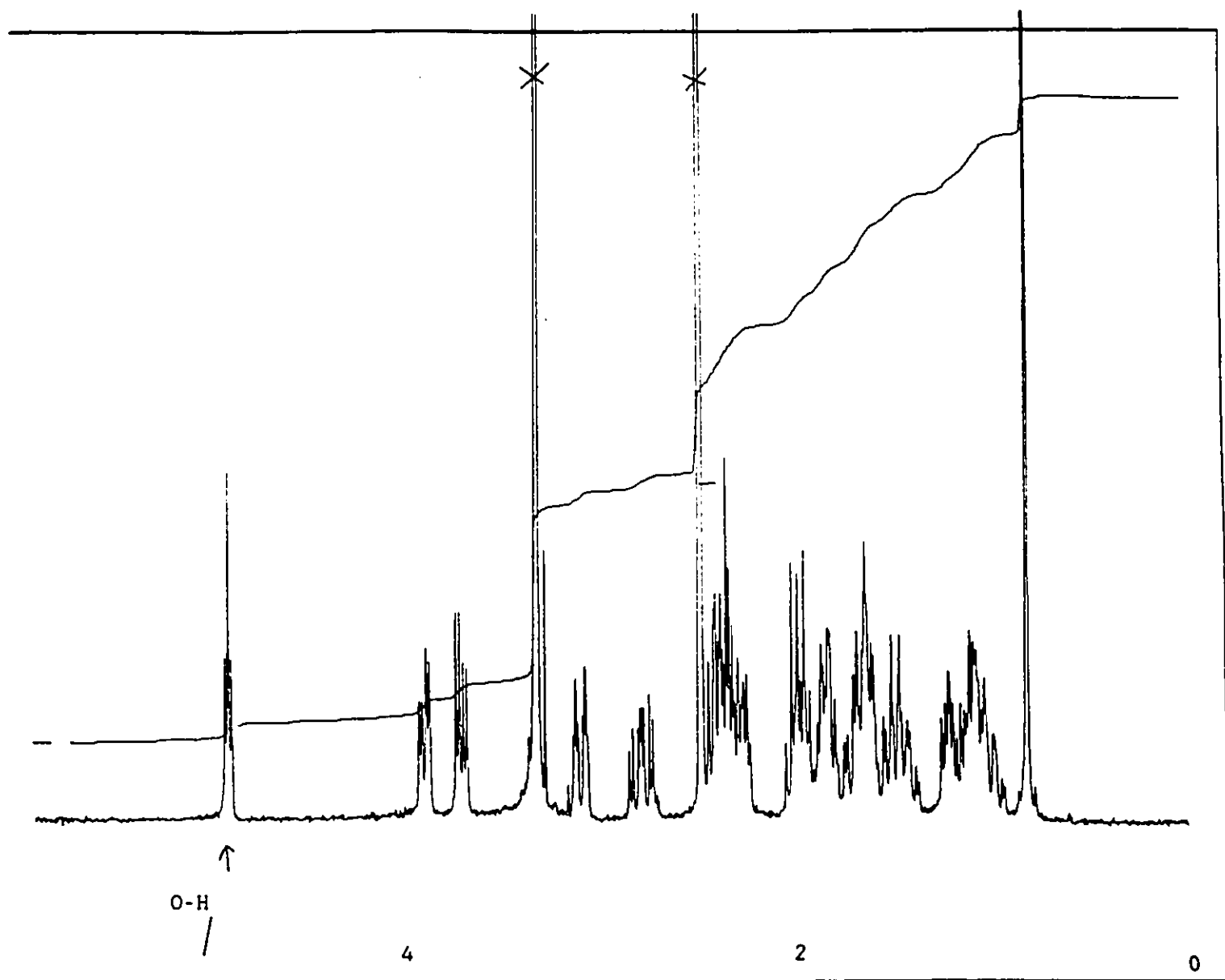
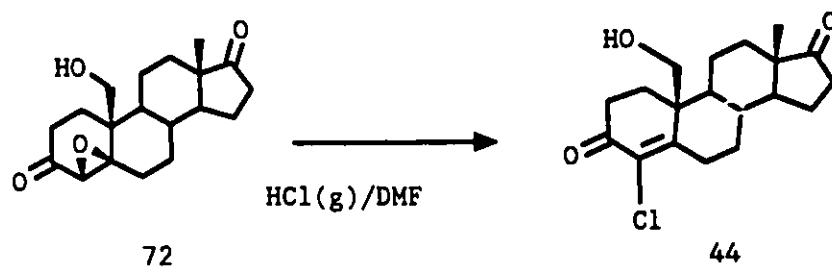


Figure 3.1.5:  $^1\text{H}$  NMR of  
4-chloro-androst-4-ene-3,17-dione-19-ol.

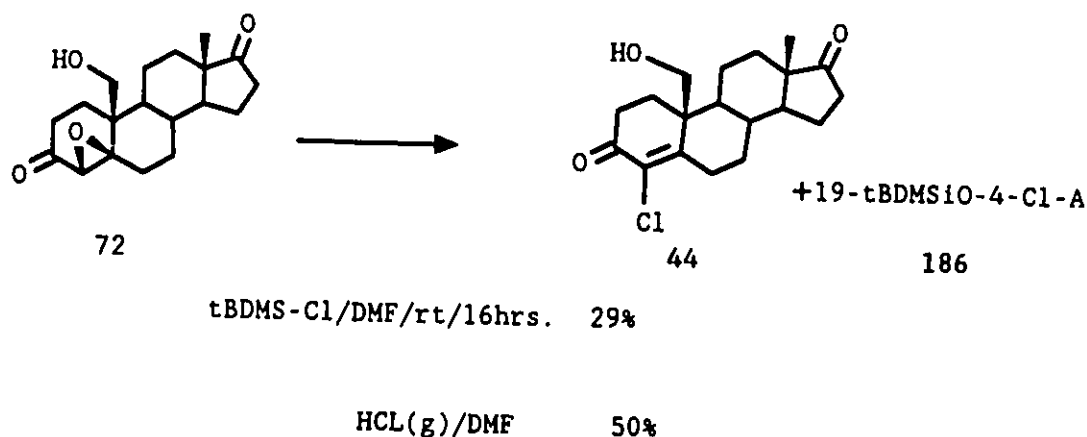


An attempt at isolating the putative 183a from the thermal degradation of 19-acids was carried out by treatment in the presence of a silylating agent, in the hope trapping the 3-one-4-alcohol 183 as a 4-silyl ether before it equilibrates to the more stable 3-alcohol-4-one. Unfortunately the 4,5-epoxides tested seemed to rearrange before thermal degradation occurred. A simulation with the thermally stable 19-alcohol showed that an epoxide rearrangement accounted for the products.

### 3.1.5 Reaction of 72 with tBDMSi-Cl/imidazole: formation of 186 and 44.

Epoxide 72 was treated with tBDMSi-Cl in DMF at room temperature. Chromatography gave two products; 19-silyloxy-4-chloro-androstenedione 186 and the parent alcohol 4-chloro-androst-4-ene-3,17-dione-19-ol 44.

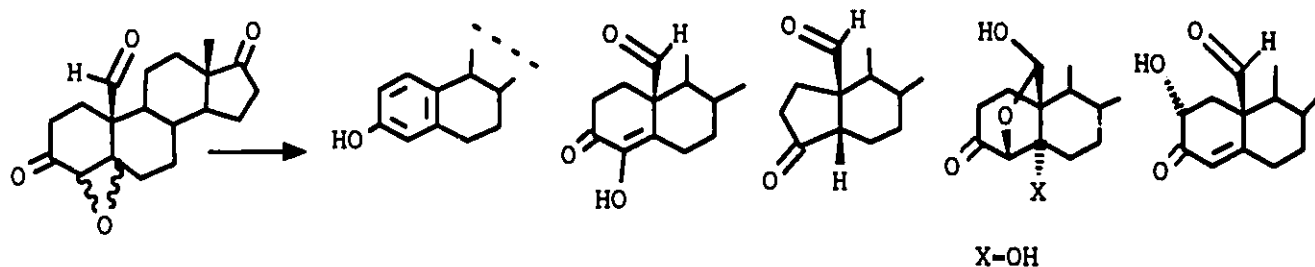
The less polar 186 19-tBDMSi-4-Cl-A showed an AB quartet for the 19-methylene, a weak parent ion at 450, the 73 peak typical of the tBDMS ethers and a M-57 cracking at mass  $m/z=393$ . The 4-chloro compounds presumably arose from the hydrogen chloride acid present in silyl chloride reagents.



**Scheme 3.1.6**

### 3.1.6 Reactions of 19-oxo-4 $\xi$ ,5-epoxy-5 $\xi$ -androstande-3,17-dione with acids and base.

Mastalerz and Morand<sup>5</sup> have shown that 19-oxo-4,5 $\xi$ -epoxy-5 $\xi$ -androstande-3,17-dione (5 $\alpha$  or 5 $\beta$ ) can deformylate to estrone under acid and basic conditions. When deformylation occurs it goes directly to estrone without isolation of the 4-alcohol 183. By adjusting the acid and the solvent it is possible to obtain good yields of 32, an analogue of the postulated enzyme intermediate where the C-5-X is an unspecified group X-, possibly a nucleophilic amino acid or electron rich species capable of stabilizing the developing charge at the C-5 position. We have incubated this compound with aromatase and found that it does not cause inhibition of the enzyme. Under those test conditions, this implies that compound 32 has no affinity for the active center and does not fit in the enzyme cavity.



|               |                 | 13 | Estrone | 188 | 189 | 32 | 10 |
|---------------|-----------------|----|---------|-----|-----|----|----|
|               | Rx. Conditions. |    |         |     |     |    |    |
| 4,5- $\alpha$ | 1               |    | 12%     | 47  |     | 26 |    |
| 4,5- $\beta$  | 1               |    | 3%      |     |     | 43 | 8  |
| 4,5- $\beta$  | 2               |    | 3%      |     |     | 82 |    |
| 4,5- $\alpha$ | 2               |    | 13%     | 47  |     | 17 |    |

Conditions 1: 20% H<sub>2</sub>SO<sub>4</sub>/Acetone

2: 20% HClO<sub>4</sub>/THF

**Scheme 3.1.7:** Reactions of 19-oxo-4,5-epoxides with acids.

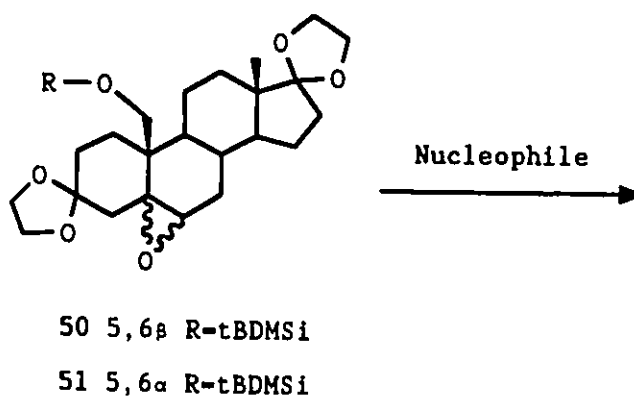
### Conclusion to the attempted preparations of 183.

Bioorganic and chemical methods to reach 183 failed because of the reactivity of 19-oxygenated intermediates. Further attempts at preparing 183 were abandoned.

#### 3.1.7 Suitability of 5,6 epoxide in affinity labels:

The nucleophilic susceptibility of both isomers of 19-tertbutyldimethylsilyloxy-3,17-bisethylenedioxy-5,6-oxido-androstane.

As seen in the previous chapter, it is of biological interest to use epoxides as trapping agent for active site nucleophiles. Nucleophilic addition to 50 and 51 were briefly investigated, since epoxide traps have been reported to react with the P<sub>450</sub> ring. Ideally, these electrophilic affinity labels should not react when they are out of the enzyme active center. However, when in the androgen binding site, an affinity label should form a strong covalent bond.



**Scheme 3.1.8: Nucleophilic susceptibility of 5,6-epoxides: simulations of active center nucleophiles.**

**Table 3.1.1: Nucleophile addition to epoxides**

|                                                   | 51 $\alpha$ -epoxide | 50 $\beta$ -epoxide |
|---------------------------------------------------|----------------------|---------------------|
| <u>1.1 eq. NaN<sub>3</sub></u>                    |                      |                     |
| RT, 3 hrs.                                        | NR                   | NR                  |
| $\Delta$ , 9 hrs.                                 | NR                   | NR                  |
| 70 eq. $\Delta$ , NH <sub>4</sub> Cl <sup>+</sup> | 190                  | 191                 |
| <u>NaBr/MeOH</u>                                  |                      |                     |
| RT, 2 days                                        | NR                   | NR                  |
| <u>Ph<sub>3</sub>H, EtOH</u>                      |                      |                     |
| RT, 3 hrs.                                        |                      | NR                  |
| $\Delta$ , 1 hrs.                                 | NR                   | NR                  |

<sup>+</sup>: solubilized in CH<sub>3</sub>OCH<sub>2</sub>CH<sub>2</sub>OH. NR: no reaction.

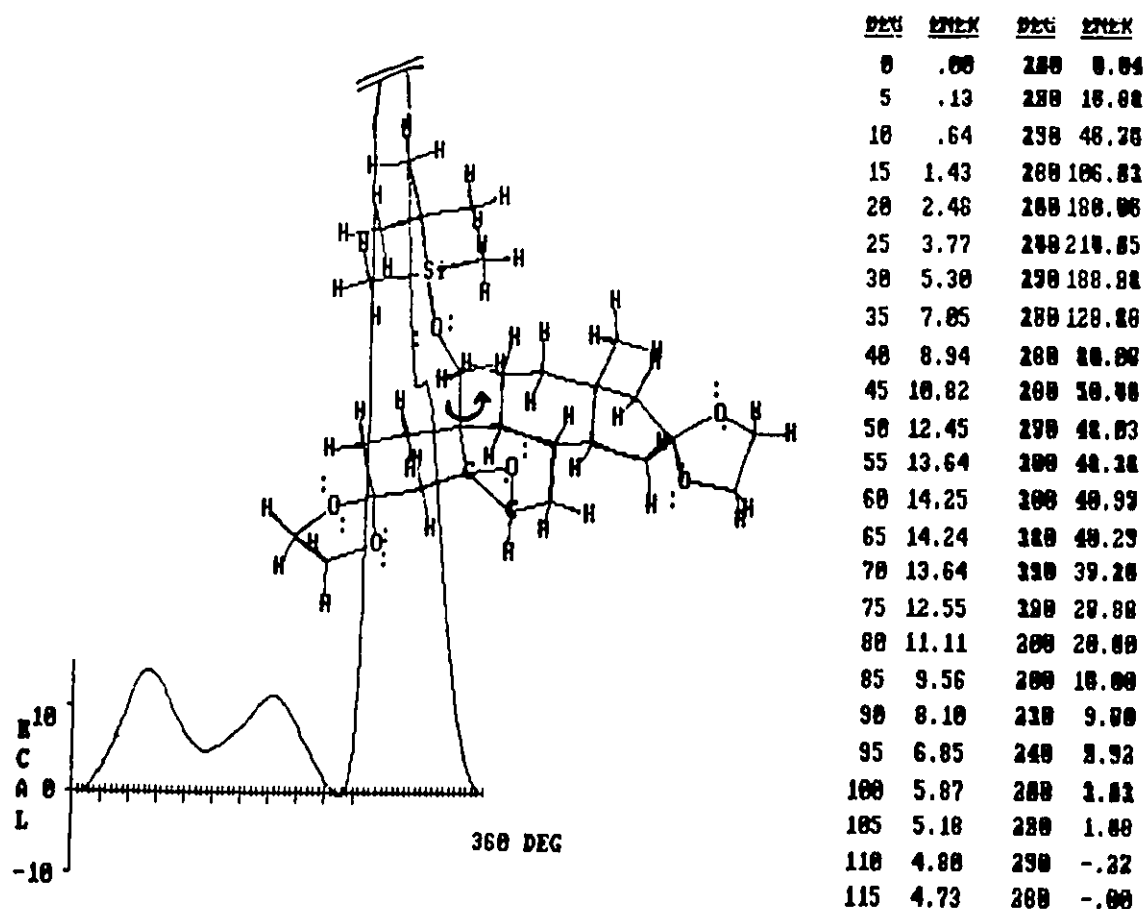
**Formation of 190; Reaction of 19-tertbutyldimethylsilyloxy-3,17-bisethylenedioxy-5,6 $\alpha$ -oxido-5 $\alpha$ -androstandane 51.**

The reaction mixture resulting from addition of sodium azide to  $\alpha$ -epoxide 51 was chromatographed on basic Woelm alumina to yield 16 mg of a product with a high mass ion at 535, a characteristic strong ketal rearrangement peak at m/z 99, silane rearrangement products 506 (M-57) and m/z at 478. The IR shows a sharp, strong absorption at 2110 cm<sup>-1</sup> corresponding to the 6- $\beta$ -N<sub>3</sub> asymmetric stretch, and an absorption at 1110 cm<sup>-1</sup> corresponding to azide C-N<sub>3</sub> symmetrical stretch. The <sup>1</sup>H NMR spectra is unusual, demonstrating a broad 3.32 ppm peak found to be a fine split quartet assigned to the 6 $\alpha$ -H that is coupled to the C-7 methylene hydrogens. The <sup>1</sup>H NMR also displays a sharp singlet at 4.43 assigned to the hydroxyl, and a doublet at 3.63 (J=11 Hz) assigned to one of the C-19 methylene. C-19 methylene often give AB quartets with coupling constants between 10 and 11 Hz.

This is particularly evident in 19-tBDMSi derivatized steroids. One of the 19-hydrogens is often buried in the ketal envelope. That there is such strong differentiation between the two C-19 protons is not surprising when the barrier to rotation of the tBDMSi group alone is considered. The added steric constraints due to the B ring substituent, specially a  $\beta$ -azide, would further separate these 19-hydrogens.

### Rotation barrier of the 19-tBDMSiO group

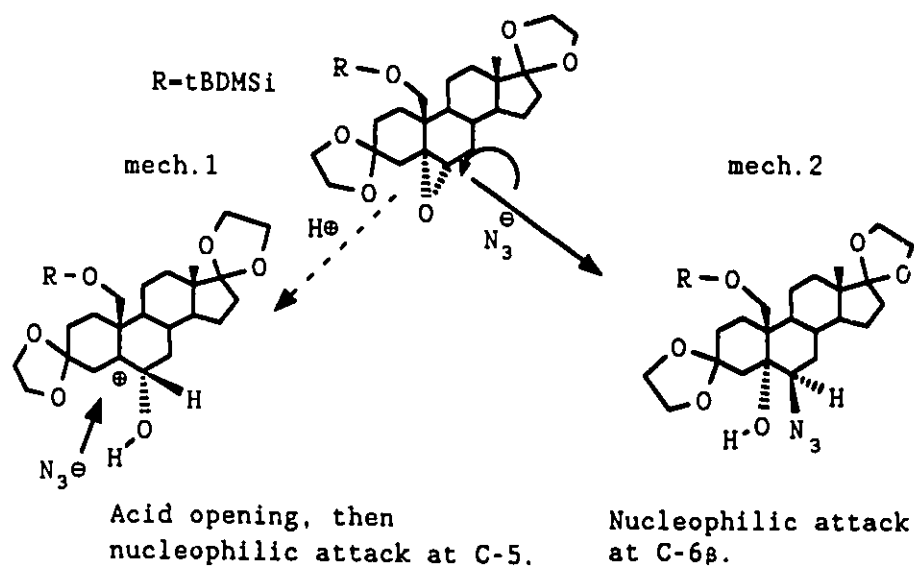
Figure 3.1.6: Rigid rotor approximation: rotation around bond C-10 and C-19.



The nucleophilic addition to epoxide **51** can follow two courses:

- 1) acid opens the epoxide, and the azide anion can add at the C-5 carbocation, or
- 2) epoxide opens as a result of nucleophilic attack at the C-6 $\beta$  position.

Nucleophilic attack at the C-6 position seems a more probable course, as the mild acid ammonium chloride is not expected to generate a carbocationic intermediate.



**Scheme 3.1.9**

**$^1\text{H}$  NMR pattern expected from mechanism 1:**

If the  $\text{OH}$  were situated at the C-6 $\alpha$ -position, it could be stabilized by the azide; coupling with the C-6 $\beta$ -hydrogen would be expected in this very stable  $\text{O-H}$ , resulting in a **doublet**, and causing the expected 6-hydrogen doublet of doublets (caused by the 7- $\text{CH}_2$ ) to be further split into a multiplet.

**<sup>1</sup>H NMR pattern expected from mechanism 2:**

If the  $\alpha$ -OH is situated at C-5, it can hydrogen bond to the lone pairs of the ketal 3 $\alpha$ -oxygen, stabilizing it against D<sub>2</sub>O exchange. It does not, however, couple with other hydrogens, and the C-6 $\alpha$ -hydrogen would then result in a doublet of doublets due to the C-7 methylene protons. MM2 calculations on the lowest energy conformer were used to generate the angle between these nuclei. From these conformers the Karplus equation can estimate the coupling constants of the C-6-hydrogen with the C-7 $\alpha$ -hydrogen at 2.6 and with the C-7 $\beta$ -hydrogen at 4.08 Hz. The actual coupling constants observed are  $J=1.25$  and  $J=1.68$  Hz.

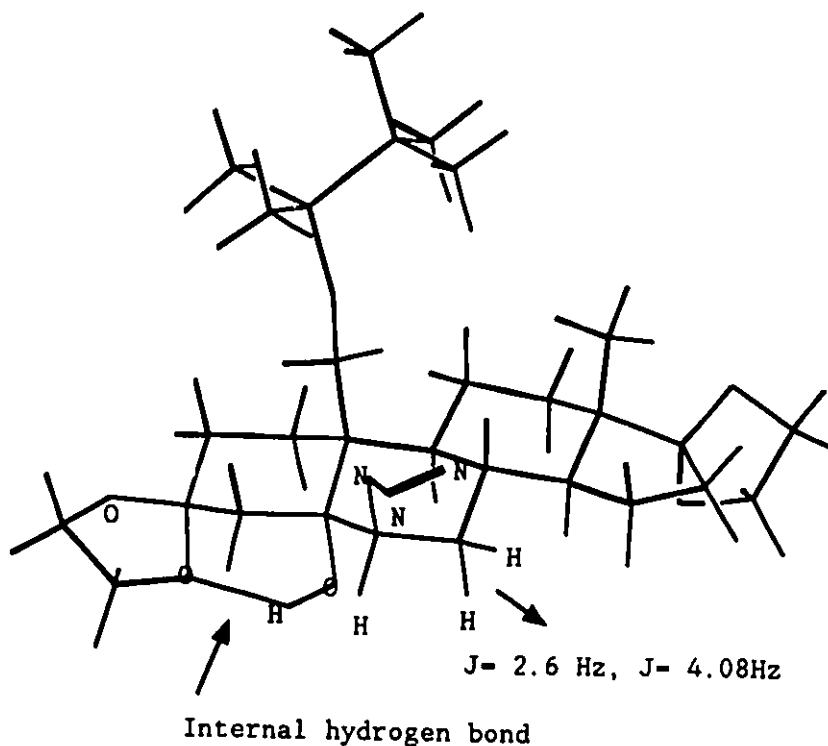
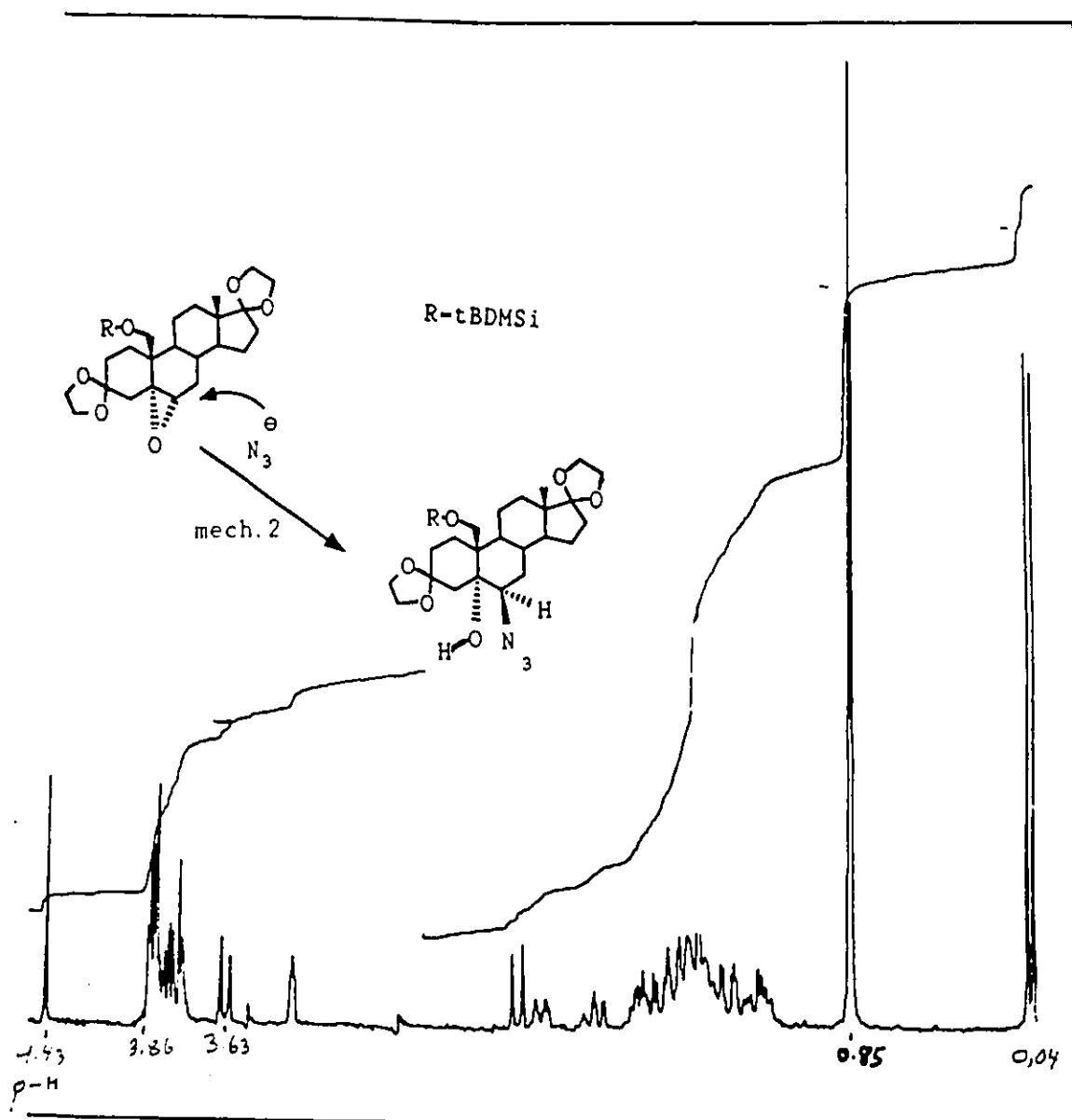


**Figure 3.1.7: MM2 minimized structure of compound 190.**

The NMR pattern expected from mechanism 2 is observed, and the correct structure for 190 is assigned as the 5 $\alpha$ -hydroxyl-6 $\beta$ -azido-derivative.

Figure 3.1.8:  $^1\text{H}$  NMR of 19-tertbutyldimethylsilyloxy-3,17-bisethylenedioxy-5 $\alpha$ -hydroxy-6 $\beta$ -azido-5 $\alpha$ -androstande 190.



### **Suitability of 5,6 epoxide in affinity labels: conclusion.**

These bioorganic experiments revealed that a 5,6-epoxide moiety could provide a suitable affinity label because the epoxide does NOT react under stoichiometric conditions until heat is applied. It would therefore not exhibit non-specific side reactions characteristic of strong alkylating agents when used in vitro.

