STUDIES IN BIOCHEMISTRY:
STEROID SYNTHESIS, P-450 ENZYMATOLOGY
AND RENAL LITHOGENESIS

By
JEREMY D. SELENGUT

Submitted to the Department of Chemistry in Partial Fulfillment of the Requirements for the Degree of

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ABSTRACT

I. A method for the synthesis of lanosterol (Lanosta-8,24-dien-3β-ol) is developed such that isotopes of hydrogen can be specifically introduced into the C-32 position of the molecule. Lanosterol labeled with tritium in this way is suitable for use in recently developed assay methods for lanosterol-14α-demethylase, a P-450 enzyme responsible for the common step in the synthesis of cholesterol and ergosterol from lanosterol. This enzyme is known to be the target of an important class of anti-fungal drugs and therefore has been the focus of considerable industrial, clinical and scientific interest. This synthesis is the first to preserve both of the double bonds in the lanosterol molecule and thus provide specifically labeled samples of the natural substrate for the mammalian and yeast enzymes, and additionally provides access to new molecules modified at the C-32 position and possessing the proper side-chain configuration. The synthesis is based on previous syntheses of C-32-modified dihydrolanosterol (Lanost-8-en-3β-ol) and significant improvements to several literature methods are provided.

II. The inhibitor (20R)-20-(1,5-hexadiynyl)-pregn-5-en-3β,20-diol has been shown to be a mechanism-based inactivator of the cholesterol side-chain cleavage enzyme, P-450scc, which is responsible for the conversion of cholesterol into pregnenolone in steroidogenic tissues. Furthermore, this inactivator has been shown to covalently bind to the protein during the course of this inactivation. These properties have been confirmed in the present work. Preliminary studies towards the identification of the site or sites of covalent linkage between the steroid inactivator and the putative active-site peptide(s) are presented. Evidence suggests that the steroid is attached to the φ2 tryptic fragment of the protein which is believed to contain the heme- and substrate-binding domain. Technical difficulties with further progress are analyzed. Studies with related mechanism-based inactivators having only one alkynyl group (such as (20R)-20-(1-hexynyl)-pregn-5-en-3β,20-diol), indicate that these molecules do not covalently bind to the protein. Speculations on the chemical differences between the inactivation modes of these two classes of molecule are discussed.
III. The granulocyte protein, calprotectin, is extracted from an unusual matrix stone (a large-volume, amorphous, soft kidney stone) and identified by N-terminal peptide sequencing and immunochemical (ELISA) methods. This protein has recently been identified with numerous inflammatory diseases including cystic fibrosis, rheumatoid arthritis, psoriasis, and skin cancers. Additionally, calprotectin has been found to possess considerable antimicrobial properties versus several important human infectious strains of fungi as well as bacteria. Struvite kidney stones, which are strongly associated with urinary tract infection, are analyzed for calprotectin content and found to contain the protein in considerable amounts. Speculations on the origin of this material and its significance for the process of renal lithogenesis are discussed and proposals for future research are outlined.

Thesis Supervisor: Dr. William H. Orme-Johnson
Title: Professor of Chemistry
DEDICATION

This thesis is dedicated to my family to whom I am indebted for the opportunity to have reached this goal.

To my mother, the artist, who taught me reading from the New York Times, algebra on her knee at the kitchen table and philosophy ever since.

To my father, the engineer, who gave me my first chemistry set, would not accept a poorly-reasoned answer, and kept me pondering for my entire seventh year when he asked me what was different about the way sight and sound and smell reached my eyes and ears and nose.

To my grandparents, you are my inspiration. To you I owe my love of words, books, music, history, biology and building something beautiful where before there was nothing at all.
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If my time at MIT has been successful and rewarding it is due in large portion to those who were around to help and support me, to you all a hearty thanks.

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Dr. Steve Dretler was instrumental in focusing our attention on the problem of the protein componant of kidney stones (the subject of chapter 4 of this thesis), and has provided...
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CHAPTER 1

CYTOCHROME P-450 ENZYMES:
AN OVERVIEW
Preface

Chapters 2 and 3 of this thesis describe research on the investigation of P-450 enzymes, specifically, lanosterol 14α-demethylase (P-450\textsubscript{14DM}) and the side-chain cleavage enzyme, cholesterol 20,22-lyase (P-450\textsubscript{scC}). The properties and biological importance of P-450 enzymes are summarized below to provide a context for the subsequent discussion of the experimental work. There have been many recent and comprehensive reviews of this field, so this discussion will be brief; the relevant reviews are cited in the text.

Cytochromes P-450: Definition and Significance

The name "P-450" is woefully uninformative, and does nothing to convey a sense of the catalytic versatility or biological importance of this class of enzyme. The name, deriving from "Pigment 450 nanometers," arose to signify a peculiar spectroscopic signal that serves as a unique thumb-print for the enzyme.\textsuperscript{(38)} When samples containing the enzyme are reduced with dithionite and treated with carbon monoxide (CO), an intense band arises at or near 450 nm compared to dithionite reduced samples.\textsuperscript{(12, 29)} Because of this intense signal it is a fairly straightforward matter to detect even dilute samples containing P-450's and this has contributed to the intensity of research in this area ever since its discovery. First noted in mammalian liver microsomes, it became apparent that the enzyme, like the other cytochrome pigments, contains heme (i.e., an iron porphyrin).\textsuperscript{(38)} The heme in P-450 enzymes is non-covalently bound and differs from other heme-containing proteins in that one of the iron ligands, perpendicular to the plane of the pyrrole nitrogens of the heme, is cysteine thiolate. This cysteine is responsible for the unique CO-mediated spectral signal.\textsuperscript{(49)}

Prior to the discovery of P-450's, it was recognized that CO could inhibit the metabolism of steroids in adrenal cortex,\textsuperscript{(43)} and this led to the discovery that P-450's are, in fact, the terminal oxidases of microsomal electron transport chains leading to steroid hydroxylation.\textsuperscript{(10)} Not long afterward a second independent function was discovered, namely the oxidation of exogenous substances in the liver.\textsuperscript{(7)}

The distribution of P-450's in biological systems is exceptionally broad. In mammalian species, P-450's are found wherever steroids are synthesized (adrenal, corpus leuteum, placenta, testes, ovaries, etc.) and in the liver, where the catabolism of endogenous and exogenous substances takes place, as well as in lung and intestinal
tissue. Furthermore, P-450 enzymes have been characterized in bacteria, fungi, corals, echinoderms, plants, insects, mollusks, fish and avians. There is every reason to believe that P-450's are nearly ubiquitous in all organisms, and the comparative study of P-450 genes (collectively characterized as a "superfamily") among species has become a significant field of research.

With the exception of the bacterial enzymes, P-450's are associated with membranes; in mammals, those of the microsomal fraction in liver tissue and mitochondria in steroidogenic tissues. This fact has made the characterization and study of mammalian P-450's challenging. No membrane-bound P-450 has yet been successfully analyzed by X-ray crystallography. Although crystals of the side-chain cleavage enzyme have been grown, they have, thus far, been of low quality as judged by spectrophotometry which indicates a significant portion of the protein exists in the denatured "P-420" form.

As mentioned above, P-450 enzymes can be divided into two groups, based on the type of molecule on which they act: endogenous or exogenous. P-450's acting on endogenous compounds in mammals are generally biosynthetic and are primarily involved in the formation of cholesterol and the steroid hormones (see introductions to chapters 2 and 3), although they also function in the biosynthesis of vitamin D, fatty acids, prostaglandins, bile acids and perhaps other endogenous compounds. Yeast also possess at least two P-450's involved in the biosynthesis of the fungal steroid ergosterol. P-450's may also be involved in the specific catabolism of endogenous compounds, although these pathways are less well understood. In plants there are many examples of P-450 enzymes involved in the biosynthesis of natural products such as amino acid derivatives (p-coumaric, caffeic and sinapic acids), fatty acids, flavinoids (apigenin, pisatin, marmesin and psoralin), and terpenoids (phaseic acid, kaurenoic acid and digitoxigenin). The possible roles for P-450 in the oxidation of bacterial natural products (especially terpenoids, which are first cyclized as hydrocarbon products which are often further elaborated by oxidation) have not been thoroughly evaluated. These P-450's are characterized by high substrate specificity and regioselectivity of catalytic action. Generally, only a single, specific atom is oxidized among many chemically equivalent sites.

P-450's acting on exogenous compounds possess quite different properties. Adapted, as they are, to the detoxification of many different exogenous chemicals, hepatic P-450's are not specific for a particular compound, but rather, groups of compounds with similar molecular shapes. Detoxification is arranged by making fat-soluble compounds with low solubility in the aqueous serum and urine more water-soluble by using successive hydroxylations, de-alkylations and other oxidative chemistry. Without this process, these
fat-soluble chemicals could build up in the membranes and fatty cells of the body to dangerous concentrations. It has been found that individuals express multiple hepatic P-450's which have somewhat overlapping substrate specificities. In this way evolution has arranged a system that can act on a general class of compounds including new chemicals such as man-made pesticides, that no previous member of the species has ever encountered. The only limitation to this activity is that these P-450's must not act on natural endogenous compounds indiscriminately; these activities have surely been screened out by natural selection. (This is in contrast to the other major mammalian defensive mechanism, the immune system, in which a nearly infinite selection of specific agents is screened by each individual to eliminate antibodies which react with the "self." ) The distribution of these multiple hepatic P-450 genes is not uniform throughout a species, there is considerable inter-individual variation in the suite of genes carried and the regulation and expression of these genes.\(^{(19)}\) This variability explains in part a long noted individuality in the response of human patients to drug treatments as well as the sensitivity of some individuals to certain chemicals at levels much lower than the norm.\(^{(30)}\) The interaction of pharmaceuticals with various P-450's is a major concern of drug researchers and manufacturers, as inopportune "detoxification" of the active form of a drug can reduce the amount of time the drug stays at a useful level in the blood stream, thereby requiring more frequent treatments or larger doses, and risking increased side-effects.\(^{(9, 26)}\) Alternately, determination of the biochemical mode of action of a drug may depend on knowledge of the activity of P-450's on the compound because the form administered may not be the active agent.

As has been realized from nearly the first report of this type of P-450, while carcinogens and mutagens can be inactivated by P-450 enzymes, essentially non-toxic compounds can also be "activated" to carcinogenic or mutagenic forms by the very same enzymes.\(^{(14)}\) Thus the study of P-450's has been intimately linked to the study of cancer as well as chemotherapy.\(^{(16)}\)

Bacteria seem to possess similar types of relatively broad specificity P-450's that act on exogenous substances. Apparently, the role of these P-450's is not the detoxification of non-polar compounds, but rather it is the first step in the use of these compounds as sources of carbon. Bacteria seem to use P-450's as digestive enzymes when they find themselves in environments high in hydrocarbons and low in carbon sources that are easier to digest. Examples include the camphor and linalool hydroxylases of *Pseudomonas putida*. Several species have P-450's which act on simple linear alkanes, as do several yeast species. Generally these enzymes are not constitutively expressed, but are induced by the conditions of the medium in which they are growing. It is possible
however, that these enzymes have another biological role entirely and have only been selected for such a digestive role by the manipulations of researchers growing these strains on unusual carbon sources.\(^{(2, 47)}\)

**The P-450 Catalytic Cycle**

Much of what is known about the catalytic cycle of P-450 enzymes is based on the studies of the camphor hydroxylase from *Pseudomonas putida* (P-450\(_{\text{cam}}\)). Thanks to this enzyme's soluble nature, it was the first of this class to be crystallized and studied by X-ray crystallography. Many of the biophysical experiments that have been performed to investigate the catalytic mechanisms of P-450's in general have focused on P-450\(_{\text{cam}}\) because of the insights to be gained from comparison with the available three-dimensional structures. A schematic of the P-450\(_{\text{cam}}\) catalytic cycle, as it is now understood, is shown in Figure 1. In brief, the steps of the cycle are as follows: 1) substrate (S) binds to the enzyme in its resting state where the iron is in the ferric (3+) state; 2) a protein electron carrier (in P-450\(_{\text{cam}}\), putidaredoxin) reduces the heme iron to the ferrous (2+) state; 3) Oxygen binds to the iron atom; 4) a second electron is delivered yielding a highly unstable intermediate which 5) rearranges, splitting the oxygen-oxygen bond with the addition of two protons and releasing one oxygen atom as water (the timing of this release is unclear); 6) the remaining oxygen bound to the iron acts on the substrate with the formal insertion of the oxygen into a carbon-hydrogen bond, forming an alcohol; 7) product release regenerates the resting state of the enzyme. Each of these steps is discussed in detail below.

In the resting state of P-450\(_{\text{cam}}\) the iron is low-spin ferric state and the sixth ligand position is filled by water or a hydroxide. Binding of the substrate results in the displacement of the sixth ligand and rearrangement of the iron ligand field from an octahedral geometry to square pyramidal. This rearrangement has two effects: first, the spin state changes from low-spin to high-spin, and second, the reduction potential of the protein is raised from -300 mV to -170 mV, allowing rapid electron transfer from the biological reductant, putidaredoxin. In effect, this acts as an electron gate, only allowing reduction of the heme after the substrate has bound (Scheme 1).

The high-spin ferrous heme formed by this reaction then rapidly binds dioxygen forming a low-spin, hexacoordinate complex. The ferrous dioxygen complex is somewhat stable and has been isolated and characterized spectroscopically. Stopped-flow spectrophotometry of the rat liver microsomal P-450\(_{\text{LM2}}\) indicates that the initially formed ferrous iron-dioxygen complex (k > 1000 s\(^{-1}\)) is converted to the form observed in the
Figure 1: Generalized P-450 catalytic reaction cycle; individual steps are discussed in the text. A schematic of the iron ligation in the resting state of the enzyme is at the center.

steady state with a rate of 210 min\(^{-1}\). Studies of this steady state complex by Mössbauer spectroscopy indicate that the iron is in a state similar to that of oxyhemoglobin. These observations suggest that the steady-state electronic structure of this intermediate is best described as a ferric superoxide complex (Scheme 2). At this stage the overall charge on the complex is -1 (Fe = +3, O\(_2^-\) = -1, heme = -2, thiolate = -1). This complex is the final one that can be isolated and studied spectroscopically because subsequent steps are exceedingly rapid. After this stage in the mechanism, all information about the structure of the intermediates has been gained indirectly from comparison with other heme proteins, model compounds, and the products of P-450 catalysis.

The chemically active oxygen intermediate is generated by the addition of a second electron and two protons into the system. Through these additions one of the oxygens of the bound superoxide anion is converted to water and released. However, this is only known to be true because of the overall stoichiometry of the reaction; it is not known if the oxygen atom is converted directly to a molecule of water by protonation and elimination,
the simplest mechanism (Scheme 3a), or by another avenue. Other possibilities include dioxygen bond cleavage via acylation of the terminal oxygen atom by an active site carboxylate, or formation of a cyclic peroxoiron which is the active species which forms water during the hydroxylation chemistry (Scheme 3b and c). Ortiz de Montellano argues(39) that although there is no concrete evidence to support any of these possibilities, the acyl-intermediate hypothesis should be ruled out because no carboxylic cofactors are required for any known P-450's, the crystal structure of substrate bound P-450cam does not indicate any acylating functional group in the active site(41) and dioxygen bond cleavage in peroxidases and metalloporphyrin models does not require any acylating group. Furthermore, the cyclic peroxo-structure should be ruled out because model compounds with similar structure are chemically unreactive and, because P-450's can be made to turn over to identical products using the single oxygen atom transfer reagent iodosobenzene, the active intermediate most likely contains only one iron-bound oxygen atom. Therefore, in the absence of compelling evidence to the contrary, the simple protonation-elimination scheme (3a) should be used as a working model.

What, then, is the working model for the actual oxidizing equivalent of cytochrome P-450? Assuming that water has indeed left from the complex, we can tally up the electrons in the complex as follows: direct removal of H₂O from the doubly protonated ferrous superoxide species would leave the proximal oxygen bearing two lone pairs and one-half of the shared pair with iron to give 5-electrons and a positive charge; the iron would still be ferrous with two positive charges. Adding in the heme and thiolate ligands gives an overall neutral charge on the complex (this checks with the previous calculation of -1 charge on the ferric superoxide intermediate since 1 electron and 2 protons have been added while neutral water has left). This is only a formal counting and not meant to suggest that the 5-electron oxygen atom is a stable configuration for the system. In fact, it is only one of quite a few

Scheme 2: Oxygen binding to pentacoordinate, ferrous heme P-450
Scheme 3: Possible mechanisms for dioxygen cleavage after the final electron addition to P-450.
possible electronic configurations that must be considered. The basic configurations include \([\text{Fe}^{2+}][\text{O}^+], [\text{Fe}^{3+}][\text{O}], [\text{Fe}^{4+}][\text{O}^-] \text{ and } [\text{Fe}^{5+}][\text{O}^{2-}]\) (Scheme 4, 1-6). In addition, there exists the possibility that one electron can be drawn in from the porphyrin ligand to give a heme cation radical as is observed in "Compound I" of peroxidases (see below) yielding \([\text{P}^+][\text{Fe}^{3+}][\text{O}^-] \text{ and } [\text{P}^+][\text{Fe}^{4+}][\text{O}^{2-}]\) (Scheme 4, 7 and 8) to round out the list.

\[\begin{array}{cccc}
\text{Fe}^{II} & \text{Fe}^{III} & \text{Fe}^{IV} & \text{Fe}^{V} \\
\text{1} & \text{2} & \text{3} & \text{4} \\
\text{Fe}^{II} & \text{Fe}^{III} & \text{Fe}^{IV} \\
\text{5} & \text{6} \\
\text{Fe}^{III}-\text{Heme}^+ & \text{Fe}^{IV}-\text{Heme}^+ \\
\text{7} & \text{8}
\end{array}\]

\textbf{Scheme 4:} Possible electronic configurations for the P-450 iron-oxygen atom complex responsible for substrate activation

* At this time it is important to discuss the various conventions in the depiction of iron oxygen complexes. It has been common in the literature to refer to an \([\text{FeO}]^{3+}\) complex with the shorthand figure \([\text{Fe}^V=\text{O}]\). This formalism can be confusing if one concludes from it that the oxygen, as if it were in a carbonyl, was equally sharing two electron pairs with the iron and possessed only two lone pairs (and thus would be at an oxidation state of 0, not -2). This use of the double bond symbol represents the donation of two lone pairs to the metal, since, in inorganic chemistry, all usual oxygen atom ligands to metals are "oxo" and at the -2 oxidation state. This figure has found wide use in the metalloprotein literature because it appears to be an adequate representation of the properties of Compound I and II of peroxidases. As will be discussed in the main text, however, it very likely is a poorer representation of the electronic properties of the P-450 iron-oxygen complex, and it will not be used in this discussion. In its place, a single-headed arrow will be used to represent the donation of one lone pair, while a single line always represents a shared bond.
Several heme-containing protein systems have iron-oxygen intermediates which are stable enough to characterize spectroscopically and have been discussed as models for P-450. The peroxidases are heme-containing enzymes which catalyze reduction of peroxide to water with the accompanying oxidation of various organic substrates. The fifth ligand in peroxidases is the imidazole nitrogen of a histidine residue instead of the cysteine thiolate found in P-450's. Peroxidases, like P-450's, have iron-oxo intermediates, denoted Compound I and Compound II (I is reduced to II by the addition of an electron). There is considerable evidence from Mössbauer, magnetic susceptibility, electron absorption, NMR and X-ray absorption methods that Compound I of horseradish peroxidase is composed of a "ferryl" iron (FeIV) and a heme π-cation radical. The oxygen, then, has a complete octet (O²⁻, Scheme 4, 8). EXAFS methods show the iron-oxygen bond length to be short (at 1.65Å), indicating considerable double bond character. (Thus, configuration 8 might be even more accurately represented with two dative bonds from oxygen to iron.) Compound II is quite similar, lacking only the heme π-cation radical. Studies of cytochrome c peroxidase show that this enzyme differs mainly in the presence of a protein-centered radical instead of the heme-centered radical in Compound I. The catalases have a tyrosine fifth ligand but have very similar spectroscopic properties to the peroxidases. Although they have no naturally occurring Compound II-like state, the Compound I-like state of catalase is almost identical to that of the peroxidases. Finally, chloroperoxidase, which has a thiolate fifth ligand like the P-450's (but a peroxidase-like polar heme environment), also achieves a Compound I-type iron-oxo complex, although the electronic ground state seems to be different from the peroxidases and catalases.¹

The iron-oxygen complex in P-450 enzymes must be distinctly different from these systems to explain the powerful chemical reactivity it possesses and the extremely short lifetime of its intermediate forms. Nevertheless, the seeming uniformity of [FeO]³⁺ complex structures among the three related enzyme types, including the thiolate-containing chloroperoxidase, suggests that the difference may not be large. As will be discussed in the next section, the overwhelming evidence concerning P-450 chemistry suggests that radicals are involved. All of the electronic structures under consideration here have an odd number of electrons, and thus are capable of the one-electron oxidations and proton abstractions that characterize P-450 chemistry. The question is, which configurations place unpaired electron density on the oxygen atom where it can initiate the chemistry?

The Compound I intermediate (e.g. Scheme 4, 8) places the radical on the porphyrin. This is appropriate for peroxidase, for instance, since electron transfer to organic substrates is believed to take place at the heme edge in this system. For P-450, however, it seems unlikely that an oxygen with a full octet and bearing a -2 charge will be
involved in radical chemistry. The hydrophobic nature of the P-450 heme pocket differs markedly from the environment of the other oxo-iron enzyme systems; very likely this environment favors a less electron-rich oxygen, disfavors the ionic $\pi$-cation radical, and high-valent iron oxidation states (note also that the total charge on the P-450 complex is neutral, while in peroxidase it is +1). Champion has suggested that the cysteine thiolate contributes significant extra electron density to the iron through $\pi$-bonding between the S(sp$^2$) and Fe(d$\pi$) orbitals. By thus occupying the d$\pi$-orbitals, the corresponding overlap between the oxygen lone pairs and the d$\pi$-orbitals is decreased, as is the iron-oxygen bond order.$^{(6)}$ This should result in a slightly longer Fe-O distance, and theoretical calculations have indicated that a longer distance stabilizes an oxy-radical species which lies within 0.7 kcal/mol of the Compound I-like ground state.$^{(32)}$ Champion further suggests that the Compound I electronic configuration (e.g. Scheme 4, 8) is in equilibrium$^*$ with this low-lying oxy-radical state ([Fe$^{4+}$][O$^-$], seven electrons on oxygen, e.g. Scheme 4, 6) in all of the iron-oxygen heme enzymes, the differences arising from the position of the equilibrium constant. In peroxidase and catalase, the Compound I form is strongly favored. In chloroperoxidase the cysteine thiolate alters this balance only slightly. But in P-450, with a non-polar environment and a cysteine thiolate, the oxy-radical is strongly favored (Scheme 5). Of course, this remains a topic wide open for differences of opinion; in particular, some may prefer the ferric iron-oxygen atom complex [Fe$^{3+}$][O] which would be expected to be a stronger radical reagent because of its weaker dipole (e.g. Scheme 4, 5).

Based on the observations of stereochemical scrambling$^{(45)}$ and high intrinsic isotope effects,$^{(15)}$ it has been proposed that the initial step in aliphatic carbon atom hydroxylation is a radical hydrogen atom abstraction to give an iron-bound hydroxyl and a carbon-centered radical. This radical then recombines with the oxygen (dubbed the "rebound" mechanism), generating the hydroxylated product and the ferric iron resting state of the enzyme (Scheme 6). The rate of this radical recombination has been estimated by the observation of partitioning between hydroxylation and radical ring opening in strained systems whose intrinsic ring opening rates are known. This method is known as the "radical clock." Although the ring opening of methylcyclopropane was not observed (ring opening rate = $10^8$ s$^{-1}$), partitioning was observed in the bicyclo[2.1.0]pentane system, indicating a recombination rate on the order of $10^{10}$ s$^{-1}$.$^{(40)}$ There are now many examples of reactions that could only result from the initial formation of substrate radicals, some of which are presented below.