The epoxide moiety WILL react, however, under high nucleophilic concentrations, which are typical of protein binding sites where high local concentration effects are known to occur.

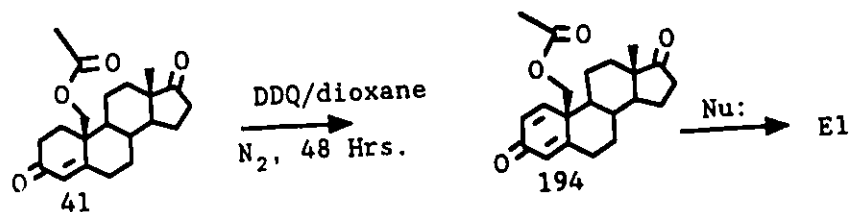
### **3.1.8 Suitability of $\Delta^{1,4}$ steroids as mechanism-based inhibitors: the chemical reactivity of 19-oxygenated-androsta-1,4-diene-3,17-dione.**

Many aromatase inhibitors rely on the presence of the  $\Delta^{1,4}$ -3-keto functionality for aromatase inhibitory activity despite the fact that the parent  $\Delta^{1,4}$ -androstadienedione is a chemically stable compound.

Since aromatase carries out oxidations at the C-19 position, it was of interest to investigate the chemical reactivity of 19-oxygenated steroids, which could mimic potential mechanism-based intermediates of aromatase.

### **Preparation of 19-acetoxy-androsta-1,4-diene-3,17-dione. 194**

Androstenedione-19-acetate **41** was oxidized with DDQ in refluxing dioxane for one day. The resulting  $\Delta^{1,4}$ -19-acetate **194** exhibited characteristic spectral data.



**Scheme 3.1.8: Synthesis of 194.**

**Attempted preparation of 19-hydroxy-androsta-1,4-diene-3,17-dione. 195**

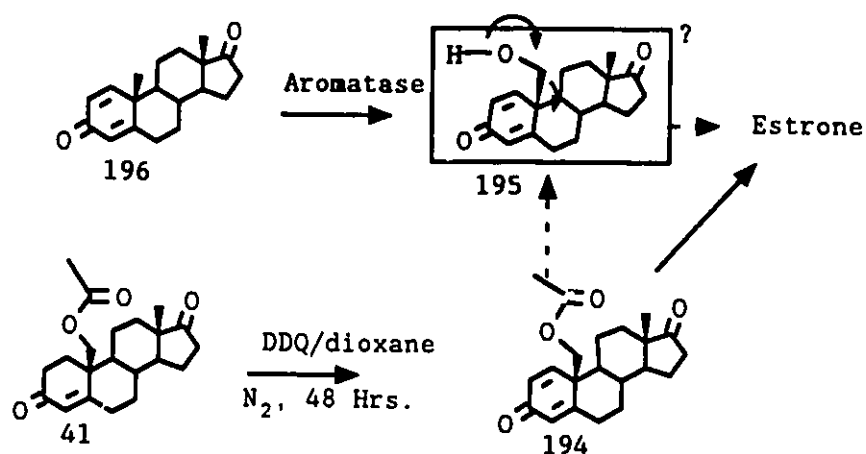
Unexpectedly, the 19-acetate group of compound 194 proved impossible to remove under mild acid, mild basic and mild nucleophilic conditions. In all the cases listed, either no reaction occurred or aromatization (to form estrone) was observed. The putative 19-alcohol 195 intermediate could not be isolated; it seemed to decompose to estrone, presumably via deformylation.

**Table 3.1.2: Reaction conditions to deprotect 194.**

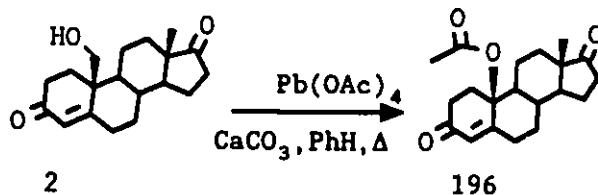
| Temperature                                                   | 0°C | RT        |
|---------------------------------------------------------------|-----|-----------|
| NaCN/MeOH                                                     |     | E1        |
| NaHCO <sub>3</sub> /MeOH/N <sub>2</sub> ;                     | NR  | E1        |
| HCl/H <sub>2</sub> O/MeOH                                     | NR  | NR        |
| HCl/H <sub>2</sub> O/MeOH                                     | NR  | NR        |
| HCl/H <sub>2</sub> O/MeOH                                     |     | Reflux:E1 |
| K <sub>2</sub> CO <sub>3</sub> /MeOH/H <sub>2</sub> O/Benzene |     | E1        |
| LiOOH                                                         | NR  |           |

Attempted deprotection of the 19-acetate 194.

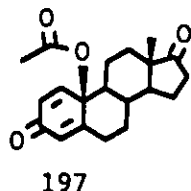
Based on the chemical reactivity of the 19-acetate, and (presumably) of the 19-alcohol **195**, we propose here that the aromatase based oxidation of the C-19 methyl group generates a 19-alcoholic intermediate which can then quickly deformylate.



### 3.1.9 The chemical reactivity of 10-oxyacetyl-estra-1,4-diene-3,17-dione **197**.



Similarly, the 10-acetate 197 is a postulated intermediate analogue in the biosynthesis of estrogen. We found that it decomposes readily under a variety of mild conditions: thermal degradation alone (in refluxing THF) is sufficient to generate a 46% yield of aromatic steroid.



**Conclusions: The bioorganic chemistry of oxygen bearing steroids.**

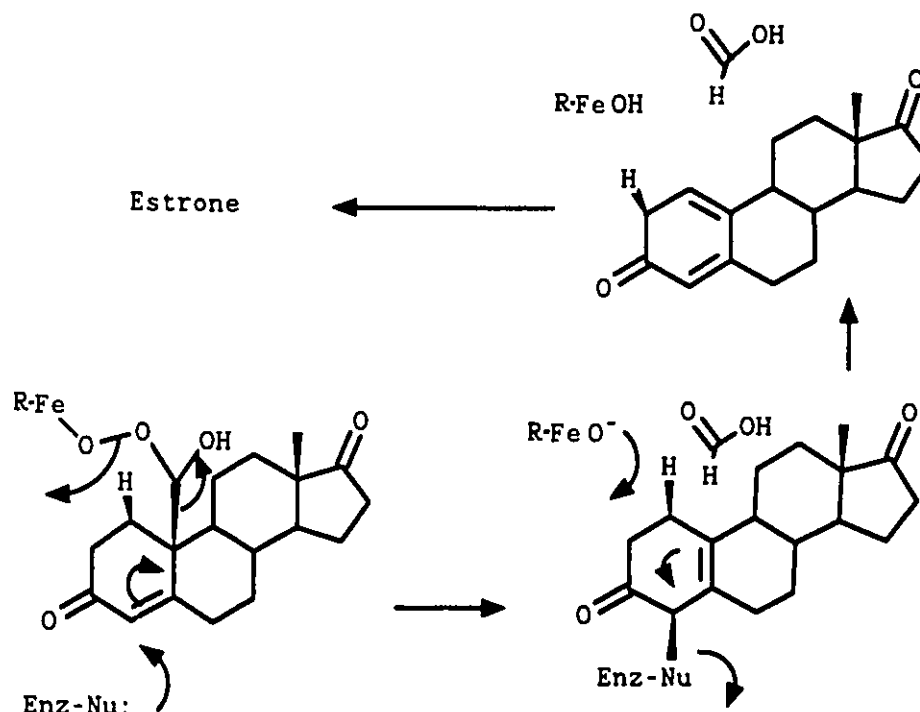
The  $\Delta^{1,4}$  pharmacophore imparts lability to the C-10-formyl and the C-10-methylene-oxyacetyl-estra-1,4-diene-3,17-dione. If these are indeed intermediate in the biochemical process it is expected that they would aromatize readily. When nucleophiles were present, aromatization was preferred to addition-elimination sequence.

Model 5,6-epoxides displayed desirable reactivity in the presence of nucleophiles and were evaluated as potential affinity labels earlier. They did not, however, cause irreversible inactivation in the aromatase active center.

## The Bioorganic chemistry of sulfur bearing compounds.

## 3.2.1 Attempted addition of nucleophile at C-19

Models were sought to test the possibility of enzyme-assisted elimination of a C-19 intermediate (such as hydroperoxide) which have been invoked as a possible mechanism of the enzyme aromatase. These models would approach the combined addition at the steroidal C-4 carbon with displacement of an iron-C-19-hydroperoxy analogue.

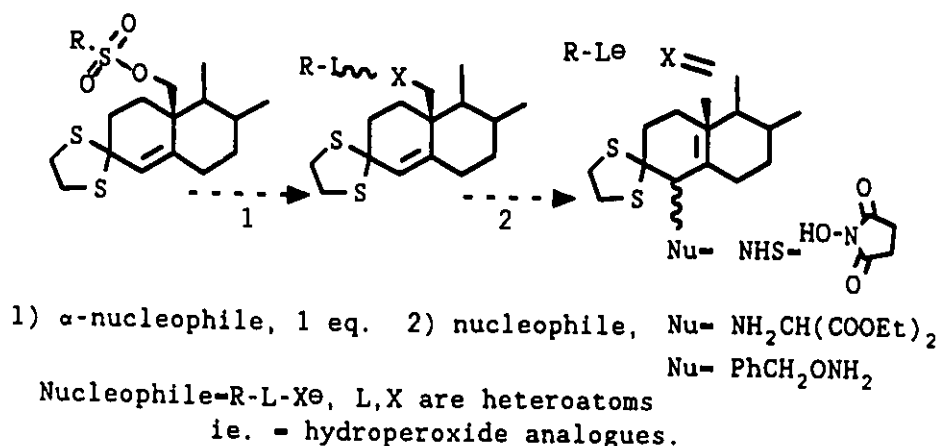


Scheme 3.2.1

The chemical reactivity of a nucleophile to  $\alpha, \beta$ -unsaturated ketones usually involves addition either directly to the ketone [1,2] or to the conjugated alkene [1,4]; the outcome depends on the nature of the nucleophile. [3,4] addition occur with electrophilic reagents (ex:mCPBA), but none are reported for nucleophilic reagents.

The aromatase enzyme must therefore modify the usual reactivity of the C-4 position. This can be accomplished by distorting the electron cloud of the alkene or by actually modifying the  $\alpha, \beta$ -unsaturated-3-ketone to an  $\alpha, \beta$ -unsaturated-3-hemiketal.

19-sulfonate derivatized-3-thioketal-steroids were thought to be adequate model for two reasons; 1) they seemed convenient precursors of desired C-19 hydroperoxide models, and 2) the modified reactivity of the 3-ketone would then model the enzyme 3-hemi- thioketal reactivity at C-4. Thus, susceptibility to nucleophilic attack at C-4 could be tested without complications due to Michael additions.



### Scheme 3.2.2

This two-pronged approach would model the C-4 incoming nucleophile hypothesis, via C-19 leaving groups or hydroperoxide analogues. The results, however, were not as anticipated.

Indeed, numerous nucleophilic additions were attempted with potential  $\alpha$ -heteroatoms (hydroperoxide analogues), and the C-19 position proved too unreactive. Results of some typical reactions are found below.

**Table 3.2.1:** Reaction of nucleophiles with thioketal derivatives.

|                                        | ()-Dithiolane-19-Ms |                    | 3,17-Bis-dithiane     |                  |
|----------------------------------------|---------------------|--------------------|-----------------------|------------------|
|                                        | 55-(3,17)           | 56-(3)             | 198 19Ms-             | 58 19Ts-         |
| NaI/acetone                            | 240                 | 239                | NR: $\Delta$ , 27days | --               |
| PhCH <sub>2</sub> O-NH <sub>2</sub>    | --                  | --                 | NR                    | $\Delta$ d.      |
| NH <sub>2</sub> CH(COOEt) <sub>2</sub> | NR                  | NR(rt) $\Delta$ d. | TLC, 6days            | $\Delta$ d.diox. |
| NHS                                    | 235 X-ray           | 236                | NR 45°/12days         | --               |
| $\Delta$ pyr                           | 237, 238 2D-J       | 233, 234 HETCOR    | 2 cmpds               | 224              |

NHS: N-hydroxy-succinimide; pyr:pyridine; TLC:new products were seen by TLC, but only starting material could be re-isolated;  $\Delta$ d.: decomposes on heating NR: No Reaction. X-ray, HETCOR and 2D-J resolve refer to the spectroscopic method used to identify the product in addition to the usual ADEPT, <sup>1</sup>H NMR and <sup>13</sup>C NMR spectroscopy.

#### Nucleophilic addition to tosyl and mesyl dithiane:

Although some neo-pentylic C-19 sulfonates are inert to nucleophilic displacement<sup>23</sup> (due to possible 1,3 interactions), the sulfonate leaving groups should allow displacement by the potent  $\alpha$ -effect nucleophile salts.

Nevertheless, attempts to displace the dithiane 19-mesylate 198 or 19-tosylate 58 failed at room temperature. No nucleophilic addition occurred at room temperature, or, on increased heating, the reaction products afforded non-polar compounds of unknown structure.

Steric hindrance was thought to increase the steroid  $\beta$ -face crowding resulting in alternative reaction pathways. We analyzed the minimized conformations of the dithianes synthesized, using a modified MM2 molecular mechanics computer program. This generates lowest energy structures, allows rotamer analysis, and displays steric hindrance about the  $\beta$ -face of the A ring.

With the exception of compound 239, the C-19 tosylate and mesylate were inert to nucleophiles below 45 °C and decomposition occurred above this temperature. This could be followed closely by chromatography (TLC). Experiments were then conducted to understand the spectral data on the thermal degradation products.

**Preparation of 224: Thermal degradation of 58,  
19-hydroxy-4-androstene-3,17-bispropylenethioketal  
19-toluenesulfonic ester.**

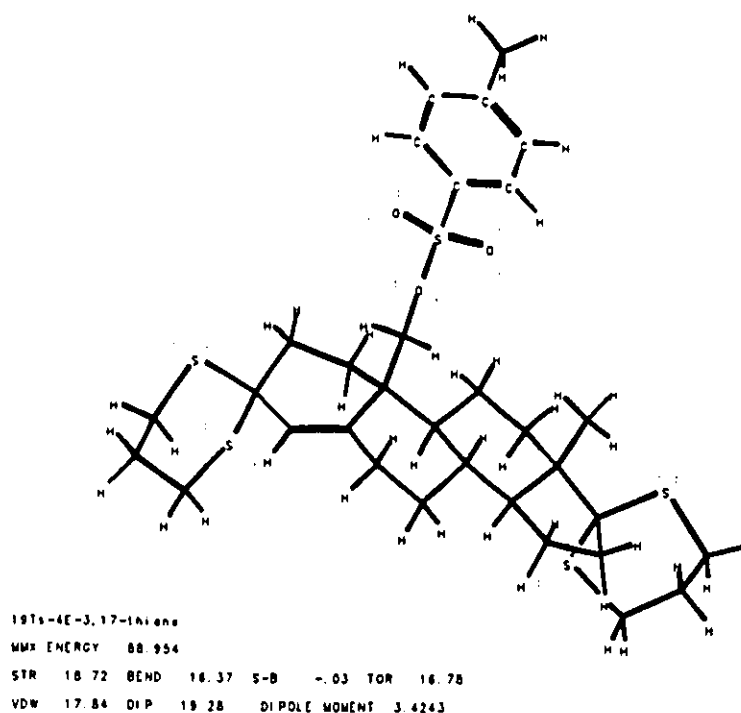


**Figure 3.2.1: 3-dimensional structure of 58 according to MMx computations.**

A suspension of 58 in 10 ml pyridine was refluxed for one day. Workup and chromatography gave one compound, 224. The  $^1\text{H}$ -NMR spectra was characterized by a collapse of the 19-CH<sub>2</sub>O to a sharp singlet which integrated to one proton. Two new doublets appeared at 0.24 and 0.76 ppm, each integrating to one proton ( $J=4.6$  Hz). No tosyl peaks were present. The alkenyl hydrogen at 5.8 was a doublet of doublet ( $J=2$  Hz). Two protons, appearing to be a multiplet, (complex triplet), were centered at 2.9 and 3.1 ppm. Additional information was required to elucidate this structure.

#### The Nuclear Overhauser Effect (nOe).

The nOe is a through-space effect indicating proximity of the dipoles irradiated with the dipole affected. Four readily separable protons were irradiated. The effect is summarized below.

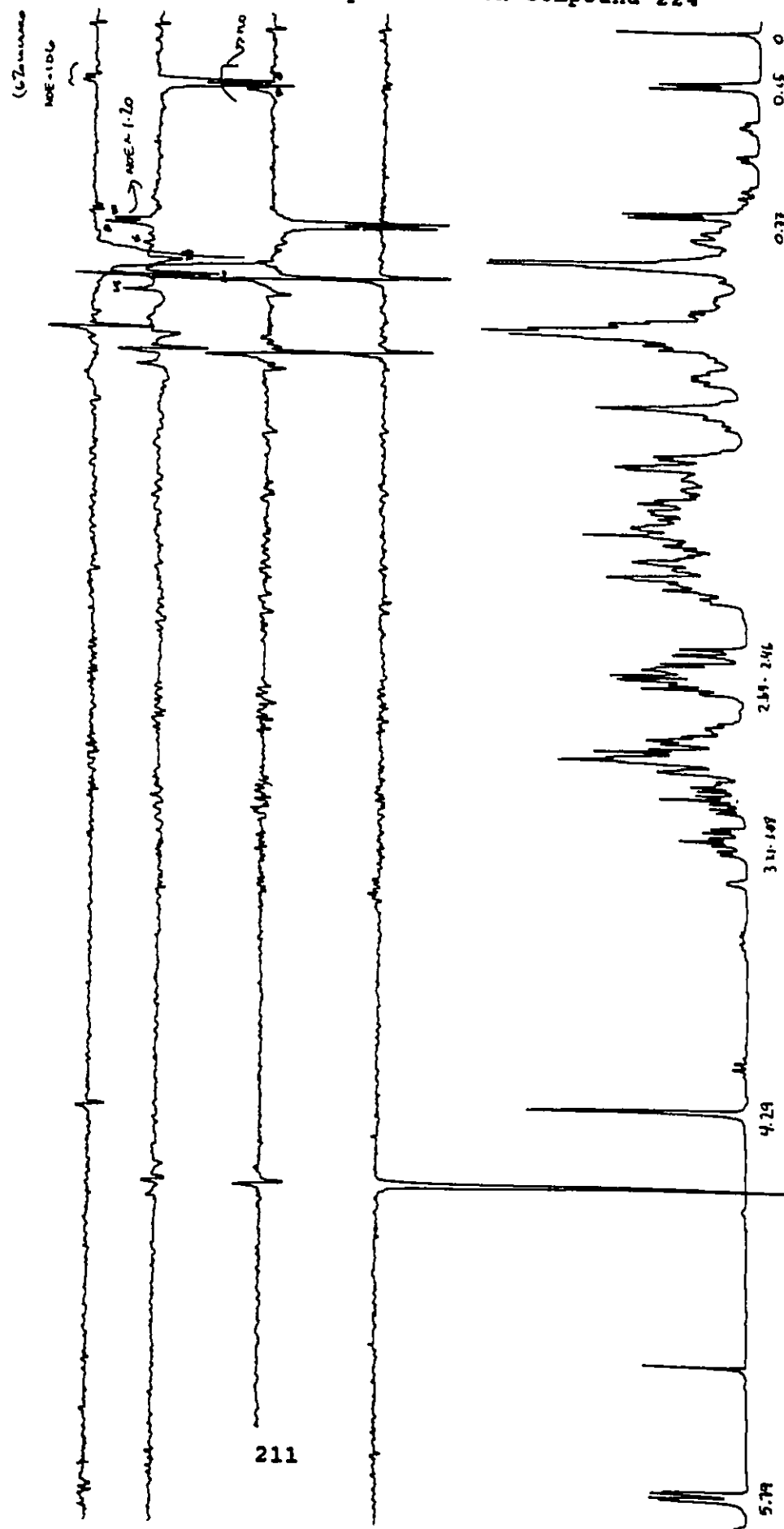
**Table 3.2.2:** Results of the nOe experiments on 224.

| Irradiate<br>↓ $\delta$ (ppm) | effect (%) |         |     |      |       |
|-------------------------------|------------|---------|-----|------|-------|
|                               | 0.2        | 0.7     | 4.2 | 5.77 | other |
| 0.25 doublet                  | --         | 15 (22) | w   | w    | 3.1 m |
| 0.77 doublet                  | 8 (21)     | --      |     | w    | 3.1 m |
| 4.2 singlet                   |            | 1 (2)   | --  | w    |       |
| 18-CH <sub>3</sub> singlet    | (6)        | (-3)    |     | w    | 1.3 m |

w: weak effect; values in parenthesis are range of effect when a doublet is affected. Observable effects indicate proximity between the hydrogens.

Figure 3.2.2: Results of the NOE experiment on compound 224

224 : Results of the NOE experiments on 224.



## Correlation Spectroscopy (COSY)

Correlation spectroscopy identifies coupling between nuclei. This technique was applied to the structure elucidation.

**Table 3.2.3:** Results of the COSY experiments on 224.

| <sup>1</sup> H NMR δ (ppm)                       | Coupled to |       |        |       |
|--------------------------------------------------|------------|-------|--------|-------|
| 0.25 doublet                                     | 0.77d      | 2.15m |        |       |
| 0.77 doublet                                     |            | 0.22m | 2.46m  |       |
| 4.29 singlet                                     |            | 5.79s | 2.46 m | 2.15m |
| 5.79 quartet                                     |            | 4.2s  | 2.46 m | 2.15m |
| 2.85-2.91 m CH <sub>2</sub> S 4-5H includes 1-CH |            |       |        |       |
| 2.46-2.64 m CH <sub>2</sub> S 4-5H includes 1-CH |            |       |        |       |
| 3.08-3.21 m CH <sub>1</sub> S 1H                 |            |       |        |       |
| 2.9-3.04 m CH <sub>1</sub> S 1H                  |            |       |        |       |
| -2.15 m CH <sub>2</sub> CS                       |            |       |        |       |

# Results of the COSY experiments on 224.

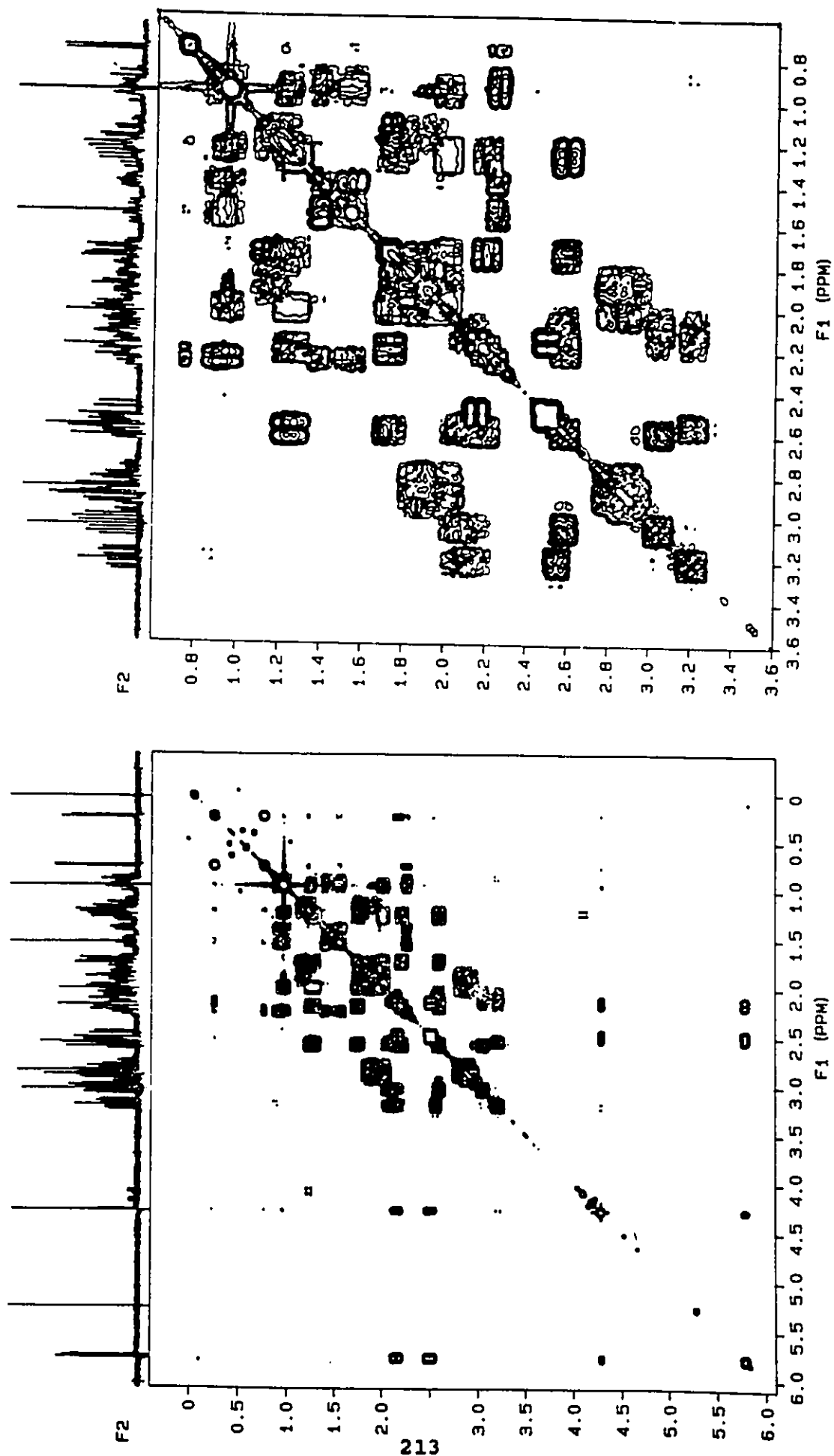
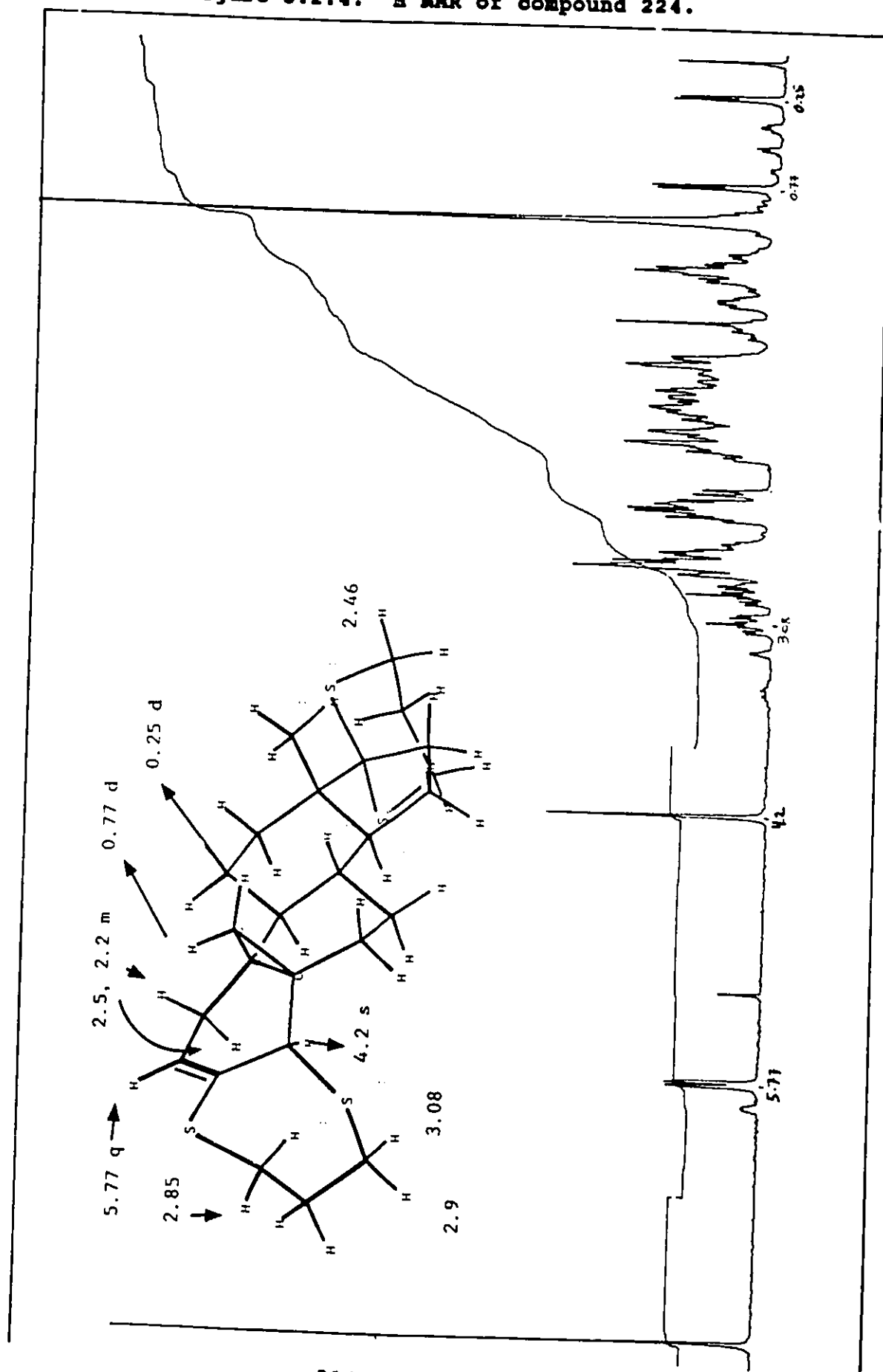


Figure 3.2.3: Results of the COSY experiment on compound 224.