* The difference between discrete chemical states in equilibrium, which implies a difference in atomic configuration and an energy barrier between the states, and resonance among electronic configurations in this system is a point for further debate.
Scheme 5: Proposed equilibrium between Compound I type (left) and oxy-radical (right) electronic configurations in P-450's (top) and peroxidases (bottom).
(After Champion, JACS, 111, p. 3433-3434, 1989)

Scheme 6: Radical rebound mechanism for aliphatic hydroxylation by P-450
P-450 Chemistry: scope of reactivity

There is a delicate balance in P-450 catalysis between the reactive oxygen-iron center, the chemical structure of the substrates and the steric constraints of the various enzyme active sites that results in a very wide variety of reaction types. Although the oxygen "rebound" mechanism is adequate to describe the chemistry of a large number of P-450 reactions, there are classes of reactions for which it is clearly incomplete. The salient factor that binds all of the observed chemistry is the ability of the reactive species to abstract hydrogen atoms or, if no hydrogen atom is available or the reduction potential of the substrate is particularly high, perform one-electron oxidations. For instance, if the binding of a substrate does not place any protons close enough to the activated oxygen, electron transfer, which can occur over longer distances, may take place. The subsequent chemistry of these substrate-radical species is governed by several factors: the intrinsic recombination rate (a property of the metallo-center and the distance separating it from the substrate radical), the number of atoms over which the substrate-radical can delocalize (a property of the substrate) and finally, the intrinsic rates of the pathways for rearrangement of the substrate radical (a combined property of the substrate and the active site of the protein which binds it in a particular, perhaps strained, conformation). With this rationale in mind, the remarkable diversity of P-450 reactions presented below should seem less surprising.

Under "basic reactions" (Scheme 7) are listed the building blocks which underlie many P-450 reactions. The addition of oxygen to carbon is listed separately from the heteroatomic reactions because they are believed to proceed through distinctly different mechanistic intermediates. The typical rebound mechanism is in effect for carbon atoms, but nitrogen hydroxylations are believed to involve a three-step sequence involving initial 1-electron oxidation followed by proton transfer and hydroxyl radical rebound. This mechanism is suggested by the partitioning of some systems between nitrogen hydroxylation and dealkylation (see below). In the formation of N-oxides and sulfoxides, where no hydrogen atoms are involved, electron transfer from the heteroatom is followed by oxygen atom transfer. Aliphatic hydroxylations are typical of the steroid hydroxylases, while the heteroatomic oxidations have been characterized in the metabolism of heteroatom-containing drugs and toxins such as amphetamine and pyridine. In addition to the hydroxylamine and N-oxide forms shown in Scheme 7, P-450's can catalyze the formation of nitrone (C=NR→O), oximes (C=NOH), nitroso- (RN=O) and nitro-compounds (RNO₂).
**Elementary Processes:**

- **Single Electron Oxidations**
  \[
  \begin{array}{c}
  \text{H} \\
  R_1 \quad R_2
  \end{array}
  \xrightarrow{\text{N}}
  \begin{array}{c}
  \text{H} \\
  R_1 \quad R_2
  \end{array}
  \xrightarrow{\text{N}^+}
  \begin{array}{c}
  \text{H} \\
  R_1 \quad R_2
  \end{array}
  \rightarrow \text{Various Products}
\]

- **Hydrogen Atom Abstractions**
  \[
  \begin{array}{c}
  \text{H} \quad \text{H} \\
  R_1 \quad R_2
  \end{array}
  \xrightarrow{\text{C}}
  \begin{array}{c}
  \text{H} \\
  R_1 \quad R_2
  \end{array}
  \xrightarrow{\text{C}^+}
  \begin{array}{c}
  \text{H} \\
  R_1 \quad R_2
  \end{array}
  \rightarrow \text{Various Products}
\]

**Basic Reaction Types:**

- **Aliphatic Hydoxylation**
  \[
  R-\text{CH}_3 \rightarrow R-\text{CH}_2\text{OH}
  \]

- **Heteroatom Oxidation**
  \[
  \begin{array}{c}
  \text{H} \\
  R_1 \quad R_2
  \end{array}
  \xrightarrow{\text{N}}
  \begin{array}{c}
  \text{OH} \\
  R_1 \quad R_2
  \end{array}
\]

- **Olefin Epoxidation**
  \[
  R_1 \quad R_2 \xrightarrow{\text{O}} \begin{array}{c}
  \text{O} \\
  R_1 \quad R_2
  \end{array}
  \]

- **Dehydrogenation**
  \[
  R_1 \quad R_2 \xrightarrow{\text{H}} R_1 \quad R_2
  \]

- **Peroxidation**
  \[
  R-\text{CH}_3 \xrightarrow{\text{O}} R-\text{CH}_2\text{OOH}
  \]

**Scheme 7: P-450 Reaction Types**
Olefin epoxidation is a distinct reaction type which is also found in reactions with aromatic and acetylenic carbon atoms, forming intermediate oxarenes and oxirenes, respectively, preceding rearrangement. Reactions of this type are believed to be initiated by the formation of a π-complex, but proceed through 1-electron steps to either a radical cation or a radical σ-complex, leading to products. However, the exact nature of multiple-bond oxidations by P-450's remains controversial. The action of P-450's on acetylenic compounds has received much attention due to the apparent formation of electrophilic species which act as "suicide" inactivators. These will be discussed in detail in Chapter 3 which concerns the action of such an inactivator on the side-chain cleavage enzyme.

Dehydrogenation reactions are examples of P-450 reactions in which no oxygen atom is transferred to the product. An initial proton abstraction is followed by either a radical β-elimination or electron transfer to form a carbocation which eliminates via an E1-like mechanism. P-450-mediated desaturation of alkanes (X = H) has been suggested in the microsomal metabolism of lindane (hexachlorocyclohexane) and testosterone and in the biosynthesis of ergosterol by yeast species and has been conclusively shown in the metabolism of the anti-epileptic drug, valproic acid. It is likely that many more examples of this mode of reactivity will come to light in the future; a recent report suggests that fatty acid desaturases may be P-450 enzymes and the P-450-mediated lyase activity in the synthesis of psoralin (Scheme 8) can be understood as a β-elimination of an isopropyl radical. The P-450-mediated oxidation of alcohols to ketones or aldehydes has also been proposed to involve desaturation in some cases. The oxidation of epitestosterone to androstenedione, for instance, was found to proceed without the

![Scheme 8: A possible mechanism for the P-450-mediated biosynthesis of psoralin from (+)-marmesin.](image)

introduction of any $^{18}O$ from $^{18}O_2$, while the oxidation of testosterone was found to involve the gem-diol mechanism discussed below, with concomitant incorporation of $^{18}O$.

The peroxidation of aliphatic carbon atoms is relatively rare, but peroxo-attack on carbonyl carbons appears to be more common (see below). The chemistry of P-450-supported peroxide chemistry has been reviewed.$^{35}$

The "elaborated" reactions (depicted in Scheme 9) are those in which the initial oxidation event is believed to be one of the basic types, but further rearrangement (or reaction of that product) disguises the underlying chemistry. One mechanism for the oxidation of alcohols to aldehydes or ketones is merely the hydroxylation of a carbon already bearing an alcohol; the resulting product is a gem diol, a hydrated carbonyl. The most well-known examples of this reaction are the second step in the $14\alpha$-demethylation of lanosterol by $14\alpha$-demethylase and the second step in the $19$-demethylation of androstenedione by aromatase (see below, and references in the following two chapters). A special case of this type of reaction is the oxidative cleavage of esters in which, instead of $\text{OH}^-$ leaving from the tetrahedral intermediate to form water, a carboxylate anion leaves to form an acid $[\text{R}_1\text{CH}_2\text{OOCR}_2 \rightarrow \text{R}_1\text{CH(OH)OOCR}_2 \rightarrow \text{R}_1\text{CHO} + \text{R}_2\text{COOH}]$.\textsuperscript{22}\n
Similarly, heteroatom de-alkylation, oxidative deamination and dehalogenation are examples of hydroxylations followed by elimination from a tetrahedral intermediate (Scheme 9, reaction 2). These types of reactions are numerous in the area of xenobiotic metabolism.$^{17}$

Aromatic hydroxylation is an elaboration of oxygen insertion into double bonds. Rearrangement of the intermediate oxarene to the phenol requires the 1,2-migration of one of the two groups adjacent to the oxygen on the aromatic ring (Scheme 9, reaction 4, R\textsubscript{2}), this has been called the "NIH shift" and has been observed using deuterated aromatic compounds, as well.$^{39}$ The final reaction depicted is an enzymatic version of the Baeyer-Villiger rearrangement in organic chemistry. Here, a putative heme-iron bound peroxide acts as a peracid equivalent. Notable examples of this reaction are found in the final step of the $14\alpha$-demethylase sequence$^{11}$ and (possibly) in the $17,20$-lyase reaction forming androstenedione from progesterone.$^{33}$ In the case of aromatase, however, the intermediacy of a formate ester has been ruled out.$^{162}$ Hepatic P-450's have also been found which catalyze this reaction on cyclohexanecarboxaldehyde.$^{50}$

The reaction sequences of $14\alpha$-demethylase and the side chain cleavage enzyme (the topics of Chapters 2 and 3) are shown in schematic form (in Scheme 10) as examples of P-450's which act multiply on single substrates to effect even more elaborate chemistry. The demethylase pathway includes hydroxylation, dehydrogenation (via the gem diol).
Alcohol Oxidation (Dehydrogenation)

\[ R-\text{CH}_2\text{OH} \rightarrow \begin{bmatrix} \text{OH} \\ \text{R-C-OH} \end{bmatrix} \rightarrow R-\text{CHO} + \text{H}_2\text{O} \]

Heteroatom Dealkylation (\(X = \text{NH or O}\))

\[ R_1-X-\text{CH}_2 \rightarrow \begin{bmatrix} \text{OH} \\ R_1-\text{CH}-\text{OH} \end{bmatrix} \rightarrow R_1-\text{XH} + R_2\text{CHO} \]

Oxidative Deamination or Dehalogenation (\(X = \text{NH}_2\) or halide)

\[ R_1-\text{CH}-R_2 \rightarrow \begin{bmatrix} \text{OH} \\ R_1-\text{C}-R_2 \end{bmatrix} \rightarrow \text{R}_1\text{R}_2 + \text{HX} \]

Aromatic Hydroxylation (with accompanying 1,2-shift)

\[ \text{R-} \rightarrow \begin{bmatrix} \text{O} \\ \text{R}_1-\text{O-R}_2 \end{bmatrix} \rightarrow \text{R}_1\text{R}_2 \]

Ester formation (Baeyer-Villiger Oxidation)

\[ \text{R}_1\text{R}_2 \rightarrow \begin{bmatrix} \text{O} \\ \text{R}_1\text{R}_2 \end{bmatrix} \rightarrow \text{R}_1\text{R}_2 \]

**Scheme 9:** Elaborations of P-450 Reactions

Demethylation

\[ \text{R-} \rightarrow \text{R-} \rightarrow \text{R-} \rightarrow \text{R-} \rightarrow \text{R-} \]

Chain Cleavage (with glycol cleavage)

\[ \text{R-} \rightarrow \text{R-} \rightarrow \text{R-} \rightarrow \text{R-} \rightarrow \text{R-} \]

**Scheme 10:** Multiple P-450 lyase reactions
Baeyer-Villiger oxidation and α-elimination. The side-chain cleavage reaction sequence illustrates yet another activity, glycol cleavage.

In Scheme 11, four examples of P-450-initiated radical reactions and rearrangements are presented. Reaction 11a, a ring expansion, represents only one of several ways that cyclobutylamines can react with P-450, others include hydroxylation, ring opening and covalent modification of the heme. Similar reactivity is found with cyclopropylamines. One of the strongest proofs of an electron abstraction mechanism for heteroatom oxidation is reaction 11b in which 1,4-dihydropyridines are oxidized to pyridines with the expulsion of hydrogen and alkyl radicals. Reaction 11c shows the proposed mechanism for the P-450-mediated rearrangement-hydroxylation of liquiritigenin to 2,7,4′-trihydroxyisoflavanone which is subsequently dehydrated to give isoflavone, a parent compound for many plant isoflavonoid phytoalexins having antimicrobial activity. The final reaction, 11d, the biosynthesis of a gibberellin (GA12 aldehyde) from ent-hydroxykaurenoic acid has been tentatively proposed to be P-450 mediated and involves a ring contraction and an overall alcohol dehydrogenation.

Finally, Scheme 12 shows three examples of a novel mode of P-450 action involving the radical rearrangement of alkyl peroxides. In reaction 12a, the hydroperoxide (derived from lipoxygenase action on linoleic acid) is transformed into an allene oxide by allene oxide synthase, isolated from flax seed. The reaction is formally a dismutation as one atom of the peroxide is reduced to water, while the other is oxidized to the allene oxide. Allene oxides are unstable and, in this case, it was detected by the isolation of its decomposition products, an α-ketol, a γ-ketol and a cyclopentenone (t1/2 decomposition 9s at 10 C, pH 7). Flax seed contains an allene oxide cyclase which specifically catalyzes the conversion of this transient compound into a precursor of the plant growth regulator, jasmonic acid. Allene oxides are also known in several species of marine invertebrates. Similarly unusual are the reactions of thromboxane (TXA) synthase and prostacyclin (PGI) synthase (reactions 12b), both of which act on the prostaglandin endoperoxide (PGH₂). In all of these reactions the chemistry is initiated by the homolytic cleavage of a peroxide by iron and the subsequent rearrangement is apparently controlled by the precise conformation of the bound substrate. It is notable that in this class of reaction no dioxygen or reducing equivalents are required; these are provided entirely by the substrate. Accordingly, the rates of these reactions are significantly faster than the ordinary type of P-450 mechanisms.
Scheme 11: P-450-mediated radical reactions
Scheme 12: P-450-mediated rearrangement of alkyl peroxides
Conclusions

It is clear from the above discussion that, as a versatile catalyst, the combination of dioxygen, heme- and thiolate-ligated iron, reducing equivalents and a conformationally restricted hydrophobic protein binding pocket is impressive. The power of P-450 enzymes to activate unfunctionalized carbon atoms regio- and stereospecifically is particularly attractive to chemists interested in the development of new oxidative catalysts for organic chemistry. The development of biomimetic catalysts with oxidative and molecular recognition properties similar to P-450 has become a significant sub-field, \(^{(34)}\) while others have attempted to use P-450 enzymes as organic chemical catalysts directly. \(^{(5)}\) The prospects for the large-scale production of fine chemicals by the use of P-450-expressing bacteria and fungi (natural and recombinant) are considerable. In fact, the raw materials for such applications, microbial P-450's, are one of the fastest-growing segments of the P-450 superfamily genetic list. The genetic engineering of P-450-containing microbes designed to cleanse the environment of toxins and pollutants is also a distinct possibility.

The study of P-450 enzyme catalysis has mainly focussed on the bio-inorganic aspects of the system in the determination of the basic chemical mechanisms. However, it seems that the contribution of the substrate binding pocket to the course of catalysis to particular products is critical. The information desired is: in what conformation and with what relative orientation will a particular substrate bind to a particular P-450 enzyme? Of course, with most P-450 enzymes presently unavailable for study by X-ray crystallography and too large for NMR techniques, this presents a considerable challenge to researchers interested in characterizing these interactions.

As the number of known P-450 gene sequences increases (and it is very difficult to guess what the eventual total will be) the possibilities for determination of P-450 structure by comparison with the soluble, crystallizable, forms and the use of computer-based methods become more feasible (see introduction to Chapter 3). In addition, the determination of the amino acid residues lining the active site of P-450 enzymes by chemical means with mechanism-based inactivators should aid in this process. Finally, genetic manipulation of P-450 sequences by various directed and combinatorial mutagenesis methods to modulate the substrate specificity should, in the future, be an important method in the investigation of these matters and the development of new catalytic activities.

Considering the nearly ubiquitous distribution, variety and importance of biochemical roles, and diversity of genetically related sequences, it seems likely that the study of P-450's will become even more important to the fields of bioorganic chemistry, molecular evolution, molecular recognition, pharmacology and toxicology as technical difficulties continue to be surmounted.
References


CHAPTER 2

THE SYNTHESIS OF SPECIFICALLY ISOTOPE-LABELED LANOSTEROL FOR THE ASSAY OF CYTOCHROME P-450\textsubscript{14DM} (LANOSTEROL 14\textalpha-DEMETHYLASE)
Introduction: The study of P-450 14α-Demethylase

General Background

The motivator for this research is the P-450 enzyme, lanosterol 14α-demethylase, which is responsible for the oxidative removal of carbon 32 from the quaternary position 14 of lanosterol (see Scheme 1)*. To answer the question "Why?" requires a somewhat broad foray into biochemistry, specifically the areas of bio-organic (or natural products) chemistry and pharmacology. Lanosterol (and the closely related compound cycloartenol in plants) holds a central position in the biosynthesis of steroids. The distinctive tetracyclic ring structure of all steroids first arises in these molecules from the linear precursor, squalene (see Figure 1). Steroids are widely distributed throughout the natural world. They are synthesized by all animals, plants, sponges and fungi. Some bacteria and other unicellular organisms are also capable of steroid biosynthesis. Organisms such as mollusks and insects obtain their steroids from their diet. Clearly, these molecules have, from extremely ancient times, evolved indispensable roles in the processes of life. Although the conventional wisdom of public discourse may only recognize "steroid" as referring to certain controlled substances, these chemicals are relevant to our daily lives, from the ubiquitous reports on cholesterol in diet and disease to the birth control "pill" and the sex hormones testosterone and estrogen, from the anti-inflammatory drug cortisone to anti-asthmatic breathers and other chemotherapeutics.

With very few exceptions, all steroids in nature are modified such that the methyl group attached to carbon-14 of lanosterol (or cycloartenol) is replaced by a hydrogen atom. This process is mediated by 14α-demethylase. In mammals, this begins a 22-step enzymatic cascade that results in the formation of cholesterol. Cholesterol, then, is further

* Notes on steroid nomenclature: The steroid nucleus is composed of four rings which are denoted A through D from left to right as pictured in Scheme 1. The molecule is roughly planar, and thus a distinction can be made between the two "faces" of the molecule. By convention, the axial methyls at C-10 and C-13 (C-19 and C-18, respectively) are said to be on the β-face, and the methyl group at C-14 (C-32) is said to be on the α-face. Proper names are based on the carbon skeleton having the same number and configuration of carbons. Thus, a steroid lacking the 4α, 4β, and 14α carbons of lanosterol would be named based on "cholestane", while a similar molecule having an additional carbon at position 24 would be named based on "ergostane". Double bonds are referred to either as numbers imbedded in a name preceding "ene" or, less formally, with a "Δ" followed by a number preceding a common name. For instance, for a particular isomer of lanosterol; Lanosta-7,24-dien-3β-ol or Δ7-lanosterol. Solitary double bond numbers always refer to bonds from the carbon enumerated to the next one in sequence; double bonds between carbons not in sequence are denoted by a second number in parentheses. For instance, Δ7,17-lanosterol refers to a double bond between carbons 7 and 8, while Δ24(28)-ergosterol refers to a double bond between carbons 24 and 28 in the side-chain.
modified to yield all of the various steroid hormones found in these organisms. In plants cycloartenol is generally converted into sitosterol (24α-ethylcholest-5-enol), and in many fungal species 24-methylene-24,25-dihydrolanosterol (or lanosterol itself in yeasts) is converted to ergosterol (24β-methylcholesta-5,7,22-trienol, see Figure 1). Fungi and algae have considerable heterogeneity in the identity of their sterols from species to species; with only rare exception, however, all possess 14α-demethylases.\(^{(154)}\)

A considerable attraction to the study of this enzyme is its chemistry. Like the cholesterol side-chain cleavage enzyme, P-450 14α-demethylase belongs to the class of P-450's which effect complex chemical transformations. The power of P-450 enzymes to regio- and stereospecifically hydroxylate unfunctionalized carbon atoms has been the envy of organic chemists for decades, but the ability of these "complex" P-450's to act multiple times on a single substrate makes them truly exceptional catalysts.

In the area of steroid biosynthesis at least six P-450's fall into this category (Figure 2), many other "simple" P-450 hydroxylases are known in this area as well. P-450\(_{scc}\) hydroxylates once, hydroxylates again one carbon away from the first and then performs an oxidative glycol cleavage.\(^{(89)}\) P-450\(_{17α}\) (17,20 lyase) hydroxylates once \(α\) to a ketone and then cleaves the bond between them.\(^{(108)}\) Aromatase hydroxylates twice on the same methyl carbon and then cleaves it from the steroid nucleus accompanied by aromatization of
Figure 1: The biosynthesis of steroids in various kingdoms
Figure 2: P-450 enzymes in Steroid Biosynthesis
the steroid A-ring.\(^{81, 140, 141}\) 14α-demethylase, like aromatase, hydroxylates twice on the same carbon and cleaves it from the steroid nucleus as formate after the equivalent of a Baeyer-Villiger oxidation (see mechanistic discussion below). P-450\(_{11\beta}\) (aldosterone synthase) is believed to hydroxylate three times, once at the 11 position and twice at the 18 position of deoxycorticosterone, to generate a hydroxy-aldehyde which cyclizes to a hemiacetal, although the possibility remains that these activities are mediated by more than one closely related P-450's.\(^{111}\) Finally, from the biosynthesis of ergosterol in yeast, Δ-22-desaturase (presumably) hydroxylates once and then catalyzes the β-elimination of that hydroxyl group.\(^{67, 165}\)

These reactions form a mechanistically related group that appear to elaborate on the straightforward hydroxylation chemistry defined for P-450\(_{CAM}\) (see previous chapter). Subtle protein-mediated differences in active site structure, however, create this complexity in oxidative chemistry. The nature of these transformations suggests that there is a great deal to learn about how a single enzyme can control these sequential processes and exhibit distinct mechanistic modes on different intermediate states, and how different P-450's can exhibit such different chemical reactivities. The evolutionary efficiency exhibited in the use of a single active-oxygen species and a single binding site to effect these multi-step, multi-electron oxidations is truly intriguing.

P-450 14α-demethylase is the most widely distributed P-450 of this class; a fact which allows for comparisons between widely separated species. The enzyme has been successfully purified from rat liver microsomes,\(^{146}\) pig liver microsomes,\(^{132}\) and \textit{Saccharomyces cerevisiae}.\(^{157, 163, 166}\) Because the enzyme is found in yeast, the opportunity arises for the relatively straightforward cloning and genetic manipulation of the enzyme. The genetic sequence of the protein has been determined from \textit{S. cerevisiae}, \textit{Candida albicans} and \textit{C. tropicalis}.\(^{36, 79, 88}\) This has already been exploited to yield homologous overexpression in \textit{S. cerevisiae}\(^{153}\) and the expression of the \textit{C. tropicalis} gene in \textit{S. cerevisiae}.\(^{37}\) Mutant cells having altered or absent demethylase activity have been obtained using Chinese hamster ovary cells and \textit{S. cerevisiae}.\(^{38, 83}\) Recently, the study of lanosterol 14α-demethylase from human liver microsomes has begun.\(^{120}\) For these reasons, 14α-demethylase is a particularly accessible system to investigate the detailed issues of structure, function and mechanism of the complex P-450's.

Besides these considerations of basic science in chemistry and enzymology, this research is motivated by the very practical prerogatives of clinical and agricultural pharmacology. 14α-demethylase has emerged as a central player in the area of anti-fungal drug discovery and design. The connection between the enzyme and anti-fungal drugs is described in detail below.
Fungi and the higher eukaryotes are similar in many ways in terms of their basic biochemical pathways. The metabolic adaptations and structural innovations that separate fungi from bacteria are generally conserved in all eukaryotes including mammals. This vast biochemical gulf between mammals and bacteria are the reason why most bacterial infections can be successfully treated by drugs (anti-bacterials); what may kill a bacterial cell may very well have no effect on a mammal because the affected function in the bacterium may not exist or is fundamentally different in the eukaryotic cell. Although the effort to discover anti-bacterial agents has been long and arduous, once discovered, these compounds have been notable for their effectiveness and lack of severe side-effects.\(^{(53)}\)

Fungal cells, on the other hand, are quite another story. Although in general the mammalian immune system is effective at preventing fungal infection, when that system is compromised either by disease, immune system weakness, or the mutation of an infectious species to a new and unrecognized form, anti-fungal drugs are considerably less effective than their anti-bacterial counterparts.\(^{(53)}\) Discrimination between the biochemical machinery of fungal cells and that of host cells is difficult and usually only partial, resulting in side-effects, often of a very serious nature. Considering the most common internally administered antifungal drugs, amphotericin B, 5-fluorocytosine, miconazole and ketoconazole, possible side effects include chills, fever, rigors, nausea, diarrhea, pain, convulsions, anemia, renal toxicity, liver toxicity, electrolyte imbalance, anaphylaxis and cardiac arrhythmia.\(^{(158)}\) What may harm a fungal cell, it seems, will often harm a human cell as well, perhaps only less so. In recent times, with the prevalence of aggressive chemotherapy, AIDS and the use of immunosuppressive drugs, corticosteroids and broad-spectrum antibiotics during transplant surgery, the occurrence of systemic mycotic infections has become a significant concern.\(^{(161)}\) In fact, fungal infection has become a major cause of death among cancer patients and transplant recipients, and the frequency of fungal infection in patients with cancers of the blood more than doubled in the 1980's.\(^{(26)}\) AIDS, in particular has presented a challenge to medical science, life-threatening fungal infections that are extremely rare in all other categories of illness are prevalent among AIDS patients.\(^{(19)}\) The agricultural industry, as well, desires to find anti-fungal pesticides that are less susceptible to inducing drug-resistant strains and have lower human toxicity. Many universities, hospitals and pharmaceutical companies have invested considerable time and money in the effort to discover improved anti-fungal drugs and treatments. Pharmaceutical companies such as Du Pont de Nemours, Smith Kline & French, Shionogi, Sumitomo and Schering Plough, in particular have been at the forefront of basic research on anti-fungal therapies.\(^{(40, 47, 52, 80, 102, 124, 130, 167)}\)
The anti-fungal drugs that have shown the most selectivity have been found, almost exclusively, to affect the process of steroid biosynthesis or function. Of the fungicidal inhibitors acting on steroidogenic enzymes, a considerable number target the 14α-demethylase. Why should this system prove to be the fungal Achilles' heel when mammalian cells also have steroids as well as their own 14α-demethylases? As mentioned above, the sterol biosynthesis pathways in yeast and the higher eukaryotes diverge subsequent to lanosterol demethylation (and in filamentous fungi immediately after lanosterol cyclization from oxidosqualene) to yield ergosterol and cholesterol. Thus, one possible reason is that as omnivorous creatures we do not rely solely on our biosynthetic enzymes to provide a supply of cholesterol for steroid biosynthesis; we consume a considerable amount in our diet. Fortunately, the intermediates of cholesterol biosynthesis between lanosterol and cholesterol do not seem to be necessary for health. Fungi living in the environment of the human body, however, are not supplied with ergosterol or any other metabolic precursor to it, and cannot use cholesterol for growth.

Why this dichotomy between the steroid requirement of fungi and higher eukaryotes exists is probably intimately related to the basic evolutionary schism that separated the kingdom Animalia from the fungal world, namely multicellularity and differentiation. The functions of steroids in the higher eukaryotes are two-fold. They function as hormones, carrying information between cells, tissues and organs. In general, only those steroids beyond cholesterol in the biosynthetic pathway function in this mode. The second function is the modification and maintenance of the physical properties of the cell's phospholipid bilayer membranes. This function is shared by all eukaryotes although ergosterol fulfills this role in fungi and cholesterol in animals. The precise three-dimensional shape of the membrane steroid molecules have been selected for complementarity with the shape of the membranes' constituent phospholipids. Over the course of evolution these molecules have varied together resulting in membranes with physical properties appropriate to their biochemical niche - the particular external environmental conditions in which they find themselves. The surfaces of animal cells, in contrast, do not have to come in contact with the outside environment and therefore have evolved different physical properties than fungal cells.

Whatever the reason, interference with the fungal steroid biosynthetic pathways is one of the few handles that we have on the problem of systemic fungal infection, and the drugs presently available are clearly inadequate. The definition, on the molecular level, of the origin of anti-fungal drug binding specificity and species selectivity is fundamental to the design of improved drugs and treatments. For this reason, laboratories in academia and
industry have increasingly focused on illuminating the structural and mechanistic properties of the fungal steroid biosynthetic machinery such as the lanosterol 14α-demethylases.

**Anti-fungal drugs and the Inhibition of 14α-Demethylase**

The problem of side effects in enzyme-targeted pharmacology can be divided into three regimes. First, an administered drug may have systemic effects unrelated to its target enzyme; gastro-intestinal imbalance or liver and kidney toxicity, for instance, may result from many different types of chemicals. Second, the drug designed to mimic a particular substrate of a particular enzyme may effect other enzymes that act on similar substrates, or closely related enzymes that act on different substrates. The more effectively a drug binds to a particular protein target, however, the less likely there will be interferences of this type. Third, there may be specific overlap of the target enzyme in the infecting organism with an analogous enzyme in the host organism. All three effects are relevant to the design and development of anti-fungal drugs. Clearly, as mentioned above, there is overlap between the 14α-demethylase of fungi with that of plants and mammals, however, much of the problem with side effects in anti-fungal drugs may result from these drugs not discriminating between the numerous other P-450's present in mammalian tissues. For instance, it is known that many anti-fungal drugs have broad spectrum effects on P-450 enzymes (see below).

The clinical inhibition of an enzymatic activity requires a tight binding inhibitor (that is, one with strong complementarity of shape and electronic structure with the active site of the enzyme). The stronger the binding of the inhibitor relative to the natural substrate of the enzyme the lower the dosage of inhibitor will be required to completely inhibit the enzymatic activity. Low dosage is an important factor in a potential drug because this will lessen the severity of any side-effects. Clearly the optimal case is where the inhibitor binds considerably more tightly than the natural substrate. Is this physically possible? It is, and the logic behind this conclusion is developed in the following paragraphs.

In receptors and binding proteins, full advantage can be taken of the binding interaction between a protein and its target. Consider, for example, the exquisitely strong binding interactions of avidin for biotin or immunoglobins for their antigens. Apart from the intrinsic dissociation rate, release of bound molecules in receptor proteins can be initiated by an external signal causing a conformational change in the protein, if necessary. In an enzyme, generally, the only specific signal to release the product is the relative difference in shape between the product and the starting material or between the chemical states of cofactors, which are often not dramatic (enzymes often bind their substrates in
conformations approximating the transition state of the chemical process\(^{(23)}\). Thus, enzymes cannot bind their substrates as tightly as might be physically possible, to do so would lower the off-rate of the product and slow down the overall rate of catalysis.\(^{(85)*}\)

Using the example of steroid-metabolizing enzymes, the interactions between substrate and active site are believed to be mediated by a small number of discrete hydrogen bonds and a much larger number of non-polar contacts constituting the hydrophobic effect. For "perfect" binding (maximal energy release on binding) the active site would trace every curve of the non-polar regions of the substrate with close contacts excluding the maximal number of water molecules from the hydrophobic binding pocket, and all hydrogen bonds would be at optimal distances and with optimal angular orientations. Conversely, in order to avoid having too strong a binding constant, there must be gaps between the protein and substrate in places, clashes causing strain in others and hydrogen bonds having less than optimal parameters. The consequence of this state of affairs is that it should always be possible, in principle, to find or design a molecule which will bind to an enzyme active site considerably more tightly than the natural substrate.

The possibility for differentially inhibiting an enzyme like 14α-demethylase in various fungi versus humans can be explained in part by divergent evolution. Evolution will select enzyme primary sequences which fold to form an active site which is optimal for that particular biochemical niche; the binding of the substrate will be as strong as possible to give a high selectivity balanced against an off-rate sufficient to produce enough product for the organism's needs. As other biochemical pathways have changed throughout the divergence of fungi and mammals, so will have this optimal value. Furthermore, evolution will select sequences that yield optimal values as conditions change, but there will be no selective pressure on the precise identity of the sequences that yield this value. Thus, in enzymes separated by as much divergence time as the fungal and mammalian 14α-demethylases, even though the mechanism of the enzyme may have been exquisitely conserved and many catalytically important residues remain unchanged as well, the precise nature of the binding interactions can be expected to vary considerably. Where in one homolog, for example, there may be close contacts all around the 10β-methyl, strain-causing interactions with 13β-methyl and extra room around the 4,4-dimethyl group, in another homolog these interactions may be quite different: as long as the binding constant is appropriate, the details are unimportant as far as evolution is concerned. Following from

* It might be inferred from this argument that the slower an enzyme needs to go, as defined by its biochemical niche, the tighter it will bind its substrates in order to gain in specificity. This would be expected to be especially applicable to enzymes like 14α-demethylase which bind discrete intermediate states non-covalently and are relatively sluggish.
this argument, the inhibitor which binds most tightly to a particular species' enzyme may not bind nearly so well to that of a distantly related species.*

The fact that presently available anti-fungal drugs acting as 14α-demethylase inhibitors are somewhat selective in their toxicity even though both human and fungal cells utilize this enzyme suggests that there may be considerable heterogeneity in the three-dimensional shape of the substrate-binding cavities. Detailed comparisons between the binding constants of these inhibitors for purified fungal and mammalian 14α-demethylases have not yet been carried out in a systematic way. Almost exclusively these compounds, discovered by screening methods, contain aromatic nitrogenous ligands (generally, pyridines, diazoles and triazoles) meta-substituted with lipophilic groups of varying structure. The nitrogen atoms of these molecules are believed to interact directly with the heme iron resulting in a type II spectral binding shift. These compounds are known as DeMethylation Inhibiting fungicides or DMI; examples include ketoconazole, miconazole, itraconazole, buthiobate, fenarimol, and prochloraz (Figure 3). It is believed that these inhibitors cause an accumulation of C-14-methylsterols in the cell membrane and, because of the protruding axial methyl group (see side-on view of lanosterol, Scheme 2) the cell membrane integrity is affected. Recent advances in the development of anti-fungal drugs have been extensively reviewed.

* This brings up the possibility, of course, that continued drug warfare between species will cause convergent evolution of the target enzymes with respect to their detailed active site shapes through the growth of drug-resistant strains. Is it possible that this has already happened in mammalian fungal parasites of archaic lineage through biological drug warfare? The discovery of unexpectedly large conservation of detailed active site shape would imply a co-evolution mediated by natural anti-fungal enzyme inhibitors.
Figure 3: Some Demethylation Inhibiting Fungicides (DMI)
The chemically homogeneous nature of the DMI suggests that at least part of the selectivity of these drugs may be due to secondary effects such as differential concentration in fungal cells relative to mammalian cells or processing of these drugs that mitigate their effect once they reach the mammalian cell. However, no chemical or physical basis for such secondary effects have been shown. On the other hand, drug screening and development is by no means a random process and most work has clearly focused on the improvement of presently available DMI compounds; this may in part explain the restricted nature of the chemical groupings in DMIs. Enzymatic studies show that these drugs have considerable cross-reactivity with P-450 enzymes in general, a fact which may account for many of the observed side-effects.

In contrast to the classical technique of drug discovery, there has recently been an increasing focus on logical drug design based on enzyme active sites target. This technique holds promise in being able to produce inhibitors that are tailored to particular active sites thus minimizing side effects through non-specific inhibition. As noted above, the effectiveness of the DMI fungicides makes 14α-demethylase an attractive target for this "engineered" approach. Unfortunately, 14α-demethylases are membrane bound proteins and have resisted attempts to acquire X-ray crystal structures. The only available crystal structure of a P-450 enzyme is that of camphor hydroxylase which, as a bacterial enzyme acting on a very different substrate, cannot be expected to yield much information of a specific nature about the binding properties of lanosterol to 14α-demethylase by comparison.

Thus, researchers must resort to deduction of the structural features of the active site indirectly, through comparative binding studies. Analysis of the numerous N-heterocycle inhibitors (DMIs) has led to little structural information due to the very wide variety of lipophilic groups that define the set of effective fungicides. This effort is complicated by the fact that effectiveness against one fungal species or another is a complex phenomenon dependent not only on enzymatic binding but also on secondary factors such as permeability and processing; these effects have not generally been adequately controlled in published studies. Furthermore, considering the possibility of multiple potential conformations and binding orientations in many of the drugs it is difficult to model the shape of the bound inhibitors. Several studies have attempted to quantify structural parameters of these non-steroidal inhibitors and other, more simplified, model compounds using purified or partially purified enzyme in spectroscopic studies or competitive turnover assays. Although several generalizations about the active site of 14α-demethylases have been deduced from this work, specifics are in short supply. Some useful information has come from studies of inhibitors and probes structurally related to the
natural substrate, lanosterol. It has been found, for instance, that the 3β-hydroxyl group in
the A-ring is essential for orientation and processing of the substrate, presumably due to
specific hydrogen bonding interactions, that removal of more than two of the side chain
carbons results in significant loss of activity, and that the enzyme will metabolize
substrates lacking the 4,4-dimethyl group.

The direct approach of placing heme-ligating groups into the 14α-methyl position
of lanosterol has led to some notably strong binding constants (Table 1). For comparison, lanosterol has a Km of 165 μM and a Kd of 6.9 μM. However, the variety of potential anti-fungals accessible by this method is limited, reducing the possibility of finding candidates that posse all of the features of useful drugs such as solubility, permeability, oral activity and, importantly, low cost of synthesis. Bossard has recently reported that the compound, 32-ethynyllanosterol, like the acetylene-containing compounds discussed in Chapter 3 in regard to P450scc, is a time-dependent mechanism-based inactivator of purified rat liver 14α-demethylase (Table 1, final entry). Similarly, the 15α-hydroxy-32-aldehyde derivative of lanosterol has been found to inactivate the enzyme. The placement of the double bond at the B-C ring junction is critical for enzyme binding and turnover, Δ7 compounds are metabolized considerably slower and have poorer binding characteristics than the natural Δ8 substrate, Δ6 steroids are not turned over by the enzyme at all.

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<tr>
<td>Inhibitors based on modification of Lanosterol</td>
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</table>

<table>
<thead>
<tr>
<th>R</th>
<th>Inhibition</th>
<th>Versus</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Structure" /></td>
<td>IC50 = 30 nM</td>
<td>various dermatophyte fungal species</td>
<td>(41)</td>
</tr>
<tr>
<td><img src="image" alt="Structure" /></td>
<td>Ki = 0.61 μM</td>
<td>Rat liver microsomes</td>
<td>(143)</td>
</tr>
<tr>
<td><img src="image" alt="Structure" /> (also Δ15)</td>
<td>IC50 = 2 nM</td>
<td>Human hepatoma cell line</td>
<td>(96)</td>
</tr>
<tr>
<td><img src="image" alt="Structure" /></td>
<td>Ki=1.2 μM, K\text{inact}=0.15 \text{ min}^{-1}</td>
<td>Purified enzyme from rat liver</td>
<td>(27)</td>
</tr>
</tbody>
</table>
Recent work by Wright and Honek has combined the use of small imidazole-containing molecules to bind to the heme iron with the technique of photoaffinity labeling. Attaching aromatic azides to the imidazole ligand with short linkers of varying lengths they have found a molecule which, when irradiated at 313 nm, covalently labels the active site of the 14α-demethylase from *Saccharomyces cerevisiae*.\(^{160}\) In future work the active site residue(s) to which the probe is linked will be discovered, and very likely, new photoaffinity probes will be found that attack different active site residues. Since these probes have only one free imidazole nitrogen with which to bind the heme iron, modeling of the interaction may, in a fairly straightforward manner, yield distance constraints between these residues and the heme iron.

Since the primary structures of these P-450's are well known, the potential exists for computer modeling of their three-dimensional structure. In fact the first attempts at this process have begun based on secondary structure prediction, hydropathy and alignment with the P-450\(_{cam}\) structure,\(^{107}\) much in the same vein as those performed by S. Joardar on P-450\(_{scc}\) in our laboratories (see introduction to Chapter 3 of this thesis).\(^{73}\) In combination with distance constraints acquired from the photoaffinity labeling experiments mentioned above and other techniques, a much clearer picture of the active site structures of these and other P-450's may be forthcoming in the near future.

Effective anti-fungal drugs of the future may very well be chemical hybrids havingazole-based heme-binding groups, steroid-mimicking regions fine-tuned for exploitation of inter-species enzyme heterogeneity and pendant groups that do not interact with the enzyme but target the drug for differential entry into fungal cells.

**Proposed Mechanism: An Historical Overview**

The delineation of the enzymatic mechanism of lanosterol 14α-demethylation has been an active research area for over 35 years. The literature in this field has been complicated by false starts and misleading information and made difficult by technical complexities. Looked at in historical perspective, however, this research (along with that on the enzymes aromatase and the side-chain cleavage enzyme) represents a monumental effort to define the properties of a powerful, complex and highly specific oxidative machine. The last five years have been extremely fertile in this respect, culminating in the isolation and convincing structure determination of the final oxidative intermediate preceding demethylation.\(^{52}\) An overview of the developments over the last three decades is presented below.
Figure 4: $^{14}$C-labelling of Lanosterol and Cholesterol from acetate
By 1957 Cornforth and Popjak had completely determined the pattern of $^{14}$C-incorporation into lanosterol and cholesterol by methyl- and carboxylate-carbon labeled $^{14}$C-acetate (Figure 4).\(^{(41-43)}\) Using this information Olson and co-workers showed that $^{14}$C-labeled lanosterol is converted to cholesterol in rat liver homogenates with the release of at least three equivalents of radioactivity as $^{14}$CO$_2$, corresponding to the $4\alpha$, $4\beta$ and $14\alpha$-methyl groups of lanosterol. No $^{14}$C-formaldehyde was detected, even though consideration was given to enzymatic conversion of formaldehyde to CO$_2$, perhaps because the trapping reagent used inhibited the enzyme. Decarboxylation is direct: amino acids are not intermediate 1-carbon carriers.\(^{(112)}\) Years later it was shown that the $14\alpha$-methyl is, in fact, released as formate, as will be discussed below.

Gautschi and Bloch showed that $\Delta^{8,24}$-4,4-dimethylcholestadienol is a $14$-demethylated intermediate by chromatographic isolation and synthesis of an authentic standard.\(^{(56)}\) Chromatographic techniques alone could not differentiate between the double bond isomers. Oxidation by osmium tetroxide and lead tetraacetate, however,\(^{(34)}\) yielded a ten-membered ring diketone matching that produced by oxidation of the synthesized $\Delta^{8}$-compound (Scheme 3).\(^{(57)}\)

This isolated intermediate seemed to be one step removed from the actual decarboxylation. Two proposals were considered for this elimination (Figure 5): a) double bond migration to the $8(14)$ position and b) oxidation of the 15-position to yield an

**Scheme 3:** Degradation of some isomeric demethyllanosterols
Figure 5: Early Mechanistic Proposals for Lanosterol 14-Demethylation
8,14-diene. Several groups showed that one of the hydrogen atoms at C-15 is removed in the biosynthesis of cholesterol from [2RS-3H2]-mevalonic acid and the Δ8,14 steroid is converted to cholesterol. It was later shown that the proton lost at the 15 position is the 15α-proton, and that no protons are lost from the adjacent 16-position. This information supports the C-15 oxidation mechanism for demethylation. However, a two-step isomerization of the 8(14)-olefin to the 8(9)-olefin through the intermediate 8,14-diene remained a possibility (Figure 5, pathway b'). Considerable uncertainty existed on this point for several years, but the issue was settled by preparation of the putative Δ8(14)-lanosterol and demonstration that it is not an efficient precursor to cholesterol when incubated with rat liver homogenates, and that while Δ8-lanosterol yields a Δ8,14 intermediate, the Δ7 isomer yields a Δ7,14-diene intermediate. The nature of the oxidation at carbon-15 was alternately considered as a hydroxylation, hydrogen atom abstraction and a hydride elimination.

In 1972 research by Alexander and co-workers showed that the original work on the oxidation state of the released 14α-methyl carbon atom was in error, and the carbon is in fact released as formic acid, not carbon dioxide. At around the same time it was discovered that the process of lanosterol demethylation required oxygen, NADPH and was inhibited by carbon monoxide (in both rat liver and yeast) and was thus very likely mediated by P-450 enzymes. These discoveries precipitated a reevaluation of the previously proposed mechanisms, and the possibilities considered are summarized in Figure 6.

Mechanism 6a involves base catalyzed β-elimination after hydroxylation at position 15. The observed loss of the 15α-proton strongly suggested that such an alcohol must also be in the alpha orientation since all steroid hydroxylations investigated at that time (and since) had been shown to involve retention of configuration, and therefore the reaction must involve a syn elimination. Mechanism 6b hypothesizes an enzymatic Baeyer-Villiger oxidation, insertion of an atom of oxygen between a carbonyl carbon and an aliphatic carbon. In synthetic chemistry this rearrangement is preceded by the attack of a peroxide anion on the carbonyl carbon; the enzymatic reaction would presumably be similar. However, in organic chemistry it is observed that the hydrogen atom of aldehydes is much more likely to migrate than the alkyl group, resulting in the formation of a carboxylate, not a formate ester. Thus there must be some distinct mechanistic differences between the enzymatic and chemical reactions. Syn elimination of the resulting formate ester could be catalyzed by an enzymatic base. Non-enzymatic elimination of this ester would be expected to result in the energetically more favorable anti elimination. Mechanism c is similar to b in proposing a peroxy-intermediate. As shown in the figure, elimination could be achieved
Figure 6: Proposed Mechanisms for P-450 14α-Demethylase based on Elimination of Formate
by a radical mechanism, but also could be considered to arise from a heterolytic or even electrocyclic mechanisms.\textsuperscript{(1)} Mechanisms analogous to both 6b and 6c had been proposed in relation to C-19 demethylation in the biosynthesis of estrogen.\textsuperscript{(3)}

By 1978 it had been shown that all three oxidative steps require oxygen and NADPH. The fact that the oxidation of the 32-alcohol to the aldehyde was apparently not catalyzed by the common alcohol dehydrogenase-type mechanism suggested that the entire demethylation may be catalyzed by a single enzyme.\textsuperscript{(1)} Some reports, however, suggested that more than one oxidase might be responsible for the several oxidative steps.\textsuperscript{(63)} The one-enzyme hypothesis was later confirmed when 14α-demethylase was purified to homogeneity,\textsuperscript{(12, 144, 163)} as well as through the discovery of demethylase-deficient strains of yeast and Chinese hamster ovary cells.\textsuperscript{(11, 144)}

A series of elegant experiments, initially using intact rat liver microsomes and later purified lanosterol 14α-demethylase, showed that several oxidized intermediates could be accumulated in solution at the expense of demethylated products. Conditions for this mechanistic interruption included limiting the supply of NADPH, low enzyme and high substrate concentrations, competitive inhibition by non-steroidal inhibitors, short incubation times and high pH. It was also found that cholesterol treatment of intact microsomes caused intermediates to accumulate. These intermediates were conclusively demonstrated to be the 32-aldehyde and 32-alcohol compounds predicted by the proposed mechanisms. These experiments suggest that oxidized demethylation intermediates are freely diffusible from the enzyme active site but, under normal turnover conditions, are not released because of tight binding and the speed of subsequent chemical steps.\textsuperscript{(129, 144-146)} Similar results were observed with the enzyme isolated from \textit{S. cerevisiae}.\textsuperscript{(14, 17)}

Synthesis of these intermediates and incubation of them with the purified enzyme systems provided the relevant kinetic constants (Table 2, adapted from\textsuperscript{(51)}). The measured maximal rates for dihydrolanosterol and the corresponding 32-alcohol and 32-aldehyde clearly indicate kinetic competence ($V_{\text{max}} = 0.64$, 7.00 and 20.6 nmol min$^{-1}$ mg$^{-1}$, respectively).