Figure 3.2.4:  $^1\text{H}$  NMR of compound 224.



The following structure was minimized with MM2, and would fit the correlation data as follows:

The 2-CH,1-CH<sub>2</sub> angles can be established from the minimized structure, and the Karplus equation yields the coupling constants: this computes as;

2-H/1 $\beta$ -H coupling of 5.75 Hz, for an angle of 27° and

2-H/1 $\alpha$ -H coupling of 2.60 Hz, for an angle of 89°.

This defines a pattern similar to that observed for the alkenyl proton.

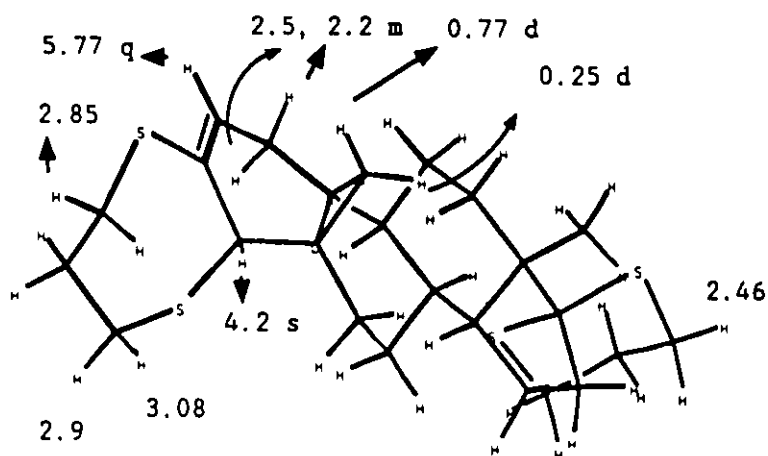


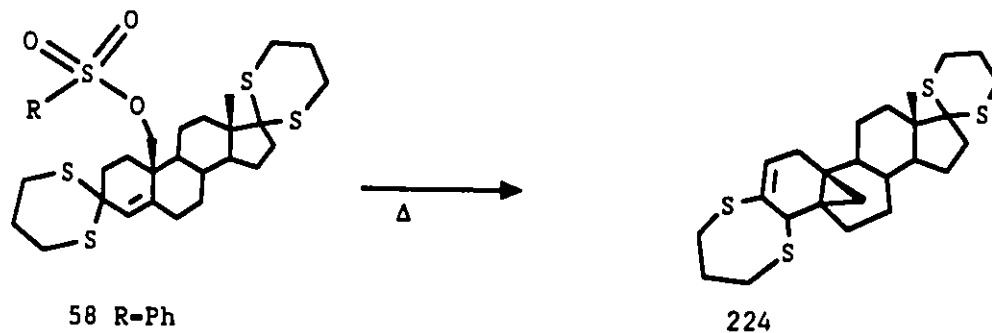
**Figure 3.2.5: Structure of compound 224, with assignments of key protons.**

Allylic hydrogens in positions  $\alpha$  and  $\beta$  are often found in the 2.8-3.1 region, and they can be quite distinct. The COSY experiment clearly indicates that no measurable coupling occurs within the 2.8-3.8 ppm region. It is linked instead to the 2.46-2.64 m region, normally assigned to CH<sub>2</sub>S, but exceptionally demonstrating a high integration. The downfield multiplex usually related to the second set of CH<sub>2</sub>S also exhibits a high integration: thus it seems probable that the allylic protons, are embedded in the 2.2 and 2.5 multiplets. The low field multiplets at 3.08-3.21 and 2.9-3.04, integrating for one hydrogen each, could be hydrogens from the CH<sub>1</sub>S, shifted by the presence of the alkene. The nOe experiments also confirm this pattern.

Though there would be an expected effect between geminal protons (0.25 and 0.77), no effects are observed with coupled hydrogens otherwise. Through space effects with the 0.77 peak, over the A ring, would appear, but none would appear with the 0.25 singlet situated over the B ring. With this assignment then, the coupled 1-CH<sub>2</sub> would show no effect, and the hydrogen 3-CH<sub>1</sub>S closest to the  $\beta$ -19 propane would exhibit some effect, and it does.

**Conclusion to nucleophilic addition at the C-19 position of dithiane 58.**

The C-19 sulfonates investigated are unreactive to the nucleophiles tested. Instead of the desired addition, rearrangement products are produced. These could occur as a result of the  $\pi$ -bond assisted C-19 elimination, followed by stabilization of carbocations and either addition or rearrangements at the reactive center. Characterization of 224 seems to confirm this sequence, via stabilization of the carbocation by the sulfur atoms, and collapse by loss of the 2-hydrogen to form the alkene.

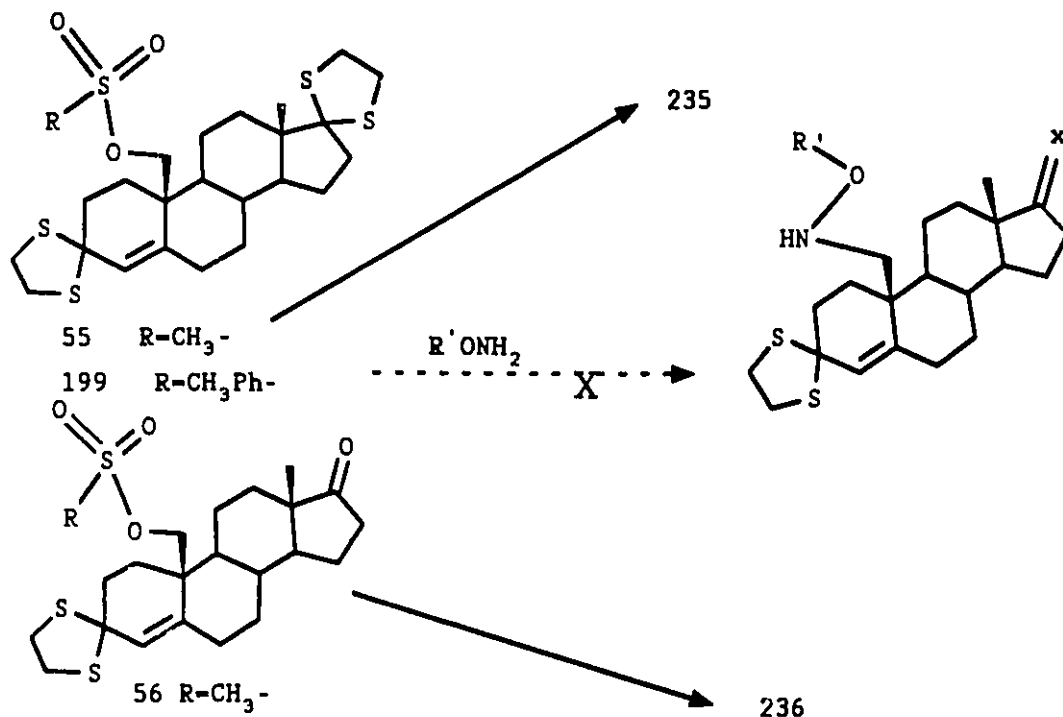


**Scheme 3.2.3**

In order to decrease the steric hindrance above the A-ring, and perhaps facilitate C-19 displacement, the smaller 55 and 56 mesylate dithiolane derivatives were used.

# Investigations of the nucleophilic addition to mesyl bis-dithiolane.

The smaller dithiolane derivatives should decrease the hindrance to nucleophilic attack at the C-19 position. Reactions with these were investigated.



Scheme 3.2.4

## Reaction of NHS with (55) 19-hydroxy-4-androstene-3,17-bisethylenethioketal 19-methanesulfonic ester.

Though 19-mesyl-bis-dithiolanes seemed more reactive than the dithianes towards nucleophiles, the product was not the desired addition product. The spectral characteristics of the N-hydroxy-succinimide addition products 235 and 236 could not be accounted for.

Although the mono-dithiolane 56 displays less steric hindrance on the  $\beta$ -face, the addition products could not be identified readily either.

The thermal sensitivity of the mesyldithiolane was assessed.

**Preparation of 237 and 238: The thermal degradation of (55)  
19-hydroxy-4-androstene-3,17-bisethylenethioketal  
19-methanesulfonic ester.**

Two products were generated during the thermal degradation of 55 in refluxing pyridine; the less polar product 237 corresponds, (TLC,  $^1\text{H}$  NMR,  $^{13}\text{C}$  spectra), to compound 235, the result of NHS reaction. This confirmed that thermal rearrangement occur before significant nucleophilic addition can take place at the hindered C-19 position. A more potent leaving group was sought.

**Preparation of 239: Reaction of NaI with (56)  
19-hydroxy-4-androstene-3-ethylenethioketal-17-one  
19-methanesulfonic ester.**

The transformation of the 19-mesylate to an iodide was thought to increase the chances of reacting the N-O-based alpha effect heteroatoms (NHS-, BnNO-) at the C-19 position, as it is often useful in carbohydrate chemistry to replace the sulfonate groups with iodo leaving groups,

Nevertheless, addition of NaI in acetone to the sulfonate 56 resulted in no product after two days. Changing the conditions to refluxing isopropanol for one day gave a product with a mass spectra indicating a parent ion incorporating an added iodide ( $P^+ = 488$ ) and a corresponding peak at ( $M-128$ ) corresponding to loss of the iodide. The infrared revealed the absence of  $\text{SO}_2$  and  $\text{SO}$  bands and, surprisingly, exhibited no C-I stretch band at  $500\text{ cm}^{-1}$ .  $^1\text{H}$ -NMR confirmed that a simple addition at C-19 had NOT occurred as there was no  $\text{CH}_2\text{I}$  peak, and the product had lost

the C-4 alkene proton. A multiplet at 3.28 integrating for one proton and a new singlet at 0.648 integrating for two hydrogens characterized the spectra.  $^{13}\text{C}$ -NMR confirmed that the rearrangement product had an intact ketone, two quaternary alkene carbons, and had lost the  $\text{CH}_2$  peak expected in the 50 to 75 ppm carbon-heteroatom region.

**Preparation of 240:**

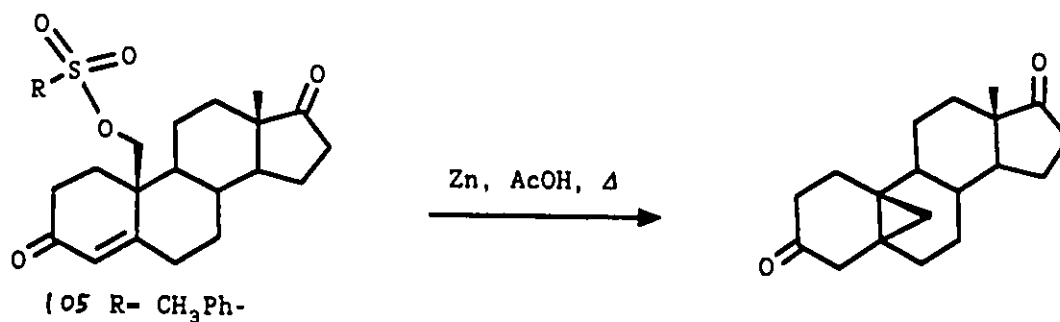
**Reaction of NaI with (55) 19-hydroxy-4-androstene-3,17-bisethylenethioketal 19-methanesulfonic ester.**

Similarly, compound 55 was treated with NaI at increasing temperatures until reaction occurred. The products, however, consisted of a pattern of quaternary carbons unlike that of thermal rearrangement products 237 or 238, though the  $^1\text{H}$  NMR at the low field looked similar.

Since release of steric hindrance by changing from bis-dithiane to bis-dithiolane to mono-dithiolane did not prove sufficient, and use of C-19 leaving groups such as tosyl, mesyl or the smaller iodide were also plagued with rearrangement, it was decided to elucidate the structure of the thermal rearrangement products.

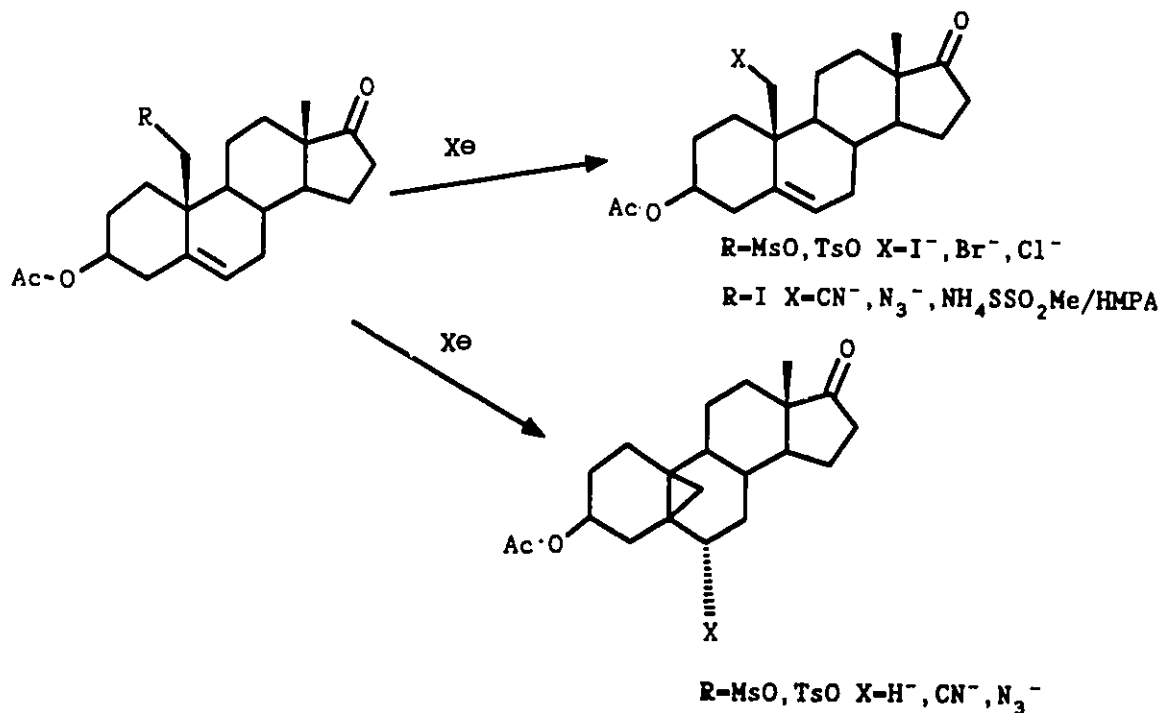
**Review: Reactivity of the 19-sulfonate groups.**

Steroids with C-19-sulfonate are known to rearrange. In these, a double bond at C-4,5 or C-5,6 can participate in the elimination of the 19-sulfonate, with stabilization of the resulting C-5 tertiary carbocation through various rearrangements. For example, reducing the tosylate with zinc powder yields a cyclopropane<sup>27</sup>. A similar reaction product was described earlier as a product of the thermal degradation of the bis-dithiane tosylate 58.



**Scheme 3.2.5**

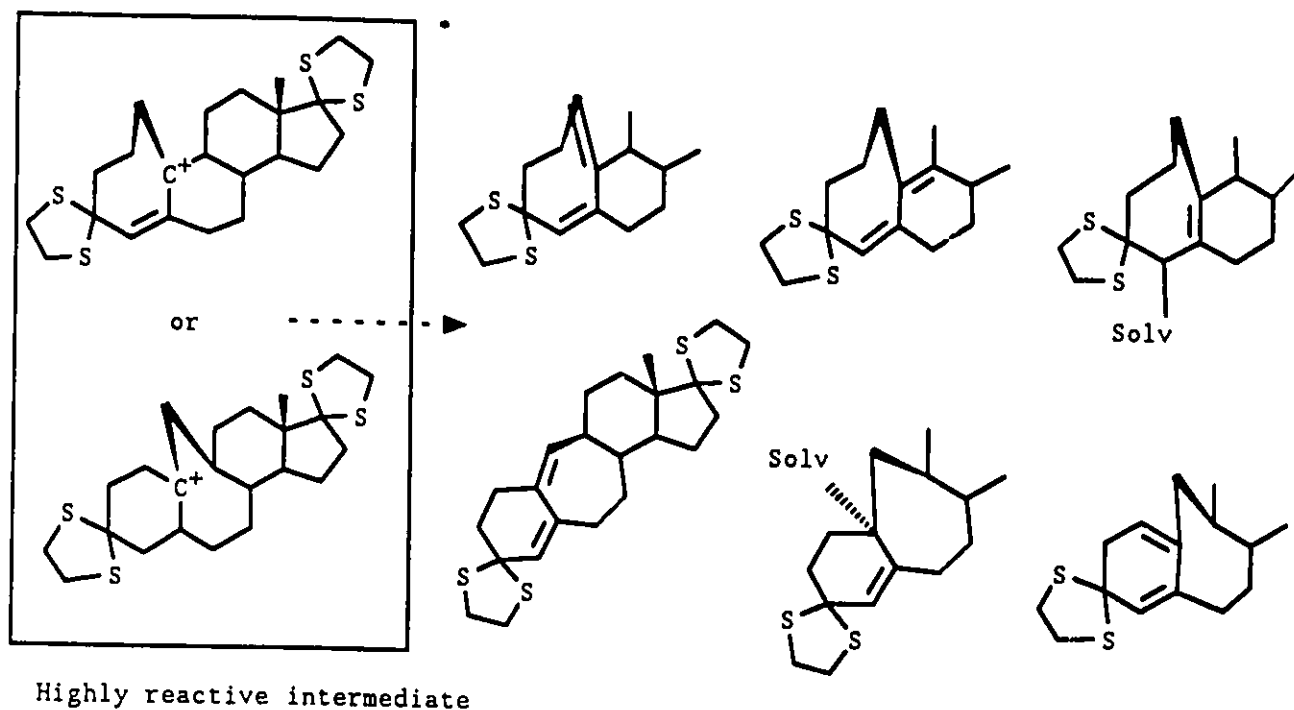
The presence of a 5,6 double bond activates the C-19 position. Nucleophilic additions to these can proceed either by direct addition to the C-19 leaving group, or by quasi "vinylogous" addition to the C-6 alkene, forming the 5,10 cyclopropane<sup>28-31</sup>.



**Scheme 3.2.6: reactivity of  $\Delta^5$ -19-leaving groups with nucleophiles.**

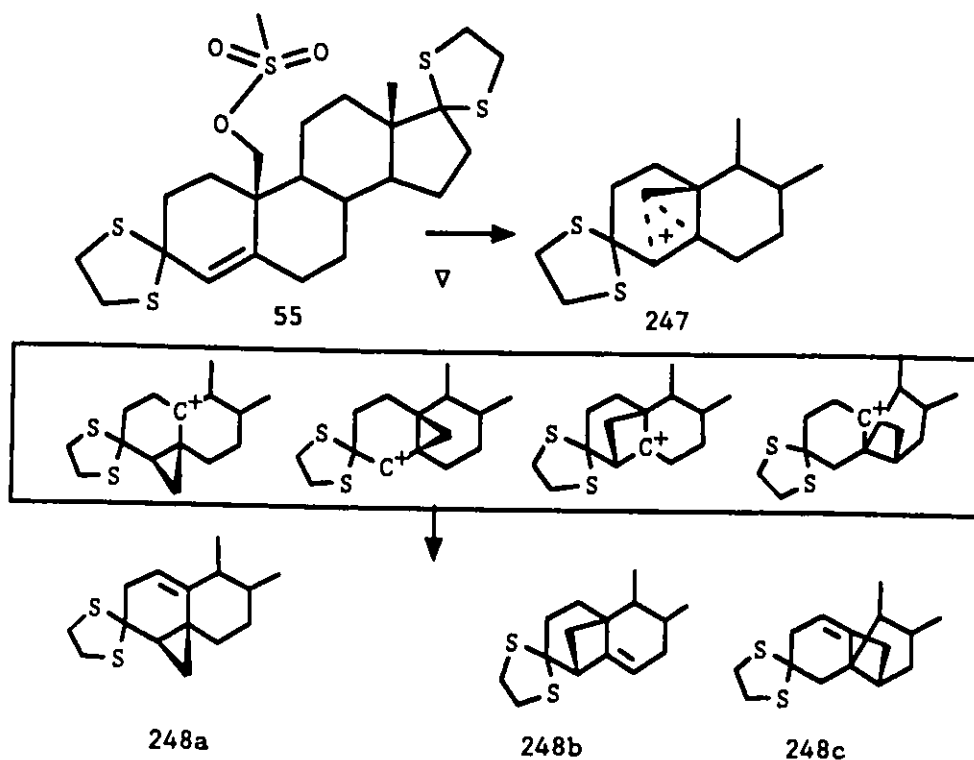
## Analysis of the thermal rearrangement of bis-dithiolanes

By analogy with the published reactions of steroidal- $\Delta^5$ -19-sulfonates, the following products are likely in the thermal rearrangement of 19-mesylate-bis-dithiolane **55**.



**Scheme 3.2.7**

A reasonable speculation is that internal rearrangement occurs prior to the nucleophile addition since spectral analysis indicates that **237** and **238** are not addition products. The reactive rearrangement intermediates could have the following structures.



**Scheme 3.2.8: Possible rearrangements products of the thermal degradation of 19-mesylate 55.**

#### **Spectral interpretation of compounds 237 and 238.**

237 shows six quaternary and three tertiary carbons by  $^{13}\text{C}$  NMR. The  $^1\text{H}$  NMR shows no alkene hydrogens and no C-19-methylene-sulfonate. The mass spectrum is similar to starting material. Spectroscopic data on the product of the thermal rearrangement of 55 could be satisfactorily explained by structure 248a, the improbable anti-Bredt's compound 248b or the strained 248c.

#### **Mass Spectra of the rearrangement compounds**

Both rearrangement products show a strong parent ion at 436 and no peaks at higher mass. The two spectra differ slightly in the lower molecular weight range.

An analogy to the thermal rearrangement can be made with the process occurring in the injection port of a GC-MS: The high temperatures there generate the first kinetically more stable species, which are then separated and ionized. The MS of the starting material **55** thus, indicates a parent ion at 532 with a strong degradation peak at (M-96), which corresponds to loss of (M-CH<sub>3</sub>SO<sub>2</sub>O,H), the C-19-methane sulfonate.

#### **<sup>1</sup>H 300 MHz NMR of the rearrangement compounds**

The <sup>1</sup>H 300 MHz NMR spectrum of the starting mesylate is characterized by a doublet for the CH<sub>2</sub>-19-methylene (4.45 ppm, 2H, d), the mesyl (3.0 ppm, 3H, s) and the alkene singlet (5.73 ppm, 1H, s).

**These are all absent in the products.**

**237** displays no alkenyl peaks, no mesyl and no C-19 in the usual region.

**238** displays a quartet in the alkene region (5.98 ppm, 1H, ab quartet, 2.3Hz), no mesyl and a triplet corresponding to one hydrogen at (4.01 ppm, 1H, 1.7Hz).

Thus, both products have a rearranged A ring and seem to have lost integrity of the C-19 position.