The $K_{\text{m}}$ of 32-hydroxylanosterol is notably lower than that of lanosterol and is clearly part of the enzymatic strategy for avoiding premature release of the intermediates.\textsuperscript{(14, 51)} It should be noted also that release of the product after demethylation is surely promoted by the planarization of carbons 14 and 15, which can be expected to cause a distinct change in the conformation of the D-ring and thus, the disposition of the C-18 methyl and the side-chain with respect to the rest of the steroid nucleus. Molecular modeling of the substrate and product of lanosterol 14α-demethylase indicate a distinct
curvature of the molecule arises from demethylation (this effect is well represented by the distance between carbons 18 and 19 which increases from 4.16Å to 4.78Å, see Scheme 4, a and b)*

The nature of the third and final oxidative intermediate prior to demethylation remained enigmatic for many years. Although mechanism a (Figure 6) initially seemed the most parsimonious, it was shown that, of the two possible synthetic 15-hydroxylanost-7-enols, only the $^{15}\beta$ diastereomer could be converted to cholesterol by rat liver homogenate. Thus, either the $^{15}\beta$-alcohol is only fortuitously converted to cholesterol and is not a significant intermediate in its biosynthesis, or the activation at C-15 represented a novel hydroxylation mechanism involving inversion of stereochemistry.\(^{(101, 136, 137)}\)

Considering what is known about the mechanism of P-450-mediated hydroxylations, this latter possibility seems remote.\(^{(66)}\) Significantly later it was shown that, while both of the 15-hydroxylanosterols are, in fact, inhibitors of the demethylase, the $^{15}\alpha$-alcohol is extremely potent in this respect. This evidence suggests that one of the other two mechanisms is operational;\(^{(106)}\) however, the inference was somewhat tentative because synthesis of the 15-hydroxy-32-aldehydes had remained elusive.

Recently, considerable light has been shed on this process in an outstanding report by Fischer, Trzaskos and co-workers at DuPont.\(^{(52)}\) Extending their initial work on the accumulation of oxidized intermediates, it was found that by incubating the 32-aldehyde with purified enzyme and using a slight modification of their product extraction procedure, a third intermediate could be isolated. Infrared, $^1$H-NMR, EI and FAB mass spectra of the intermediate are consistent with the 14$\alpha$-formyUlanosterol structure predicted by Bayer-

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**TABLE 2**

Kinetic constants for several 14$\alpha$-demethylase substrates

<table>
<thead>
<tr>
<th>Compound</th>
<th>$K_m ($µM)</th>
<th>$V_{max}$</th>
<th>$V_{max}/K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lanosterol</td>
<td>165</td>
<td>3.39</td>
<td>20.5</td>
</tr>
<tr>
<td>Lanost-8-en-3$\beta$-ol</td>
<td>312</td>
<td>0.64</td>
<td>2.07</td>
</tr>
<tr>
<td>Lanost-7-en-3$\beta$-ol</td>
<td>338</td>
<td>0.09</td>
<td>0.26</td>
</tr>
<tr>
<td>Lanost-8-en-3$\beta$,32-diol</td>
<td>56</td>
<td>7.00</td>
<td>125</td>
</tr>
<tr>
<td>3$\beta$-hydroxylanost-8-en-32-al</td>
<td>368</td>
<td>20.6</td>
<td>56</td>
</tr>
</tbody>
</table>

---

\* Minimizations were performed using MM2 on Chem3D Plus from Cambridge Scientific Computing
Scheme 4a: An MM2 minimized space-filling model of Lanosterol. The 14α-methyl protrudes from the bottom face of the molecule at the center of the drawing.

Scheme 4b: An MM2 minimized space-filling model of 4,4-dimethylcholesta-8,24-dienol. Note the relative curvature of the steroid nucleus (the side-chains are represented with equivalent conformations for comparison).

Villager rearrangement (Scheme 6, mechanism b). Previously the steroid fraction had been saponified during the extraction procedure, precluding the isolation of this ester. Re-incubation of the HPLC purified intermediate with enzyme confirm that it is a kinetically competent precursor to the 8,14 diene. Reducing equivalents and oxygen are not necessary for this final elimination. In addition, the putative intermediate of mechanism a, 15α-hydroxy-32-aldehyde, was successfully synthesized and shown not only to not be a competent intermediate but also to be a time-dependent inactivator of the enzyme. Another intriguing observation was that the final elimination of the formate ester, though efficiently catalyzed by the enzyme, was not inhibited by the competitive P-450 inhibitor ketoconazole which binds to the heme iron or by the 15α-hydroxy-32-aldehyde. This suggests that a
second site on the enzyme may be responsible for this transformation. A similar suggestion has been made with respect to the final step in the mechanism of aromatase.\(^{(105)}\)

The mechanism of the final oxidative transformation as it is now understood is summarized in Figure 7. Although the Baeyer-Villiger rearrangement is not a general mechanism in P-450 lyases (it has been ruled out as a mechanism in aromatase \(^{(162)}\)), the attack of a peroxide equivalent on carbonyls seems to represent a fundamental mechanistic mode parallel to the radical rebound mechanism for hydroxylation and oxygen atom insertion into multiple bonds (see discussion in Chapter 1). Aside from 14α-demethylase, peroxide attack has been implicated in the mechanisms of aromatase, 17,20-lyase, and the metabolism of xenobiotic aldehydes by P-450 \(LM_2\),\(^{(39, 96, 150)}\) and may be involved in the final lytic step of P-450\(_{scC}\).\(^{(90)}\)

**Assays of Lanosterol 14α-demethylase**

The simplest way to measure the affinity of a substrate or inhibitor for a P-450 enzyme is to monitor the spectroscopic changes that occur upon binding of the molecule. This method is very effective for inhibitors that yield a type II binding spectrum, that is, those like the azole antifungal drugs, which coordinate to the heme iron through a heteroatom.\(^{(122)}\) Type I spectral changes occur when the binding of a substrate or inhibitor causes a change in the heme iron spin state from low-spin to high-spin resulting in a blue-shift of the Soret maximum.\(^{(156)}\) In difference spectra this appears as an absorption minimum near 420 nm and a maximum around 388-390 nm with an isosbestic point at 407 nm.\(^{(110)}\) This effect is generally less pronounced than the type II change. In the case of lanosterol 14α-demethylase the effect is small, 1 \(\mu M\) enzyme results in only an ~0.036 AU maximal change (in the difference spectrum) even though the dissociation constant of lanosterol is 6.9 \(\mu M\).\(^{(9, 17, 163)}\) In addition, competition experiments based on these spectral changes can give misleading non-linear results and artifacts if full consideration is not given to the detailed binding kinetics of the system.\(^{(109)}\)

The first assay based on the turnover of lanosterol to demethylated products was based on the mechanistic studies of Alexander and Akhtar in 1972 in which \([32^{-3}H]-24,25\)-dihydrolanost-7-ene-32,3β-diol was used as a substrate for the enzyme and radioactive water and formic acid were identified as products.\(^{(6)}\) The radioactive products in these assays were quantitated by freeze-drying the acidified incubation solutions and collecting the volatiles with a liquid nitrogen trap or by bulb-to-bulb distillation.\(^{(1, 104)}\) If desired, the formic acid could then be quantitated independent of the water by forming the non-volatile formate salt under alkaline conditions and distilling again. Considering the complicated
Figure 7: Proposed Mechanism for Final Oxidative Step of Demethylation
synthesis involved and the time required to perform this assay, subsequent researchers found it quite cumbersome and soon turned to other methods.

Aoyama and Yoshida developed an assay in 1978 using gas chromatography. After addition of an internal standard steroid, the enzymatic incubation is saponified and (non-radiolabeled) steroids are extracted with 9:1 petroleum ether-diethyl ether or methylene chloride. The solvents are dried over sodium sulfate and removed by evaporation, after which the residue dissolved in and trimethylsilylated with a small amount of N-trimethylsilyl imidazole and then chromatographed. Quantitation is achieved by integration of the resulting peaks. The accuracy of this method suffers somewhat from high and variable backgrounds due to irrelevant impurities in the protein, cofactor and buffer preparations.

In 1984 researchers at Du Pont developed an improved assay using the newly developed HPLC/liquid scintillation flow-through detector which has found considerable use. By using radio-detection, problems of baseline interference are eliminated because only metabolites of the substrate are detected. General labeling of dihydrolanosterol is achieved by catalytic tritiation of a crude mixture of lanosterol and dihydrolanosterol or by sodium borotritide reduction of dihydrolanosterone. Although the extraction procedures must still be done, no trimethylsilylation is necessary. However, one must acquire a radio-flow type detector to take advantage of this assay.

In both of these types of chromatographic assay, if the demethylase preparation used is not sufficiently pure, contaminating enzymatic activities may complicate the results due to further processing of the demethylated product. For instance, in order to assay demethylase activity in microsomal preparations, reduction of the demethylase product Δ^8,14 diene by a specific 14-reductase occurs rapidly with saturating amounts of NADPH and cyanide must be added to inhibit the action of the 4-demethylase enzymes. Although little time is invested in the preparation of substrates for these assays, the throughput of the assay is limited by the time it takes to perform the extractions and chromatography steps. Estimating from procedures described in the above references it seems that no more than 10-15 determinations can be made per chromatograph per day; thus, these assays are very labor intensive. Although quite appropriate for the analysis of metabolic intermediates or the products resulting from alternative substrates or inhibitors (especially in combination with mass spectroscopy), these methods are not well suited to routine assays, the screening of inhibitors, variations in multiple parameters for kinetic studies or other uses requiring many data point determinations.
In a return to the earliest assay procedures, Bossard and co-workers at Smith Kline & French Laboratories have updated the tritiated formate/water quantitation assay creating a procedure that is both rapid and accurate.\(^{(28)}\) The technique is straightforward. Enzyme incubations with 32-[\(^3\)H]-dihydrolanosterol are quenched with 1% Emulgen 913 (a detergent), 40% trichloroacetic acid (to precipitate the proteins) and 0.25% formate as a cold carrier. After centrifugation the supernatant is passed through a Sep-Pac C\(_{18}\) cartridge (Waters Associates) with two water washes and is counted by scintillation. Samples can be processed in large batches each taking only a few minutes. The Sep-Pac cartridges are regenerated by washing with methanol and re-equilibration with water. This technique eliminates both baseline noise and interference due to further processing of the demethylated steroids. Assuming that 14\(\alpha\)-demethylase is the only enzyme acting on the 14\(\alpha\)-methyl and there is no enzymatic reincorporation of the released formate into a non-polar phase, then this assay should be just as accurate in a crude system as it is in measuring the activity of purified enzyme. For high-throughput applications this assay is several hundred times faster than previous methods. The publication of this procedure has placed a new importance on research towards the efficient production of 32-labeled lanosterol substrates, as the time and effort required to produce this material is the only significant impediment to its general utility.

**Research goals**

In order to investigate 14\(\alpha\)-demethylase the most basic requirement is the ability to measure its presence. The choice or design of an assay is often crucial to the success of future experimentation. It is desirable to use an assay which is easy, sensitive, accurate and quick (determinations per hour) to perform, even if this requires a long initial period of work, since this laboratory is committed to an extended project in this direction. As summarized above, the major difference in assays in the case of 14\(\alpha\)-demethylase is whether the substrate for the assay requires a difficult multi-step synthesis (i.e. specific radio-labeling), a brief, simple synthesis (i.e. general radio-labeling), or even no synthesis at all. The time required to perform a single determination of each of these assays seems to be inversely proportional to the difficulty in preparing the substrate. The publication of the Bossard tritium-release assay\(^{(28)}\) just prior to the commencement of this project was fortuitous, and the commitment was made to use this method along with its synthetic challenges.
The choice of synthetic strategy to use in the preparation of the assay substrate, however, was also based on several longer-term research goals. Since the synthesis of various potential inhibitors and probe molecules were contemplated, the techniques to be initially developed needed to afford the most versatile suite of intermediates possible so as to limit the time spent in the preparation of these other targets. Some of these considerations are discussed below.

As has been accomplished in our laboratory in the past for the side-chain cleavage protein, we intend to define the precise relationship between bound substrate and inhibitor molecules and the heme plane using ESEEM and EXAFS studies.\(^{(74)}\) Using ESEEM, the distances between the heme iron and the deuterium atoms of specifically deuterated steroids can be determined.\(^{(70)}\) It is desirable to find synthetic procedures which activate lanosterol at several of the positions on the \(\alpha\)-face of the steroid which are strategically located around the point of action of the demethylase such that the required deuterium atoms can be introduced (Figure 8). Since the enzyme sequentially converts lanosterol into 32-hydroxy-, 32-aldehyde- (or 32,32-dihydroxy-) and 14-formyllanosterol intermediates, in order to assess changes in the binding of these oxidized intermediates relative to the substrate it is also desirable to use synthetic procedures that provide easy access to some or all of these intermediates in regiospecifically deuterated forms.

Nearly all assays published to this point have used 24,25-dihydrolanosterol as the assay substrate even though this is not the natural substrate for either the mammalian or

\[
\text{Figure 8: View of the } \alpha\text{-face of Lanosterol showing the protons surrounding the } 14\alpha\text{-methyl}
\]
fungal proteins. Dihydrolanosterol has a five-fold lower \( V_{\text{max}} \) compared to lanosterol and a two-fold higher \( K_m \) (i.e. a ten-fold lower specificity, \( V_{\text{max}}/K_m \)) for the rat liver enzyme (see Table 2).\(^{(51, 146)}\) This choice, which is made to ease the synthetic burden, does not significantly impair the assay's utility in terms of relative measurements. The synthesis of authentic, labeled lanosterol was desirable for several reasons. First, although the synthetic procedures would surely require modification, it seemed reasonable that the side-chain double-bond could be preserved without undue added complexity, yielding an assay with greater sensitivity. Second it would be beneficial to develop the synthetic techniques necessary to control that functionality so that it could be used in the design of inhibitors and probes. Furthermore, although in yeast species and mammals the substrate of the demethylase is lanosterol, in filamentous fungi 24-methylene-lanosterol is the natural substrate. It has been shown that, even in yeast, the enzyme has a preference for 24-methylene-lanosterol compared to the enzyme from rat liver.\(^{(10, 15, 16)}\) The development of the techniques for the preservation of the side-chain double bond through the synthesis of specifically labeled lanosterol allows for the study of 24-methylene-lanosterol derivatives in the future. Assays using the authentic substrates in each of these systems may aid in the development of anti-fungal inhibitors that discriminate between the various enzymes by the exploitation of this difference in side-chain preference.\(^{(103)}\)

The synthetic work described here represents the initial phase of a larger project in this laboratory aimed at creating selective, effective 14α-demethylase inhibitors and understanding the molecular basis of P-450 chemistry and selectivity. A number of proposals for future research in this laboratory based on the work presented here are given at the end of this chapter.

**Previous syntheses of Lanosterol that are Amenable to Specific Labeling at the C-32 Position**

**Synthesis of Dihydrolanosterol from Cholesterol**

The synthesis of lanostenol (dihydrolanosterol) from cholesterol in 1957 by the research labs of Woodward and Barton\(^{(159)}\) provides the basis for the preparation of all specifically-labeled dihydrolanosterol for the assay of lanosterol 14α-demethylase found in the literature.\(^{(1, 27, 28, 104)}\) Although several of the steps of the synthesis have been supplanted by more efficient methods,\(^{(84, 115)}\) the overall strategy remains remarkably unchanged. In brief, the strategy, requiring ten steps, is as follows: the cholesterol alcohol
Figure 9: Synthesis of dihydrolanosterol from cholesterol, original method by Barton with modern modification by Parish. (a) (see Feiser, JACS, 1953, 75, p.5421); (b) t-BuOK, t-BuOH, CH$_3$I; (c) LiAlH$_4$, Et$_2$O; (d) Pyridine/Ac$_2$O; (e) N-bromosuccinamide, CC$_4$I, Δ, hv; collidine; (f) HCl, CHCl$_3$, -40°C; NH$_3$, MeOH, -60°C; (g) monoperphthalic acid, Et$_2$O; NaOH, EtOH, reflux; (h) HCl, EtOH, reflux; (i) Pyridine, benzoyl chloride; (j) TMSCl, Et$_3$N, DMF, 140°C; (k) benzyltrimethylammonium fluoride, CH$_3$I; (l) hydrazine hydrochloride, triethylene glycol; NaOH, 210°C. [Barton, 1957; Bossard, 1991; Bossard, 1989; Parish, 1977]
is converted to a conjugated ketone for the introduction of the geminal methyl groups at C-4 on the steroid A-ring; a second double bond in the B-ring is formed and the double bonds are isomerized to the $\Delta^{7,14}$ position where they are oxidized-isomerized to form another conjugated ketone which is used to introduce the 14$\alpha$-methyl; the ketone is then removed by Wolff-Kishner reduction and the double bond is isomerized to the proper $\Delta^{8}$-position (Figure 9).

In recent preparations for use in enzymatic studies, the label is introduced in a straightforward manner as $^3$H methyl iodide and the overall yield is ~2%. Several disadvantages of this synthetic strategy are evident, however. If one desires to use authentic lanosterol ($\Delta^{8,24}$), the only possible way to do so is to build up the side chain from pregnenolone or progesterone.* This modification would require additional steps to protect the shortened or completed side-chain from the various reagents used. A more pressing concern is the lack of lanosterol-like intermediates in the synthesis that could be used as starting materials for inhibitors or structural probes. Since the 14$\alpha$-methyl group is introduced at the end of the synthesis, any variations (other than the introduction of functionality at C-15 or at the 14$\alpha$-methyl position itself$^{(139)}$) are limited by the requirement of being compatible with the remainder of the synthesis. A minor drawback is the fact that the introduction of the label is not the last step; the radioactivity must be handled through two synthetic steps as well as the purification of the mixture of double bond isomers ($\Delta^{8,24}$ from $\Delta^{7,24}$).

A recently published variation on this strategy$^{(18)}$ proceeds via epoxidation of the 3-hydroxylanost-14-ene isomer (as opposed to the 7,14 diene) which can be prepared from dihydrolanosterol in ~25% yield in 9 steps$^{(41)}$. Overall, this variation is more complex (14 steps) but somewhat higher yielding at ~4.3%.

**Synthesis of 32-Hydroxylanosterol from Lanosterol**

A second strategy is based on the work of numerous researchers interested in the mechanism of 14$\alpha$-demethylase and specifically the 32-oxidized intermediates of lanosterol. Dihydro-analogs of these intermediates (32-hydroxy- and 32-oxo-lanost-8-enol) have been synthesized and appeared to be suitable precursors for the synthesis of specifically-labeled lanosterol via reduction of the functional group at C-32 with the introduction of tritium ($^3$H). The basic strategy is the oxidative activation of the allylic C-7 position of lanosterol to form a 7$\alpha$-hydroxylanostane derivative. The axial 7$\alpha$-hydroxyl is

* Cholenic acid, a starting material lacking only the terminal isopropyl carbons, is prohibitively expensive at $\$125$/gram as is 25-hydroxycholesterol at $\$1,250$/gram (Steraloids, 1992 catalog).
positioned to be in close proximity to the axial 14α-methyl (a bond formed between the hydroxyl oxygen and one of the methyl protons would form a six-membered ring, Scheme 4). From this vantage the 7α-hydroxyl is used to effect a through-space radical oxidation of the otherwise inaccessible neopentyl methyl (details of these techniques are presented below). It is required that the Δ8 double bond of lanosterol be reduced to carry out these oxidations. Otherwise, with the double bond in place, the angle defined by carbons 7, 8, and 14 expands several degrees placing the hydroxyl and methyl groups too far away from each other to effectively form a bond.\(^{(68, 131)}\)

There are several advantages to this strategy over the cholesterol-based approach. Being based on lanosterol, the possibility of retaining the natural Δ24 unsaturation in the final product requires no extra synthetic effort, although extra steps may be necessary to preserve the olefin throughout the synthesis. Almost all of the synthetic intermediates of this route may be used as bases for the construction of lanosterol-like inhibitors and structural probes. Since the 32-hydroxy group is used as a handle for the introduction of the radiolabel, this transformation can be postponed until the very last step in the synthesis, obviating the need for multiple radiochemical transformations. Although no procedures for the reduction of 32-oxidized lanostenols to lanosterol have been published, many methods exist for this functional group transformation.\(^{(98)}\) For these reasons the lanosterol-based route was chosen for this project.
**Synthetic strategy**

**Methods of Synthesizing 7α–Hydroxylanostanols**

The earliest synthesis of this lansoterol derivative was achieved via a low-yielding allylic oxidation of the tetrasubstituted Δ⁸ bond to the conjugated 7-ketone with chromic acid. This was subsequently reduced by lithium in ammonia followed by catalytic hydrogenation in low yield. This method was replaced in 1965 by one incorporating the isomerization of Δ⁸ lanosterol to the Δ⁷ isomer followed by epoxidation with meta-chloroperoxybenzoic acid and reductive ring opening with lithium metal in ethylamine. A more recent version of this route resulted in a 6.2% yield.

In 1977, Sonoda published an improved method based on the 7-keto-8-ene. Using peroxide and sulfuric acid to make the ketone, it was found that the 7α-alcohol could be produced directly via catalytic hydrogenation (overall yield, 18%). This method suffers, however, for our application because the catalytic hydrogenation will reduce the side chain double bond as well. Conversion of the double bond to the dibromide does not protect it from this reduction (see Synthetic Tactics section, below).

It was our goal, therefore, to develop a new method based on stepwise dissolving metal and hydride reductions, neither of which should affect the side-chain double bond (see Tactics section below). This endeavor has resulted in a procedure that is far superior to that reported by previous researchers; by using lithium in ammonia-toluene followed by lithium tri(t-butoxy)aluminohydride we have produced the 7α-alcohol in 65% yield from the 7-keto-8-ene (which would correspond to ~45.5% from dihydrolanosterol for comparison).

**Methods of Activating the 32-Methyl Position**

The activation of the angular methyl groups of steroids and like compounds via radical chemistry has wide application in chemistry and has been especially useful in the study of enzymes in the steroid biosynthetic pathway such as aromatase and aldosterone synthase. The classical method was pioneered by Barton and co-workers in the early 1960's. Using nitrite esters of alcohols in a 1,3-diaxial relationship with a methyl group, it was found that photolysis generated the corresponding oxime. Mechanistic studies suggest the intermediacy of alkoxy and carbon-based radicals.

This method was applied to the synthesis of 32-oxygenated lanostanol derivatives by Bentley, McGhie and Barton in 1965. The 7α-hydroxylanostane was converted to
the corresponding nitrite with nitrosyl chloride which, when photolyzed in benzene, yielded the 7α-hydroxy-32-oxime. The 7α- and oxime hydroxyl groups were eliminated via their methane sulfonates yielding the 14α-nitrilo-7-ene which can be reduced to the corresponding 32-aldehyde or alcohol.\(^{(24)}\) (Scheme 5)

\[ \text{Scheme 5: Nitrite photolysis activation of C-32} \]

This method has been used to efficiently synthesize inhibitor and probe molecules functionalized at C-32 and containing the \( \Delta^7 \) double bond.\(^{(40, 148, 149)}\) However, use of this method for the synthesis of \( \Delta^8 \) lanostenols requires extra steps to isomerize the double bond. Directing the isomerization of this double bond is somewhat of a hit-or-miss process; for instance, Cooper found that neither the 14α-nitrile or 32-alcohol would isomerize to the \( \Delta^8 \) isomer, while the 32-amine isomerized completely at pH 4.5.\(^{(40)}\) It is possible to create a 70/30 mixture of \( \Delta^8/\Delta^7 \) from dihydrolanosterol using HCl in chloroform.\(^{(100)}\) Furthermore, it is only possible to isomerize the \( \Delta^7/\Delta^8 \) system of lanosterol once the \( \Delta^24 \) olefin is protected as the dibromide.\(^{(58)}\) Separation of the resulting mixture of \( \Delta^7 \)- and \( \Delta^8 \)-lanosterol isomers requires high-performance techniques such as HPLC or gas chromatography.

A second method (published, incidentally, side-by-side with Bentley's communication in 1965) has become much more widely used due to its simplicity and higher yield.\(^{(54, 114, 135)}\) Lead tetraacetate is used in place of the nitrite to generate an alkoxy radical. After hydrogen abstraction from the nearby 14α-methyl and trapping by the lead triacetate, the hydroxyl group attacks the carbon displacing the lead and forming a tetrahydrofuran ether ring (Scheme 6).\(^{(68)}\) This ether is converted by acetyolytic cleavage with refluxing pyridine hydrochloride in acetic anhydride to a mixture of 32-hydroxylanostadienes consisting of 22\% \( \Delta^8 \) and 38\% \( \Delta^7 \). Because of the simplicity of this method it was selected for use in this work.
Very recently a method of generating lanost-7-en-32-al using the hypoiodite method\(^{(68)}\) has been reported.\(^{(133)}\) However, despite generating the aldehyde in only two steps from the 7α-alcohol, the overall yield of this transformation is low (~10%) and the conditions of the hypoiodite method are incompatible with an unprotected or dibromide-protected side-chain double bond.