#### **Spectral analysis of the rearrangement products.**

High resolution spectra and HETCOR indicate the following: the high field shows an unusual AB quartet (0.6ppm, 2H, abq, 4.4Hz) attached to a CH<sub>2</sub> at 27.7 ppm, a multiplet with a sharp doublet at (0.8 ppm, 3H, d/d/d, 7Hz) attached to the carbon CH<sub>2</sub> at 27.6 ppm. There is a quaternary carbon at 24.9 ppm. This region shows one more distinct hydrogen at (0.75p, 1H, d, 10Hz) attached to a 32.3 ppm CH. The C-18 methyl appears to be a very fine split doublet, (0.914 ppm, 3H, d, 0.6Hz). The C-17 quaternary bisdithiolane carbon remains at the usual 80.5 ppm,

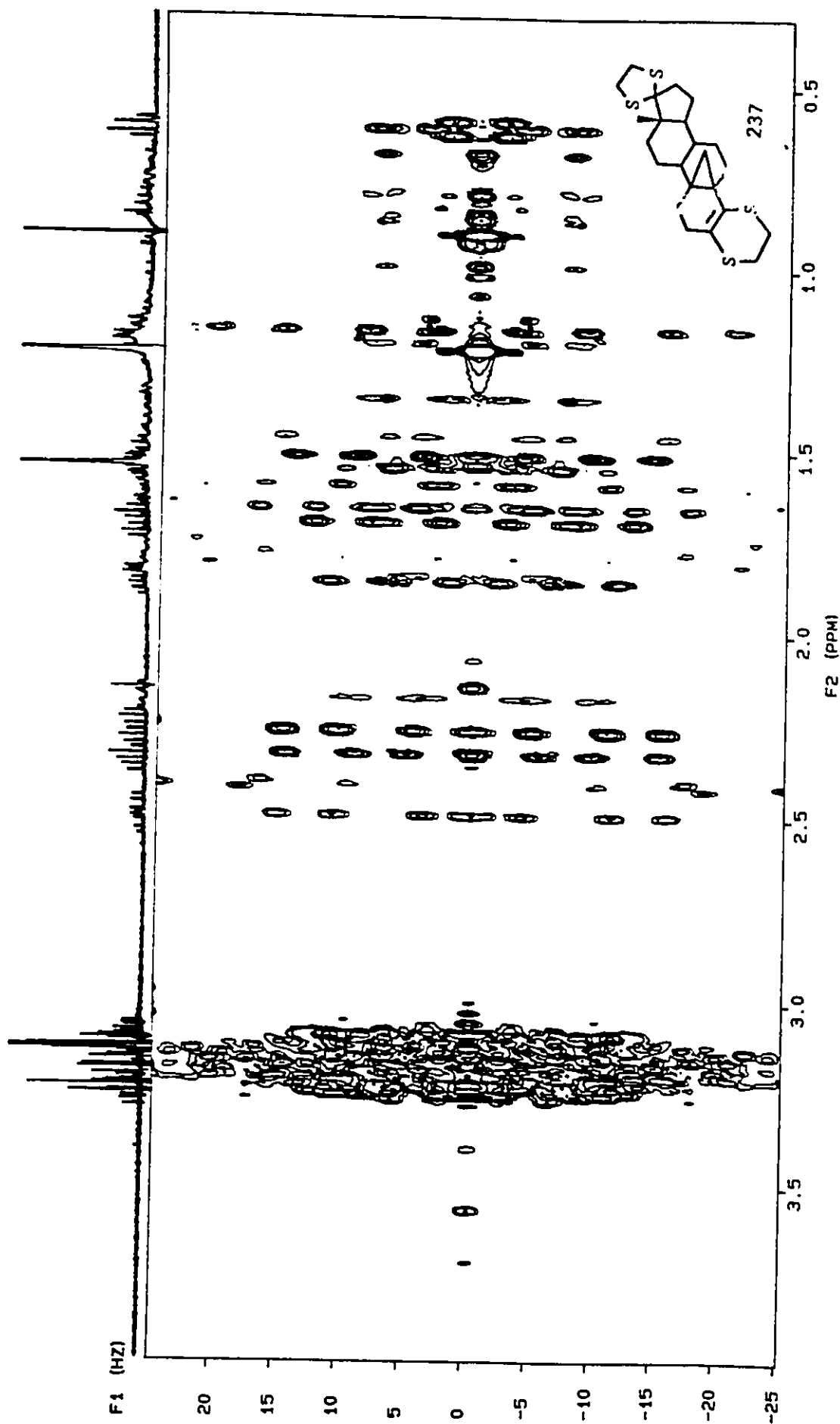
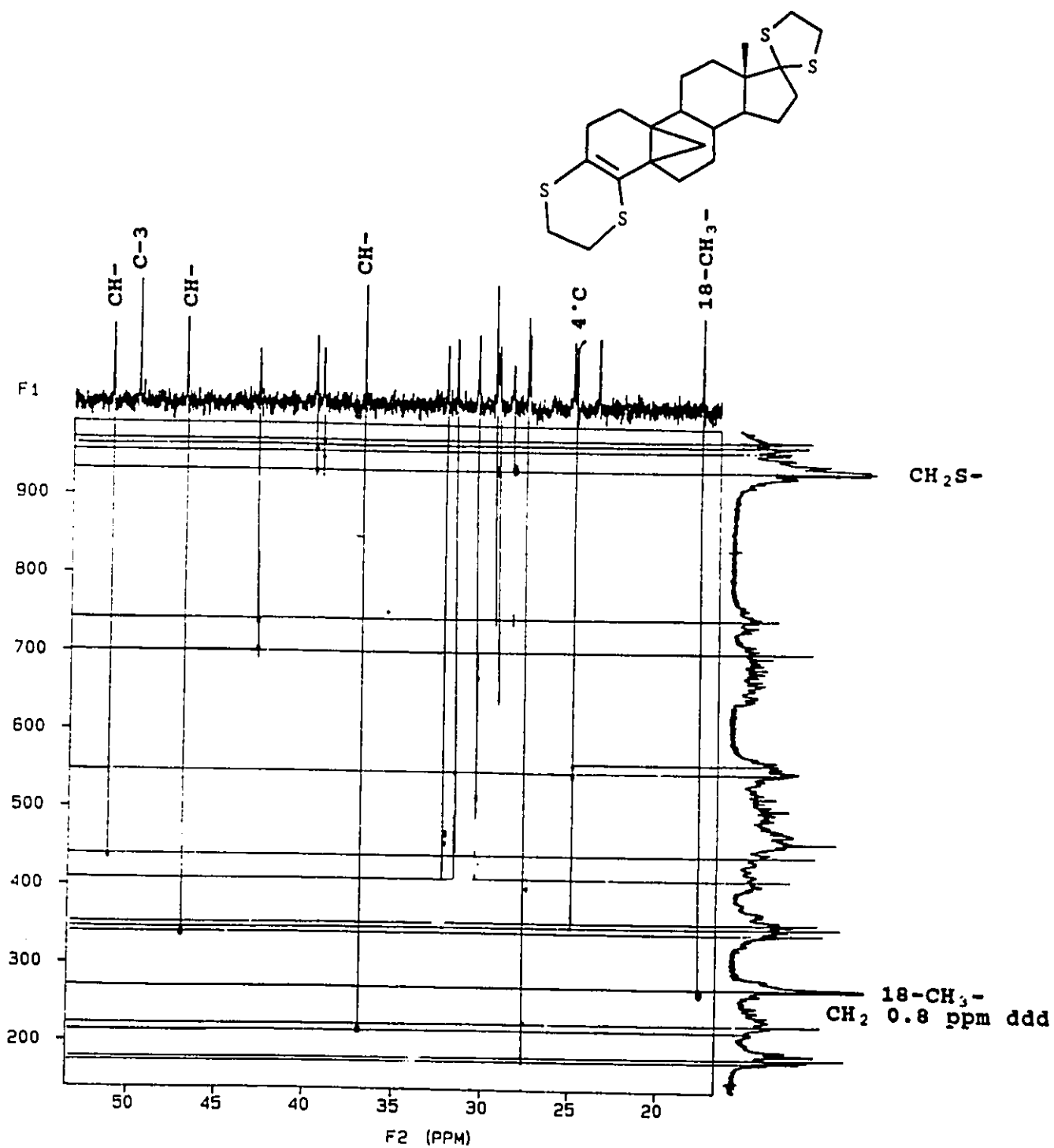


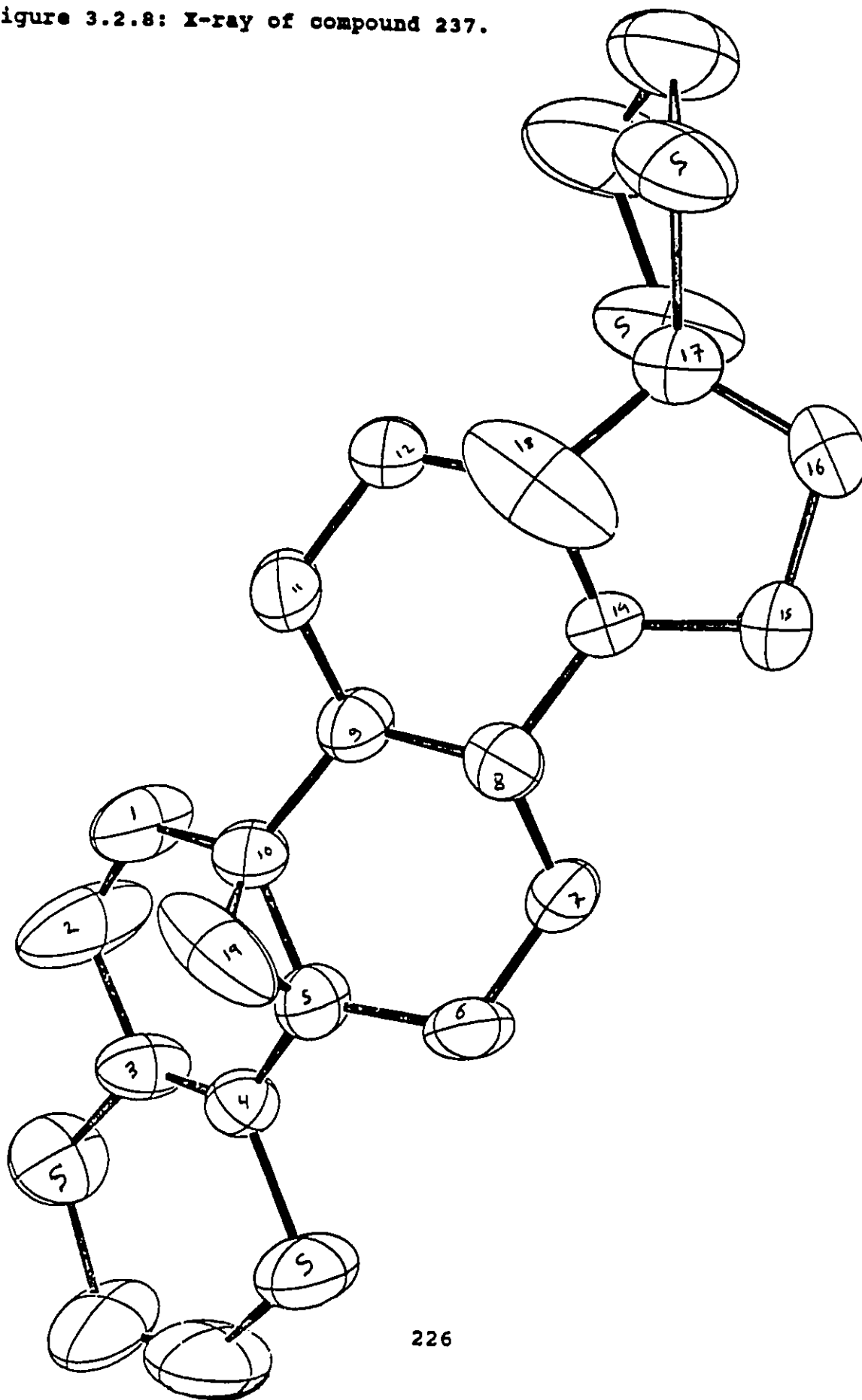
Figure 3.2.6: 2-D J-resolve spectrum of compound 237.

Figure 3.2.7:  $^{13}\text{C}$ -HETCOR spectrum of compound 237.



The structure of 237 was confirmed by X-ray spectroscopy.

Figure 3.2.8: X-ray of compound 237.



**Analysis of the thermal degradation of  
19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androsterone-3,17-dione (78) by  
"flash" pyrolysis in the presence of silylating agents and  
chromatographic separation.**

Silylation and direct injection in a pre-heated injection port (200°C) of a gas chromatograph attached to the mass spectrometer afforded a convenient method of analysis of the pyrolysis products of 78. GC separation was effected on a 6% OV-101 column with a temperature ramp of 10°C/min. This experiment afforded three peaks in the GC.

A. The more volatile corresponds to estrone treated in a similar fashion, with appropriate isotopic abundance for  $C_{21}H_{30}O_2Si_1$  and the following ion fragmentation: MS m/z: calc:342; 342(P $\oplus$ ,100%), 257 (26%), 218(24%)

B. The second compound to be separated corresponds to silylated 184, with appropriate isotopic abundance for the major breakdown product of the parent ion and the following ion fragmentation:  $C_{21}H_{32}O_3Si_1$  calc:360; MS m/z: 360(P $\oplus$ ,2%), 345(M-15, 100%), 244(61%)

C. The third compound to be separated corresponds, at least in natural abundance and molecular weight, to doubly silylated estr-3,5(10)-diene-3,4-diol-17-one 185, with appropriate isotopic abundance for the major breakdown product of the parent ion.

Though the following ion fragmentation is by no means proof of the elusive 185, the data is indicative.  $C_{24}H_{40}O_3Si_1$  calc:432; MS m/z: 432 (P $\oplus$ ,10%), 430 (92%), 416(22%), 360(11%, could be TMSi of compound 184), 358(100%), 345 (82%, major breakdown of compound 184), 342(76%, could be TMSi-estrone)), 345(M-15, 100%), 244(61%).

**Reaction of 72 with tBDMSi-Cl/imidazole:**

**Formation of 4-chloro-4-androsten-19-ol-3,17-dione (44)  
and 4-chloro-4-androsten-19-ol-3,17-dione,  
19-tert-butyltrimethylsilylether(186).**

The epoxide 72 160 mg (0.507 mM) was added to a solution containing 2.2 eq. of tBDMSi-Cl in 10 ml of DMF. After one day, two UV positive compounds appeared by TLC. Work up involved pouring in water, extracting in  $\text{CH}_2\text{Cl}_2$  and washing with water. This afforded two compounds which were chromatographed on a short  $\text{SiO}_2$  column in 30% ethyl acetate/hexane:

**4-chloro-4-androsten-19-ol-3,17-dione,  
19-tert-butyltrimethylsilylether(186).**  $\text{C}_{25}\text{O}_3\text{H}_{38}\text{Cl}_1\text{Si}_1$  (26 mg, 11% yield), MS  $m/z$ : 450 ( $\text{P}^+$ , (0.2%), 422/420, 395/393 (M-57), 357 (M-Cl), 306, 193, 89, 73 (typical of tBDMSi derivatives)  $R_f=0.9$  in E/H 4/6;  $^1\text{H}$  NMR ( $\text{DMSO}-d_6$ )  $\delta$  3.83-3.94 (19- $\text{CH}_2\text{O}$ , AB q, 2H,  $\delta_a=3.92$ ,  $\delta_b=3.85$ ); 0.82 (18- $\text{CH}_3$ , s, 3H); ADEPT  $^{13}\text{C}$ : 26 (tBuSi); 14 (18- $\text{CH}_3$ ); -6 ( $\text{CH}_3\text{Si}$ ); 220 (17-C=O); 191 (3-C=O), 160 (5-C=C), 129 (4-CH); IR ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ , KBr) 3440 br, 3500 (br) 1745 (17-C=O), 1685 (3-C=O), 1590 (C-O)

**4-chloro-4-androsten-19-ol-3,17-dione (44)**  
(50 mg, 29%) fluffy white needles from E/H. HRMS for  $\text{C}_{19}\text{O}_3\text{H}_{25}\text{Cl}$  calc: 336.1490 found: 336.1474 and MS 336 ( $\text{P}^+$ , 336/338 (30/10%), 306/308 (100/33%, M-30); no peak at 73;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.68-3.93 (19- $\text{CH}_2\text{O}$ , m, 2H,  $\delta_a=3.90$ ,  $\delta_b=3.71$ ,  $J_{ab}=2.47$ ,  $J=0.6$ ,  $J=5.5$  Hz d of d of AB quartet); 0.82 (18- $\text{CH}_3$ , s, 3H); 4.89 (OH, t, 1H,  $J=5.5$  Hz);  $^1\text{H}$  NMR in acetone- $d_6$  loses the OH triplet at 4.89 and alters the 19- $\text{CH}_2$  to an AB quartet at 4.0 ( $\delta_a=4.1$   $\delta_b=3.9$   $J_{ab}=11$  Hz).  $\lambda_{\text{max}}$  (MeOH) = 256 nm,  $\epsilon=13,357$  calculated at 259 nm. MS ADEPT  $^{13}\text{C}$  spectra shows 4 quaternary carbons ADEPT  $^{13}\text{C}$ :  $\delta$  190 (3-C=O), 219 (17-C=O), 162 (5-C=C), 127 (4-Cl-C=), ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ , KBr) 3510, 3500 (br) 1725 (17-C=O), 1675 (3-C=O), 1580 (4-C=C), 1055 (19-C-O).

**Reaction of 72 with HCl/DMF: formation of 44.**  
**Synthesis of 4-chloro-4-androsten-19-ol-3,17-dione (44)**

To 112 mg of 19-OH-4,5 $\beta$ -epoxy-A 72 add DMF saturated with HCl gas. after 30 minutes add 20 ml (9% aqueous) K<sub>2</sub>CO<sub>3</sub>, filter, wash with water, pick up in CH<sub>2</sub>Cl<sub>2</sub>. Insoluble in EA, CHCl<sub>3</sub>, Bz. Chromatography (1/40/40: DMF/EA/Hex.) yields 60 mg product (50%). MS 336/338 (P<sub>0</sub>, 30/10%), 306/308 (100/33, M-30); <sup>1</sup>H NMR (DMSO-d<sub>6</sub>)  $\delta$  3.68-3.93 (19-CH<sub>2</sub>O, m, 2H,  $\delta$ , 3.90,  $\delta$ , 3.71, J<sub>ab</sub>=2.47, J=0.6, J=5.5 Hz d of d of AB quartet); 0.82 (18-CH<sub>3</sub>, s, 3H); 4.89 (OH, t, 1H, J=5.5 Hz). ADEPT <sup>13</sup>C spectra with 4 quaternary carbons (190, 219, 162, 127) assigned respectively to C-3, C-17, C-5, C-4. IR ( $\gamma_{max}$ , cm<sup>-1</sup>, KBr) 3510, 3500 (br) 1727 (17-C=O), 1675 (3-C=O), 1580 (4-C=C), 1055 (19-C-O). This compound is identical to 44 obtained from the reaction of the epoxide with tBDMSi-Cl.

**Reactions of 19-oxo-4 $\xi$ ,5-epoxy-5 $\xi$ -androstande-3,17-dione (77) with acids and base.**

These reactions were performed in our laboratory and are documented in the doctoral thesis of Harold Mastalerz, 1981, University of Ottawa.

# EXPERIMENTAL SECTION 6: SPECTROSCOPIC DATA OF OTHER INHIBITORS

## Preparation of $\text{estra-1(10)-5(6)-diene-3-}\beta\text{-ol-17-one}$ (91)

This compound was prepared by Mastalerz, (Ph.D. thesis, University of Ottawa, 1981). It is further characterized here. The off white fluffy needles display a HRMS for  $\text{C}_{18}\text{H}_{24}\text{O}_2$  calculated at 272.1748 and found to be 272.1756. MS m/z: 272 ( $\text{M}^+$ , 100%), 254 ( $\text{M}-18$ , 9%), 197 (18%);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.54 (1-CH, d, 1H,  $J_{1,2}=3\text{Hz}$ ), 0.86 (18- $\text{CH}_3$ , s, 3H), 5.36 (6-CH, br s, 1H), 4.05 (3-CH, br  $\text{W}_{1/2}=12\text{Hz}$ ).

The following compounds were synthesized by co-workers in this laboratory and were accepted for biological testing with matching high resolution MS and one spot on TLC. Major ions are indicated.

| Compound |    |    |   |   | HRMS |            |                                         |
|----------|----|----|---|---|------|------------|-----------------------------------------|
| Code     | C  | H  | O | N | X    | calculated | observed MS m/z                         |
| 31       | 21 | 29 | 4 |   |      | 364.20499  | 364.20850: 305, 304                     |
| 32       | 19 | 26 | 5 |   |      | 334.17803  | 334.17760: 305 (M-29), 287, 249         |
| 33       | 23 | 33 | 6 |   | F    | 424.22612  | 424.22480: 364 (M-60), 304              |
| 35       | 20 | 28 | 5 |   |      | 348.19368  | 348.19290: 316 (M-MeO), 275             |
| 64       | 19 | 31 | 2 |   | Br   | 370.15074  | 370.15030: 372/370 (1:1), 313/311 (1:1) |
| 65       | 19 | 29 | 2 |   | Br   | 368.13509  | 368.13290: 368/370, 326/324 (1:1), 311  |
| 69       | 21 | 36 | 2 | 2 |      | 348.27768  | 348.27200: 316 (M-MeO), 300, 216, 101   |
| 70       | 19 | 30 | 3 |   |      | 306.21950  | 306.21480: 291 (M-Me), 278              |
| 77       | 19 | 24 | 4 |   |      | 316.16746  | 316.16610: 287 (M-CHO), 288 (M-CO), 259 |
| 81       | 19 | 32 | 1 | 2 |      | 304.25146  | 304.25190: 111                          |
| 89       | 21 | 32 | 3 |   |      | *316.20385 | 316.20160: 298 (M-16-18), 229,          |
| 90       | 21 | 28 | 2 |   |      | 312.20893  | 312.20570: 298 (M-14), 215              |
| 159      | 21 | 34 | 3 |   |      | 334.25080  | 334.24990: 302 (M-MeOH), 288, 101       |

\* High resolution was determined on the M-16 peak.

The following compounds were synthesized by co-workers in this laboratory, have no HRMS and were accepted for biological testing. They do not possess significant parent ion on the MS, but exhibit intelligible MS, and one spot on TLC. The mass ion spectra was examined and shown to be homogeneous. Major ions are indicated

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Compound

| Code | C  | H  | O | X  | P <sup>+</sup> | MS m/z                                                             |
|------|----|----|---|----|----------------|--------------------------------------------------------------------|
| 34   | 20 | 28 | 5 |    |                | 348 (P <sup>+</sup> , 1.5%), 316 (M-MeOH), 272 (M-76, 30%)         |
| 36   | 25 | 40 | 5 | Si |                | (448)na, 391 (M-57, 40%), 373 (M-Me <sub>2</sub> SiOH, 26%)        |
| 62   | 25 | 34 | 7 |    |                | (446)na, 386 (M-60, OAc), 344 (M-60-44, 33%), 284, 271             |
| 63   | 27 | 44 | 4 | Si |                | (460)na, 343 (M-57-60), 73 (silane)                                |
| 71   | 19 | 31 | 2 | Br |                | 370/372 (P <sup>+</sup> , 1.6/1.3), 290 (M-Br, 44%), 273 (M-OH-Br) |
| 80   | 23 | 32 | 5 |    |                | (388)na, 386 (1%), 344, 313, 328 (M-60, 80%), 284, 271, 253        |

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The following compounds were synthesized by co-workers in this laboratory, have no HRMS and were accepted for biological testing though they exhibit small amounts of impurities by TLC. Major MS ions are indicated

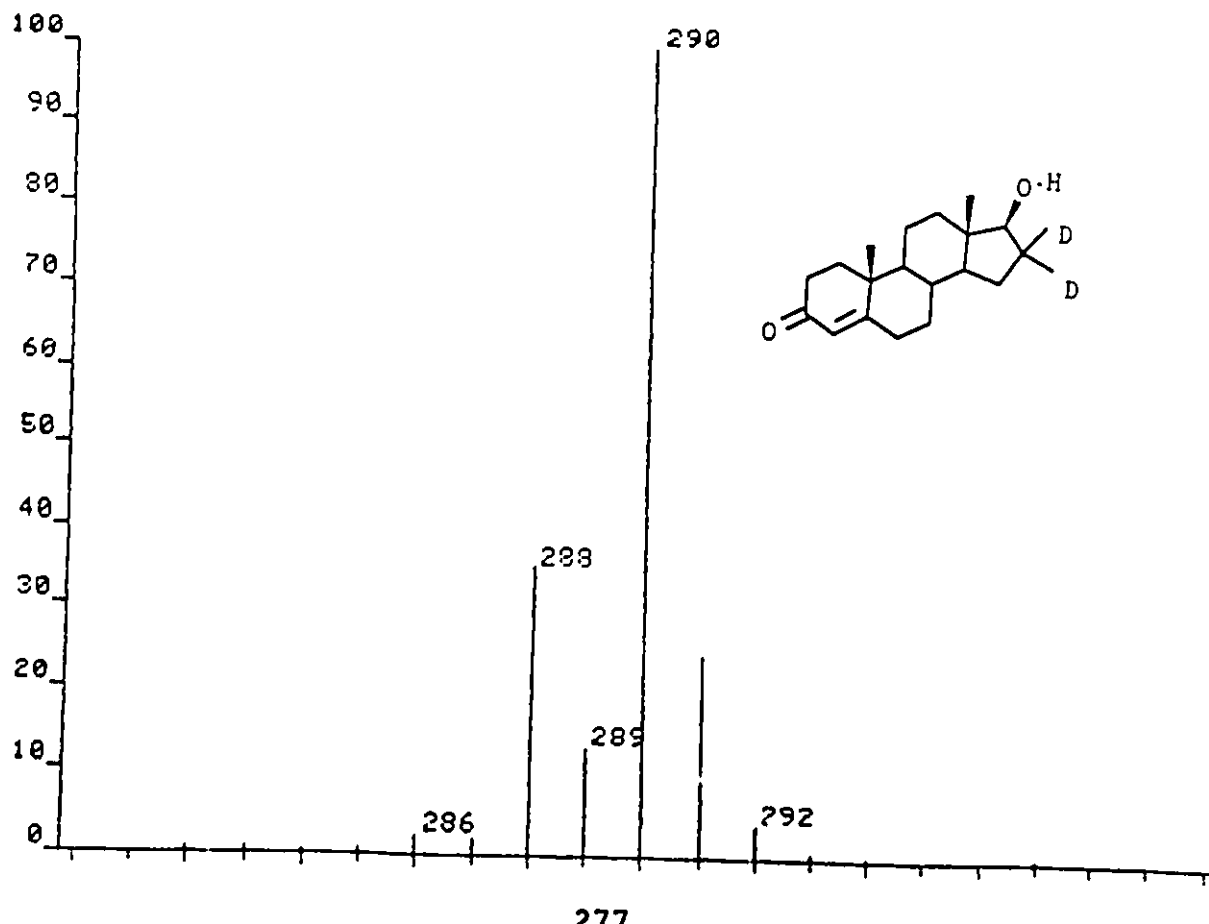
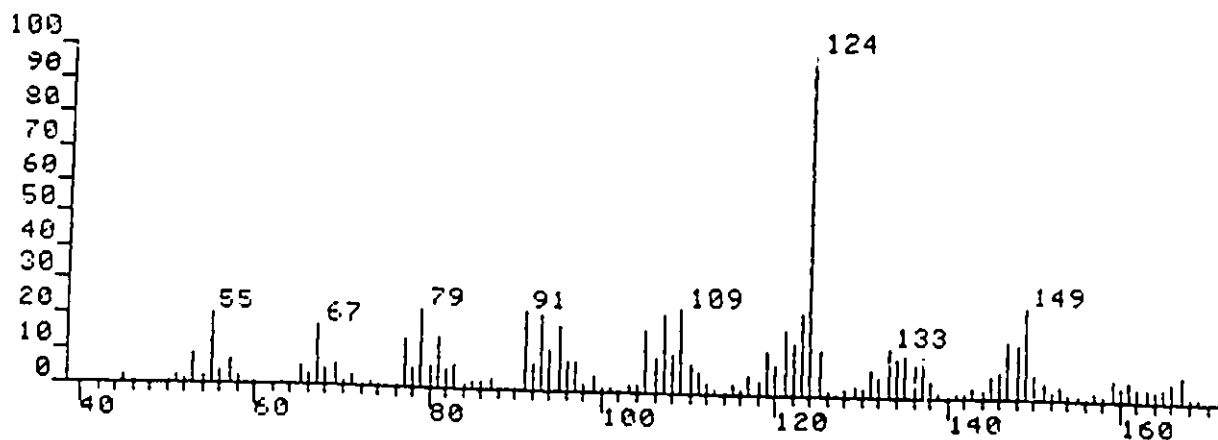
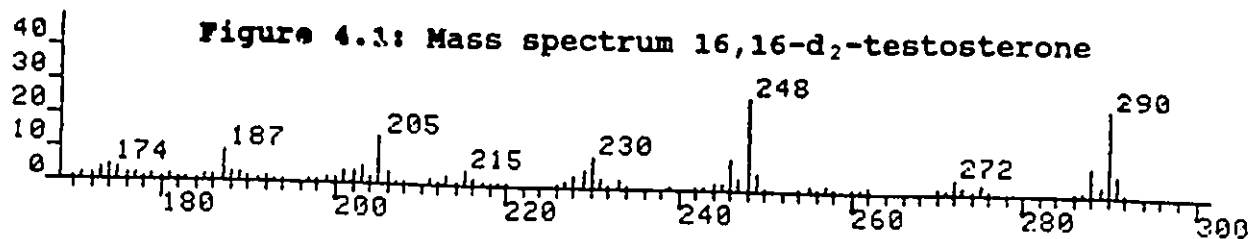
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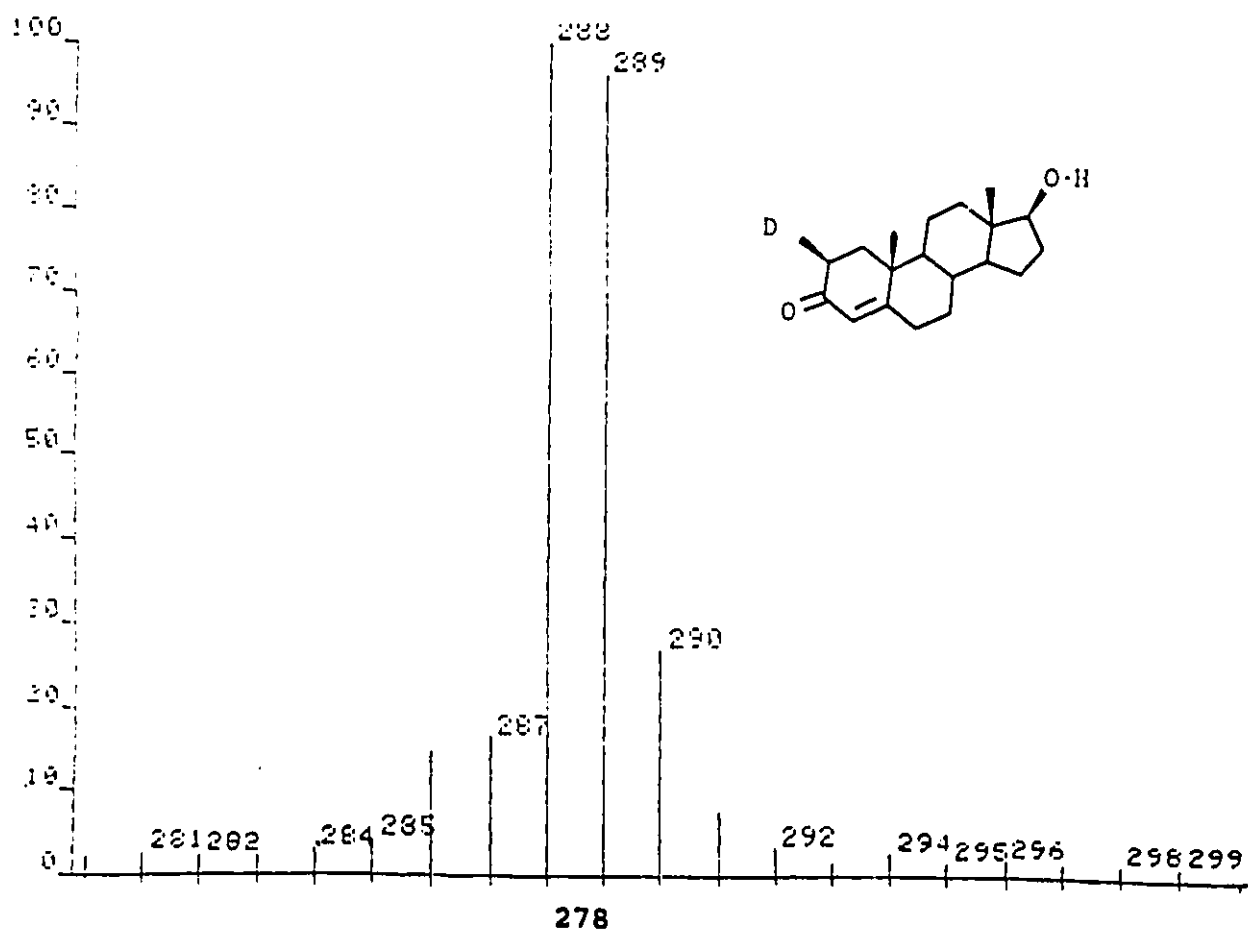
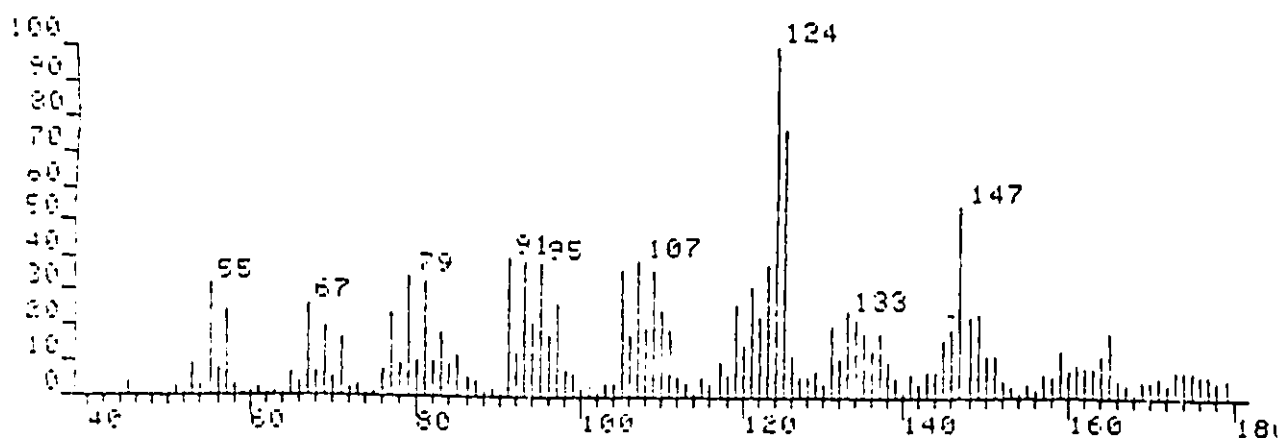
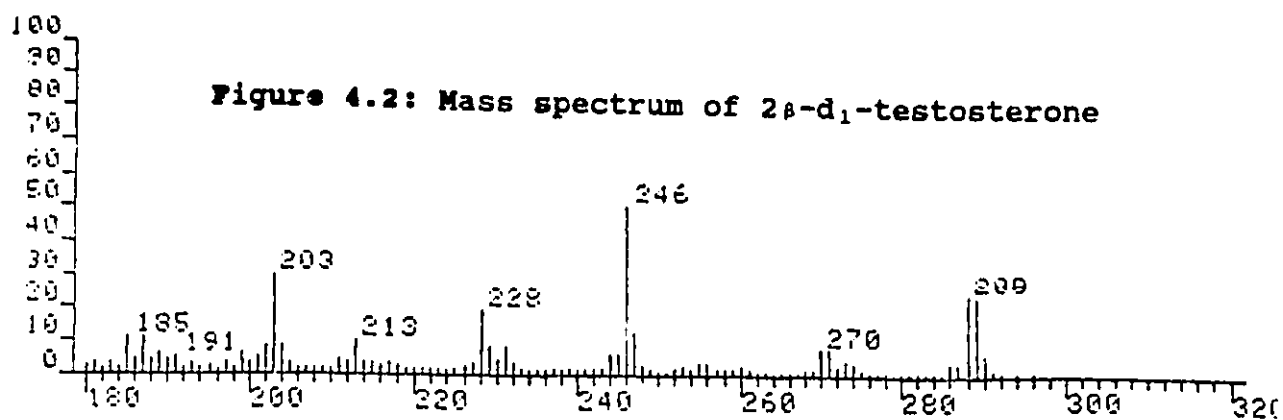
Compound