### Strategies for Reduction of 32-hydroxylanosterol to Lanosterol

Although there are no published procedures for the conversion of 32-hydroxylanosterol to lanosterol many synthetic routes are known for this type of transformation.\(^{(98)}\) The factors to consider in the choice of method in this case are varied. Although residing on a primary carbon which protrudes noticeably from the steroid α-face in stick-figure models of 32-hydroxylanosterol (Scheme 2), the position is neo-pentyl and quite hindered as can be seen from space filling models (Scheme 4a and Figure 8). For this reason, \(S_N2\) substitutions are expected to be difficult. Similarly, reactions having considerable cationic character in the transition state may be slow (primary carbon) or lead to rearrangement (since it is α to a quaternary carbon and homoallylic). The method chosen needs to be compatible with the side chain double bond. Thus, strongly acidic, electrophilic or free radical reagents should be avoided. In order to introduce tritium at the 32-position for the enzymatic assay, the tritium source should be inexpensive and in as little excess as possible. Furthermore, the labeling with tritium should be specific; no other protons in the steroid can be equilibrated with the tritium source. Of course, high yield is at a premium as this reaction is at the end of rather lengthy synthesis.

Although at a neopentyl position there are some nucleophilic substitution reactions which might be expected to proceed productively. Hydride reagents, in particular, may be able to access the proper position to displace a suitable leaving group. Most often used are
sulfinate esters such as methanesulfonyl (mesyl), toluenesulfonyl (tosyl) and trifluoromethanesulfonyl (triflyl). It is possible that these reagents may be effective in polar aprotic solvents such as hexamethylphosphoramide (HMPA) or dimethylsulfoxide (DMSO). Unfortunately, sodium borotritiide, while a relatively inexpensive source of tritium, is probably too mild a reagent for this transformation, very likely requiring a large excess if it is to be successful at all. Lithium aluminum tritiide, on the other hand, is a more expensive source of tritium.

Even though radical-forming reagents may react with the side-chain double bond, the action of tri-alkyl tin hydrides on alkyl bromides and iodides have been reported to be significantly faster than the addition to and reduction of double bonds. Tritium can be supplied easily and inexpensively by reducing tri(n-butyl)tin chloride in situ with sodium borotritiide. The challenge, then, is the conversion of the alcohol to either of these halides. Direct conversion of the alcohol to the halide, using HX or PBr₃ for instance, generally will result in considerable rearrangement of the neopentyl carbon. Several less common reagents have been reported to support halide formation with minimal rearrangement including NaX, KX or NH₄X in polyhydrogen fluoride-pyridine solution, (RO)₃PRX and R₃PX₂. The substitution of sulfinate esters by nucleophiles such as iodide and bromide ion has been effected at neopentyl positions using HMPA.

A most promising method is the reduction of the corresponding 32-aldehyde by a modification of the Wolff-Kishner method. The alcohol can be oxidized to the aldehyde by the mild Swern oxidation using DMSO and oxalylchloride at -60°C. The aldehyde can then be converted to the tosylhydrazone and reduced by one of several methods. In one method, using catecholborane, tritium can be supplied in the water used to decompose the catecholborane-tosylhydrazone complex (Scheme 7). In another variation, sodium borohydride in methanol or lithium aluminum hydride in dioxane are used; these two reductions proceed by quite distinct mechanisms. In this way, since one of the hydrogens added to the carbon comes from the solvent (or quench solution) and the other from the reagent, two equivalents of tritium can be added at once.

A fourth technique, particularly well-suited to hindered alcohols, is the dissolving metal reduction of phosphorodiamidates. The phosphorodiamidate is easily formed by the reaction of N,N-dimethylphosphoramidodichloridate (DMPADC) with the n-butyllithium- or lithium diisopropylamide-generated anion of the alcohol. The resulting monochloride is converted to the N,N,N',N'-tetramethylphosphorodiamidate (TMPDA) by the addition of dimethylamine prior to work-up of the reaction. The TMPDA is then reduced by lithium in ammonia, methyamine or ethylamine. This method is much less likely to be affected by steric factors, (consider, for instance, the reduction of the highly
hindered intermediate in the synthesis of palauolide by Piers and Wai\textsuperscript{(117)}, but has the drawback of having no direct way of introducing tritium at high specific activity. The availability of tritiated ammonia, methyl- or ethylamine is poor and their use would be extremely wasteful of the isotope. One way around this situation is to first introduce the tritium by a two-step oxidation-reduction path via the aldehyde using, for instance, Swern oxidation followed by reduction with tritiated sodium borohydride.

**Protecting groups**

Several possibilities exist for the protection of the side-chain double bond through the course of the synthesis. The most basic, and the one used in this work, is to protect the double bond as the dibromide\textsuperscript{(75, 93)} through the oxidation at position C-7 and then to avoid using techniques which would adversely affect the unprotected double bond subsequently (Figure 10). Other possibilities exist, however, and are worthy of note here. Ozonolysis of the side chain is possible in the presence of the Δ\textsuperscript{8} olefin due to the
hindered nature of that ring-juncture. This leads to 25,26,27-trisnor-24-lanosteroic acid which can then be esterified or reduced to the alcohol and protected. Lanosterol could be reformed at the end of the synthesis by reduction or oxidation to the aldehyde and elaboration of the side-chain terminus by Wittig reaction with isopropyl ylide.

Epoxidation of the side chain double bond has been carried out followed by boron trifluoride-catalyzed rearrangement to 24-ketolanosterol. This ketone can be protected by standard methods and then reduced to the Δ22 olefin by the Shapiro reaction (conversion of the ketone to the tosylhydrazone and reduction with two equivalents of alkyllithium). This olefin could then be isomerized to the more hindered Δ24 position with borane. (It is interesting to note that this 24-ketone is an excellent precursor to the substrate of the fungal 14α-demethylase, 24-methylenelanosterol, via a Wittig reaction with methyl ylide.)

Alternatively, the epoxide could be hydrolyzed to the diol (or the olefin oxidized to the diol directly) and protected. Several methods exist for the dihydroxy elimination of vicinal diols to reform the olefin.

The double bond can be converted to the 25-hydroxylanost-8-enol by hydroxymercuration or simply by treatment with aqueous acid. The alcohol can be reverted to the olefin via elimination of the corresponding bromide (the more hindered Δ24 olefin should result considering Zaitsev's rule).

As for the protection of the 3β-alcohol, the acetate group proved to be adequate for the needs of this synthesis. Although removed during reduction of the C-7 ketone as well as during deprotection of the 32-alcohol it does resist side-reactions during peroxide oxidation, lithium-ammonia reduction, and acetolytic ether cleavage. As will be discussed below, it was convenient to switch to a silyl ether for the last few steps of the synthesis in order to efficiently use some 3,32-diol which was protected as the 32-acetate (the diol is a minor product of the selective deprotection of the 3,32-diacetate). This change had no effect on the synthetic strategy.

**Synthetic Tactics**

The chemical compounds involved in the following synthesis are numbered according to Scheme 8. Due to the variations in side-chain structure, protecting groups, double bond location, functional group orientation and isotopic labeling, the following system has been adopted: only the basic steroid forms are numbered, the above variations
Scheme 8: Numbering scheme for molecules mentioned in this chapter. (Prefixes indicate side-chain structure, α/β orientation and double bond position, suffixes indicate protecting groups. For instance, \( \Delta^7\-\text{6d} \) corresponds to 3β,32-dihydroxylanosta-7,24-dienyl diacetate)
Figure 10: Synthesis of 32-[D]-Lanosterol from a commercial mixture of lanosterol and dihydrolanosterol. (a) Br₂, NaOAc, AcOH, Et₂O; (b) Pyridine, Ac₂O; separation of dihydro- from dibromo-compounds; (c) AcOH, H₂SO₄, H₂O₂; (d) Zn, AcOH, Et₂O; (e) Li, NH₃-Toluene (1:1); (f) Li(t-BuO)₃AlH, Et₂O; LiAlH₄, Et₂O; Pyridine, Ac₂O; (g) Pb(OAc)₄, Benzene, Toluene; (h) Pyridine-HCl, Ac₂O; LiAlH₄, Et₂O; (also Pyridine, Ac₂O or TBDMS-triflate, CH₂Cl₂, Pyridine, see text); Purification of Δ⁷ from Δ⁸ isomers; (i) DMSO, Oxalyl chloride, CH₂Cl₂; (j) NaBD₄, EtOH, (k) LDA, 1,2-DME, TMEDA; DMPADC (dimethylphosphoramidodichloridate); dimethylamine; (l) Li, MeNH₂.
are indicated by prefixes (DH and DB for dihydro- and dibromo- side-chains, α and β for alcohol orientations and D for deuterium labeling) and suffixes (protecting groups, see Scheme 8).

**Preparation of pure starting material and use of protecting groups**

Lanosterol is isolated in considerable quantity from wool-fat, the steroid fraction of which contains lanosterol (1a), dihydrolanosterol (DH-1a), agnosterol and gamma-lanosterol. Presently, lanosterol can be purchased from Sigma as mainly a 3:2 mixture of lanosterol and dihydrolanosterol. The separation of this mixture is quite difficult (see, for instance, early work by Ruzicka\(^{(151)}\) and earlier papers). Faced with this problem, many researchers have converted the mixture to pure dihydrolanosterol by catalytic hydrogenation.\(^{(100)}\) Others, who required only small amounts of pure lanosterol have used preparative gas chromatography and preparative HPLC.\(^{(12, 145)}\) Both of these methods are appropriate for applications where unmodified lanosterol or dihydrolanosterol are used as the assay substrates (small amounts of material need to be prepared periodically, whenever assays need to be done). For use as synthetic starting materials these methods are generally inappropriate because a large amount of material needs to be prepared very infrequently; ideally, this would only have to be done once to provide enough for the whole synthesis.

The lanosterol mixture obtained from Sigma was considerably more pure than that which earlier researchers used, so separation of the isomers by recrystallization was attempted. Recrystallization from methanol and methanol/ethyl acetate mixtures under various conditions failed to discriminate between lanosterol (1a) and dihydrolanosterol (DH-1a). Purification of the isomers by silica gel chromatography could be effected by multiple elutions on analytical thin-layer chromatography plates. Column chromatography using very low polarity solvent mixtures (like 100:1 hexanes ethyl acetate) could partially separate the material but the excessive amount of solvents necessary were deemed impractical.*

Two methods exist in the literature for the purification of lanosterol (1a) via reversible derivatization of the side-chain double bond. In the first, 24-bromolanosterol is

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* Near the end of the work presented in this chapter the t-butyldimethylsilyl (TBDMS) ether of lanosterol (1e) was prepared for comparison with that synthesized by reduction of the 32-TMPDA ester (see forward). This extremely non-polar derivative was found to separate from the dihydro-isomer (DH-1e) by up to 0.12 \(R_f\) units on analytical thin-layer chromatography in hydrocarbons such as pentane, heptane, isopentane and cyclohexane. Although the \(R_f\)'s in these solvents were higher than is generally optimal for column chromatography (~0.5), the use of large silica columns may provide a simple method for large scale purification of lanosterol.
formed,\(^{(45)}\) separated from the crude mixture and then the bromine is removed with lithium in tetrahydrofuran to regenerate lanosterol.\(^{(95)}\) This methodology may be useful in the preparation of 24-methylene lanosterol, the natural substrate of the 14\(\alpha\)-demethylase in filamentous fungi.

The second method uses bromine to generate 24,25-dibromolanosterol (DB-1a) which is separated from the crude mixture, crystallized as the acetate (DB-1b), saponified, and debrominated with zinc to regenerate lanosterol (DB-1a).\(^{(58, 75)}\) This method was judged to be more applicable to our needs as the protected molecule, dibromolanosterol acetate, should be suitable as a starting material for the synthesis of 32-[\(^3\)H]-lanosterol. As has been summarized above, this scheme calls for the oxidation of the \(\Delta^8\) double bond of lanosterol to a conjugated ketone, and the reduction of that group to an alcohol. It was reasoned that the vicinal dibromide group would be resistant to the oxidation by peroxyacetic acid, and might be sufficiently resistant to reduction by hydrogen over platinum to serve as a protecting group for the double bond through these transformations.

In a test experiment it was found that a slight excess of bromine over lanosterol yielded the 24-\(R\) and 24-\(S\) diastereomers of dibromolanosterol in a 1:1 ratio by HPLC using a C-18 reverse phase column with methanol as the mobile phase. All of the lanosterol was consumed and none of the \(\Delta^8\) double bond reacted. Purification of the product by recrystallization was achieved more easily after formation of the acetate with pyridine/acetic anhydride. It should be noted that chromatography of these dibromides is somewhat complicated by the diastereomerism, since the mobility of these isomers is different enough to significantly broaden any eluted bands.

**Activation of C-7 and C-32 positions**

**Allylic oxidation at C-7**

The conversion of lanost-8-enes to lanost-8-en-7-ones has been traditionally carried out by oxidation with chromic acid in poor yield. Since 1970 however, an improved method using dilute peroxide and sulfuric acid has been in wide use.\(^{(118)}\) This method gives yields of 60-80%.

In thinking about whether this reaction would be compatible with the dibromide protecting group, a working model for the mechanism of this reaction was developed (Figure 11). This mechanism is based on several observations: first, peracetic acid, an epoxidizing agent, is formed by the mixture of sulfuric acid, acetic acid and peroxide; second, the acidic conditions will allow partial isomerization of the \(\Delta^8\) double bond to the
**Figure 11**: Proposed mechanism of allylic oxidation by peroxide and sulfuric acid on lanosteryl acetates

Overall Reaction:  S.M. + 2H₂O₂ $\rightarrow$ Product + 3H₂O
\[ \Delta^7 \text{ and } \Delta^9(11) \text{ positions; and third, the C-7 position is considerably less hindered than the C-11 position.} \]

In carrying out the reaction using the conditions described by Pinhey (acetic acid as solvent, 0.3\% sulfuric acid, 1\% peroxide, room temperature, 17 hours) very poor conversion to product was observed. Even after 46 hours the reaction was not complete, and that which had reacted was distributed over 10 product compounds. Addition of ethyl acetate to improve the solubility of the starting material and increased oxidant concentration were not found to improve the situation. Heating the solution, however, and lowering the reaction time greatly simplified the product distribution and drove the reaction nearly to completion. Apparently, the side reactions involving degradation of the dibromide are less sensitive to rate enhancement with increasing temperature. The optimal conditions were found to be 70°C for 1 hour, yielding nearly 50\% desired product. To do the reaction on large scales, however, a more concentrated reaction mixture must be used to obtain reasonable volumes. This modification reduced the yield to around 35\%. (This figure may, however, be improved by further optimization.)

Recently it has come to our attention that an alternative oxidation may be superior in this case. Paryzek reports an interesting allylic oxidation of lanosterol (1a) to 7-keto-lanosterol (2a) in 57\% yield using ozone in polar solvents such as ethyl acetate (the 7\(\alpha\),8\(\alpha\)-epoxide is also formed in 22\% yield).\(^{116}\) Although this yield is lower than the 60-80\% reported for peracetic acid oxidation of dihydrolanosterol, it is better than the yield obtained here with dibromolanosterol. Furthermore, the workup of this reaction is considerably simpler and purification of the product should be easier than with the peracetic acid reaction. The dibromide protecting group should be quite stable under these conditions.

**Reduction of the unsaturated ketone**

The method of Sonoda (hydrogenation over platinum\(^{135}\)) for the formation of the 7\(\alpha\)-alcohol (\(\alpha\)-4) directly from the 24,25-dibromo-7-oxo-lanosterol acetate (DB-2b) was attempted first. As a test of the method the reaction was carried out on a sample of 7-oxo-dihydrolanosterol acetate (DH-2b) and, as reported, 7\(\alpha\)-hydroxylanostane (DH-\(\alpha\)-4a) was obtained in \(\sim\)20\% yield. When this reaction was attempted with the dibromo-protected compound (DB-2b), however, it was found that the bromines were reduced significantly faster than the conjugated ketone, and no brominated compounds could be recovered.

The dibromide protecting group is incompatible with reduction by lithium in ammonia, but the unprotected double-bond is insensitive to this treatment. Therefore, the
dibromide was removed by treatment with zinc/acetic acid in diethyl ether. Initially, standard conditions were used for the dissolving metal reduction: the steroid (2b) was dissolved in diethyl ether and t-butyl alcohol was added as a proton source. This was then added to an excess of lithium in liquid ammonia. The reaction was quenched with aqueous ammonium chloride. This procedure resulted in a very complex mixture of products from which the desired saturated ketone (3) could be isolated in only about 20% yield. Probably the presence of t-butyl alcohol caused over-reduction of the ketone to the alcohol by protonation of the initially formed, and otherwise stable, lithium enolate. Under such equilibrating conditions the equatorial β-alcohol (β-4) will be formed. This can also result from the addition of water in the quenching solution. (A thorough discussion of lithium-ammonia reductions of steroidal ketones can be found in Fried(48))

Changing the conditions to include toluene (to aid in the solubilization of the steroid in the ammonia), omit t-butyl alcohol and quench the reaction with ethylene dibromide to prevent over-reaction caused by premature protonation in the presence of excess lithium, resulted in a considerably cleaner reaction. It was found that reaction for considerable periods of time with excess lithium would not fully reduce the 3β-acetate to the corresponding alcohol. Furthermore, the desired products (acetate and alcohol) were contaminated in a 1:1 ratio with two side products which were shown by chemical and spectroscopic techniques to be most consistent with the α-hydroxy ketone, 3β,8β-dihydroxy-7-oxolanost-24-enol and its corresponding 3β-acetate. This assignment of structure is quite surprising, and possibly unprecedented in lithium-ammonia reductions. Due to the unusual nature of the assignment, a detailed discussion of the supporting evidence is presented in the Appendix to this chapter. The possibility that this anomalous product was due to trace water impurity was eliminated by using freshly distilled toluene and running the reaction under argon. It seems quite likely that the oxygen is introduced either in the work-up of the reaction, from O₂ dissolved in the toluene, or even from peroxides in the ether, since there is no other source of oxygen in the reaction itself (besides that small amount from any cleaved acetates). The mechanistic origin of this compound remains a mystery since it is generally believed that reduction of conjugated ketones results in the lithium enolate of the ketone prior to quenching with a proton source, and in any case, the position alpha to a ketone should be electron-rich, requiring an electrophilic oxygen source.

Whatever the mechanistic cause, the practical solution to the problem involved the relative solubility of the steroids in the ammonia and toluene phases of the reaction. Fortuitously, it was found that running the reaction in 1:1 ammonia-toluene and completely emulsifying the two immiscible phases with vigorous stirring (overhead stirring at
maximum speed in a baffled flask) completely eliminated the production of the side product.

Reduction of the ketone at position 7 with lithium aluminum hydride resulted in an unfavorable 3:1 mixture of the 7β- and 7α-alcohols (β-4, α-4). This result is consistent with previous reports in the lanosterol literature as well as the general trend for steroidal 7-ketones. Wheeler, for instance, reports that hydride reduction of 7-keto-cholestanes results in ~70% β-alcohols whether the reductant is LAH, NaBH₄ or the hindered reagent lithium tri(t-butoxide)aluminohydride. Analysis of MM2-minimized models of 7-ketolanost-24-enol (3a, Scheme 9) indicated that the primary steric block to the approach of reagents to the β-face are the two angular protons at C-8 and C-6 adjacent to the carbonyl. The two nearby β-methyls (C-18 and C-19) appear to be too far away to have much of an effect. The α-face, on the other hand, has two angular protons which are two carbons removed from the carbonyl at C-9 and C-5. In this respect it is relatively uncrowded, and seems to explain the preference of hydride reduction in the cholestane series. In lanostanes, however, the presence of the angular 14α-methyl should come into play by blocking the approach of more hindered reagents, although the smaller lithium aluminum hydride seems to be unaffected by it. Using lithium tri(t-butoxy)aluminohydride, the 7α-alcohol (α-4) was formed in a 3.75:1 ratio (79% to 21%). When this reagent was used on the crude product of the lithium/ammonia reduction, the residual acetate at C-3 was undisturbed yielding a mixture of 3β-alcohols and acetates. To complete the reaction then, an aliquot of lithium aluminum hydride was added prior to work-up to yield only the mixture of 3β,7α and 3β,7β alcohols.

The yield of 7α-alcohol (α-4) from these combined reduction steps is 65%. Another 17% is 7β-alcohol (β-4) which can, without much difficulty, be converted into the proper isomer. This compares quite favorably with the 26% yield of the literature preparation using catalytic hydrogenation.

Reprotection of the 3β-alcohol in the presence of the 7α- and 7β-alcohols is easily achieved by acetylation with acetic anhydride in pyridine. The two equatorial alcohols, 3β and 7β, react much more rapidly than the axial 7α-alcohol. This has the effect of selectively protecting the 3β-alcohol while simultaneously purifying the 7α-alcohol from the 7β-alcohol. For instance, when a mixture containing 79% 7α-alcohol (α-4a) is allowed to react until 90% of the starting diols are consumed (4 hours with 25 alcohol-equivalents of acetic anhydride in 1:2 anhydride-pyridine) the 7-hydroxy-3-acetate that is isolated in 43% yield is entirely the 7α isomer (α-4b), while the 3,7-diacetate is found to be 40% 7α (α-4d, 34% yield). By modeling the reaction as two independent first-order reactions and using the data from several trials, the optimal reaction time for maximizing the production of the 7α-monoacetate (α-4b) from a 79% 7α mixture of diols was found to be roughly 4.4 hours.
Scheme 9: MM2-minimized space-filling models of 7-oxolanost-24-enol (3a), comparison of \( \alpha \) and \( \beta \)-faces. Notice the partial occlusion of the carbonyl by the 14\( \alpha \)-methyl group. In both representations the carbonyl and its alpha carbons are constrained to the plane of the paper.

Formation of 32-alcohol

When the lead tetraacetate cyclization of 3\( \beta \)-acetoxylanost-24-en-7\( \alpha \)-ol (\( \alpha \)-4b) is carried out under the conditions specified by Parish\textsuperscript{[114]} for the saturated compound (\textit{i.e.} 24-hours refluxing benzene, 5.5 equiv. lead tetraacetate) a very poor yield (14\%) of 7\( \alpha \)-32 cyclic ether (5b) is formed. NMR of the various side-products all indicate that although the desired tetrahydrofuran ring is in evidence in all products, the side-chain double bond has been oxidized in various ways. Clearly, these results indicate that the degradation of the
side-chain is a slower process than the cyclization and, therefore, reducing the reaction time should improve the situation. It was found that reaction for only 2.5 hours resulted in a 59% yield of the desired product. 20% of the starting material was reclaimed unchanged; 10% was isolated as 7-oxolanost-24-enyl-3β-acetate (3b), while only 11% was lost to side-chain degradation. Acetolytic cleavage of this ether by pyridine hydrochloride and acetic anhydride proceeded exactly as reported by Parish and Schreopfer,(114) yielding a mixture of Δ6, Δ7 and Δ8 diacetates (6d, 1:6:3 ratio).

The diacetates formed at this stage are conveniently cleaved to a mixture of 3β-monoacetates and diols by partial reduction with lithium aluminum hydride. The primary 32-acetate is cleaved significantly faster than the secondary 3β-acetate such that only trace amounts of 3β-hydroxy-32-acetates are formed.

At this stage, the separation of the double bond isomers is most easily performed using chromatographic techniques. Apparently the disposition of the 32-alcohol is affected by the position of the double bond in the C-ring, because the isomers show small but significant mobility differences on polar chromatography media (much better than that displayed by Δ8-lanosterol (1a) and Δ7-lanosterol, for instance. Sonoda achieved separation of the diols using a Waters µ-Bondpak-NH2 HPLC column in 1:1 chloroform-hexane, while Parish used a combination of silica MPLC chromatography (1:3 EtOAc-Toluene) and silica flash chromatography (97:2.5:0.5 chloroform-ether-acetic acid).(114, 135) It was found in the present study that separation of the 3β-monoacetates was relatively straightforward using Chromatatron radial thin layer chromatography (Harrison Research Co.) in 8:1 hexane-EtOAc.