| Code | C  | H  | O |                | P <sup>+</sup> | MS m/z                                                                                      |
|------|----|----|---|----------------|----------------|---------------------------------------------------------------------------------------------|
| 66   | 19 | 33 | 2 | N              |                | 307 (57%), 290 (M-NH <sub>3</sub> or OH, 38%), 275, 259                                     |
| 68   | 19 | 32 | 2 | N <sub>2</sub> |                | 320 (100%), 303 (M-NH <sub>3</sub> or OH), 305 (M-15)                                       |
| 75   | 19 | 33 | 2 | N              |                | 307 (50%), 290 (M-17, NH <sub>3</sub> or OH 29%), 272 (M-H <sub>2</sub> O-NH <sub>3</sub> ) |
| 83   | 19 | 28 | 2 |                |                | 288 (P <sup>+</sup> ) 100%, 244, 231, 217, (290, 30%)                                       |
| 84   | 27 | 42 | 3 |                |                | 414 (59%), 396 (M-H <sub>2</sub> O), 386, 372 (M-43)                                        |

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Testosterone deuteride were synthesized in the laboratory of H. Holland and reported in Can.J.Chem., 59,2809 (1981). The mass spectra used to determine the isotopic abundances are reported below.





## Chapter 5: Biochemistry Experimental.

### SECTION 1. PREPARATION OF PLACENTAL TISSUE/MICROSOME FRACTION:

Fresh human placenta was obtained within 20 minutes of delivery and immersed in ice cold 0.15 M KCl. Subsequent manipulations were conducted in the cold room to limit tissue degradation. Connective tissue was removed, the placenta blotted dry, homogenized in a Waring Blendor with 1.5 volume ice cold 0.05M NaHPO<sub>4</sub> and 0.25 M sucrose at a pH of 7.0 and centrifuged at 10,000g for 30 minutes. The supernatant was decanted then centrifuged at 105,000g for 60 minutes. Protein concentration was assayed according to Biuret. Final concentrations were adjusted to 0.10 mg/ml with 50 mM sodium phosphate buffer at pH=7.0, and stored at -80 C°. This procedure is a modification of Brodie et al. (1986) J.Steroid Biochem. 25, 859-865.

#### ASSAY CONDITIONS:

Aromatase preparation (100 µg microsomal protein/ml) at -80 C° was warmed at room temperature for one hour. The thawing was completed in a water bath at room temperature for 10 minutes. After the addition of all other components at room temperature, the incubation flask was placed in the shaking bath at 37 C°. The enzyme activity was calculated using Reed's\* approach, from the tritium radioactivity released in the aqueous phase during the aromatization of [1 $\beta$ ,2 $\beta$ -<sup>3</sup>H]-Testosterone: 600 µL of a 3 to 5 mM NADPH solution (in 0.1 M phosphate buffer) and 700 µL of the aromatase preparation was added to 20 µL [1 $\beta$ ,2 $\beta$ -<sup>3</sup>H] Testosterone (0.1 µCi, assay concentration of 1.7nM)], 40 µL testosterone [assay concentration 2000 nM to 400 nM] and 40 µL test steroid in ethanol. Vortexing for 20 seconds started the incubation, which was carried out in the presence of air at 37 °C for 30 min in a shaking water bath. The incubation was terminated by adding 10 ml CHCl<sub>3</sub>, vortexing for 20 seconds and centrifugation at 600g for 10 min. 250 to 400 µL of the media was added to a counting vial containing 9 ml of Triton-X-scintillation cocktail. The tritium was counted over a 10 minute period.

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\* Reed K.C., Ohno S. (1976) J.Biol. Chem. 251,1625-1631  
Siiteri et al. 1974, J. Biol. Chem. 5364 (249).

## SECTION 2. KINETICS OF AROMATASE WITH STANDARD SUBSTRATE

The aromatase preparation was assayed for activity by incubating increasing concentrations of testosterone between 2 and 1970 nM, and measuring released tritium in aliquots at time intervals between 15 and 45 minutes. Analysis of specific activity against time defined the initial velocity parameters required in subsequent assays, and the testosterone concentration adequate in test steroid assays.

A typical enzyme preparation displayed a  $V_{max}$  of  $0.0055 \pm 0.0001$  n mole tritium /min/mg and a  $K_m$  of  $767 \pm 23$  nM for testosterone.

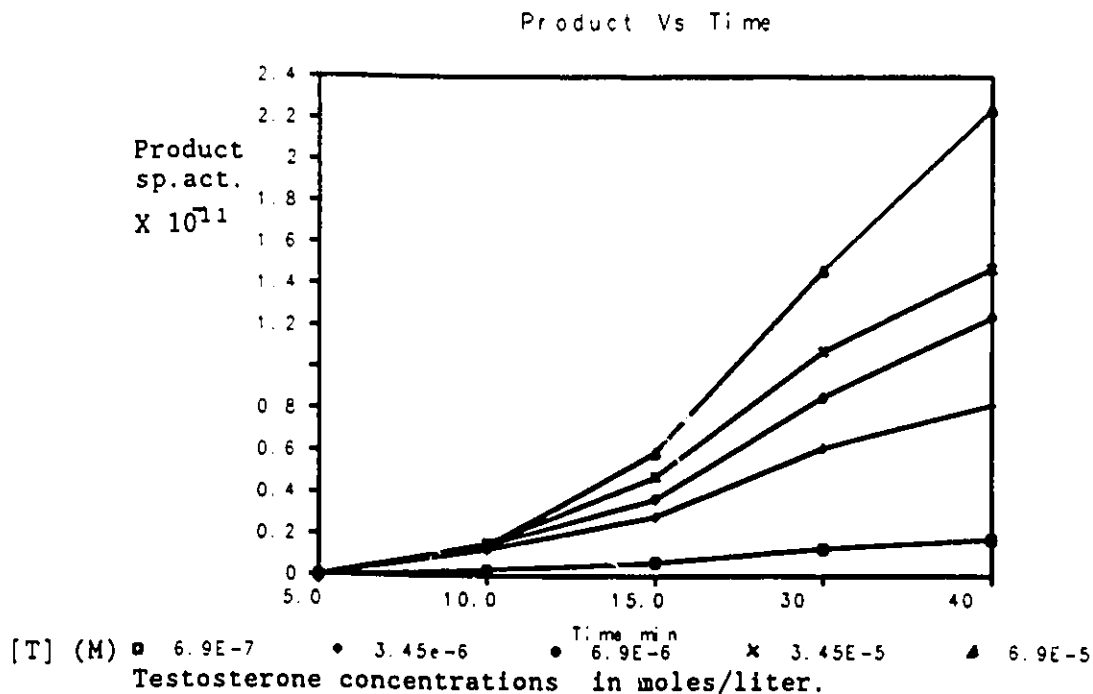


**Figure 5.1 Product formation (as specific activity) Vs. Time at various testosterone concentrations.**

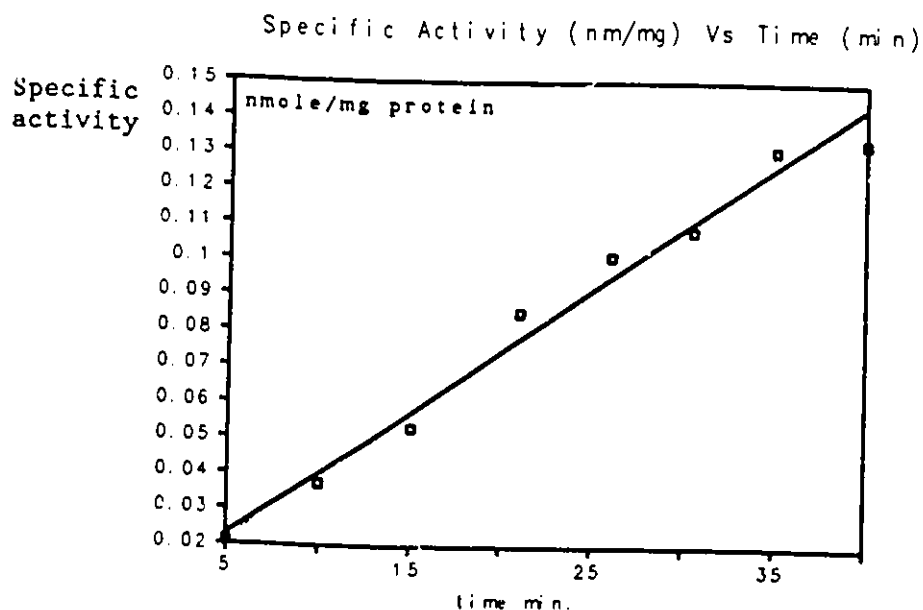


Figure 5.2: Specific activity vs. time

### SECTION 3. METHODS 1 AND 2 SCREENING TEST STEROIDS: COMPETITIVE ASSAY AGAINST TRITIATED TESTOSTERONE:

#### Preliminary Screening procedure:

The screening procedure is similar to Johnston (Johnston J.O., Wright C.L., Metcalf B.W. (1984) Endocrinology 115,776-785) with the following exceptions; steroids were tested in ethanol only, and excess reduced co-factor NADPH was supplied instead of a re-generation system.

#### 1. Screening Procedure;

Approximately 10 mg of the test steroid was dissolved in ethanol, then further diluted with ethanol to provide final assay concentrations between  $10^{-4}$  M and  $10^{-9}$  M. **Incubation:** the procedure was the same as for enzyme kinetics (see above) except for the addition of 20  $\mu$ L [ $1\beta, 2\beta$ - $^3$ H]-Testosterone (0.1  $\mu$ Ci, 1.7nM) and 40  $\mu$ L test steroid in ethanol, followed by incubation for 30 minutes.

The resulting tritium was expressed as a percent of control (%V). The simultaneous presence of tritiated testosterone and a steroid interacting with the enzyme would be observed as a decrease in the total conversion during initial kinetics. A low conversion demonstrated the important information that the steroid interacts with the aromatase.

This observed inhibition, however, did not yield information on the mechanism. Once an inhibitor was known to interact, incubations at decreasing concentrations were required to obtain the  $IC_{50}$ , the inhibitor concentration required for 50% enzyme activity. This set the stage to determine the type of inhibitor interaction observed.

#### SECTION 4: DETERMINING THE TYPE OF INTERACTION BETWEEN INHIBITOR AND AROMATASE: Method 3

These were performed under limiting enzyme conditions, such that the enzyme-substrate complex was at a steady state and there was low product formation. Incubation involved varying testosterone concentrations with a fixed inhibitor concentration. A linear plot of the data results in the type of inhibition observed. The apparent  $K_i$  was obtained, and the mode of interaction was deduced. The rate of inhibitor inactivation is  $K_{cat} = \ln 2 / t_{1/2}(\text{sec.})$

##### Incubation:

The procedure was the same as for enzyme kinetics but steroids concentrations were different: add 20  $\mu\text{L}$   $[1\beta, 2\beta\text{-}^3\text{H}]\text{-Testosterone}$  (0.1  $\mu\text{Ci}$ , 1.7 nM), 40  $\mu\text{L}$  inhibitor at a low concentration, and 40  $\mu\text{L}$  testosterone at concentrations varying from 2000 nM to 400 nM. Incubation lasted for 30 minute.

- The  $K_m$  and  $V_{max}$  were obtained from control incubations (without the inhibitor).
- The  $K_{app}$  and  $V_{app}$  were obtained by statistical analysis of the co-incubation data.
- The  $K_i$  was determined according to whether there was an effect observed on  $K_m$ ,  $V_{max}$  or both:
- Observed "Uncompetitive" effect (KV) were a special case of "Mixed" effects where both K and V were affected in the same ratio.

For the purposes of this thesis, the terms UNCOMPETITIVE INHIBITORS and MIXED INHIBITORS are not to be understood in the strict formalism of Michaelis-Menten single substrate kinetics; Aromatase is a multistage enzyme exhibiting complex kinetics.

The screening approach described here does not differentiate between uncompetitive/mixed inhibitors and irreversible inhibitors of aromatase. The purpose of the screenings performed in this thesis was to determine the apparent affinity of over 80 steroids for aromatase. See pages 46 and 47 for a more elaborate treatment of this question.

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**V effect;            Non-competitive:**

$$K_i = I \cdot V_{app} / (V_m - V_{app}) \qquad V_{app} = V_m \qquad K_{app} = K_m$$

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**K, V effect; Uncompetitive:**    can also be irreversible.

$$K_i = I \cdot K_{app} / (K_m - K_{app}) \qquad V_{app} = V_m \qquad K_{app} = K_m$$

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**K effect;            Competitive:**

$$K_i = I \cdot K_m / (K_{app} - K_m) \qquad V_{app} = V_m \qquad K_{app} = K_m$$


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Time dependence was assessed by co-incubating testosterone with inhibitor and measuring the released tritium in aliquots taken between 0 and 60 minutes. The inhibition data was calculated from the relative enzyme activity.

## CLAIMS TO ORIGINAL RESEARCH:

For the purposes of brevity, the term "Biochemistry" in this "Claims to original research list" refers to the determination of human placental aromatase affinity constants. For all of the test steroids incubated in this work, no affinity constants for aromatase have been reported in the literature. The affinity constants for all of these are claims to original research. As there are over 80 test steroids incubated with aromatase during this work, most of them will not be listed here. They are detailed in chapter 2.

- 1) Synthesis and biochemistry of 4-androsten-19-ol-3,17-dione, 19-tertbutyldimethylsilyl ether (37)
- 2) Synthesis and biochemistry of the novel 4-androsten-19-ol-3,17-dione, 19-trimethylsilyl ether (38)
- 3) Synthesis and biochemistry of the novel 4-androsten-19-ol-3,17-dione, 19-methoxymethyl ether (39)
- 4) Synthesis and biochemistry of the novel 4-androsten-6 $\alpha$ ,19-diol-3,17-dione, 19-tertbutyldimethylsilyl ether (40)
- 5) Biochemistry of 4-androsten-19-ol-3,17-dione, 19-acetate (41)
- 6) Biochemistry of 4-androsten-19-ol-3,17-dione, 19-methanesulfonyl ester (42)
- 7) Synthesis and biochemistry of the novel 4-androsten-19-ol-3,17-dione, 19-trityl ether (43)
- 8) Synthesis and biochemistry of the novel 4-androsten-4-chloro-19-ol-3,17-dione (44)
- 9) Synthesis of the novel 4-androsten-19-ol-3,17-dione, 19-benzyl ether (102)
- 10) Synthesis of the novel 5-androsten-19-ol-3,17-dione, 19-tert-butyltrimethylsilyl ether (130)
- 11) Biochemistry of 4-androsten-19-ol-3,17-dione, bisethylenethioketal, (52)
- 12) Biochemistry of 4-androsten-19-ol-3,17-dione, 3-ethylenethioketal, (53)
- 13) Synthesis and biochemistry of 4-androsten-3,17,19-trione, 3,17-bisethylenethioketal (54)
- 14) Synthesis and biochemistry of 4-androsten-19-ol-3,17-dione, bisethylenethioketal, 19-methanesulfonyl ester (55)
- 15) Synthesis and characterization of the thermal degradation product (237) 5,10-cyclopropyl-3,4-[S,S-ethylenethiane]-androsta-3(4)-en-17-one, 17-ethylenethioketal.

# CLAIMS TO ORIGINAL RESEARCH:

- 16) Synthesis and characterization of the thermal degradation product(238): 5,10-cyclopropyl-3,4-[S,S-ethylenethiane]-androsta-2(3)-en-17-one, 17-ethylenethioketal.
- 17) Synthesis and biochemistry of 4-androsten-19-ol-3,17-dione, 3-ethylenethioketal, 19-methanesulfonyl ester (56)
- 18) Synthesis and characterization of the thermal degradation product (233) 5,10-cyclopropyl-3,4-[S,S-ethylenethiane]-androsta-3(4)-en-17-one.
- 20) Synthesis and characterization of the thermal degradation product (234): 5,10-cyclopropyl-3,4-[S,S-ethylenethiane]-androsta-2(3)-en-17-one. (234)
- 21) Synthesis and biochemistry of 4-androsten-19-ol-3,17-dione, bispropylenethioketal (57)
- 22) Synthesis, biochemistry and chemistry of the novel 4-androsten-19-ol-3,17-dione, bispropylenethioketal, 19-toluenesulfonic ester (58)
- 23) Synthesis and characterization of(224): 5,10-cyclopropyl-3,4-[S,S-propylenethiane]-androsta-2(3)-en-17-one, 17-propylenethioketal.
- 24) Synthesis and biochemistry of the novel 4-androsten-3,17,19-trione, 3,17-bispropylenethioketal (59)
- 25) Synthesis of the novel 4-androsten-3,17,19-trione, 3,17-bispropylenethioketal, 19-toluenesulfonylhydrazone (226)
- 26) Synthesis of the novel 4-androsten-3,17,19-trione, 3,17-bispropylenethioketal, 19-[oxime-o-benzyl] (227)
- 27) Synthesis of 4-androsten-19-ol-3,17-dione, bispropylenethioketal, 19-methanesulfonic ester (198)
- 28) Synthesis and characterization of the novel A-homo-androst-5,10-ene-17-one-4 $\alpha$ -iodo-3-ethylenethioketal. 239
- 29) Synthesis and partial characterization of novel 4 $\alpha$ -hydroxy-estr-5(10)-ene-4,17-dione (183a).
- 30) Mass spectra experiments on the thermal degradation of 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstan-3,17-dione (78).
- 31) Biochemistry of epoxide isomers of  
4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstan-19-ol-3,17-dione, (73)  
4 $\beta$ ,5-epoxy-5 $\beta$ -androstan-19-ol-3,17-dione, (72)
- 32) Biochemistry of 4 $\beta$ ,5-epoxy-5 $\beta$ -androstan-19-ol-3,17-dione, 19-tertbutyldimethylsilyl ether (74)

# CLAIMS TO ORIGINAL RESEARCH:

- 33) Synthesis, biochemistry and chemistry of 19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstand-3,17-dione. (79)
- 34) The biochemistry of all ketals and all epoxides reported in this thesis is original to this work. See chapter 2 for details.
- 35) Synthesis of the novel 19-tertbutyldimethylsilyloxy-5 $\alpha$ -hydroxy-6 $\beta$ -azido-androstand-3,17-bisethylenedioxiide.
- 36) Chemical reactivity of 19-acetoxy-androstand-1,4-diene-3,17-dione.
- 37) Biochemistry and kinetic analysis of 2-deuterated testosterone incubation with aromatase.
- 38) Calculations and normalization of the affinity constants and binding contribution for substituent change for the reported androgen steroid inhibitors of aromatase.
- 39) Topological Considerations of Aminoglutethimide.
- 40) Aromatase inhibition by derivatives at C-19.
- 41) Aromatase inhibition by heteroatom derivatives at C-19.
- 42) Aromatase inhibition by derivatives at C-3 and C-17.
- 43) Aromatase inhibition by 3 and 17 dimethyl and ethylene ketals.
- 44) Aromatase inhibition by 3 and 17 thioketals: dithiolane and dithiane.
- 45) Aromatase inhibition: Screening of 19-bridged ethers: 19,4-oxa and 19,6-oxa derivatives.
- 46) Structure Activity Relationship: Mapping of the active center.

(17-C=O), 199.5 (3-C=O), 166.9 (5-C=C); 143.1 (aromatic C=C); 129, 128, 127 (aromatic CH), 126 (4-HC=); 87.4 (C-Ph<sub>3</sub>); 65.7 (19-CH<sub>2</sub>O); 13.7 (18-CH<sub>3</sub>).

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-tertbutyldimethylsilylether 37.**

Treatment of 19-OH-A (2) (8.99 g) with tBDMSi-Cl (2.5 eq., 5.1 g) and imidazole (1.2 eq., 5.3 g) in 40 ml DMF at 74 °C under N<sub>2</sub> for 13 hours gave 12 grams of crude 37 after work up (neutral, CH<sub>2</sub>Cl<sub>2</sub>). Chromatography (SiO<sub>2</sub>, 20% E/H) afforded 71% product (8.85g). 19-tBDMSiO-A; white plates from E/H. M.P.=158-159 °C; C<sub>25</sub>H<sub>40</sub>O<sub>3</sub>Si<sub>1</sub> MS m/z: 416(P<sup>+</sup>, 2%), 386 (25%, M-53, characteristic of tBDMSi), 359(91%), 73(100%, characteristic of tBDMSi); R<sub>f</sub>=0.3 in E/H 2/8; IR (γ<sub>max</sub>, cm<sup>-1</sup>, KBr): 1740(17-C=O), 1665(3-C=O), <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 3.86 (19-CH<sub>2</sub>O, d, 2H, J<sub>a,b</sub> = 2.3 Hz), 0.897 (18-CH<sub>3</sub>, s, 3H), 5.85 (4-CH, s, 1H), 0.027, 0.020 (CH<sub>3</sub>Si, 2xs, 6H), 0.835 (tBuSi, s, 9H); ADEPT <sup>13</sup>C: δ 220.1(17-C=O), 199.7(3-C=O), 167.3(5-C=C), 125.99(4-HC=), 65.8(19-CH<sub>2</sub>O), 13.8(18-CH<sub>3</sub>), -5.73 and -5.79 (CH<sub>3</sub>-Si), 17.99(tBu-Si);

One peak only by gas chromatography on a packed column with 3% OV-101, injector at 250°C, starting at 200°C with a temperature ramp at 15°C/min.

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-trimethylsilylether 38.**

1 gram of 19-OH-A (2) was dissolved in 11 ml of DMF and 10 ml ET<sub>3</sub>N at room temperature. 2 ml of TMSiCl were added by syringe, under N<sub>2</sub>. Work up (acidic, CH<sub>2</sub>Cl<sub>2</sub>) after 14 hr., followed by chromatography (Chromatotron, SiO<sub>2</sub>) gave 0.14 g starting material and 0.84 g (77%) of the desired 38. Fluffy needles from E/H. C<sub>22</sub>H<sub>34</sub>O<sub>3</sub>Si HRMS calc: 374.2275; found: 374.2281; MS m/z: 344(M-30), 329, 103, 73(100%, characteristic of silanes); R<sub>f</sub>=0.8 in E/H 3/7; IR (γ<sub>max</sub>, cm<sup>-1</sup>, KBr) 1730(17-C=O), 1670(3-C=O), 1610(4-C=C)

1085 (19-C=O);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.9-3.8 (19- $\text{CH}_2\text{O}$ , AB q, 2H,  $\delta_a=3.89$   $\delta_b=3.83$ ,  $J_{ab}=9.9\text{Hz}$ ); 0.90 (18- $\text{CH}_3$ , s, 3H); 5.85 (4-CH, s, 1H); 0.07 ( $\text{CH}_3\text{Si}$ , s); ADEPT  $^{13}\text{C}$  spectra shows 220 (17-C=O); 199 (3-C=O), 167 (5-C=C), 126 (4-CH); 14 (18- $\text{CH}_3$ ); 65 (19- $\text{CH}_2$ ), -0.6 ( $\text{CH}_3\text{Si}$ )

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-p-toluenesulfonylester 105.**

Compound (2) 19-OH-A (0.54 g) and recrystallized p-toluenesulfonyl-chloride (0.55 g) were reacted in 5 ml pyridine at room temperature for 2 hrs. The reaction mixture afforded 0.7 g of crude product after acidic work up.. Chromatography (Chromatotron,  $\text{SiO}_2$ , 15% E/H) gave 0.16 g recovered starting material 2 and 0.211 g of the desired tosylate 105; (34% based on recovered s.m.)  $\text{C}_{26}\text{H}_{32}\text{O}_5\text{S}$  needles from E/H.; MS m/z: 456( $\text{P}^+$ )  $R_f=0.5$  in E/H 3/7, blue color chromogen  $\text{H}_2\text{SO}_4/\text{SiO}_2$ ; IR ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$  (KBr): 1740 (17-C=O), 1665 (3-C=O);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  4.13 (19- $\text{CH}_2\text{O}$ , t, 2H), 0.86 (18- $\text{CH}_3$ , s, 3H), 5.66 (4-HC=C, s, 1H), 2.36 ( $\text{CH}_3\text{Ts}$ , s, ~3H), 7.1-7.63 (Ph, AB q, ~4H). Reported by Holland et al., Can. J. Chem. (1981) vol.59, p.2809.

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-methanesulfonylester 42.**

To 2 grams of 19-OH-A (2) in 10 ml pyridine at 0 °C was added, dropwise, 2ml of mesyl chlorides under  $\text{N}_2$ . After addition, the reaction was brought to room temperature for 5 hours. Work up (acidic, cold  $\text{CH}_2\text{Cl}_2$ ) afforded 2.9 g brown solid. Flash chromatography ( $\text{SiO}_2$ , 70%E/H) afforded 1.5 g (68%) yellow-white powder.  $\text{C}_{20}\text{H}_{28}\text{O}_5\text{S}_1$  MS m/z: 380( $\text{P}^+$ , 100%), 285(M- $\text{CH}_3\text{SO}_3$ , 16%), 284 (M-96, 65%), 271(24%),  $R_f$ = slightly less polar than starting material; detect by blue color chromogen  $\text{H}_2\text{SO}_4/\text{SiO}_2$  in E/H 7/3;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  4.43 (19- $\text{CH}_2\text{O}$ , AB q, 2H,  $\delta_a=4.23$   $\delta_b=4.50$   $J_{ab}=10\text{Hz}$ ), 0.80 (18- $\text{CH}_3$ , s, 3H), 5.83 (4-HC=C, s, 1H), 2.9 ( $\text{CH}_3\text{SO}_3$ , s, 3H).

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-acetoxylester 41.**

Compound 2 (19-OH-A) (10.45 g) dissolved in 10 ml Et<sub>3</sub>N and 30ml CH<sub>2</sub>Cl<sub>2</sub> was added dropwise slowly to acetic anhydride (15 ml) and 50 ml Et<sub>3</sub>N. The reaction was kept in a water bath to prevent overheating. Stirring at room temperature for one day, then work up (acidic) afforded 41 as a transparent oil. An analytical sample (0.6 g) was chromatographed on SiO<sub>2</sub> with 40/60 ethyl acetate / hexane. Colorless oil C<sub>21</sub>H<sub>28</sub>O<sub>4</sub> MS m/z: 344(P<sup>+</sup>, 272(100%), R<sub>f</sub>=0.3 in E/H:4/6, (blue color chromogen); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 4.38 (19-CH<sub>2</sub>O, ABq, 2H, δ<sub>a</sub>=4.13 δ<sub>b</sub>=4.63 J<sub>a,b</sub>=12 Hz), 0.94 (18-CH<sub>3</sub>, s, 3H), 5.83(4-HC=C, s, 1H), 2.03(CH<sub>3</sub>CO, s); ADEPT <sup>13</sup>C: δ 34.6 (\*2-CH<sub>2</sub>), 198.8(3-C=O), 126.9(4-HC=), 164.6(5-C=C), 32.9(\*6-CH<sub>2</sub>), 31.6(7-CH<sub>2</sub>), 35.8(8-CH), 54.1(9-CH), 41.9(10-C), 47.5(13-C), 51.2(14-CH), 219.5(17-C=O), 13.9(18-CH<sub>3</sub>), 66.6(19-CH<sub>2</sub>O), 20.9(CH<sub>3</sub> OAc), 170.4(C=O OAc)

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-trifluoromethanesulfonylester 103.**

103 was prepared by reaction of 19-OH-A 2 with triflic anhydride (CF<sub>3</sub>SO<sub>2</sub>)<sub>2</sub> at 0°C under N<sub>2</sub> according to the method of Nelson et al, Journal of Med. Chem., 1985, p. 775. An unstable analytical sample was examined by MS-fast atom bombardment (FAB) and NMR. Chromatography yields 0.27 g (37% product based on recovered starting material. The material is a white-yellow powder with no peaks corresponding to the starting material in the mass spectra. C<sub>20</sub>H<sub>23</sub>S<sub>1</sub>O<sub>5</sub>F<sub>3</sub> MS-FAB m/z: 435 (P<sup>+</sup>+1, 30%), 369 (16%), 277(73%), 185 (100%); R<sub>f</sub>=0.5 in E/H 1/1; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 4.75(19-CH<sub>2</sub>O, ABq, 2H, δ<sub>a</sub>=4.83 δ<sub>b</sub>=4.67 J<sub>a,b</sub>=10Hz), 0.908(18-CH<sub>3</sub>, s), 5.99(4-CH, s, 1H); ADEPT <sup>13</sup>C: δ 218.9(17-C=O), 197.3(3-C=O), 127.9(5-C=C), 161.4(4-HC=), 78.4(19-CH<sub>2</sub>O), 13.8(18-CH<sub>3</sub>).

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-benzylether 102.**

Compound 2 (19-OH-A) (0.22 gram) was dissolved in 15 ml benzene. To this was added  $\text{AgNO}_3$  (0.18 g, 1.5 eq.) benzylbromide (0.15 g, 1.2 eq.) and 0.1 ml  $\text{Et}_3\text{N}$ . The reaction mixture was refluxed for 1 week. Filtration of the reaction mixture through a pad of Florisil, evaporation of the solvent, acid/base work up from the  $\text{CH}_2\text{Cl}_2$  organic phase indicated 5 products by TLC. Column chromatography ( $\text{SiO}_2$ , 30 % E/H) afforded 36 mg recovered 19-OH-A and 50 mg (28%) desired 102.  $\text{C}_{26}\text{H}_{32}\text{O}_3$  MS m/z: 392 ( $\text{P}^+$ , 13%), 362 (M-30, 3%), 91 ( $\text{PhCH}_2^+$ , 100%),  $R_f=0.9$  in E/H 3/7;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  3.70 (19- $\text{CH}_2\text{O}$ , s, 2H), 0.80 (18- $\text{CH}_3$ , s, 3H), 5.9 (4- $\text{HC}=\text{C}$ , s, 1H), 7.46 (Ph, s), 4.43 (Ph- $\text{CH}_2\text{-O}$ , s, 2H).

**Synthesis of 4-androsten-19-ol-3,17-dione,  
19-methoxymethylether 39.**

The general procedure of Stork et al. (1977) J. Am. Chem. Soc., 99, p.1275 for making MOM ethers with diisopropylamine was modified as follows.

To 2 (19-OH-A) (0.23g) dissolved in 10:10:0.5 ml  $\text{Et}_3\text{N}:\text{CH}_2\text{Cl}_2:\text{DMF}$  was added, at room temperature 10 equivalent of methoxymethylchloride (0.25 ml, 10 eq.) under  $\text{N}_2$ . Heating to reflux (40 deg.) for 2 days and evaporation of the solvent, generated a mixture of three compounds. Chromatography on  $\text{SiO}_2$  afforded 46 % starting material and 12 mg (5%) desired 39.  $\text{C}_{21}\text{H}_{30}\text{O}_4$  MS m/z: 346 ( $\text{P}^+$ , 1%), 346 (M- $\text{CH}_2\text{O}$ , 27%), 286 (M- $\text{CH}_2\text{OCH}_2\text{O}$ , 8%), 271 (14%); 60 MHz  $^1\text{H}$  ( $\text{CDCl}_3$ ): ether region poorly resolved.  $\delta$  3.9 (19- $\text{CH}_2\text{O}$ , d of d, 2H,  $\delta_a=4.2$   $\delta_b=3.7$   $J_{ab}=6\text{Hz}$ ), 0.96 (18- $\text{CH}_3$ , s, 3H), 5.87 (4- $\text{HC}=\text{C}$ , s, 1H), 4.67 (MOM  $\text{OCH}_2\text{C}$ , br, 2H), 3.4 (MOM  $\text{CH}_3\text{O-}$ , s 3H).

**Attempted preparation of 4-androsten-19-ol-3,17-dione,  
19-tert-butyldiphenylsilylether 104.**

19-OH-A 2 (0.32 g) in 5 ml DMF was heated at 70 °C with tert-butyldiphenylsilyl chloride (0.7 ml, 2.5 eq.) in the presence of DMAP catalyst (0.1 wt%) and imidazole (0.2 g, 1 eq.) for 1 week. Work up (acidic) and chromatography (Chromatotron, SiO<sub>2</sub>, 30 %E/H) afforded two non-polar compounds. These could not be properly characterized, and were not of further synthetic use. The NMR are reported below for C<sub>35</sub> H<sub>44</sub>Si<sub>1</sub>O<sub>3</sub>.

**Compound 1:** 160 mg Mass spectra reveals major ions at 199, 139 but no high end. 60 MHz <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 3.9 (19-CH<sub>2</sub>O, broad), 1.03 (18-CH<sub>3</sub> and tBu), 5.8 (4-HC=C, s, 1H), 7.7 (Ph, br)

**Compound 2:** 53 mg: 60 MHz <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 3.73 (19-CH<sub>2</sub>O, s, 2H), 1.0 (18-CH<sub>3</sub> and tBu, 12H), 6.5 (4-HC=C, s, 1H), 7.5 (Ph, br).

**Reaction of 19-OH-A 2 with tBUOK.**

The general procedure of Ringold et al. (1962) Tet.Lett., 15, p.669 or Williams (1974) Synthesis, p.3332 or Williams (1981) J.Org.Chem., 46, p.1127 gave poor results and were modified as follows.

Compound 2 (0.38 g) 19-OH-A was added to a freshly prepared solution of 0.39 g potassium metal in degassed (N<sub>2</sub>) tert-butanol (25 ml) and stirred for 3½ hours. The resulting pale orange enolate solution was quenched with 10% acetic acid. Neutralization with 10 % K<sub>2</sub>CO<sub>3</sub> to pH=7 and extraction in diethylether resulted in 0.15 g yellow powder with no ether protons in the <sup>1</sup>H NMR spectra, a broad peak at 3.3 ppm, (indicating a loss of the 19-CH<sub>2</sub>O) and no alkenyl protons (implying loss of alkene). Chromatography yields yellow powder; MS m/z: 412 (M<sup>+</sup>, 1%), 323 (9%), 272 (1%), 222, 196, 91, 59

did not FAB; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1740 (17-C=O), 1650, 1660 (3-C=O, br), 1450 (4-C=C, br.), 3.3 (C-O, br.);  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  no peaks corresponding to 19-CH<sub>2</sub>O, 0.8 (18-CH<sub>3</sub>, m), no peak corresponding to 4-CH;  $^{13}C$  NMR:  $\delta$  poor resolution, most peaks are broad, with peaks about 220 (17-C=O), none between 200 and 75 (no 3-C=O, no 5-C=C and no 4-HC=). The product was assumed to be a polymer and not further characterized.

#### Reaction of 19-OH-A 2 with Sodium isopropoxide.

While bubbling N<sub>2</sub> in a solution of 0.1 g. sodium metal dissolved in 6 ml isopropanol, compound 2 (0.11 g) was added. Stirring at room temperature for 22 hrs., addition of acetic acid (10%), and work up afforded 45.9 mg of a yellow powder that is insoluble in ether and ethyl acetate, but soluble in methanol and slightly soluble in CHCl<sub>3</sub> or CH<sub>2</sub>Cl<sub>2</sub>. MS m/z: 241(M<sup>+</sup>), 185, 124; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1740 (17-C=O), 1660 (3-C=O), 1450 (4-C=C), 1260 (C-O);  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  showed no peaks above 3 ppm. no alkene peaks, 0.8-1 (18-CH<sub>3</sub>, m), and no ether peaks were resolved. The steroidal methylene shell present but not resolved.  $^{13}C$  NMR: 220.6 and 220.2 peaks indicate ketone, no alkene peaks, no CH<sub>2</sub>O peaks. The high field peaks are poorly resolved.

#### Synthesis of 5-androsten-19-ol-3,17-dione, 19-tert-butyldimethylsilylether 130.

The enone 37 19-tertbutyldimethylsilylether of 4-androsten-19-ol-3,17-dione (0.38 g) was added to 0.4 g potassium metal dissolved in 23 ml degassed (N<sub>2</sub>) tBuOH at RT for 2½ hrs. The resulting enolate solution was quenched with 10% acetic acid, neutralized with saturated NaHCO<sub>3</sub> then extracted in ether. Evaporation of the solvent gave 45 % of the desired 130. Recrystallized from CH<sub>2</sub>Cl<sub>2</sub>, the compound is soluble in MeOH, and slightly soluble in acetone or diethylether.  $^1H$  NMR of crude shows two peaks corresponding

to alkene. Purification by chromatography yields 130 with the following characteristics.  $C_{25}H_{40}O_3Si_1$  MS m/z: 416 ( $P^+$ , 3%), 386 (M-30), 359 (M-57, 100%), 284, 89, 73 (characteristic of silane);  $R_f=0.8$  in E/H 3/7 less polar than starting material;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  3.8 (19- $CH_2O$ , AB q,  $J_{ab}=15.7$  Hz), 3.25 (4 $\beta$ -CH, d, br, 1H,  $J=6$  Hz), 2.86 (4 $\alpha$ -CH, d, sharp, 1H,) 0.83-0.92 (18- $CH_3$ , tBuSi, m, 12H), 0.056-0.026 (MeSi, m, 6H), 5.56 (6-CH, br,  $\approx 1$  H).

## EXPERIMENTAL SECTION 2: THIOKETAL DERIVATIVES.

**Preparation of 4-androsten-19-ol-3,17-dione, 3-propylenethioetal (161) and 4-androsten-19-ol-3,17-dione, 3,17-bispropylenethioetal (57).**

The general procedure of Seebach et al, (1975) J.Org.Chem., p.231 for making dithiane derivative was modified as follows.

### **Preparation of 4-androsten-19-ol-3,17-dione bispropylenethioetal.57**

Compound 2 (19-OH-A, 1 gram) in 30 ml  $CHCl_3$  was treated with propanedithiol (5 eq. 1.8 ml). The reaction mixture was cooled to  $-20^\circ C$ , and 150  $\mu L$   $BF_3 \cdot Et_2O$  was added. The reaction mixture was warmed to room temperature, and monitored for 3 days, adding more of the volatile catalyst every day until only one spot showed by TLC. Work up by washing with base (KOH 10%) then water afforded one compound, a white foam becoming a glassy oil at about  $120^\circ C$ .

$C_{25}H_{38}O_1S_4$  HRMS calc: 482.1805 found: 482.1803 MS m/z: 482 ( $P^+$ , 24%), 452 (M-30, 29%), 145 ( $C_6S_2H_9^+$ , 38%);  $R_f=0.9$  in E/H 3/7, purple color chromogen on  $H_2SO_4/SiO_2$ ; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 3427 (O-H)  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  3.95/3.92 (19- $CH_2O$ , ABq, 2H,  $s_a=3.94$ ,  $s_b=3.63/3.59$ ,  $J_{ab}=10.6$  Hz), 0.967 (18- $CH_3$ , s, 3H),

5.76 (4-CH,s,1H), 2.57-2.94 (CH<sub>2</sub>S,m, 8H), 2.46-2.58 (CH<sub>2</sub>-C-S,m,4H) ADEPT <sup>13</sup>C: δ 50.14(17-C=S<sub>2</sub>), 63(3-C=S<sub>2</sub>), 143.2(5-C=C), 126.5(4-HC=), 65.8(19-CH<sub>2</sub>O), 16.37(18-CH<sub>3</sub>); 53.17 (9-CH), 49.46(14-CH), 43.2 (10-C), 48.28(13-C), 39.27(6-CH<sub>2</sub>).

**Preparation of 4-androsten-19-ol-3,17-dione,  
3-propylenethioketal 161 .**

The mono-dithiane 161 was obtained by halting the derivation procedure after a few hours and chromatographic separation. An analytical sample of the glassy oil C<sub>22</sub>H<sub>32</sub>O<sub>2</sub>S<sub>2</sub> was obtained. HRMS found:392.1849 calc:392.1836 MS m/z: 392(P<sup>+</sup>,100%), 362(M-30, 32%), 318(55%), 107(87%); R<sub>f</sub>=0.5 in E/H 3/7, (green color chromogen on H<sub>2</sub>SO<sub>4</sub>/SiO<sub>2</sub>); MS m/z: 392(P<sup>+</sup>,100%), 362(M-30,32%), 318(M-55%), 107(87%); <sup>1</sup>H NMR (CDCl<sub>3</sub>):δ 3.73(19-CH<sub>2</sub>O, ABq,2H,δ<sub>a</sub>=3.83 δ<sub>b</sub>=3.56 J<sub>a,b</sub>=10 Hz), 0.86(18-CH<sub>3</sub>,s,3H),5.70(4-HC=C,s,1H), 2.5-2.9(CH<sub>2</sub>-S,m,4H).

**SYNTHESIS OF DITHIANES DERIVED AT C-19:**

**Preparation of  
4-androsten-19-ol-3,17-dione,bispropylenethioketal,  
19-toluenesulfonylester.58**

To 0.47 g of the 19-alcohol 57 dissolved in 5 ml pyridine and cooled at 0 °C was added 2.5 eq. (0.46 g) p-toluenesulfonyl-chloride (pTsCl). After addition, the reaction was warmed to room temperature, then heated at 60 degrees for 2 hours to finish the reaction: caution must be taken not to overheat as the desired tosylate degrades thermally (see reaction 224 below). Pouring onto 100 ml water, stirring 5 minutes, filtration and trituration with water afforded 76% (0.47g) white powder 58. C<sub>32</sub>H<sub>44</sub>O<sub>3</sub>S<sub>5</sub> no parent ion at P<sup>+</sup>=636 (ether chemical ionization) MS m/z: 464

(M-171, M-pTSA), 172 (pTSA), 145 ( $C_6S_2H_9$ , characteristic of D ring dithianes);  $R_f=0.6$  in E/H 2/8, (green color on  $H_2SO_4/SiO_2$ ),  $R_f \approx SM$ ;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  4.14 (19- $CH_2O$ , d, 2H,  $J=1.96$  Hz), 0.91 (18- $CH_3$ , s, 3H), 5.54 (4-CH, s, 1H), 2.76-2.92 ( $CH_2S$ , m, 8H), 2.46-2.58 ( $CH_2-C-S$ , m, 4H); ADEPT  $^{13}C$ :  $\delta$  49.99 (17- $C=S_2$ ), 62.9 (3- $C=S_2$ ), 142.3 (5- $C=C$ ), 127.8 (4- $HC=$ ), 71.8 (19- $CH_2O$ ), 16.31 (18- $CH_3$ ); 53.55 (9-CH), 49.53 (14-CH), 41.2 (10-C), 48.14 (13-C), 41.09 (6- $CH_2$ ), 39.61 (8-CH), 21.7 ( $CH_3Ph$ ), 144.66 (aromatic Me- $C=$ ), 132.7 (aromatic H- $C=$ ).

**Preparation of 4-androsten-19-ol-3,17-dione  
bispropylenethioketal, 19-methanesulfonylester. 198**

Compound 57 (0.25 g) was dissolved in 10 ml pyridine and 5 ml  $CH_2Cl_2$  and cooled at 0 °C. Methanesulfonyl-chloride (0.25 ml) was added dropwise, then the reaction mixture was warmed to room temperature and heated at reflux ( $\approx 42^\circ C$ ) for 15 minute. The reaction could not be monitored by TLC easily, as the product is only slightly less polar than the starting 19-alcohol. The color chromogen is, however, characteristically green. Work up involved pouring onto 75 ml water, stirring 5 minutes, picking up in  $CH_2Cl_2$  and washing with water. This afforded 97% (0.28g) of a white/yellow foam. An analytical sample was chromatographed on Chromatotron ( $SiO_2$ , 10% E/H)  $C_{27}H_{40}O_3S_5$  MS m/z: 560 ( $P^+$ , 1%), 464 (M- $CH_3SO_3H$ ), 96 (MSOH), 145 ( $C_6S_2H_9$ );  $R_f=0.6$  in E/H 2/8, (green color  $H_2SO_4/SiO_2$ ),  $R_f \approx SM$ ; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1350 1170 (S=O), 950, 530;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  4.4 (19- $CH_2O$ , AB q, 2H,  $\tau_a=4.49$   $\tau_b=4.26$   $J=9.6$  Hz), 0.97 (18- $CH_3$ , s, 3H), 5.63 (4-CH, s, 1H), 2.56-2.96 ( $CH_2S$ , m, 8H), 3.00 ( $CH_3SO_2$ , s, 3H) ADEPT  $^{13}C$ :  $\delta$  50.02 (17- $C=S_2$ ), 62.9 (3- $C=S_2$ ), 142.1 (5- $C=C$ ), 127.8 (4- $HC=$ ), 70.8 (19- $CH_2O$ ), 16.35 (18- $CH_3$ ); 53.41 (9-CH), 49.52 (14-CH), 41.2 (10-C), 48.1 (13-C), 39.65 (6- $CH_2$ ), 37.03 (8-CH), 37.45 ( $CH_3SO_2$ ).

**Preparation of 224: Thermal degradation of 58,  
4-androsten-19-ol-3,17-dione, bispropylenethioketal,  
19-toluenesulfonyl ester.**

Compound 58 (100 mg) was suspended in 10 ml pyridine (b.p.=114 °C) and heated to reflux for 24 hours. Work up in brine, extraction in CH<sub>2</sub>Cl<sub>2</sub>, evaporation and Chromatotron separation (SiO<sub>2</sub>) afforded 37 mg of compound 224. C<sub>25</sub>H<sub>36</sub>S<sub>4</sub> MS m/z: 464(P<sup>+</sup>,58%), 390(9%), 357(14%) 145 (C<sub>6</sub>S<sub>2</sub>H<sub>9</sub>,100%), no peak at 172 corresponding to pTOSH; R<sub>f</sub>=0.5 in 4%E/H; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 0.77 and 0.25 (19-CH<sub>1</sub>-: d of d : over A ring, s<sub>a</sub>=0.77 d, 1H, and 19-CH<sub>1</sub>- s<sub>b</sub>=0.25 over B ring, d, 1H, J<sub>a,b</sub>=4.6 Hz), 0.96 (18-CH<sub>3</sub>,s,3H), 5.79 (2-HC=,1H,d of d, s<sub>a</sub>= 5.77 s<sub>b</sub>= 5.80 J<sub>a,b</sub>=2 Hz), 2.85-2.91 (CH<sub>2</sub>S and 1β-CH,m, 5H), 2.46-2.64 (CH<sub>2</sub>S and 1α-CH,m,5H), 4.29 (4-HC,s,1H), 3.08-3.21 (4-S-CH<sub>1</sub>β, tm,1H) 2.9-3.04 (4-S-CH<sub>1</sub>α, tm,1H);

**Characterization of 224:**

ADEPT <sup>13</sup>C: δ 50.54 (17-C=S<sub>2</sub>), 130.64(3-C=), 16.4 (18-CH<sub>3</sub>); 122.64(2-HC=), 51.2 (9-CH), 48.27(14-CH), 39.7(10-C), 47.16 (13-C); <sup>1</sup>H NMR: nOe experiments indicate the following protons are nearby:

Hydrogens at 0.2 and 0.7 show strong 22% effect.

Hydrogens at 4.2 and 0.7 show a 2% effect.

Hydrogens at 0.2 and 0.7 both affect a multiplet at 3.2,

**Preparation of 4-androsten-3,17,19-trione,  
3,17-bispropylenethioketal (59): Oxidation of  
4-androsten-19-ol-3,17-dione, bispropylenethioketal 57.**

Compound 57 (0.3 g) was dissolved in 10 ml CH<sub>2</sub>Cl<sub>2</sub>. 3 eq. of pyridinium chlorochromate (PCC) (0.405g) and sodium acetate (0.75g) was added and stirred for an hour at RT. Pouring the reaction mixture over Florisil column, triturating with CH<sub>2</sub>Cl<sub>2</sub> and flash chromatography (10% E/H) afforded 0.143 mg desired 59 aldehyde (48% yield) as a white powder (E/H). C<sub>25</sub>H<sub>36</sub>O<sub>1</sub>S<sub>4</sub> MS m/z: 480 (P<sup>+</sup>), 452 (M-28), 145 (C<sub>6</sub>S<sub>2</sub>H<sub>9</sub>); TLC R<sub>f</sub>=0.5 E/H 15/85; IR (γ<sub>max</sub>,cm<sup>-1</sup>,KBr): 1710

(19-C=O), 1420 (4-C=C);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  9.79 (19-CHO, s, 1H), 0.953 (18-CH<sub>3</sub>, s, 3H), 5.75 (4-CH, s, 1H), 2.8-2.9 (CH<sub>2</sub>S, m, 12H), ADEPT  $^{13}\text{C}$ :  $\delta$  54.3 (17-CS<sub>2</sub>), 62.9 (3-CS<sub>2</sub>), 139.3 (5-C=C), 126.8 (4-HC=), 202.6 (19-CHO), 16.1 (18-CH<sub>3</sub>), 37.67 (8-CH), 53 (9-CH), 49.8 (14-CH), 47.82 (10-C), 49.9 (13-C).