The diols formed by over-reduction are easily reconverted to the diacetates for repetition of the above procedure, however, at this stage the 32-alcohol can be selectively protected as the acetate (6c) by treatment with acetic anhydride in pyridine with monitoring by TLC (Yield ~65%). The 3β-alcohol can then be quantitatively protected as the TBDMS ether (6f) using TBDMS trflate in methylene chloride with pyridine as base. Saponification with 1 N NaOH in THF/Methanol gives the isomeric 3β,32-dihydroxylanostadienyl-3β-TBDMS ethers (6e) in excellent yield. The double bond isomers in this case are separated by silica flash chromatography in 5:2 cyclohexane-chloroform.

Since the isomers are easily separated at this stage, the possibility of converting the Δ7 isomer (Δ7-6e) into the Δ8 isomer (Δ8-6e) was explored. The standard method for this isomerization in 24,25-dihydrolanosterol (DH-1a) uses HCl gas passed through a solution of the steroid in chloroform (100). Treatment of the Δ7-32-alcohol (Δ7-6e) in this manner resulted in numerous compounds by TLC. By cooling the solution to -30°C in a
bromobenzene/liquid nitrogen bath a cleaner reaction was observed that by TLC appeared to result in a 60/40 $\Delta^8/\Delta^7$ ratio. Analysis by NMR, however, showed that although the desired double bond isomerization had taken place, the side chain olefin was extensively modified. This trial does, however, indicate that the presence of the 32-hydroxyl is no impediment to the desired isomerization. A search of the literature showed a report of the isomerization of $\Delta^7$-lanosterol (DH-1a) by first protecting the side-chain double bond again as the dibromide (DB-1a)$^{(58)}$. Thus, by a three step procedure, the $\Delta^7$ isomers should be convertible to usable $\Delta^8$ products.

**Reduction of the 32-alcohol (6) to Lanosterol (1) with the Introduction of Isotopes at the C-32 Position**

The primary goal in this section was to show that the reduction of 32-hydroxy lanosterol to lanosterol is possible. Throughout attempts towards this goal, the work was carried out on only milligrams of material at a time, because that was all that was available. Because this conversion was expected to be problematic, our strategy was to utilize the $\Delta^6$ and $\Delta^7$ isomers of the alcohol whenever possible to test the reactions in order to preserve the proper $\Delta^8$ isomer for those conditions which looked promising. Of course, this may have precluded some methods for which these "test isomers" were unsuitable while the proper isomer may have been more successful. Furthermore, we did not have the luxury of being able to extensively vary the reaction conditions of each method in order to tease out the desired products out of seemingly unfavorable results. Four basic methods were attempted. The first three were deemed unsuccessful due to either rearrangement or steric hindrance. The fourth method, however, was quite successful (and the discovery of which coincided with the exhaustion of our last batch of 32-hydroxylanosterol).

**Hydride reduction of Sulfonate Esters**

The tosylate of $\Delta^6$-32-hydroxylanosteryl-3β-acetate ($\Delta^6$-8b) was reacted with lithium aluminum hydride in THF for 3.5 days and yielded three major products and several minor ones. The only product with an $R_f$ near that of lanosterol was shown by NMR to have undergone some kind of rearrangement. It is possible that reaction in HMPA or some other polar aprotic solvent might yield an unrearranged product, but this was not attempted.
Iodide substitution of Mesylate

The mesylate of Δ7-32-hydroxylanosteryl-3β-acetate (Δ7-7b) was treated with sodium iodide in HMPA and heated to 100 C for 4.5 hours. Repeated thin layer chromatography under optimized conditions revealed that the initially identified product spot was, in fact, composed of five compounds, two of which correspond to solvolysis products of the mesylate. The solvolysis products appear to contain three double bonds by NMR and mass-spectroscopy, and probably resulted from D-ring expansion. One of the products contains a cyclopropane ring (presumably by formation of a bond between carbons 8 ands 32) as evidenced by a pair of doublets in the H1-NMR between 0 and 1 ppm. The last two products contain iodine, but lack the vinyl resonance from C-7 in the NMR, and probably result from iodine trapping of rearranged cations. Figure 12 shows some of the possible rearrangement products and their mechanisms of formation; many other possibilities exist. It is possible that reduction of the temperature, shorter reaction times or the use of the smaller bromide ion instead of iodide might yield the desired product, but this was not attempted here.

Reduction of 32-Tosylhydrazone

The 32-aldehyde (9) was easily formed from the corresponding alcohol via the Swern oxidation using DMSO and oxalyl chloride with yields in excess of 95%. The tosylhydrazone (10) was formed by refluxing the aldehyde with toluene sulfonhydrazide in ethanol for several hours. Recovery of the product was difficult if too much reagent had been used, but yields approached 85%.

Reduction of the tosylhydrazone (10) using excess sodium borohydride in refluxing methanol after the method of Caglioti, yielded what appeared to be a mixture of lanosterol (1a) and lanosteryl acetate (1b) by TLC. After workup, reduction of the acetate with LAH, and purification of the product, TLC showed a mixture of a UV-transparent spot which co-eluted with lanosterol and a more polar UV-absorbing spot in a 1:1 ratio. NMR of the less polar compound, unfortunately, does not match with authentic lanosterol. The identity of this product has not yet been determined.

Reduction of the tosylhydrazone (10) with catecholborane by the method of Kabalka (-10 C, 20 minutes, followed by quenching with sodium acetate trihydrate) resulted in no reaction. Increasing the length of reaction, amount of reagent and temperature had no effect (the reagent was shown to be pure by comparison of the NMR with published standards, the solvents were confirmed to be dry, and the starting material was shown to have the proper structure after recovery from the reaction mixture). Initial modeling of the starting material and reagent using the HyperChem package (AutoDesk,
Figure 12: Some possible rearrangement products of the C-32 cation
Inc.) seemed to indicate that there was little steric impediment to the approach of the boron and its attached hydrogen into position alongside the carbon-nitrogen bond which is initially reduced (Scheme 7). After the negative result, the molecule was reexamined using a plastic CPK-type model. This model clearly illustrated that the rehybridization of the proximal nitrogen to sp\(^3\) in the intermediate catecholborane adduct hinders the formation of that adduct because of clash between the catechol group and the steroid nucleus.

**Reduction of TMPDA**

Conversion of the \(\Delta^7\)-alcohol (\(\Delta^7-6e\)) to the corresponding tetramethyl-phosphonodiamidate (TMPDA, \(\Delta^7-11e\)) was carried out by the method of Liu\(^{(94)}\) using dimethylphosphoramidic dichloride (DMPADC) after formation of the lithium alkoxide of the alcohol with LDA. The reaction was quite clean and gave a 79% yield. Reduction of the TMPDA with lithium in methyamine (without a proton source to inhibit reduction of the double bonds\(^{(117)}\)) gave three products in 6:3:1 ratio. The most abundant product was consistent with the desired compound, \(\Delta^7\)-lanosterol-TBDMS ether (\(\Delta^7-1e\)), however this could not be confirmed for lack of an authentic standard. The second most abundant product appears to contain a cyclopropane ring by NMR as well as, perhaps, a \(\Delta^6\) olefin. Both of these compounds show the expected weight of desired product by mass spectroscopy.

Reaction of the \(\Delta^8\)-alcohol (\(\Delta^8-6e\)) with DMPADC was problematic, giving only a 12% yield under the standard conditions; using excess reagent only reduced this yield. The consumption of the starting material is accompanied by the formation of a considerably less polar side-product which is unidentifiable by NMR, but presumably results from a rearrangement-elimination of the intermediate monochlorophosphoramidate. This reactivity difference between the two double bond isomers is surprising. Varying the conditions will probably improve the yields, but lack of material has precluded such a study at this time. Reduction of the TMPDA (\(\Delta^8-11e\)) with lithium in methyamine, however, resulted in the clean production of a single product (>95% yield by TLC). This product co-spotted with authentic lanosterol-TBDMS ether in several solvent systems, showed identical \(^1\)H and \(^{13}\)C-NMR's, and gave the same retention time and fragmentation patterns by GC-MS.

In order to introduce tritium at the C-32 position the alcohol can be oxidized to the aldehyde and reduced with tritiated sodium borohydride. As a model for this process, the aldehyde (\(\Delta^8-9e\), see above) was reduced with sodium borodeuteride. NMR shows >60% incorporation of deuterium into the 32 position.

\(^1\)H-NMR of these compounds indicates that there has been no degradation or isomerization of the side-chain double bond throughout these transformations.
Conclusions: Comparison of yield and complexity with dihydro-syntheses

The synthesis described above formally represents the first synthesis of lanosterol isotopically labeled at the 14α-methyl position. The conversion of 32-hydroxylanosterol (6a) to lanosterol (1a), described in the previous section, provides a second independent route to these labeled lanosterols in parallel to the route from cholesterol to 24,25-dihydrolanosterol (DH-1a, Figure 9). Furthermore, this work demonstrates the feasibility of preserving the side-chain olefin of lanosterol through a complex series of synthetic transformations.

Compared to the corresponding synthesis of 32-hydroxydihydrolanosterol (DH-6), although no comprehensive effort was made to optimize yields for all steps, preservation of the double bond clearly results in some loss of overall yield. These additional losses arise mainly during the preparation and purification of dibromolanosteryl acetate (DB-1b), and the oxidation of this compound to the 7-keto derivative (DB-2b). As mentioned above, the purification of lanosterol may be improved in the future by chromatographic separation of the silyl ethers, and the allylic oxidation may be improved by using an ozonolysis methodology. The lead tetraacetate activation of the C-32 position resulted in some losses due to double bond reactivity, but by lowering the reaction time, this was mainly eliminated. This results more in a loss of research time than yield, as the incompletely reacted mixture must be purified and the remaining starting material re-oxidized. Although the penultimate reaction synthesizing the TMPDA derivative of 32-hydroxylanosterol is problematic at this time, further investigations will likely alleviate this difficulty.

The predictable failure of the hydrogenation of the dibromo-7-ketolanosterol (DB-2) to selectively reduce the (hindered) conjugated ketone in the presence of the dibromide was fortuitous as it prompted us to investigate the two-step reduction path using lithium-ammonia and lithium tri(t-butyl)aluminoxydride. This new reduction method is far superior to previously published methods in yielding 7α-alcohols and is applicable to both lanosterol- and dihydrolanosterol-based syntheses.

Overall, this synthesis compares favorably in terms of yield and complexity with the cholesterol based synthesis, and may, in time, prove to be a superior method for the synthesis of labeled dihydrolanosterols. Presently it remains the sole method for obtaining specifically-labeled lanosterol.
Materials, Methods and Data

Materials and Equipment

All chemicals were purchased from Aldrich chemical company, were of the highest grade available and were used without further purification unless noted otherwise. All NMR's were taken in deuterochloroform with tetramethylsilane as an internal standard unless otherwise noted. Spectra were acquired on the MIT Chemistry department's Varian XL-series and Unity 300 MHz spectrophotometers. Mass spectra were taken using either a Finnegan System 8200 quadrupole mass spectrometer in electronic ionization mode or an Hewlett Packard 5890-series gas chromatograph with HP-1 100% dimethylpolysiloxane (gum) column equipped with a Hewlett Packard 5971-series mass selective detector. Infrared spectra were acquired using a Perkin Elmer 1600 series FT-IR spectrophotometer with samples as thin films on NaCl plates. Melting Points were determined in sealed tubes using a Hoover Mel-Temp apparatus.

Synthetic Preparations

Lanosteryl and Dihydrolanosteryl Acetates (1b, DH-1b). A mixture of lanosterol (la, 62%) and dihydrolanosterol (DH-la, 38%) purchased from Sigma (40 g, 92 mmol) was added to a 2 liter round-bottom flask. 800 mL of freshly distilled pyridine and 150 mL fresh acetic anhydride were added. The solution was allowed to stir overnight. The solution volume was reduced by distillation of the volatile solvents under reduced pressure. The remaining solution was dissolved in methylene chloride and extracted with 2 N HCl, distilled water and saturated brine. The organic phase was dried over magnesium sulfate, filtered and concentrated under reduced pressure. When the volume of methylene chloride reached approximately 400 mL, 400 mL of methanol were added. Evaporation of the solvents continued until cooling resulted in crystal formation. Two batches of recrystallized product yielded 36.5 g (83% yield). This material was relatively enriched in lanosterol acetate (68%) compared to dihydrolanosterol acetate as assayed by integration of the 24-vinyl proton relative to the 3α-acetoxy proton (see below). The mother liquor was reserved for further purification during future preparations (accounting for this process, the overall yield is approximately 90%). $^{13}$C-NMR (CDCl$_3$): δ 15.78 (C-18), 16.53 (C-30), 17.63 (C-27, 1b), 18.12 (C-6), 18.63 (C-21, 1b), 18.74 (C-21, DH-1b), 19.20 (C-19), 21.00 (C-11), 21.33 (C-3", Ac-CH$_3$), 22.55 (C-26,
DH-1b, 22.86 (C-27, DH-1b), 24.18 (C-2; C-23, DH-1b), 24.24 (C-32), 24.94 (C-23, 1b), 25.73 (C-26, 1b), 26.39 (C-7), 27.91 (C-31), 28.00 (C-25, DH-1b), 28.20 (C-16), 30.82 (C-15), 30.99 (C-12), 35.29 (C-1), 36.26 (C-20, 1b), 36.37 (C-22, 1b), 36.47 (C-20, DH-1b; C-22, DH-1b), 36.89 (C-10), 37.80 (C-4), 39.54 (C-24, DH-1b), 44.46 (C-13), 49.82 (C-14), 50.40 (C-17, 1b) 50.49 (C-5; C-17, DH-1b), 80.91 (C-3), 125.24 (C-24, 1b), 130.85 (C-25, 1b), 134.22 (C-9), 134.48 (C-8), 170.97 (C-3', Ac-C=O). 13C-NMR assignments are based on the data published in (49).

24,25-Dibromolanosteryl acetate (DB-1b).
36.5 g of a mixture of lanosteryl acetate (1b, 68%, 24.82 g, 52.87 mmol, 1 equiv.) and dihydrolanosteryl acetate (DH-1b, 32%) was dissolved in 640 mL anhydrous diethyl ether in a 1 liter round-bottom flask. The solution was chilled to 0 C in an ice water bath. 5.5 g of sodium acetate (69.98 mmol, 1.32 equiv.) was dissolved in about 200 mL of acetic acid. To this acetate solution was added 3.0 mL of bromine (58.32 mmol, 1.1 equiv.). This solution was added to the reaction flask slowly with a dropping addition funnel over 2 hours. When the stirred solution remained yellow for more than one minute the excess reagent was quenched with several drops of cyclohexene. The mixture was washed with bicarbonate until the ether layer tested neutral with pH paper and then washed with distilled water. The combined organic layers were dried over magnesium sulfate, filtered and concentrated. The crude product was recrystallized repeatedly from methanol/methylene chloride yielding 11.72 g of >97% pure DB-1b (44% yield). The mother liquors were combined with those from other preparations and recrystallized. The overall yield is estimated at 70%.

7-oxo-24,25-dibromolanosteryl acetate (DB-2b). 15.8 g. of dibromolanosteryl acetate (DB-1b, 25.2 mmol, 25 mM) was placed in a 2-liter round-bottom flask affixed with a reflux condenser and dissolved in 965 mL of acetic acid. The solution was heated to ~70 C until all of the dibromide dissolved. The flask was flushed with argon and 4 mL of sulfuric acid (3 equiv., 0.4% final concentration) in 5 mL of acetic acid were added slowly through an addition funnel over 20 minutes. When this addition was complete, 33.4 mL of 30% hydrogen peroxide (13 equiv., 1% final concentration), was added all at once. The reaction was stirred at ~70 C for one hour and then poured into 1 liter of ice water. The resulting precipitate was filtered and washed with methanol. The crude product was recrystallized from methanol and methylene chloride to yield 6.51 g of product. The mother liquor was chromatographed on alumina using a step-gradient of heptane, 50:1 heptane/EtOAc, 25:1 heptane/EtOAc, 9:1 heptane/EtOAc, and 100% EtOAc.
This yielded fractions which were recrystallized to yield 1.2 g of product. Final yield, 7.7 g, 49%.

**DB-2b:** $^1$H-NMR (CDCl$_3$): $\delta$ 4.46 (dd, $J_1$=4.6Hz, $J_2$=11.2Hz, C-3, 1-H), 4.11 (dd, $J_1$=1.2Hz, $J_2$=11.1, C-24 {one diastereomer}, 0.4-H), 4.06 (dm, $J$=9.3Hz, C-24 {one diastereomer}, 0.6-H), 2.00 (s, acetate, 3-H), 1.93 and 1.92 (s, C-26 {two diastereomers}, 3-H), 1.76 and 1.75 (s, C-27 {two diastereomers}, 3-H), 1.34 (s, C-19, 3-H), 0.91 (d, C-21, 3-H), 0.90 (s, C-30, 3-H), 0.87 (s, C-32, 3-H), 0.83 (s, C-31, 3-H), 0.62 and 0.61 (s, C-18 {two diastereomers}, 3-H).

**7-oxolanosteryl-3β-acetate (2b).** 18.3 g of 7-oxo-24,25-dibromomolanosterol acetate (DB-2b, 28.5 mmol) was added to a 3 liter round-bottom flask along with 2 liters of anhydrous diethyl ether. The mixture was stirred with an overhead stirring blade. The ether was heated to reflux and 25 mL of acetic acid were added (420 mmol, 14.7 equiv.) followed by 4.66 g of zinc dust (71.2 mmol, 2.5 equiv.) added cautiously. The solution was allowed to stir at reflux until all of the dibromide had dissolved and TLC showed complete reaction (approximately one hour). The solution was allowed to cool and was filtered to remove the zinc dust. The ether was evaporated under a stream of air to about 300 mL. 1 liter of methanol was added to precipitate the steroid which was filtered and washed with methanol. The filtrate was evaporated further to yield additional steroid. The total yield of this reaction was 80%.

**2b:** $^1$H-NMR (CDCl$_3$): $\delta$ 5.09 (tt, C-24, 1-H), 4.51 (dd, C-3, 1-H), 2.36 (m, C-8, 1-H), 2.29 (d-br, C-6, 2-H), 2.06 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.19 (s, C-19, 3-H), 0.96 (s, 3-H), 0.92 (d, C-21, 3-H), 0.91 (s, 3-H), 0.88 (s, 3-H), 0.65 (s, C-18, 3-H).

**3β-hydroxy-7-oxolanost-24-ene (3a).** 13.6 g of 7-oxolanosteryl acetate (2b, 28.2 mmol) was dissolved in 500 mL of dry toluene. A 2 liter baffled round-bottom flask was placed in a 10 liter bucket and equipped with an overhead stirrer, dewar condenser, gas inlet and dropping addition funnel. The apparatus was cooled to -78 C in a dry-ice/acetone bath and approximately 500 mL of anhydrous ammonia was condensed into the flask. 2.5 g of lithium metal (360 mmol, 12.8 equiv.) were added to the ammonia in small pieces. When the metal had dissolved (deep royal blue solution), the steroid solution was added over 10 minutes through the addition funnel. The reaction mixture was stirred vigorously for three hours and then quenched by the addition of 10 mL dibromoethane. 10 mL of acetic acid in 20 mL of methanol were added over 30 minutes and the ammonia was evaporated under a stream of argon over the next 24 hours. The remaining solution was
partitioned between water and methylene chloride. The aqueous phase was extracted three
times with methylene chloride and the combined organic phases were washed with water
and brine, dried over sodium sulfate and evaporated to yield the crude product (13.1 g,
96% crude yield), a white solid. TLC shows primarily the desired product contaminated
somewhat with 3β-acetoxy-7-oxolanost-24-ene (3b).

3a: $^1$H-NMR (CDCl$_3$): $\delta$ 5.09 (tt, C-24, 1-H), 3.25 (dd, C-3, 1-H), 2.36 (m, C-8,
1-H), 2.29 (d-br, C-6, 2-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.08 (s, C-19, 3-
H), 0.95 (s, 3-H), 0.90 (s, 3-H), 0.88 (d, C-21, 3-H), 0.83 (s, 3-H), 0.75 (s, C-18, 3-
H). $^{13}$C-NMR (CDCl$_3$): $\delta$ 13.08 (C-19), 14.70 (C-18), 15.01 (C-31), 17.10 (C-32),
17.58 (C-27), 18.61 (C-21), 20.46 (C-11), 24.84 (C-23), 25.66 (C-26), 27.39 (C-2),
27.68 (C-30), 28.21 (C-16), 31.67 (C-12), 33.77 (C-15), 35.74 (C-20), 36.60 (C-10),
36.40 (C-22), 36.95 (C-1), 39.00 (C-4), 39.76 (C-6), 45.83 (C-13), 46.34 (C-14), 48.68
(C-9), 49.59 (C-17), 53.11 (C-5 and C-8), 78.50 (C-3), 125.08 (C-24), 130.82 (C-25),
211.65 (C-7).

3b: $^1$H-NMR (CDCl$_3$): $\delta$ 5.09 (tt, C-24, 1-H), 4.49 (dd, C-3, 1-H), 2.37 (m, C-
8, 1-H), 2.29 (d-br, C-6, 2-H), 2.05 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-
27, 3-H), 1.11 (s, C-19, 3-H), 0.91 (d, C-21, 3-H), 0.90 (s, 6-H), 0.83 (s, 3-H), 0.76
(s, C-18, 3-H). $^{13}$C-NMR (CDCl$_3$): $\delta$ 13.10 (C-19), 14.70 (C-18), 15.97 (C-31), 16.97
(C-32), 17.58, (C-27), 18.60 (C-21), 20.47 (C-11), 23.80 (C-2), 24.83 (C-23), 25.65
(C-26), 27.49 (C-30), 28.19 (C-16), 31.63 (C-12), 33.78 (C-15), 35.74 (C-20), 36.47
(C-10), 36.39 (C-22), 36.60 (C-1), 37.82 (C-4), 39.52 (C-6), 45.82 (C-13), 46.34 (C-
14), 48.55 (C-9), 49.56 (C-17), 53.10 (C-5 and C-8), 80.17 (C-3), 125.08 (C-24),
130.82 (C-25), 211.16 (C-7).

3β,7α-dihydroxylanost-24-ene ($\alpha$-4a) and 3β,7β-dihydroxylanost-24-
enone ($\beta$-4a). 13.1 g of crude 3β-hydroxy and 3β-acetoxy-7-oxolanost-24-ene (~29
mmol) was transferred to a 1 liter round-bottom flask and dissolved in 600 mL anhydrous
diethyl ether. 23 g. of lithium tri(t-butoxy)aluminohydride (90.4 mmol, 3.1 equiv.) was
added to the solution which was allowed to stir over night. TLC showed that reduction of
the 7-keto group was complete by this time. To convert all of the product to the 3β-
hydroxy species, lithium aluminum hydride was added and the solution allowed to stir
until TLC showed a single product. The reaction was quenched by the addition of 10%
aqueous ammonium chloride. The solution was filtered to remove the reagents, the filter
cake washed with ether, and the combined filtrates dried over sodium sulfate. Evaporation
of the ether yielded 10.7 g of product (82% yield from compound 2b) which was found to
be 79% α ($\alpha$-4a) at the 7-position by proton NMR.
\(\alpha\)-4a: \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 5.09 (tt, C-24, 1-H), 4.06 (s-br, C-7, 1-H), 3.26 (dd, C-3, 1-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.08 (s, C-19, 3-H), 0.96 (s, 3-H), 0.92 (s, 3-H), 0.87 (d, C-21, 3-H), .80 (s, 3-H), 0.74 (s, C-18, 3-H).

\(\beta\)-4a: \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 5.09 (tt, C-24, 1-H), 3.58 (dt, \(J_1=4.8\)Hz, \(J_2=10.2\)Hz, C-7, 1-H), 3.19 (dd, C-3, 1-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.97 (s, C-19, 3-H), 0.92 (s, 3-H), 0.90 (d, C-21, 3-H), 0.88 (s, 3-H), .81 (s, 3-H), 0.78 (s, C-18, 3-H).