**Preparation of 4-androsten-3,17,19-trione,  
3,17-bispropylenethioketal, 19-toluenesulfonylhydrazone. 226**

Reaction of the 19-aldehyde 59 with 1.1 eq. p-TOS-NNH<sub>2</sub> in refluxing ethanol and Chromatotron separation affords 49 % of the desired 19-derivative. C<sub>32</sub> H<sub>44</sub> O<sub>2</sub> S<sub>5</sub> N<sub>2</sub> calculated 685. MS m/z: No parent ion, 534 (4%), 477 (M-TosNH<sub>2</sub>, M-171), 450 (100%); R<sub>f</sub>=0.3 in E/H 2/8; IR ( $\nu_{\text{max}}$ , cm<sup>-1</sup>, KBr): 3130 (NH), 1370 (SO<sub>2</sub>), 1160 (SO, strong), 1440 (4-C=C), 670;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  7.20/7.27 (19-H-C=N, each s), 0.74 (18-CH<sub>3</sub>, s, 3H), 5.59 (4-CH, s, 1H), 2.8-2.9 (CH<sub>2</sub>S, m, 12), 2.41 (CH<sub>3</sub>Ph, s, 3H); 7.29-7.78 (aromatic Ph);

**ADEPT Characterization of 4-androsten-3,17,19-trione,  
3,17-bispropylenethioketal, 19-toluenesulfonylhydrazone. 226**

ADEPT  $^{13}\text{C}$ :  $\delta$  49.1 (17-C=S<sub>2</sub>), 63 (3-C=S<sub>2</sub>), 141.6 (-C=C), 135 (4-HC=), 152.7 (19-HC=N), 15.97 (18-CH<sub>3</sub>); 53.4 (9-CH), 49.7 (14-CH), 45.9 (10-C), 39.4 (13-C), 21.67 (CH<sub>3</sub>-Ph), aromatic peaks: 144-141.

**Preparation of 4-androsten-3,17,19-trione,  
3,17-bispropylenethioketal, 19-[oxime-O-benzyl]. 227**

Compound 58 (57 mg) was suspended in 3 ml pyridine and 10 ml CH<sub>2</sub>Cl<sub>2</sub>. Excess (0.11g) free base O-benzyl-hydroxylamine and a catalytic amount of para-toluenesulfonic acid (3 crystals) were added. Refluxing for 36 hours showed a major compound slightly less polar than the starting material by TLC. Pouring the reaction mixture in ether, work up involved evaporating the solvent and triturating the solid with water. Chromatotron (SiO<sub>2</sub>, 10% E/H) afforded 55% (39 mg) of the desired oxime. 227: C<sub>32</sub>H<sub>43</sub>S<sub>4</sub>O<sub>1</sub>N<sub>1</sub> MS m/z: 585 (P<sup>+</sup>, 2%), 477 (M-Ph-CH<sub>2</sub>OH, M-108, 11%), 450, 145 (C<sub>6</sub>S<sub>2</sub>H<sub>9</sub><sup>+</sup>), 91

(Ph-CH<sub>2</sub>, 100%); R<sub>f</sub>=0.3 in 10%E/H; IR(γ<sub>max</sub>, cm<sup>-1</sup>, KBr): 1430, 1450 (C=C), 1010 (C-O); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 7.47 & 7.33 (19-HC=O-: two singlets, cis and trans HC=N), 0.866 (18-CH<sub>3</sub>, s, 3H), 5.608 (4-HC=, 1H, s) 2.66-2.94 (CH<sub>2</sub>S, m, 8H), 5.05 (Ph-CH<sub>2</sub>O, 2H, d, J=0.8 Hz), 7.35-7.24 (16 peaks, aromatic); ADEPT <sup>13</sup>C: δ 49.8 (17-C=S<sub>2</sub>), 63.1 (3-CS<sub>2</sub>) 124.6 (4-HC=), 142.4 (5-C=), 151.9 (19-HC=N), 16.09 (18-CH<sub>3</sub>); 53.5 (9-CH), 49.1 (14-CH), 44 (10-C), 48.37 (13-C), 37.1 (8-CH), 39.52 (6-CH<sub>2</sub>), 75.7 (Ph-CH<sub>2</sub>-O), 137.8 (aromatic -C=C), 128-126 (aromatic -HC=).

**Preparation of 4-androsten-19-ol-3,17-dione,  
3-ethylenethioketal (53) and 4-androsten-19-ol-3,17-dione,  
bisethylenethioketal (52).**

Compound 2 (19-OH-A, 4g, 13 mmoles) in 100 ml methanol was treated with ethanedithiol (1.8 eq., 2.04 ml), 2 ml BF<sub>3</sub>-etherate, and stirred at room temperature for 1 hour. The solvent was evaporated and the residue (5.8g) dissolved in ethyl acetate. Work up (KOH) followed by chromatography (Chromatotron 40%CH<sub>2</sub>Cl<sub>2</sub>/60% hexane) afforded the white bisdithiolane powder 52 2.85g (48%) and a white foam monothioketal 282 2.04 grams (41%):

**Characterization of 53 and 52.**

**4-androsten-19-ol-3,17-dione, 3,17-bisethylenethioketal  
(52).**

C<sub>23</sub>H<sub>34</sub>O<sub>1</sub>S<sub>4</sub> MS m/z: 454 (P<sup>+</sup>), 424, 396, 131 (D ring fragmentation C<sub>5</sub>H<sub>7</sub>S<sub>2</sub><sup>+</sup>) TLC: CH<sub>2</sub>Cl<sub>2</sub>/Hexane 4/6; <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 3.93-3.61 (19-CH<sub>2</sub>O, ABq split: dt, 2H: δ<sub>a</sub>=3.93, J=0.7, 2.3: δ<sub>b</sub>=3.61 J=9Hz) 0.905 (18-CH<sub>3</sub>, s, 3H), 5.82 (4-CH, s, 1H), 3.03-3.4 (CH<sub>2</sub>S, m, 8H); ADEPT <sup>13</sup>C: δ 65.1 (3-CS), 129.5 (4-HC=), 140.7 (5-C=C), 38.6 (8-CH), 53.1 (9-CH), 41.9 (10-C), 49.1 (13-C), 52.3 (14-CH), 80.2 (17-CS), 17.4 (18-CH<sub>3</sub>), 65.7 (19-CH<sub>2</sub>O).

**4-androsten-19-ol-3,17-dione, 3-ethylenethioketal (53)**

$C_{21}H_{30}O_2S_2$  MS  $m/z$ : 378 ( $P^+$ ), 350, 347, 313, 287, 61, no D ring fragmentation peak at 131; TLC E/H 3/7;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  3.66 (19- $CH_2O$ , d of AB q, 2H,  $J=9Hz$ ) 0.859 (18- $CH_3$ , s, 3H), 5.83 (4-CH, s, 1H), 3.18-3.39 ( $CH_2S$ , m, 4H); ADEPT  $^{13}C$ :  $\delta$  65 (3-C), 129.6 (4-HC=), 140.6 (5-C=C), 35.86 (8-CH), 53.7 (9-CH), 41.99 (10-C), 47.7 (13-C), 51.2 (14-CH), 220.3 (17-C=O), 13.9 (18- $CH_3$ ), 65.6 (19- $CH_2O$ ).

**Preparation of 4-androsten-19-ol-3,17-dione, 3-ethylenethioketal, 19-methanesulfonylester. (56)**

To 1.8 g. 19-OH-3-dithiolane 53 in 10 ml pyridine and 10 ml  $CH_2Cl_2$  was added 4 eq.  $MsCl$  (1.5 ml) at room temperature. Reflux at 40 °C for 2 hrs. followed by work up (acid/base) gave 2.7 g of a yellow oil. Chromatography afforded 1.49 g (68%) white foam 56.  $C_{22}H_{32}O_4S_2$  MS  $m/z$ : 456 ( $P^+$ , 2%), 360 (M- $CH_3SO_3H$ , 100%), 333 (18%), 332 (65%), 267 (21%); TLC E/H 3/7; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1740 (17-C=O), 1360 ( $SO_2$ ), 1180 (S=O), 960, 840, 530;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  4.35 (19- $CH_2O$ , AB q, 2H,  $\delta_a=4.42$ ,  $\delta_b=4.28$ ,  $J_{ab}=9.8 Hz$ ), 0.875 (18- $CH_3$ , s, 3H), 5.74 (4-CH, s, 1H), 3.18-3.39 ( $CH_2S$ , m, 4H), 3.01 ( $CH_3SO_2$ , s, 3H);

ADEPT  $^{13}C$ :  $\delta$  ; 64.6 (3-C), 128.5 (4-HC=), 139.5 (5-C=C), 35.7 (8-CH), 53.6 (9-CH), [60.3] (10-C), [47.6] (13-C), 51.2 (14-CH), 219.8 (17-C=O), 13.8 (18- $CH_3$ ), 70.5 (19- $CH_2O$ ), 37.7 ( $CH_3SO$ ).

**Preparation of 4-androsten-19-ol-3,17-dione, 3,17-bisethylenethioketal, 19-methanesulfonylester. 55**

To 1.8 g. 19-OH-3,17-dithioketal 52 in 10 ml pyridine and 10 ml  $CH_2Cl_2$  was added 4 eq.  $MsCl$  (1.5 ml) at room temperature. Reflux at 40 °C for 2 hrs., work up (acid/base) and chromatography yields 55  $C_{24}H_{36}O_3S_5$  MS  $m/z$ : 532 ( $P^+$ , 1%), 436 (M- $CH_3SO_3H$ , 13%), 408 (8%), 197, 131 (33%, D

ring  $C_5H_7S_2$ ); TLC  $R_f$ =SM: IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1350 ( $SO_2$ ), 1170 ( $S=O$ ), 950, 530;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  4.45 (19- $CH_2O$ , AB q, 2H,  $\delta_a=4.45$   $\delta_b=4.24$   $J_{ab}=9.8Hz$ ), 0.914 (18- $CH_3$ , s, 3H), 5.73 (4-CH, s, 1H), 3.0-3.89 ( $CH_2S$ , m, 8H), 3.0 ( $CH_3SO_2$ , s, 3H); ADEPT  $^{13}C$ :  $\delta$  64.7 (3- $CS_2$ ), 128.4 (4- $HC=$ ), 139.8 (5- $C=C$ ), 37.6 (8-CH), 53.1 (9-CH), [48.9] (10-C), [40.11] (13-C), 52.3 (14-CH), 80.1 (17- $CS_2$ ), 17.3 (18- $CH_3$ ), 70.8 (19- $CH_2O$ ), 37.8 ( $CH_3SO$ ).

**Preparation of 233 and 234: Thermal degradation of (56)  
4-androsten-3,17-dione, 3-ethylenethioketal,  
19-methanesulfonylester**

Compound 56 (0.2 g) was refluxed in pyridine for 4 hrs. Work up and Chromatography with Chromatotron ( $SiO_2$ , 25%  $CH_2Cl_2$ /hexane) afforded two compounds. Both lost S-O bands in the IR, and the mass spectral parent ions correspond to M-96 of starting material. There are no 19- $CH_2O$  in the  $^1H$  NMR. The  $C^{13}NMR$  shows a new peak at 24.79 (5-C), loss of the mesyl, and loss of the  $HC=C$  peaks.

**Characterization of 233**

233: 38.8 mg, 36% yield.  $C_{21}H_{28}O_1S_2$  MS  $m/z$ : 360 ( $P^+$ , 100%), 332 (M- $C_2H_4$ , 99%), 196 (38%), 184, 181, 91; TLC  $R_f$ =0.3 in 25 %  $CH_2Cl_2$ /hexane; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1730 (17- $C=O$ ), 1450 br( $C=C$ ), 1050, 1010, 760 (C-S);  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  0.65 (s, 2H fine split  $J<0.1Hz$ ), 0.859 (18- $CH_3$ , s 3H), 3.16-3.11 ( $CH_2-S$ , m, 4H); ADEPT  $^{13}C$ :  $\delta$  127.9 120.4 (3,4- $C=C$ ), 220.8 (17- $C=O$ ), 14.1 (18- $CH_3$ ); 47.6 (9-CH), 50.1 (14-CH), 29.4 (10-C), 48.3 (13-C), 35.1 (8-CH) 29.34 and 23.39 ( $CH_2S$ ): 24.79 (5-C); HETCOR: 27.59  $CH_2$  correlates to the hydrogens doublet at 0.6ppm. 2-D J-resolve was also performed.

### Characterization of 234

**234:** yield 29mg (19%),  $C_{21}H_{28}O_1S_2$  MS  $m/z$ : 360 ( $P^+$ , 100%), 332 ( $M-C_2H_4$ , 43%), 196 (14%), 181 (28%), 105 (21%), TLC 25 %  $CH_2Cl_2$ /hexane; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1730 (17-C=O);  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  6.0 (1-HC=C, ABq, 1H,  $J=2.3Hz$ ), 4.04 (t, 1H,  $J=1.5Hz$ ) 0.77-0.32 (19- $CH_2$ , dd, 2H,  $\delta_a=0.77$   $\delta_b=0.34$   $J_{ab}=4.4Hz$ ), 0.85 (18- $CH_3$ , s, 3H), 3.0-3.2 ( $CH_2$ -S, m, 4H); ADEPT  $^{13}C$ :  $\delta$  127.4 (2-HC=C), 129.2 (3-C=C), 220.9 (17-C=O), 13.9 (18- $CH_3$ ); 47.9 (9-CH), 50.73 (14-CH), 48.7 (10-C), 48.1 (13-C), 35.8 (8-CH), 35.3 and 32.9 ( $CH_2$ S): 24.79 (5-C), 50.76 (4-HC-S, allylic): 26.7\* (12- $CH_2$ ), 18.11\* (19- $CH_2$ )

#### HETCOR:

26.7  $CH_2$  correlates to 0.84, 0.85 doublet methyl (12-C- $\beta$ H, W coupling), 50.76 correlates to t at 4.03 ( $J^4$  with a number of  $CH_2$  is possible), 18.11  $CH_2$  correlates to the hydrogen doublets at 0.77 and at 0.34 ppm (19- $CH_2$ ), 26.7  $CH_2$  correlates to the hydrogens multiplet at 0.84 ppm (12-C- $\beta$ H splitting methyl), 50.76 CH correlates to the hydrogens triplet at 4.03 (4-HC-S), 127.4 correlates to the doublet of doublet at 6 ppm (2-HC=); 2D-J resolve was also performed.

### Preparation of 235:

**Reaction of N-hydroxysuccinimide (NHS) with (55)  
4-androsten-19-ol-3,17-dione, 3,17-bisethylenethioketal,  
19-methanesulfonyl ester**

Compound 55 (0.17g) and 3 eq. (0.19g) N-hydroxy-succinimide (NHS) were refluxed in isopropanol for 2 days. Work up and chromatography (Chromatotron,  $SiO_2$ , 5% ethyl acetate/hexane) afforded 86.3 mg of a white compound 235. Their spectra indicate thermal decomposition of the parent compound displaying  $^1H$  NMR,  $^{13}C$  ADEPT, MS and IR consistent with the thermal rearrangements observed for the bisethylenethioketal 55 (above). In view of the importance of these rearrangements, confirmation of this structure was obtained from X-Ray.

**Reaction of NHS with (56), 4-androsten-19-ol-3,17-dione,  
3-ethylenethioketal, 19-methanesulfonylester**

4-androsten-19-ol-3,17-dione, 3-ethylenethioketal, 19-methanesulfonylester **56** (0.25g) and 3 eq. (0.19g) N-hydroxysuccinimide (NHS) were refluxed in isopropanol for 2 days. Work up and chromatography afforded compounds **233** and **234**. Their spectra indicate thermal decomposition of the parent compound displaying  $^1\text{H}$  NMR,  $^{13}\text{C}$  ADEPT, MS and IR consistent with the type of thermal rearrangements observed for the mono-ethylenethioketal **56** (above).

**Preparation of 237 and 238: Thermal degradation of (55)  
4-androsten-19-ol-3,17-dione, bisethylenethioketal,  
19-methanesulfonylester**

Compound **55**, 4-androsten-19-ol-3,17-dione, bisethylenethioketal, (0.15 g) was refluxed in pyridine for 4 hrs. Chromatography with Chromatotron ( $\text{SiO}_2$ , 5%  $\text{CH}_2\text{Cl}_2$  in hexane) afforded two compounds.

**237**: 23 mg, white plate from  $\text{CH}_2\text{Cl}_2$ . HRMS calc:436.1385 found: 436.1369 for  $\text{C}_{23}\text{H}_{32}\text{S}_4$  MS m/z:436( $\text{P}^+$ , 100%), 408 ( $\text{M}-\text{C}_2\text{H}_4$ , 95%), 196 (80%), 131 (43% D ring  $\text{C}_5\text{H}_7\text{S}_2$ );  $R_f=0.3$  in 5%  $\text{CH}_2\text{Cl}_2$ /hexane; IR( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ , KBr): 1445, 1410 (C=C), 930, 845 (C-S);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$ : 0.75(19-CH, d, 1H,  $J=10\text{Hz}$ ), 0.60-0.65 (AB q, 2H  $\delta_a=0.60$   $\delta_b=0.64$   $J_{ab}=4.4\text{Hz}$ ), 0.914(19-CH, d, 1H,  $J=0.6\text{Hz}$ ), 0.8(18- $\text{CH}_3$ , ddd, 3H,  $J=7$ ), 3.06-3.3 ( $\text{CH}_2-\text{S}$ , m, 8H); ADEPT  $^{13}\text{C}$ :  $\delta$  128 and 120 (3,4-C=C), 80.5 (17-C- $\text{S}_2$ ), 17.7 (18- $\text{CH}_3$ ); 47 (9-CH), 51.3(14-CH), 36.9(10-C), 49.8(13-C), 24.9(5-C), 9.7(19- $\text{CH}_2$ );

**HETCOR:**

32.3 CH correlates to the hydrogen doublet at 0.75ppm  
27.6  $\text{CH}_2$  correlates to the hydrogens ddd  $\text{CH}_2$  at 0.8ppm  
27.73  $\text{CH}_2$  correlates to the hydrogens quartet at 0.6ppm

The spectra for HETCOR and the 2-D J resolve are presented in the text.

238: 22 mg, white plate from  $\text{CH}_2\text{Cl}_2$   $\text{C}_{23}\text{H}_{32}\text{S}_4$  MS  
m/z: 436 ( $\text{P}^+$ , 100%), 408 ( $\text{M}-\text{C}_2\text{H}_4$ , 29%), 196 (20%), 131 (98%) (D  
ring  $\text{C}_5\text{H}_7\text{S}_2$ );  $R_f=0.5$  in 5 %  $\text{CH}_2\text{Cl}_2$ /hexane; IR ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ ,  
KBr): 1405, 1375 (C=C), 930, 810 (C-S);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  5.98  
(1-HC=C, q, 1H,  $J=2.3\text{Hz}$ ), 4.01 (t, 1H,  $J=1.7\text{Hz}$ ), 0.72-0.32  
(19- $\text{CH}_2$ , dd, 2H,  $\delta_a=0.72$   $\delta_b=0.32$   $J_{ab}=4.4\text{Hz}$ ), 0.896  
(18- $\text{CH}_3$ , s, 3H), 0.892-0.796 ( $\text{CH}_3$ , m, 3H), 2.87-3.3  
( $\text{CH}_2$ -S, m, 8H), ; ADEFT  $^{13}\text{C}$ :  $\delta$  129.4 (2-HC=), 127.5 (3-C=C),  
47.3 (4-CH-S), 24.3 (5-C), 37.7\* (8-CH), 50.9\* (9-CH), 36.9  
(10-C), 49.5 (13-C), 51.1\* (14-CH), 80.5 (17-C-S<sub>2</sub>), 17.6  
(18- $\text{CH}_3$ ): 23.9 (C).

HETCOR: The spectra for 2-D J-resolve and HETCOR resolve  
are reported in chapter 3.

#### Preparation of 239:

Reaction of NaI with (56) 4-androsten-19-ol-3,17-dione,  
3-ethylenethioketal, :9-methanesulfonylester.

4-androsten-19-ol-3,17-dione, 3-ethylenethioketal,  
19-methanesulfonylester 56 (0.25g) and 2 eq. (0.16g) NaI were  
refluxed in 25 ml acetone. After 1 day since no product  
appeared by TLC, the solvent was evaporated and the residue  
dissolved in isopropanol. Reflux for 2 days generated an  
iodo-androgen 239.

$\text{C}_{21}\text{H}_{29}\text{O}_1\text{S}_2\text{I}_1$  m/z: 488 ( $\text{P}^+$ , 7%), 360 ( $\text{M}-\text{I}$ , 84%), 332  
(100%), 196 (38%); TLC 5%  $\text{CH}_2\text{Cl}_2$ /hexane; IR ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ , KBr):  
1735 (17-C=O), no C-I stretch at 500;  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  
0.861 (18- $\text{CH}_3$ , s, 3H), 3.08-3.2 ( $\text{CH}_2$ -S, m, 4H), 3.28 (CHI, m 1H);  
ADEPT  $^{13}\text{C}$ :  $\delta$  127.8, 120.2 (5, 10-C=C), 220 (17-C=O),  
14.2 (18- $\text{CH}_3$ ); 51.1 (4-CHI), 50.1 (9-CH), 48.3 (3-CS<sub>2</sub>),  
47.6 (14-CH), 35.9 (13-C), 35.2 (8-CH).

**Preparation of 240: Reaction of NaI with (55)  
4-androsten-19-ol-3,17-dione, bisethylenethioketal,  
19-methanesulfonylester**

4-androsten-19-ol-3,17-dione, bisethylenethioketal, 19-methanesulfonylester **55** (0.16g, 0.3mM) and excess NaI were refluxed in 25ml isopropanol. Reflux for 2 days generated 37 mg of a mixture of three compounds. The presence of the iodo-androgen **240** was detected by selecting the 564 ion in the MS. The mass spectra reported corresponds to the expected fragmentation product of an iodinated-steroid.

**240**:  $C_{23}H_{33}S_4I$  HRMS calc: 564.051<sup>o</sup> found: 564.0494 m/z: 564(P<sup>+</sup>, 7%), 436(M-I, 70%), 408 (59%), 196 (53%), 5 %  $CH_2Cl_2$ /hexane; Two compounds. ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): no C-I stretch at 500;  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  0.638 (19- $CH_2$ , s, 2H) 0.885 (18- $CH_3$ , s, 3H), 3.07-3.29 ( $CH_2$ -S, m), 3.32/3.68 (CHI, dd 1H).

In view of the poor yield and presence of impurities, there were no further attempt at characterization.

**Preparation of 54: 4-androsten-3,17,19-trione,  
3,17-bisethylenethioketal.**

Compound **52** (1.08 g) was dissolved in 100 ml  $CH_2Cl_2$ , with a suspension of pyridinium chlorochromate (PCC) (3 eq., 1.5 g) and 0.6 eq. NaOAc (0.1 g)), stirred for half an hour at room temperature, poured over Florisil column, triturated with 200 ml  $CH_2Cl_2$ , 200 ml ethylacetate and chromatographed by flash chromatography. This afforded 0.61 g. (66% product based on recovered starting material) white cubes from E/H.  $C_{23}H_{33}O_1S_4$  MS m/z: 452 (P<sup>+</sup>); TLC E/H 15/85; IR ( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1710 (19-C=O);  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  9.80 (19-CHO, s, 1H), 0.889 (18- $CH_3$ , s, 3H), 5.81 (4-CH, t, 1H, J=1.5Hz), 3.06-3.39 ( $CH_2$ S, m, 8H), 2.16-2.5 ( $CH_2$ S, m, 4H) ADEPT  $^{13}C$ :  $\delta$  64 (17-CS<sub>2</sub>), 137 (5-C=C), 129 (4-HC=), 203 (19-CHO), 17 (18- $CH_3$ ), 38.3 (8-CH), 53 (9-CH), 52 (14-CH), 53 (10-C), 48.8 (13-C).

### SECTION 3: DIETHYLENEKETAL EPOXIDE DERIVATIVES.

#### Synthesis of 19-tertbutyldimethylsilyloxy- 4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstande-3,17-dione (76) and 19-tertbutyldimethylsilyloxy-4 $\beta$ ,5-epoxy-5 $\beta$ - androstande-3,17-dione (74)

To 8.45 grams of 4-androstande-19-ol-3,17-dione, 19-tertbutyldimethylsilylether 37 (20.3 mM) in 1 liter of methanol cooled at -12°C was added 51 ml 30% H<sub>2</sub>O<sub>2</sub> with vigorous stirring. Aqueous 10% NaOH (16 ml) was added dropwise, to prevent overheating and overoxidation. The reaction mixture was brought to room temperature and stirred for 20 hours. Work up involved pouring in brine, extraction with CH<sub>2</sub>Cl<sub>2</sub> and washing with water. The crude (5.71 grams, 65% yield) was purified by HPLC (Waters). Spectral data confirmed the purity of the two isomers.

H.Mastalerz reported the NMR and IR of these compounds in his Ph.D. thesis, University of Ottawa, 1981. S.Hrycko reported the results of A-ring over-oxidations for similar compounds in her Ph.D. thesis, University of Ottawa, 1988.

#### Preparation of 19-hydroxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstande- 3,17-dione. 73

To 1.2456 grams of 19-tertbutyldimethylsilyloxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstande-3,17-dione 76 in freshly distilled THF was added, under nitrogen, 4.3 ml tertbutylammonium fluoride solution (1M/THF). After 16 hours, the reaction mixture was poured in water, extracted in ethyl acetate, washed with water, brine, then dried over Na<sub>2</sub>SO<sub>4</sub> to yield 0.67 grams (73%) crude. Chromatography (SiO<sub>2</sub> plug, 50% EA/Hex) afforded the desired compound 73 in 61% yield. The compound is slightly soluble in CH<sub>2</sub>Cl<sub>2</sub>, CHCl<sub>3</sub>, MeOH, CCl<sub>4</sub>, ethyl acetate, and is insoluble in acetone.

**Characterization of 19-hydroxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -  
androsterane-3,17-dione. 73**

HRMS for  $C_{19}H_{26}O_4$  calc:318.1829 found: 318.1830 MS m/z: 318(P<sup>+</sup>), 300(24%), 288(M-30), 245 (80%) ;  $R_f=0.3$  in E/H 1/1; IR( $\gamma_{max}$ ,  $cm^{-1}$ , KBr): 1738(17-C=O), 1694(3-C=O), 1060,1040 (C-O);  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  4.05(19-CH<sub>2</sub>O, doublet of AB quartet, 2H,  $\delta_a=4.07$   $\delta_b=3.94$   $J_{ab}=10$  Hz,  $J_{ac-bc}=0.9$ Hz), 0.904 (18-CH<sub>3</sub>, s, 3H), 3.17(epoxy 4-CH, s, 1H); ADEPT  $^{13}C$  (DMSO-d<sub>6</sub>):  $\delta$  219.2(17-C=O), 205.7(3-C=O), 67.04 (5-C-O), 62.95(4-HC-O), 62.69(19-CH<sub>2</sub>O), 13.5(18-CH<sub>3</sub>). Mastalerz reported the 60 MHz  $^1H$  NMR of this compound in his Ph.D. thesis, University of Ottawa, 1981.

**GC-MS Characterization of 19-hydroxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -  
androsterane-3,17-dione. 73**

Silylation and direct injection in a pre-heated injection port at 300°C of a gas chromatograph attached to the mass spectrometer afforded a convenient method of confirmation for 73. GC separation was effected on a 6% OV-101 column with a temperature ramp of 10°C/min. This experiment afforded one readily identifiable peak in the GC. This peak corresponds to the silylation product (trimethylsilyl-73) with the following major ions for  $C_{22}H_{34}O_4Si_1$  calc:390 GC-MS m/z: 390(P<sup>+</sup>, 18%), 360(23%, M-30), 332(M-50%), 300(25%), 103(100%), 73(91%, characteristic of TMSi derivatives).