\(7\alpha\)-hydroxylanost-24-enyl-3\(\beta\)-acetate (\(\alpha\)-4b). 164 mg of 3,7-dihydroxylanostenes (\(\alpha\)-4a, 79\% and \(\beta\)-4a, 21\%); 0.368 mmol, 2 equivalents of alcohols) was dissolved in 3.5 mL of dry pyridine and 1.75 mL of fresh acetic anhydride (18.54 mmol, 50 equiv.). The solution was stirred at room temperature for four hours. The reaction mixture was poured into 300 mL diethyl ether and was washed with distilled water, cold 2 N HCl, and saturated bicarbonate. The combined organic layers were dried over sodium sulfate, filtered and evaporated to dryness. The crude product was purified using a 2 mm chromatotron rotor and yielded the following results: 60 mg 3,7-diacetates (\(\alpha\)-4d and \(\beta\)-4d, 33.6\% yield, 60\% \(7\beta\) by NMR), 78 mg 7-hydroxy-3-acetate (\(\alpha\)-4b, 43.4\% yield, 100\% \(7\alpha\) by NMR), 5 mg 3-hydroxy-7-acetate (\(\beta\)-4c, 2.8\% yield, 100\% \(7\beta\) by NMR) and 18 mg 3,7-diol (\(\alpha\)-4a, 10\% yield, 100\% \(7\alpha\) by NMR). Total yield: 89.8\%. Yield as \(7\alpha\) products: 66.8\%.

\(\alpha\)-4b: \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 5.09 (tt, C-24, 1-H), 4.06 (s-br, C-7, 1-H), 3.26 (dd, C-3, 1-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.08 (s, C-19, 3-H), 0.96 (s, 3-H), 0.92 (s, 3-H), 0.87 (d, C-21, 3-H), .80 (s, 3-H), 0.74 (s, C-18, 3-H).

\(\beta\)-4c: \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 5.09 (tt, C-24, 1-H), 4.86 (dt, \(J_1=5.1\)Hz, \(J_2=10.8\)Hz, C-7, 1-H), 3.21 (dd, C-3, 1-H), 2.00 (s, 7'-acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.96 (s, C-19, 3-H), 0.94 (s, 3-H), 0.89 (d, C-21, 3-H), 0.83 (s, 3-H), 0.80 (s, 3-H), 0.79 (s, C-18, 3-H).

\(\beta\)-4d: \(^1\)H-NMR (CDCl\(_3\)): \(\delta\) 5.09 (tt, C-24, 1-H), 4.86 (dt, \(J_1=5.1\)Hz, \(J_2=10.8\)Hz, C-7, 1-H), 4.47 (dd, C-3, 1-H), 2.04 (s, 3'-acetate, 3-H), 2.00 (s, 7'-acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.97 (s, C-19, 3-H), 0.89 (d, C-21, 3-H), 0.87 (s, 3-H), 0.85 (s, 3-H), .83 (s, 3-H), 0.79 (s, C-18, 3-H).

\(7\alpha,3\beta\)-oxolanost-24-enyl-3\(\beta\)-acetate (5b). 7.45 g of 7\(\alpha\)-hydroxylanost-24-enyl-3\(\beta\)-acetate (\(\alpha\)-4b) (5.3 mmol) were added to 2 liters of benzene and 500 mL of toluene in a 3 liter round-bottom flask. The mixture was heated to reflux and 250 mL of
benzene was distilled off to remove any residual water. The reaction was kept under an atmosphere of argon at all times. 34.82 g of lead tetraacetate (78.5 mmol, 5.1 equiv.) was added to the solution and the reaction was refluxed for 2.5 hours. The solution was allowed to cool for one hour more, and then 500 mL of 20% potassium iodide was added to quench the reaction. This caused the precipitation of a yellow solid which was redissolved by addition of 400 mL of saturated sodium thiosulfate. The organic and aqueous layers were separated after shaking in a separatory funnel. The aqueous phase was extracted three times with ether, and the combined organics washed twice with distilled water. The organic phase was dried over sodium sulfate, filtered and concentrated under reduced pressure. The concentrated solution of steroids in toluene was diluted with 10:1:1 hexanes/ethyl acetate/methylene chloride and loaded onto a flash silica column equilibrated in that solvent mixture. The column was eluted with 8:1:1 and then 5:1:1. Fractions containing mainly the desired product were recrystallized from methanol/methylene chloride. Side fractions and mother liquors were repurified by column chromatography and recrystallization. Final yields were 4.39 g, 59% yield of desired product (5b), ~20% yield of unreacted starting material (α-4b), and ~10% yield of 7-oxolanost-24-enyl acetate (3b).

5b: $^1$H-NMR (CDCl$_3$): δ 5.08 (tt, $J_1$=3 Hz, $J_2$=7.5 Hz, C-24, 1-H), 4.49 (dd, $J_1$=5.4 Hz, $J_2$=11.7 Hz, C-3, 1-H), 4.17 (s-br, C-7, 1-H), 3.97 (d, $J$=7.8 Hz, C-32, 1-H), 3.33 (d, $J$=6.9 Hz, C-32, 1-H), 2.04 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.91 (d, $J$=6.9 Hz, C-21, 3-H), 0.87 (s, C-19, 3-H), 0.86 (s, 3-H), 0.83 (s, 3-H), 0.81 (s, C-18, 3-H)

3β,32-dihydroxylanostadienyl diacetates ($\Delta^6$-6d, $\Delta^7$-6d, $\Delta^8$-6d). 0.5 g of 7,32-oxolanost-24-enyl acetate (5b, 1.03 mmol) was added to a 250 mL round bottom flask and dissolved in 90 mL acetic anhydride. To this was added 1 g of pyridinium hydrochloride (8.6 mmol, 8.3 equiv.) and the mixture was heated to reflux under an atmosphere of nitrogen and allowed to react overnight. The reaction was quenched by pouring over water-ice and allowed to stand 1.5 hours until the ice had completely melted. The resulting precipitate was filtered and dissolved in methylene chloride. TLC showed the product as a single broad spot consisting of the $\Delta^8$ ($\Delta^8$-6d), $\Delta^7$ ($\Delta^7$-6d) and $\Delta^6$ ($\Delta^6$-6d) dienyl diacetates.

3β,32-dihydroxylanostadienyl-3β-acetates ($\Delta^6$-6b, $\Delta^7$-6b, $\Delta^8$-6b) and 3β,32-dihydroxylanostadienes ($\Delta^6$-6a, $\Delta^7$-6a, $\Delta^8$-6a). The crude dienyl diacetates mixture ($\Delta^6$-6d, $\Delta^7$-6d, $\Delta^8$-6d) was dissolved in 100 mL dry diethyl ether in
a 250 mL round-bottom flask. Small aliquots of lithium aluminum hydride (LAH) were added and the progress of the reaction was followed by TLC. The reaction was allowed to proceed until the amount of 3β-monoacetates was maximized. The LAH was quenched by the addition of 10% aqueous ammonium chloride followed by ether/water extraction. The combined ether layers were dried over sodium sulfate and separated on a 2mm chromatotron rotor by the following procedure. As the solubility of the material was poor in the most efficient solvent mixture, the material was loaded onto a semi-dry rotor in 10:1:1 hexanes/ethyl acetate/methylene chloride spiked with approximately 50% methylene chloride to make a concentrated solution. The rotor was then allowed to spin completely dry under nitrogen gas. The compounds were eluted in a step-gradient of 10:1:1, 5:1:1, and 100% ethyl acetate.

In a typical preparation: 6% 7,32-oxolanost-24-enyl acetate (5b), 5% 3β,32-dihydroxylanosta-6,24-dienyl-3β-acetate (Δ6-6b), 18% a 1:1 mixture of 3β,32-dihydroxylanosta-8,24-dienyl-3β-acetate (Δ8-6d) and 3β,32-dihydroxylanosta-7,24-dienyl-3β-acetate (Δ7-6d), 20% a mixture of 3β,32-dihydroxylanosta-6,24-diene (Δ6-6a) and 7,32-oxolanost-24-ene (5a), 33% a 2:3 mixture of 3β,32-dihydroxylanosta-8,24-diene (Δ8-6a) and 3β,32-dihydroxylanosta-7,24-diene (Δ7-6a). Total yield: 82%. Yield of Δ8 and Δ7 products: 51%. Yield of reclaimed starting material: 15%.

The 1:1 mixture of Δ8 and Δ7 monoacetates was separated on a chromatotron rotor using 8:1 hexanes/EtOAc. A small percentage of methylene chloride was used in the loading mixture to provide a concentrated initial band.

Δ8-6b: 1H-NMR (CDCl3): δ 5.08 (t-br, J=6.9Hz, C-24, 1-H), 4.49 (dd, J1=4.8Hz, J2=11.7Hz, C-3, 1-H), 3.62 (d, J=11.1Hz, C-32, 1-H), 3.22 (d, J=11.1Hz, C-32, 1-H), 2.05 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.90 (s, C-19, 3-H), 0.94 (s, 3-H), 0.90 (s, 3-H), 0.87 (s, 3-H).

Δ7-6b: 1H-NMR (CDCl3): δ 5.56 (m, C-7, 1-H), 5.09 (t-br, J=6.9Hz, C-24, 1-H), 4.49 (dd, J1=4.8Hz, J2=11.7Hz, C-3, 1-H), 3.63 (dd, J1=1.5Hz, J2=10.2Hz, C-32, 1-H), 3.23 (d, J=10.5Hz, C-32, 1-H), 2.05 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.97 (s, C-19, 3-H), 0.93 (s, 3-H), 0.90 (d, J=5.7Hz, C-21, 3-H), 0.87 (s, 3-H), 0.72 (s, C-18, 3-H).

Δ6-6b: 1H-NMR (CDCl3): δ 5.82 (dt, J1=3Hz, J2=10.8Hz, C-6, 1-H), 5.64 (d-br, J=9.9Hz, C-7, 1-H), 5.08 (t-br, J=7.5Hz, C-24, 1-H), 4.51 (dd, J1=4.8Hz, J2=10.8Hz, C-3, 1-H), 4.17 (dd, J1=1.6Hz, J2=10.8Hz, C-32, 1-H), 3.43 (m-br, C-32, 1-H), 2.37 (dm, 1-H), 2.06 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.90 (s, C-19, 3-H), 0.87-0.86 (s-br, C-18, 30, 31 and 32, 12-H).
Δ^8-6a: H-NMR (CDCl₃): δ 5.09 (t-br, J=6.9Hz, C-24, 1-H), 3.62 (d-br, J=9.6Hz, C-32, 1-H), 3.2 (m, C-32 and C-3, 2-H), 2.04 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.00 (s, C-19, 3-H), 0.91 (d, C-21, 3-H), 0.89 (s, 3-H), 0.83 (s, 3-H), 0.70 (s, C-18, 3-H).

Δ^7-6a: H-NMR (CDCl₃): δ 5.37 (m, C-7, 1-H), 5.09 (t-br, J=6.9Hz, C-24, 1-H), 3.62 (d-br, J=9.6Hz, C-32, 1-H), 3.2 (m, C-32 and C-3, 2-H), 2.04 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.04 (s, C-19, 3-H), 0.98 (s, 3-H), 0.91 (d, C-21, 3-H), 0.90 (s, 3-H), 0.72 (s, C-18, 3-H).

Δ^6-6a: H-NMR (CDCl₃): δ 5.81 (dt, J₁=3Hz, J₂=10.8Hz, C-6, 1-H), 5.68 (dt, J₁=2.4Hz, J₂=9.9Hz, C-7, 1-H), 5.08 (tt, J₁=3Hz, J₂=7.5Hz, C-24, 1-H), 4.17 (dd, J₁=1.6Hz, J₂=10.8Hz, C-32, 1-H), 3.43 (d, J=11.7Hz, C-32, 1-H), 3.27 (dd, J₁=5.7Hz, J₂=11.7Hz, C-3, 1-H), 2.37 (dm), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.02 (s, C-19, 3-H), 0.87 (d, J=4.8Hz, C-21, 3-H), 0.86 (s, 3-H), 0.84 (s, 3-H), 0.80 (s, C-18, 3-H).

3β,32-dihydroxylanostadienyl-32-acetates (Δ^7-6c, Δ^8-6c). 3β,32-dihydroxylanostadienes (Δ^8-6a, Δ^7-6a) were dissolved in pyridine and 2-5 equivalents of acetic anhydride were added. The reaction was monitored by TLC until the starting material was >90% consumed, generally less than 8 hours. The reaction was quenched by pouring into water and worked up by extraction with ethyl acetate and 5% HCl, saturated bicarbonate, saturated brine and distilled water washes. The products were separated by chromatatron or silica flash chromatography. Yields of 32-acetates were >75%.

Δ^8-6c: H-NMR (CDCl₃): δ 5.08 (t-br, C-24, 1-H), 3.95 (dd, C-3, 1-H), 3.24 (dd, C-3, 1-H), 1.97 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.99 (s, C-19, 3-H), 0.90 (d, J=6.3Hz, C-21, 3-H), 0.87 (s, 3-H), 0.70 (s, C-18, 3-H).

Δ^7-6c: H-NMR (CDCl₃): δ 5.21 (m, C-7, 1-H), 5.08 (t-br, C-24, 1-H), 4.59 (dd, C-32, 1-H), 3.67 (d, J=10.8Hz, C-32, 1-H), 3.24 (dd, C-3, 1-H), 2.03 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.97 (s, C-19, 3-H), 0.87 (s, 3-H), 0.80 (s, 3-H), 0.70 (s, C-18, 3-H).

3β,32-dihydroxylanosta-7,24-dienyl-3β-acetate-32-mesylate (Δ^7-7b).

Δ^7-7b: H-NMR (CDCl₃): δ 5.30 (d-assym, C-7, 1-H), 5.08 (t-br, C-24, 1-H), 4.51 (dd, J₁=5.4Hz, J₂=10.8Hz, C-3, 1-H), 4.40 (dd, J₁=8.4Hz, J₂=very small, C-32, 1-H), 4.02 (d, J=9.3Hz, C-32, 1-H), 2.93 (s, mesylate-CH₃, 3-H), 2.05 (s, acetate, 3-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.96 (s, C-19, 3-H), 0.92 (s, 3-H), 0.86 (s, 3-H), 0.73 (s, C-18, 3-H).
3β,32-dihydroxylanostadienyl-3β-TBDMS ether (Δ8-6e, Δ7-6e).

3β,32-dihydroxylanostadienyl-32-acetates were dissolved in dry methylene chloride in a flame dried flask under argon and chilled to 0 C. To this was added 3.0 equivalents of pyridine and 1.2 equivalents of t-butyldimethylsilyltriflate (TBDMS-Tf). The reaction was essentially instantaneous. If TLC showed less than 95% conversion, another small aliquot of reagent was added to finish the reaction. The reaction was worked up by ether/water extraction and evaporated. The crude product was dissolved in tetrahydrofuran, methanol and 1 N NaOH (2:1:1) and warmed to reflux. TLC showed complete cleavage of the ester after 2 hours. The reaction was worked up by ether/water extraction, washed with water and saturated brine and dried over magnesium sulfate. Yield, >95%. The double bond isomers (Δ8-6e and Δ7-6e) were separated by flash chromatography in 4:1 hexane-chloroform.

Δ8-6e: 1H-NMR (CDCl3): δ 5.09 (t-br, C-24, 1-H), 4.63 (d, J=9.6Hz, C-32, 1-H), 3.20 (m, C-32 and C-3, 2-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.04 (s, C-19, 3-H), 0.91 (s, 3-H), 0.90 (s, 12-H), 0.70 (s, C-18, 3-H), 0.04 (s, SiCH3, 3-H), 0.03 (s, SiCH3, 3-H).

Δ7-6e: 1H-NMR (CDCl3): δ 5.36 (m, C-7, 1-H), 5.09 (t-br, C-24, 1-H), 4.63 (d, J=9.6Hz, C-32, 1-H), 3.20 (m, C-32 and C-3, 2-H), 1.69 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 1.57 (s, 3-H), 0.90 (s, C-19, 3-H), 0.89 (s, t-butyl, 9-H), 0.86 (s, 3-H), 0.72 (s, C-18, 3-H), 0.04 (s, SiCH3, 3-H), 0.03 (s, SiCH3, 3-H).

Lanosterol-TBDMS ether. Same procedure as above on a 3:2 mixture of lanosterol and 24,25-dihydrolanosterol. The products were separated by silica flash chromatography in 100% heptane.

1e: 1H-NMR (CDCl3): δ 5.10 (t-br, C-24, 1-H), 3.20 (dd, J1=4.5Hz, J2=10.8Hz, C-3, 1-H), 1.68 (s, C-26, 3-H), 1.60 (s, C-27, 3-H), 0.98 (s, C-19, 3-H), 0.91 (d, J=6.3Hz, C-21, 3-H), 0.91 (s, 3-H), 0.89 (s, 9-H), 0.87 (s, 3-H), 0.77 (s, 3-H), 0.69 (s, C-18, 3-H), 0.034 (s, SiCH3, 3-H), 0.029 (s, SiCH3, 3-H).

13C-NMR (CDCl3): δ 15.79 (C-18), 15.94 (C-30), 17.66 (C-27), 18.17 (C-6), 18.49 (CH3-Si), 18.67 (C-21), 19.21 (C-19), 21.07 (C-11), 24.26 (C-32), 24.95 (C-C-23), 25.75 (C-26), 25.95 ((CH3)3C-Si), 26.56 (C-7), 28.24 (C-16), 28.30 (C-31?), 28.44 (CH3-Si), 30.90 (C-15), 31.11 (C-12), 35.62 (C-1), 36.28 (C-20), 36.42 (C-22), 36.95 (C-10), 39.52 (C-4), 44.53 (C-13), 49.86 (C-14), 50.43 (C-17) 50.49 (C-5), 79.48 (C-3), 125.32 (C-24), 130.86 (C-25), 134.30 (C-9), 134.68 (C-8).
MS (HP GC/MS): C_{36}H_{64}OSi, M^+ = 540.49 (calc.) 540.45 (obs.), M^+-CH_3 = 525, M^+-\text{(CH}_3)_3\text{C} = 483, M^+\text{-TBDMSOH-H} = 407, M^+\text{-TBDMSOH-CH}_3 = 393

3β-acetoxylanosta-8,24-dien-32-al (Δ^8-9b). In a flame-dried 25 mL round bottom flask with spin bar and rubber septum under argon and cooled to -60°C in a chloroform dry-ice bath, was placed 700 µl of dry methylene chloride and 211 µl of 2.0 M oxalyl chloride in methylene chloride (422 µmol, 1.15 equiv.). To this solution was added a mixture of 62 µl dry dimethylsulfoxide (891 µmol, 2.4 equiv.) and 180 µl of methylene chloride dropwise via syringe. After 5 minutes, a solution of 3β-acetoxylanosta-8,24-dien-32-ol (Δ^8-6b, 178 mg, 367 µmol, 1.0 equiv.) in 890 µl methylene chloride was added by syringe. This was allowed to stir for 15 minutes and the reaction was then quenched by the slow addition of 300 µl triethylamine. The reaction was allowed to warm to room temperature and distilled water was added. The reaction was worked up by extraction of the aqueous phase with methylene chloride followed by 1% HCl, saturated bicarbonate, and saturated NaCl washes of the organic layer which was then dried over MgSO_4, filtered and evaporated to dryness. Yield: >95%.

Δ^8-9b: ^1H-NMR (CDCl_3): δ 9.43 (s, C-32, 1-H), 5.05 (tm, J=5.7Hz, C-24, 1-H), 4.46 (dd, J_1=4.5Hz, J_2=11.4Hz, C-3, 1-H), 2.05 (s, acetate, 3-H), 1.67 (s, C-26, 3-H), 1.58 (s, C-27, 3-H), 1.08 (s, C-19, 3-H), 0.90 (d, J=7.8Hz, C-21, 3-H), 0.89 (s, 3-H), 0.85 (s, 3-H), 0.75 (s, C-18, 3-H)

3β-t-butyldimethyldisilyloxylanosta-7,24-dien-32-al (Δ^7-9e). The same procedure was used as above was used on 3β,32-dihydroxylanosta-7,24-dienyl-3β-TBDMS ether (Δ^7-6e).

Δ^7-9e: ^1H-NMR (CDCl_3): δ 9.58 (s, C-32, 1-H), 5.42 (m, C-7, 1-H), 5.06 (t-br, C-24, 1-H), 3.18 (dd, J_1=4.5Hz, J_2=11.1Hz, C-3, 1-H), 1.67 (s, C-26, 3-H), 1.59 (s, C-27, 3-H), 1.25 (s, 3-H), 0.90 (d, J=6.3Hz, C-21, 3-H), 0.89 (s, 12-H), 0.85 (s, 3-H), 0.72 (s, C-18, 3-H), 0.04 (s, SiCH_3, 3-H), 0.03 (s, SiCH_3, 3-H).

[32-D]-3β,32-dihydroxylanosta-8,24-dienyl-3β-TBDMS ether (Δ^8-D-6e). 30 mg of 3β-acetoxylanosta-8,24-dien-32-al (Δ^8-9e) was dissolved in about 20 mL of anhydrous ethanol with ~2 mL benzene to aid dissolution of the relatively insoluble aldehyde. Small aliquots of sodium borodeuteride (>98% D) were added; the solution was refluxed and monitored by TLC. When all of the starting material had been consumed the reaction was quenched by the addition of aqueous ammonium chloride and extracted with ether.
3β-acetoxylanosta-8,24-dienyl-32-tosylhydrazone ($\Delta^8$-10b). 25 mg of 3β-acetoxylanosta-8,24-dien-32-al ($\Delta^8$-9b, 1.0 equiv.) was added to a screw-capped vial and dissolved in 2 mL of absolute ethanol. 11.1 mg of tolylsulfonhydrazide (1.2 equiv.) was added to the flask which was then equipped with a reflux condenser. The solution was heated to a vigorous reflux for several hours. The reaction was monitored by TLC. Generally the reaction was completed by 5 hours; occasionally additional tolylsulfonhydrazide must be added to complete the reaction.

$\Delta^8$-10b: \textsuperscript{1}H-NMR (CDCl\textsubscript{3}): δ 7.80 and 7.28 (dd, J=7.8Hz, aromatic, 4-H), 7.33 (m-br, =NNHSO\textsubscript{2}Ar, 1-H), 6.94 (s, C-32, 1-H), 5.07 (tm, J=6.9Hz, C-24, 1-H), 4.49 (dd, $J_1$=4.2Hz, $J_2$=11.4Hz, C-3, 1-H), 2.39 (s, -ArCH\textsubscript{3}, 3-H), 2.05 (s, acetate, 3-H), 1.69 (s, C-26, 3-H), 1.59 (s, C-27, 3-H), 0.99 (s, C-19, 3-H), 0.86 (s, 3-H), 0.84 (s, 3-H), 0.80 (d, J=6.3Hz, C-21, 3-H), 0.68 (s, C-18, 3-H).

3β-$t$-butyldimethylsiloxylanosta-7,24-dienyl-32-tosylhydrazone ($\Delta^7$-10e).

$\Delta^7$-10e: \textsuperscript{1}H-NMR (CDCl\textsubscript{3}): δ 7.84 and 7.28 (dd, J=8.5Hz, aromatic, 4-H), 7.70 (m-br, =NNHSO\textsubscript{2}Ar, 1-H), 7.51 (s, C-32, 1-H), 5.31 (s-br, C-7, 1-H), 5.07 (tm, J=6.9Hz, C-24, 1-H), 3.19 (dd, $J_1$=4.5Hz, $J_2$=11.1Hz, C-3, 1-H), 2.40 (s, -ArCH\textsubscript{3}, 3-H), 1.70 (s, C-26, 3-H), 1.59 (s, C-27, 3-H), 0.89 (s, 12-H), 0.85 (s, 3-H), 0.84 (s, 3-H), 0.80 (d, J=6.3Hz, C-21, 3-H), 0.65 (s, C-18, 3-H), 0.042 (s, SiCH\textsubscript{3}, 3-H), 0.038 (s, SiCH\textsubscript{3}, 3-H).