**Preparation of 19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androsterane-  
3,17-dione. 79**

To 0.52 grams (1.63 mM) of compound 73 in 180 ml of acetone in an ice bath (4 °C) was added 1.8 ml of Jones' reagent (3 eq., 2.66 moles/liter). The reaction was followed by TLC (FA/Hex 7/3) to ensure complete conversion to the aldehyde, then to the carboxylic acid. The reagent was quenched with methanol after 4 hours. Extraction in ether, washing with water and brine resulted, after evaporation, in 0.49 grams (1.47 mM, 90%) of the desired carboxylic acid 79 as a white powder.

**Characterization of 19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstan-3,17-dione. 79**

Crystals from diethylether, mp=183-184 °C. for C<sub>19</sub>H<sub>24</sub>O<sub>5</sub>.

MS (DIP 100°C) m/z 332 (P<sup>+</sup>, 86%), 314 (M-18, 19%), 288 (M-CO<sub>2</sub>, 47%), 270 (M-CO<sub>2</sub>-O, estrone, 12%),

MS(DCI, chemical ionization, ether) m/z: 333 (P<sup>+</sup>+1, 27%), 315 (10%, M-H<sub>2</sub>O), 289 (45%, M-CO<sub>2</sub>) 271 (15%, M-CO<sub>2</sub>-H<sub>2</sub>O); R<sub>f</sub>=0.3 in E/H 7/3; <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  0.95 (18-CH<sub>3</sub>, s, 3H), 3.222 (epoxide 4-HC-O, s, 1H), no peaks corresponding to the 19-CH<sub>2</sub>-O doublet found in the starting material. IR ( $\gamma_{max}$ , cm<sup>-1</sup>, KBr): 1730 br (17-C=O and carboxy), 1240 (COOH), 1185, 1060, 1030 (C-O). For pyrolysis and GC-MS experiment see section 5 below.

**Preparation of 19-hydroxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstan-3,17-dione. 72**

To 5.0117 grams of 19-hydroxyandrostane-1,16-dione (16.9 mM) in 750 ml methanol cooled at 4°C was added 30% H<sub>2</sub>O<sub>2</sub> (19 ml) with vigorous stirring. Aqueous 4N NaOH (19 ml) was added (dropwise, to prevent overheating). The reaction mixture was maintained at 4 °C and stirred for 15 hours. Work up involved pouring in brine, evaporating most of the methanol, extracting in CHCl<sub>3</sub>, washing in water and drying the organic phase with Na<sub>2</sub>SO<sub>4</sub>. Evaporation afforded crude epoxide which was recrystallized from petroleum ether/ethyl acetate to give the desired 72 (3.17 grams, 59% yield). C<sub>19</sub>H<sub>26</sub>O<sub>4</sub> MS m/z: 318 (P<sup>+</sup>, 18%), 288 (11%, M-CH<sub>2</sub>O), 271 (10%), 260 (50%) <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  3.93 (19-CH<sub>2</sub>O, ABq, 2H,  $\delta_a$ =3.73  $\delta_b$ =4.10 J<sub>ab</sub>=12 Hz), 0.90 (18-CH<sub>3</sub>, s, 3H), 2.86 (epoxide 4-CH-O, s, 1H). Mastalerz reported this compound in his Ph.D. thesis, University of Ottawa, 1981.

**GC-MS Characterization of 19-hydroxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androsterane-3,17-dione. 72**

Silylation and direct injection in the pre-heated injection port (300°C) of a gas chromatograph attached to the mass spectrometer afforded a convenient method of confirmation for 72.

GC separation was effected on a 6% OV-101 column with a temperature ramp of 10°C/min starting at 200 °C. This experiment afforded one peak in the GC-MS with the following expected fragmentation: C<sub>24</sub>H<sub>34</sub>O<sub>4</sub>Si<sub>1</sub> MS m/z: 390(P $\oplus$ ,8%), 360(11%,M-30), 345(13%), 332(15%), 103(69%), 73(100%, characteristic of MeSi).

**Preparation of 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androsterane-3,17-dione. 78**

To 3.17 grams (9.96 mM) of the 19-ol 72 in 300 ml of acetone in an ice bath (4 °C) was added 11.3 ml of Jones' reagent (3 eq., 2.66 moles/liter). The reaction was followed by TLC (EA/Hex 7/3) to ensure complete conversion to the aldehyde, then to the carboxylic acid. The reagent was quenched with 15 ml methanol after 4 hours. Extraction in ether, washing with water and brine, evaporation of the organic layer and recrystallization from petroleum ether/ethylacetate afforded 2.04 grams (6.13 mM, 62%) of the desired carboxylic acid 79 as white crystals with a melting point of 169-171 °C, and bubbling at 195°C (possibly loss of CO<sub>2</sub>) for C<sub>19</sub>H<sub>24</sub>O<sub>5</sub> MS m/z: 332(P $\oplus$ ,7%), 288 (33%,M-CO<sub>2</sub>), 270(M-CO<sub>2</sub>-H<sub>2</sub>O), 244(100%); MS(DCI, chemical ionization), (P+1+2Et<sub>2</sub>O, 15%), 333(P<sup>+</sup>+1,17<sup>+</sup>, 315(19%,P-H<sub>2</sub>O), 289(27%, P-CO<sub>2</sub>), 271(54%, P-CO<sub>2</sub>-H<sub>2</sub>O); <sup>1</sup>H NMR (CDCl<sub>3</sub>):  $\delta$  no peaks corresponding to 19-CH<sub>2</sub>O, 0.951(13-CH<sub>3</sub>,s,3H), 3.068(4-CH,s,1H). IR( $\gamma_{max}$ ,cm<sup>-1</sup>,KBr): 1700-1750br (17-C=O,3-C=O and 19-C carboxy) 1210, 1180, 1160 (C-O), 1240s, 690s (epoxy C-O). For pyrolysis and GC-MS experiment see section 5 below. Mastalerz reported this compound in his Ph.D. thesis, U. of Ottawa, 1981.

EXPERIMENTAL SECTION 4: Synthesis of  
3,17-diethyleneketal derivatives

Preparation of 5-androsten-19-ol-3,17-dione,  
diethyleneketal, 19-acetate. (47)

The 19-acetate-androgen 41 was dissolved in 40 ml benzene, 6 ml ethyleneglycol and a catalytic amount (55 mg) of para-toluene-sulfonic acid. Reflux in a Dean-Stark apparatus for 3 days, followed by washing with water and evaporation of the excess benzene resulted in 1.822 grams crude 47. An analytical sample gave the following spectral characteristics for the oil:  $C_{25}H_{36}O_6$  MS m/z: 432 ( $P^+$ , 10%), 389 (M-43), 388 (M-44), 99 (100%);  $R_f=0.8$  in E/H 6/4, (red-brown color chromogen  $H_2SO_4/SiO_2$  TLC);  $^1H$ -NMR ( $CDCl_3$ ):  $\delta$  4.53 (19- $CH_2O$ , ABq, 2H,  $\delta_a=4.43$   $\delta_b=4.1$ ), 0.90 (18- $CH_3$ , s, 3H), 2.03 ( $OCOCH_3$ , s, 3H), 5.4 (6-CH, d,  $J=10Hz$ , 1H).

Preparation of 5-androsten-19-ol-3,17-dione,  
diethyleneketal. (45)

1.822 grams (4.2 mM) of 47 was dissolved in 40 ml (5%KOH/MeOH) solution. After refluxing for an hour, the reaction mixture was poured in water. The precipitate was filtered to give 1.1974 grams (3.07 mM, 73%) compound 45. Recrystallization from acetone/petroleum ether yields yellow needles.  $C_{23}H_{34}O_5$  MS m/z: 390 ( $P^+$ , 4%), 360 (M-30, 29%), 298 (30%), 99 (100%);  $R_f=0.4$  in E/H 6/4; IR ( $\gamma_{max}$ ,  $cm^{-1}$ ,  $CHCl_3$ ): 3650 ( $-O-H$ ), 1110, 910s, 650 (C-O);  $^1H$  NMR ( $CDCl_3$ ):  $\delta$  no acetate peak. 3.62 (19- $CH_2O$ , AB quartet,  $\delta_a=3.61$   $\delta_b=3.63$ , 1H,  $J=2.5Hz$ ), 3.96 (19- $CH_2O$ , multiplet, 1H), 3.78-3.92 (ketal multiplet,  $CH_2-O$ , 8H), 5.71 (6- $HC=$ , broad AB quartet, 1H,  $J=3.1Hz$ ), 2.3 (7- $CH_2$ , ABq,  $\delta_a=2.17$   $\delta_b=2.48$ , 2H,  $J=13.4Hz$ ).

**Preparation of 5-androsten-19-ol-3,17-dione,  
19-tertbutyldimethylsilylether. (46)**

4-androsten-19-ol-3,17-dione, 19-tertbutyldimethylsilylether (37) (1.0582 g, 2.54 mM) in refluxing benzene (75ml) was treated with 2 ml ethylene glycol in the presence of paratoluenesulfonic acid catalyst (28 mg) in a Dean-Stark apparatus for 31 hours at reflux. 5Å molecular sieve were placed in the trap to remove water. Work up involved washing with aqueous Na<sub>2</sub>CO<sub>3</sub> (pH=10) then H<sub>2</sub>O, followed by chromatography (SiO<sub>2</sub>, 10%E/H). This afforded, after recrystallization in ether, the desired bisethylenedioxy derivative 46 in 47% yield (1.0444 g, 2.07mM, 81%). White needles from ether. HRMS on C<sub>29</sub>H<sub>46</sub>O<sub>5</sub>Si<sub>1</sub> calc: 504.3271, found: 504.3268, MS m/z: 504(P<sup>+</sup>, 22%), 447(M-57, 18%, characteristic of tBDMSi), 372(17%), 297(18%), 99(100%, characteristic of ketal), 73 (58%, characteristic of tBDMSi); R<sub>f</sub>=0.3 in E/H 1/9; IR(ν<sub>max</sub>, cm<sup>-1</sup>, KBr) 1470 (4-CH=), 1110, 1090 (Si-O); <sup>1</sup>H NMR (CDCl<sub>3</sub>): δ 3.65(19-CH<sub>2</sub>O, ABq, 2H, δ<sub>a</sub>=3.58, δ<sub>b</sub>=3.71, J<sub>a,b</sub>=10.8Hz), 3.8-3.97(ketals, m, 8H), 0.863 (18-CH<sub>3</sub>, s, 3H), 5.52(6-CH, d, 1H, J=4.7Hz), 2.5(7-CH<sub>3</sub>, d, 1H, J=15Hz), 0.009 and 0.003(CH<sub>3</sub>Si, two singlets, 6H), 0.848(tBuSi, s, 9H).

**Preparation of  
6β-androstan-5β,6-epoxy-19-ol-3,17-dione,  
diethyleneketal, 19-tertbutyldimethylsilylether. 50 and  
6α-androstan-5α,6-epoxy-19-ol-3,17-dione, diethyleneketal,  
19-tertbutyldimethylsilylether. 51**

To 1.0444 g 5-ene 46 (2.07 mM) in 75 ml CH<sub>2</sub>Cl<sub>2</sub> was added 1.9 eq. mCPBA (0.6697 g). The solution was refluxed for 6.5 hours. Work up involved adding 0.9 eq. of NaHSO<sub>3</sub> (0.19 g), then 30 ml aqueous 5% NaHCO<sub>3</sub> (pH=8) and stirring for 5 min. The organic layer was washed with 5% NaHCO<sub>3</sub> then brine. Evaporation afforded 1.024 g of crude (95%). Flash chromatography (SiO<sub>2</sub>, E/H=2/8) afforded two compounds in the ratio 6/4: β/α (less polar/more polar).

**6 $\beta$ -androstan-5 $\beta$ ,6-epoxy-19-ol-3,17-dione,  
diethyleneketal, 19-tertbutyldimethylsilylether. 50**

Chromatography gave 45% product (0.48 g, 0.923mM) as an amorphous white powder. C<sub>29</sub>H<sub>48</sub>O<sub>6</sub>Si MS m/z: 520 no parent ion, 463 (M-57,12%, characteristic of tBDMSi fragmentation), 371(11%), 297(15%), 99(100%, characteristic of ketal); R<sub>f</sub>=0.7 in E/H 3/7; <sup>1</sup>H 3.92-3.79(CH<sub>2</sub>-O ketals, m, 8H), 3.65 (19-CH<sub>2</sub>O, 2H,ABquartet,  $\delta_a$ =3.61,  $\delta_b$ =3.73, J=9.5 Hz), 2.93 (6-CH-O,d, J=1.6Hz), 2.51(7-CH<sub>2</sub>,d,1H,J=13.6Hz), 0.796(18-CH<sub>3</sub>,s,3H), 0.051 and 0.058(CH<sub>3</sub>Si,two singlets,6H), 0.877 (tBuSi,s,9H); IR( $\gamma_{max}$ ,cm<sup>-1</sup>,KBr): 1470, 1460, 1100 strong (Si-O) 860m,840m (C-O).

**6 $\alpha$ -androstan-5 $\alpha$ ,6-epoxy-19-ol-3,17-dione,  
diethyleneketal, 19-tertbutyldimethylsilylether. 51**

Chromatography yields 0.31g (0.596 mM,29%) of compound 51, amorphous white powder. C<sub>29</sub>H<sub>48</sub>O<sub>6</sub>Si; R<sub>f</sub>=0.1 in E/H 2/8; <sup>1</sup>H NMR (CDCl<sub>3</sub>) $\delta$  3.89-4.0(19-CH<sub>2</sub>O and ketals,m,10H), 2.88 (6-CH-O, doublet, 1H,J=3.1 Hz), 2.30(7-CH<sub>2</sub>,d,1H,J=13 Hz); 0.82(18-CH<sub>3</sub>,s,3H); 0.908 (tBuSi,s,9H); 0.095and 0.10 (CH<sub>3</sub>Si,two singlets); IR( $\gamma_{max}$ ,cm<sup>-1</sup>,KBr): 860m,775s (C-O).

**Preparation of 6 $\alpha$ -androstan-5 $\alpha$ ,6-epoxy-19-ol-3,17-dione,  
diethyleneketal. (48)**

To 0.3817 grams (0.74mM) of 19-tertbutyldimethylsilyloxy 51 in freshly distilled THF (10 ml) was added, under nitrogen, 2.5 ml tertbutylammonium fluoride solution (1M solution in THF). After 23 hours, 1 ml water was added, the reaction mixture evaporated and filtered through a SiO<sub>2</sub> plug. Recrystallization from ethylacetate gave 0.257g (0.63 mM,86%) of the desired 48 as needles with a melting point mp=204-206°C. C<sub>29</sub>H<sub>48</sub>O<sub>6</sub>; MS: 406 (P<sup>+</sup>,11%), 318 (10%), 99(100%, characteristic of ketal); R<sub>f</sub>=0.3 in EA/Pet.Ether 1/3; <sup>1</sup>H NMR (CDCl<sub>3</sub>) $\delta$  4.05-3.79(CH<sub>2</sub>O,m,10H); 2.90(6-CH-O, 1H,d,J=4.0 Hz); 2.36 (7-CH<sub>2</sub>,d,1H,J=14Hz); 0.83 (18-CH<sub>3</sub>,s,3H), no tBDMSi peaks;

**Further characterization of 6 $\alpha$ -androstan-  
5 $\alpha$ ,6-epoxy-19-ol-3,17-dione, diethyleneketal. (48)**

$^1\text{H}$  NMR (acetone- $\text{d}_6$ ) does not resolve the ether region, but a doublet corresponding to O-H appears at 2.79/2.82. It collapses in the presence of  $\text{D}_2\text{O}$ ;  $^1\text{H}$  NMR (benzene- $\text{d}_6$ ) shifts a 4.0 ppm fine split AB quartet out of the ethyleneglycol envelope. By analogy with the  $\beta$ -isomer, this probably corresponds to a C-19 methylene. IR ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ ,  $\text{CCl}_4$ , Nicolet FT-IR) 3694s (O-H), 3479br (H-bonding), 810s 865s 950s (epoxide C-O, KBr).

**Preparation of 6 $\beta$ -androstan-5 $\beta$ ,6-epoxy-19-ol-3,17-dione,  
diethyleneketal. (49)**

To 1.022 g. (1.96mM) of 19-tertbutyldimethylsilyloxy 50 in freshly distilled THF (10 ml) was added, under nitrogen, 3 eq. of tertbutylammonium fluoride solution (5.9 ml, 1M/THF). After 44 hours, 1 ml of water was added, the solvent evaporated and filtered through a  $\text{SiO}_2$  column (30% EA/Hex) to give 1.028 grams of the deprotected 49 as an amorphous powder.  $\text{C}_{23}\text{H}_{34}\text{O}_6$  MS 99(100%, characteristic of ketal);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ )  $\delta$  3.8-3.97(ketal  $\text{CH}_2\text{O}$ , m, 8H); 3.04 ( $\xi$ -CH-O, d,  $J=2.1$  Hz); 4.1(19- $\text{CH}_2\text{O}$ , 1H, doublet,  $J=11\text{Hz}$ , coupled to the peak at 3.57); 3.57(19- $\text{CH}_2\text{O}$ , ABq, 1H,  $J=11\text{Hz}$ , collapses to a doublet on addition of  $\text{D}_2\text{O}$ , confirming possible coupling to the hydroxy doublet at 3.08 and coupling to the other 19- $\text{CH}_2\text{O}$  at 4.1); 3.08 (19-OH, d,  $J=11\text{Hz}$ , collapses on addition of  $\text{D}_2\text{O}$ ); 2.51 (7- $\text{CH}_2$ , d, 1H,  $J=14$ ); 0.89 (18- $\text{CH}_3$ , s, 3-H); IR ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ , KBr): 3470 br (O-H), 810s 865s 950s (C-O)  $^1\text{H}$  NMR: (acetone- $\text{d}_6$ ) collapse the O-H. One of the 19-CH d of d is changed to a doublet, the other has been shifted into the ketal multiplet and can not be interpreted.

The fact that isomer 48 is more polar than isomer 49, and that there is such a differentiation between the 19-methylene protons of 49 argues for an internal hydrogen bond between the epoxide oxygen and the 19-hydroxyl that would result in locking the conformation. This served as a basis for assignment of the epoxide configuration.

**Preparation of 5 $\alpha$ -androstan-5 $\alpha$ -hydroxy-6 $\beta$ -azido-19-ol-  
3,17-dione, diethyleneketal  
19-tertbutyldimethylsilylether. (190)**

1.1 eq. ( $9.75 \times 10^{-5}$  M) of a 2.1 M solution of  $\text{NaN}_3$  in water was added to the  $\alpha$ -epoxide **51** ( $8.9 \times 10^{-5}$  M) in 2 ml dioxane for 3 hrs. Reflux for 12 hours did not yield products. An additional 170 mg (70 eq.) of a 2.1 M solution of  $\text{NaN}_3$  in water, 90 mg of ammonium chloride in 3 ml of  $\text{CH}_3\text{OCH}_2\text{CH}_2\text{OH}$  was refluxed for 1 day with 34 mg of 5,6 $\alpha$ -epoxide in 2 ml dioxane. Work up and chromatography (10% E/H) on basic Woelm alumina yields 16 mg of the azido **190**;  $\text{C}_{29}\text{H}_{49}\text{O}_6\text{N}_3$  calc: 563 MS m/z: no parent ion, 535 (M-28, 1%), 518 (M-45, 4%), 506 (M-57, 6%), 478, 463 (19%), 297, 160, 99 (100%, ketal), 73 (24%);  $R_f=0.6$  in E/H 2/8, blue/green  $\text{H}_2\text{SO}_4$ ); IR ( $\gamma_{\text{max}}$ ,  $\text{cm}^{-1}$ ,  $\text{CHCl}_3$ ): 3480 (O-H, broad), 2110 (6- $\beta$ - $\text{N}_3$ , strong, stretch, asym), 1110 (C- $\text{N}_3$ , stretch, sym.), 1080, 1060 (C-O, Br));  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ):  $\delta$  3.32 (6 $\alpha$ -H, broad singlet is a d of d, 1H,  $J_{ab}=1.25$  and 1.68 Hz); 0.853 (18- $\text{CH}_3$ , s); 0.863 (tBuSi, s); 3.76-3.86 ( $\text{CH}_2\text{C}$  ketal, m, 10H); 4.43 (OH?, s, 1H); 3.63 (19- $\text{CH}_2\text{O}$  d, 1H,  $J=11\text{Hz}$ , half of AB q); 0.045 and 0.07 ( $\text{CH}_3\text{Si}$ , two singlets, 6H); Addition of  $\text{D}_2\text{O}$  does not cause any change in the  $^1\text{H}$  NMR splitting patterns, indicating a possible strong internal hydrogen bond to the  $\alpha$ -hydroxy.

## EXPERIMENTAL SECTION 5: REACTIONS OF ANALOGUES

### Thermal degradation of 19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstan-3,17-dione. (79)

A few milligrams of the compound was heated, neat, at 195°C under N<sub>2</sub>. The reaction was followed by TLC on SiO<sub>2</sub> (CHCl<sub>3</sub>). This results in 5 compounds;

- #1 top, unidentified
- #2 corresponding to estrone by R<sub>f</sub>
- #3 strongly UV positive, with a red Kober chromogen in H<sub>2</sub>SO<sub>4</sub>/SiO<sub>2</sub>
- #4 UV positive, with a yellow Kober chromogen in H<sub>2</sub>SO<sub>4</sub>/SiO<sub>2</sub>
- #5 corresponding to starting material by R<sub>f</sub>.

A suspension of the acid 79 (260 mg) in freshly distilled decalin (b.p. 195°C) was heated at reflux under nitrogen for 1 hour. The reaction mixture was chromatographed by Chromatotron using CH<sub>2</sub>Cl<sub>2</sub> and MeOH (0%-5%) as the eluent. In addition to separating estrone, this approach appeared to concentrate one of the desired major compounds.

Past experience with the difficulty in separating the isomers of 258 was confirmed as an apparently clean band again showed two spots by TLC after purification on silica. Chromatography of this fraction with a different eluent system (1%CHCl<sub>3</sub>/ethylacetate 15%/ hexane 84%) allowed concentration of one of the components of the overlapping bands. Re-crystallization in E/H afforded a major product (69 mg) which was assigned to be 3 $\beta$ -hydroxy-estr-5(10)-ene-4,17-dione 184. Although the <sup>1</sup>H NMR appears clean, analysis by ADEPT <sup>13</sup>C showed this to be a mixture of both 3-hydroxy isomers, in a ratio approximately 1:1. Similar attempts with the  $\beta$ -carboxylic acid degradation (below) and by Mastalerz (Ph.D. thesis) resulted in a similar mixture of the 3-isomer.

It is tempting to postulate that these isomers are interconvertible via tautomerization of the 4-keto, and that no amount of chromatography can separate these. NMR experiments were not undertaken to confirm whether this hypothesis is valid.

**Characterization of the pyrolytic degradation of  
19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstane-3,17-dione. 79**

**3 $\xi$ -hydroxy-estr-5(10)-ene-4,17-dione 184:** White crystals from E/H.  $C_{18}H_{24}O_3$  MS m/z: 288(P $\oplus$ ,27%), 270 (4%,M-18), 244(M-44,100%); TLC: UV+ve, Red Kober chromogen in  $H_2SO_4$ /  $SiO_2$ ;  $\lambda_{max}$ (MeOH)= 247nm IR ( $\gamma_{max}$ , $cm^{-1}$ ,KBr): 1730(17-C=O), 1655 (4-C=O), 1620(5,10-C=C), 1135, 1050 (C-O), 3400 (internal O-H bond?);  $^1H$  NMR ( $CDCl_3$ ): $\delta$  4.03-4.10 (3- $CH_1$ -OH, assigned as one doublet for each isomer, or an overlap of AB quartet, both fine split by the OH, 2H,  $\delta_1$ =4.040  $\delta_2$ = 4.085,  $J_2=J_1$ =5.3Hz, probably with a 2- $CH_2$ , further coupled with the 4-hydroxyl with  $J_{a-OH}=J_{b-OH}$ =1.8Hz. The fine split coupling collapses, along with the OH peak at 3.75, on addition of  $D_2O$ ), 0.890(18- $CH_3$ ,s,3H), 3.746 and 3.756 (3-O-H, two doublets,1H, $J$ =1.8Hz), ; ADEPT  $^{13}C$  clearly shows 36 carbons, each a doublet, confirming the presence of both  $C_{18}$  isomers. The significant parts of the spectra are the presence of 6 quaternary low field carbons assigned as follows:  $\delta$  219.84 & 219.78 (17-C=O), 200.50 & 199.69(4-C=O), 159.21 & 158.18(5-C=C), 129.5 (10-C=C), 71.90 & 71.78(3- $CH_1$ -O), 14.04 & 13.92 (18- $CH_3$ ).

**MINOR COMPONENT:** impure, yellow powder 9.5 mg. tentatively assigned as **4 $\alpha$ -hydroxy-estr-5(10)-ene-3,17-dione 183a** with some 184 in the ratio 2/1.  $C_{18}H_{24}O_3$  MS m/z: 288(P $\oplus$ ,26%), 270 (3%,M-18), 244(M-44,100%); TLC: UV-ve, purple Kober chromogen in  $H_2SO_4$ /  $SiO_2$  with some 184 UV+ve); IR( $\gamma_{max}$ , $cm^{-1}$ ,KBr): 1785(3-C=O), 1730(17-C=O), 3440 (internal O-H ?);  $^1H$  NMR ( $CDCl_3$ ): $\delta$  4.00(4- $CH_1$ O,s,1H), 0.958 (18- $CH_3$ ,s,3H), no alkene peaks, 3.756(O-H,d,  $J$ =1.7Hz).

There is an additional peak corresponding to a fine split doublet in the NMR spectra at 4.09, and the presence of an extra methyl peak at 0.893. These additional peaks are assigned to the presence of compound 184 in the ratio 2:1 183a/184, by TLC and overlap of the  $^1\text{H}$  NMR spectra.

**Undefined components of the pyrolytic degradation of 19-carboxy-4 $\alpha$ ,5-epoxy-5 $\alpha$ -androstane-3,17-dione. 79**

The additional inseparable polar components correspond to two degradation products (TLC) with two distinct mass spectra (parent ions at 288 and 286), absence of H-C-O in the NMR, two singlets in the alkene region, and an AB quartet about 6.7 reminiscent of the  $\Delta$ -1,4-diene pattern for the 1-CH and 2-CH protons. It is possible that one of these is **estra-1,4-diene-3-hydroxy-17-one.**

**Preparation of 3 $\beta$ -hydroxy-estr-5(10)-ene-4,17-dione (184) by thermal degradation of 19-carboxy-4 $\beta$ ,5-epoxy-5 $\beta$ -androstane-3,17-dione. 78**

A few milligrams of the 19-acid 78 were heated, neat, at 195°C under  $\text{N}_2$ . The reaction was followed by TLC on  $\text{SiO}_2$  ( $\text{CHCl}_3$ ) results in 8 compounds, of which estrone and starting material can be identified, in addition to the mixture of 184 alcohol isomers.

A suspension of the acid 78 in decalin (b.p. 195°C) was heated at reflux under nitrogen for 2.5 hours. The solvent was evaporated under reduced pressure and the residual semi-solid was chromatographed on basic Woelm alumina grade III. 184 exhibited the same spectral characteristic as those reported by Mastalerz (Ph.D. thesis, U. of Ottawa, 1981);  $^1\text{H}$  NMR ( $\text{CDCl}_3$ ): $\delta$  (3-CH-O, fine split ABq, 1H,  $\delta_a=3.94$   $\delta_b=4.18$   $J_{ab}=13.5\text{Hz}$ ,  $J_{ac-bc}=5\text{Hz}$ ), 0.95 (18-CH<sub>3</sub>, s, 3H).  $\text{C}_{18}\text{H}_{24}\text{O}_3$  calc: 288 found MS m/z: 288(P<sub>0</sub>, 44% with appropriate isotopic abundance for  $\text{C}_{18}\text{H}_{24}\text{O}_3$ ), 244(100%).