3β-$t$-butyldimethylsiloxylanosta-7,24-dienyl-32-N,N,N',N'-tetramethylphosphorodiamidate ($\Delta^7$-11e, 3β-TBDM-tranosta-7,24-dienyl-32-TMPDA). 3β,32-dihydroxylanosta-7,24-dienyl-3β-TBDM ether (12b, 1.0 equiv.) was dissolved in 4:1 freshly distilled (from CaH\textsubscript{2}) 1,2-dimethoxyethane and tetramethylmethylenediamine (TMEDA) in a dried flask under argon with magnetic stirring. In a second dried flask under argon was added freshly distilled tetrahydrofuran and freshly distilled diisopropylamine (1 mmols) and cooled to 0°C on ice. \textit{n}-butyllithium (1 mmol of a 2.0 M solution in hexanes) was added slowly by syringe. 1.5 equiv. of the lithium diisopropylamide, thus generated, was added to the initial reaction flask slowly by syringe and allowed to stir 15 min. 5.0 equiv. of dimethylphosphorodichloridate (DMPADCl) was added to the solution by syringe and allowed to stir for several hours. When the reaction was complete by TLC (4-8 hours), the reaction flask was cooled to 0°C and an excess of dimethylamine was added by pasteur pipette. (NOTE: dimethylamine is...
extremely volatile and must be stored and used on ice at all times.) After 5 minutes the reaction was allowed to warm to room temperature and was worked up by ether-water extraction with rinses with 1% HCl, saturated NaHCO₃, and saturated brine. The combined organics were dried over magnesium sulfate and filtered. Yield, 79%.

\( \Delta^7\text{-llle:} \) \( ^1\text{H-NMR (CDCl}_3\text{): } \delta 5.24 \text{ (d-br, } J=5.4, \text{ C-7, 1-H), 5.09 \text{ (t-br, C-24, 1-H), 4.08 \text{ (dm, } J=7.2\text{Hz, C-32, 1-H), 3.60 \text{ (dd, } J_1=3.3\text{Hz, } J_2=9.3\text{Hz, C-32, 1-H), 3.17 \text{ (dd, } J_1=4.2\text{Hz, } J_2=11.1\text{Hz, C-3, 1-H), 2.62 \text{ (dd, } J_1=9.6\text{Hz, } J_2=23.7\text{Hz, P(N(CH}_3\text)\text}_2, 12\text{-H), 1.68 \text{ (s, C-26, 3-H), 1.60 \text{ (s, C-27, 3-H), 0.89 \text{ (s, t-butyl and one steroid methyl, 12-H), 0.85 \text{ (s, 3-H), 0.83 \text{ (s, C-18, 3-H), 0.71 \text{ (s, C-18, 3-H), 0.045 \text{ (s, SiCH}_3, 3-H), 0.031 \text{ (s, SiCH}_3, 3-H).}}}

3\text{β-t-butyldimethylsilyloxylanosta-8,24-dienyl-32-N,N,N',N'-tetramethylphosphorodiamidate} (\Delta^8\text{-llle, 3β-TBDMSLanosta-8,24-dienyl-32-TMPDA}). Same procedure as above. With this isomer, however, the yield is poor under these conditions (12%) apparently due to rearrangement/elimination of the TMPDA (see Tactics section above).

\( \Delta^8\text{-llle:} \) \( ^1\text{H-NMR (CDCl}_3\text{): } \delta 5.07 \text{ (t-br, C-24, 1-H), 3.84 \text{ (dd, } J_1=3.4\text{Hz, } J_2=9.2\text{Hz, C-32, 1-H), 3.67 \text{ (dd, } J_1=2.2\text{Hz, } J_2=8.8\text{Hz, C-32, 1-H), 3.11 \text{ (dd, } J_1=5.0\text{Hz, } J_2=11.0\text{Hz, C-3, 1-H), 2.65 \text{ (dd, } J_1=9.6\text{Hz, } J_2=23.7\text{Hz, P(N(CH}_3\text)\text}_2, 12\text{-H), 1.68 \text{ (s, C-26, 3-H), 1.60 \text{ (s, C-27, 3-H), 0.98 \text{ (s, 3-H), 0.89 \text{ (s, t-butyl, 9-H), 0.86 \text{ (s, 3-H), 0.77 \text{ (s, C-18, 3-H), 0.69 \text{ (s, C-18, 3-H), 0.040 \text{ (s, SiCH}_3, 3-H), 0.028 \text{ (s, SiCH}_3, 3-H).}}}

Lanosterol-TBDM ether (1e). Into a flame-dried 25ml three neck flask cooled to 0 C, with a glass-sheathed spin bar, gas inlet and dewar condenser with dry ice/acetone was condensed ~5ml of anhydrous methylamine. To this was added a small lump of lithium metal (large excess). This was allowed to stir until the solution turned deep blue/black. 8.7 mg of 3\text{β-TBDMSLanosta-8,24-dienyl-32-TMPDA} (\Delta^8\text{-llle}) was dissolved in freshly distilled THF and added to the reaction mixture by syringe. After reacting at 0 C for 5 minutes the reaction was quenched by the addition of distilled water. The mixture was extracted with diethyl ether, rinsed with water and saturated brine, dried over magnesium sulfate and filtered. TLC shows >90% reaction to a product which co-spots (0.05 Rf units) with authentic lanosterol-TBDM ether in 100% pentane. \( ^1\text{H-NMR, } ^{13}\text{C-NMR, GC retention time and mass spectroscopy are all identical with the authentic standard.} \)
Proposals for future research

Syntheses of Hemi-lanosterols as building-blocks for structural probes and drugs

In light of the need for drugs which specifically bind to the active site of lanosterol 14α-demethylase and other closely related enzymes (which bind lanosterol derivatives as discussed in the main text), and considering the scientific need to develop probe molecules which can be used to determine the amino acid residues lining the active site, we have considered the utility of a modular design. Inhibitors and probes containing either the A and B rings, or the C and D rings of lanosterol and other variable groups may have particular advantage because of their built-in binding characteristics. These modular probes have been dubbed "hemilanosterols," and several specific compounds based on them have been imagined with various uses (Figure 13).

Two routes to these hemilanosterols are proposed. The first, based on some degradative chemistry presented in the Mechanisms section of the introduction, above,\(^4\)\(^3\)\(^4\) gives access simultaneously to the precursors of both the AB and CD hemilanosterol (Figure 14). The second is based on the classic route of total steroid synthesis, the Robinson annelation (Figure 15) and accesses only the AB hemilanosterol. Both of these routes were briefly investigated by a graduate student in our labs who has since departed (S. Lokey).

Preparation of lanosterol affinity column material

The purification of 14α-demethylase is, at present, rather tedious, and would be streamlined considerably if an affinity method were available for specific adsorption of the protein. It has been shown that the binding of lanosterol is mediated, in part, by a specific interaction with the 3β-hydroxyl group.\(^1\)\(^3\)\(^2\)\(^6\)\(^\)\(^\)\(^\)\(^\)\(^\) Studies on altered side-chains of lanosterol have shown that significantly shortened side-chains impair turnover of the enzyme,\(^1\)\(^2\)\(^6\) but no studies have investigated the binding characteristics of unnaturally long side-chains. These can be synthesized using a method based on Sato's work: ozonolysis of lanosterol yields the tris-norlanosterol-24-aldehyde.\(^1\)\(^2\)\(^6\)\(^\)\(^\)\(^\)\(^\)\(^\) This could be elaborated by Wittig condensation with the phosphorus ylide of a long-chain halo-amine such as 1-amino-7-
bromooctane or 1-amino-8-bromooctane. Should this type of compound show affinity for the enzyme, it could be tethered to sepharose column material via the amino group using cyanogen bromide chemistry.
Figure 13: Some potential photo-affinity probes, mechanism-based inhibitors and radical clock probes based on hemilanosterol skeletons.
Figure 14: Proposal for synthesis of hemilanosterols AB and CD via degradation of Lanosterol. Starting material, either dihydrolanosterol or suitably protected lanosterol. (a) Pb(OAc)$_4$ followed by OsO$_4$ or modern equivalent (after Castells, 1956); (b) α-oxidation by MoO$_5$ or other oxidant; (c) CrO$_3$, separation of diacid products (all subsequent steps are shown for AB-compounds only, but they are identical for the BC version); (d) SOCl$_2$ 1 eq.; (e) MeLi; SOCl$_2$; n-BuLi; (f) NaH; R$_2$Br; BF$_3$, dithioethane; Raney nickel; (g) TsNNH$_2$; n-BuLi, R$_1$Li.
Figure 15: Proposal for synthesis of hemilanoesterol AB via Robinson annelation. (a) H₂O, 24 h.; DMSO, L-proline, 5 days; (b) HOCH₂CH₂OH, p-TsOH; Li-NH₃; MeI; (c) LAH, TBDMSOTf, AcOH, H₂O; (d) LDA, R₂Br; TsNHNH₂; n-BuLi, R₁Li.
Appendix

Evidence supporting the assignment of structure of two Li-ammonia-toluene reduction side-products as 3β,8β-dihydroxy-7-oxolanost-24-enol and its corresponding 3β-acetate.

1. Ring A and the side-chain of the steroid are unchanged as evidenced by NMR, the 3β-alcohol and 3β-acetate of the compound have both been isolated.

2. The compound is not oxidizable back to the starting material by chromium trioxide-pyridine in methylene chloride, however, standing in chloroform for several days does result in partial reversion to the conjugated ketone, probably through trace HCl catalysis (β elimination of an alcohol?).

3. The compound contains a second alcohol group. This is supported by IR of the 3β-acetate (broad peak at 3330 cm\(^{-1}\)), and by the esterification of the 3β-acetate to a less polar product by acetic anhydride in pyridine.

4. The compound contains a ketone. IR of the 3β-alcohol shows a carbonyl stretch (1708 cm\(^{-1}\)). However, there seemed to be no reaction of the compound with LAH in THF after 10 minutes (perhaps this reduction is slow, this was not investigated further).

5. \(^{13}\)C-NMR shows a ketone resonance at 206 ppm and a resonance at 94 ppm which is consistent with a hindered tertiary alcohol. The 3β-alcohol and acetate appear in their usual positions as do the vinyl carbons of the side-chain; no other double bonds are present. Proton-coupled \(^{13}\)C-NMR confirms that the 94 ppm resonance corresponds to a carbon with no attached protons.

6. \(^{1}\)H-NMR is not consistent with a 9-hydroxy-7-ketone. This compound would have a singlet due to the isolated proton at C-8. This singlet should be well down-field due to the adjacent alcohol and ketone; no such singlet is observed. In contrast, what is observed is a triplet at 2.85 ppm and a doublet at 2.25 ppm which may correspond to a 9α-proton (coupled to two protons on C-11) and one of the two C-6 protons (the other falling closer to 2.0 ppm among some other unresolved resonances), respectively. This is consistent with the assigned structure.

7. The \(^{13}\)C-NMR spectra of 7-oxolanosta-24-enol and its acetate (3a and 3b) were completely assigned by comparison with published literature spectra of lanostane derivatives,\(^{(49)}\) H-coupled \(^{13}\)C-NMR spectra and 2-D hetero-correlation (HetCor) and COSY NMR, and comparison of typical acetate-shift correlations. Comparison of this standard with the \(^{13}\)C spectrum of the unknown yielded unambiguous assignment of all of
the unknown resonances (5 ppm, 22 carbons within 1 ppm) except for carbons 8 and 9. Assignment of the compound as the 9-alcohol was inconsistent because the carbons alpha to the alcohol-bearing carbon are only shifted by -0.35, -0.42 and -1.98 ppm (C-8, C-10 and C-11, respectively), while the carbonyl at C-7 is shifted by +4.86 ppm and the C-6 carbon by +2.4 ppm compared to 3a and 3b. Assignment as the 8-alcohol yields more realistic shifts of +4.86, -4.93 and -4.17 ppm (C-7, C-9 and C-14) as well as the +2.4 ppm shift of C-6.
References


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CHAPTER 3

PRELIMINARY STUDIES ON THE
ACTIVE SITE STRUCTURE OF P-450_{Scc}:
A REPORT ON RESEARCH IN PROGRESS
Preface

The study of the cholesterol side-chain cleavage enzyme (P-450\textsubscript{scc}) has been an active research area in the Orme-Johnson research lab for many years. My contribution to this project has been brief, but I include this account here as a record for those who take on this work after me. I am indebted to my predecessors, Subhendu Joardar, Emily Miao, Atsushi Nagahisa and many others before them. The experiments described below are quite preliminary and were done in an exploratory manner. The account given here serves mainly to suggest a heretofore unappreciated complexity in the inhibition of SCC by certain alkyne-containing compounds. For this reason little has been conclusively shown, but the indications for future researchers are that certain assumptions cannot be made and experiments must be designed with considerable caution to avoid false leads and ambiguous results.

In the introduction to the thesis of Dr. Subhendu Joardar is found an extensive discussion of the properties of this enzyme, its biological niche and research on its inhibition along with a thorough list of references. My discussion here, then, will be brief and I refer readers to his thesis and several other excellent reviews referenced therein.(9)

Previous Research on the Active Site Structure of P-450\textsubscript{scc}.

The side-chain cleavage enzyme is found in the adrenal cortex, testis, ovary and placenta of mammals and is responsible for the conversion of cholesterol to pregnenolone via a three-cycle (6 electron) oxidation in which the side chain of cholesterol is removed as isocapraldehyde (Scheme 1). From pregnenolone all of the other steroid hormones are synthesized (Scheme 2). Side chain cleavage is the rate limiting step in steroidogenesis and is hormonally regulated. Thus, this step can serve as a point at which steroidogenesis can be arrested by inhibition. This has been suggested as a desirable therapeutic regimen for the treatment of prostate and other steroid hormone-dependent cancers and may have other clinical utility.

Because of the membrane-bound nature of mammalian P-450's, these proteins have resisted attempts to form crystals of suitable quality for the determination of x-ray crystal structures. Due to this difficulty, indirect methods have been devised to illuminate the three-dimensional structure of P-450 active sites. These techniques fall into three categories: chemical, spectroscopic and computational; which, together, may provide considerable insight into the matter.
Chemical techniques encompass covalent modification of the active site residues and the heme cofactor. Mechanism-based inhibitors (see below) and photoaffinity probes are designed to bind specifically to the active site and either by enzymatic turnover or activation by light, generate a highly reactive species there which will form a covalent link with the surrounding active site components. Subsequent analysis of the peptide reveals the location of the link and the identity of a segment of the protein which borders the active site. Heme-directed probes which ligate to the iron atom can be made to rearrange and modify the heme itself. Analysis of the modification pattern can reveal those areas of the heme which are exposed, and which are blocked by the protein. (23)

Spectroscopic techniques have been developed to determine the precise orientation of a bound inhibitor or substrate with respect to the heme iron. EXAFS (Extended X-ray Absorption Fine Structure) is used to determine the radial distance from the iron atom (the X-ray absorber) to nearby surrounding atoms (the backscatterers of emitted photons). This technique requires a strong source of polychromatic (white) X-rays such as that provided by Brookhaven's National Synchrotron Light Source. ESEEM (Electron Spin Echo Envelope Modulation) can be used to determine the distance between the iron atom (the paramagnetic species) and nearby NMR-active nuclei. Use of an appropriate inhibitor
Scheme 2: The biosynthesis of mammalian steroid hormones from pregnenolone
strategically labeled with deuterium, one can use these techniques in tandem to define parameters of the conformation and relative orientation of a bound inhibitor.

Since P-450's comprise a large group of related genes it is theoretically possible to model the three dimensional structure of a particular P-450 protein by comparison with one for which an accurate structure is known using computational methods. At this time, the only structure available is that of the camphor hydroxylase (P-450\textsubscript{cam}) from \textit{Pseudomonas putida}. This P-450 is, unfortunately, only distantly related to the mammalian P-450's, making accurate comparisons difficult. However, the active site regions of P-450's are apparently more closely conserved than the protein in total including the surface and membrane-binding regions, and thus, modeling of this region only can reasonably be expected to yield meaningful results.\cite{9}

In the previous ten years several effective competitive and mechanism-based inhibitors of P-450\textsubscript{scc} have been developed in the Orme-Johnson labs (Figure 1).\cite{11,14-16,29} These have been used to help implement the above program of inquiry into the active site structure of P-450\textsubscript{scc}. In particular, the mechanism-based inhibitor 20-(1,5-hexadiynyl)-5-pregnen-3β,20α-diol (5, 20-HDY), was notable because, along with time-dependent inactivation of the enzyme, it was subsequently discovered that the steroid becomes covalently linked to the protein.\cite{13} This was unexpected, because previous examples of alkyne-containing inhibitors in the literature tended to covalently label the heme.\cite{20-22,24} It is believed that this type of inhibitor is converted into an oxirene by oxygen insertion into an acetylene π-bond. This reactive species, which can be alternately viewed as an oxygen-substituted vinyl cation, vinyl radical or an α-oxocarbene, could trap weak protein nucleophiles such as threonine, serine, aspartate, glutamate, lysine or tyrosine or insert into carbon-carbon or carbon-nitrogen bonds (Scheme 3).\cite{13}

The obvious next experiment, to determine the site of label incorporation, was not performed at that time because of the absence of any model for the active site of SCC. Instead, a program to develop such a model was begun.

The mechanism-based inhibitor (not an inactivator), (20S)-22-thiacholesterol (1) has been shown to be oxidized by the action of SCC to give (20S,22R)-22-thiacholesterol-S-oxide (2), a particularly tight-binding compound (\(K_d=0.1\mu\text{M}\)).\cite{11,29} The resulting enzyme-inhibitor is considered to be a model for the binding of the first oxidative
Figure 1: P-450<sub>sc</sub> inhibitors and inactivators studied in the Orme-Johnson laboratories

(1) (20S)-22-thiacholesterol
(2) (20S,22R)-22-thiacholesterol-S-oxide
(3) (20R)-20-(1-hexynyl)-pregn-5-en-3β,20-diol (20-HY)
(4) (20R)-20-(4-methyl-1-pentynyl)-pregn-5-en-3β,20-diol (20-MPY)
(5) (20R)-20-(1,5-hexadiynyl)-pregn-5-en-3β,20-diol (20-HDY)
(6) (20S)-20-(2-trimethylsilyl-ethyl)-pregn-5-en-3β,20-diol
(7) (22R)-22-aminocholesterol
intermediate in side chain cleavage, (22R)-22-hydroxy-cholesterol. The presence of sulfur atom in the place of a carbon in the natural substrate is fortuitous, because it allows for detection of the Fe-S (3.01 Å) distance by EXAFS. Coupled with the Fe-O distance of this complex (1.99 Å), and the crystallographically determined S=O bond length of the sulfoxide (1.5 Å), it is possible to calculate the angle $\angle$Fe-O-S using the law of cosines (118.5°).\(^{(9, 10)}\) The synthesis of (20R)-20-D-sulfoxide allowed for determination of the distance between the iron and this deuterium atom as 4.2 Å using ESEEM. This distance
serves to constrain the torsions about the S=O bond and the S-C20 bond somewhat (Figure 2).\(^9\),\(^10\) Further experiments with deuterium labels at different positions will continue to refine the model for substrate binding at the SCC active site. Earlier work on C-22 deuterium-labeled (22R)-22-hydroxy-cholesterol showed a distance of 4.0Å between that deuterium and the heme iron.\(^8\)

![Figure 2: One possible conformation of the bound sulfoxide inhibitor (2) yielding a distance of 4.2Å between the deuterium and the heme iron. T\(_1\) and T\(_2\) are the torsion angles defined by Fe-O=S-C\(_{20}\) and O=S-C\(_{20}\)-C\(_{21}\), respectively. (0 = all in the same plane in cis conformation) The S=O and S-C\(_{20}\) bonds have been set to the crystallographically determined lengths of 1.5Å and 1.8Å, respectively. The ∠Fe-O=S angle has been set to 118.5°.](image)

The active site region of several steroidogenic active sites, including that of SCC, have been modeled in this lab using multiple sequence alignments based on sequence identity, conservative replacements and hydropathy-based secondary structure prediction. These techniques were used to locate the helical regions lining the active site that correspond to the analogous regions found in the crystal structure of P-450\(_{cam}\). These helices (helix I and helix L), and their neighboring stretches of peptide were then modeled by computer into the crystal structure model of the active site of P-450\(_{cam}\), first by substituting the SCC regions for the CAM regions in a series of "point mutations". After each amino acid change the side-chain torsions were modified to minimize steric clashes with the rest of the sequence. After all of the residues were mutated, the entire structure
was energy minimized. Finally, a model of (22R)-22-hydroxy-cholesterol was placed in the active site using the constraints found from the EXAFS and ESEEM studies discussed above, and the complex was minimized again.\(^{(9)}\)

From this model it has been predicted that a particular stretch of amino acids along Helix I are the most likely to be covalently modified by the alkynyl mechanism-based inhibitors. This region includes Thr290, Thr291 and Thr294 as possible nucleophiles to attack any turnover-generated electrophile from the inactivator acetylene (see Scheme 3).

**Research goals**

The goals of this research project, then, are to lay the groundwork for the determination of the site of labeling by the inhibitor, \(20-(1,5\text{-hexadiynyl})-5\text{-pregnen}-3\beta,20\alpha\text{-diol}\) (5, 20-HDY). Several points needed to be investigated to assess the feasibility of this project:

1) Reconfirm the covalent nature of the adduct between 20-HDY and the protein.

2) Assess the stability of this adduct; will it hydrolyze or otherwise cleave during the planned procedures.

3) The monoyne inhibitor \(20-(1\text{-hexynyl})-5\text{-pregnen}-3\beta,20\alpha\text{-diol}\) (3, 20-HY) has been shown to be an inactivator, although a poorer one than 20-HDY. However, this inhibitor was not assayed for its protein-labeling efficiency. The \(20-(4\text{-methyl-1-pentynyl})-5\text{-pregnen}-3\beta,20\alpha\text{-diol}\) (4, 20-MPY), which more closely approximates the natural cholesterol side chain has been synthesized but had not been evaluated as an inhibitor or inactivator. The important point to evaluate here is whether the effectiveness of 20-HDY is due to steric factors which give it a better binding constant, and thus a higher rate of inactivation, or rather the presence of the second triple bond is critical for the labeling of the protein for chemical reasons.

4) Is the labeling by 20-HDY specific to a particular site on the protein or are there a number of discrete peptide-steroid adducts.

5) Under what conditions can the protein be mildly unfolded without aggregation or precipitation such that cleavage by proteases will be exhaustive and not yield overlapping or overly large fragments.

6) What conditions are necessary to isolate the steroid-containing peptide fragment after proteolysis (since the peptide-steroid conjugate is expected to be quite hydrophobic).
Materials and Methods

Protein Purifications

P-450_{scc} was purified from bovine adrenal glands via published procedures by Orme-Johnson\(^{(18)}\) and Tsubaki\(^{(27)}\). In brief, the cortices of the bovine adrenals were separated from the internal medullar tissue, suspended in buffer, homogenized in a Waring blender, and centrifuged to remove cellular debris. This supernatant is sonicated to break the mitochondria, and ultracentrifugation yields the P-450-containing membrane fraction in the pellet. The pellet is resuspended and homogenized in buffer and then potassium cholate is added to solubilize the membranes. Centrifugation removes insoluble material, and the supernatant is partitioned by ammonium sulfate precipitation. P-450_{scc} is concentrated in the 33-45% saturation fraction. This precipitate is resolubilized in buffer and batch adsorbed onto DEAE-cellulose equilibrated in buffer containing the detergent Emulgen 913 at room temperature. This material is poured into a column and eluted with the detergent-containing buffer. The P-450-containing fractions are pooled, concentrated using an Amicon membrane cell, and loaded onto a column of hydroxyapatite in the same buffer. The column is eluted with a step gradient of 10, 20, 40 and 80 mM potassium phosphate buffer containing detergent. P-450-containing fractions are pooled, separated into 10 ml portions, frozen in liquid nitrogen and stored for use at -80°C.

P-450 content is measured by the CO-binding difference spectrum of the dithionite reduced protein at 450 nm by the method of Omura and Sato\(^{(17)}\). UV/Visible spectra were acquired using a Perkin-Elmer 557 double wavelength, double beam spectrophotometer. Total protein content was measured with the Bradford dye-binding assay\(^{(2)}\) using a kit supplied by BioRad. Measurements were made using a Hewlett Packard 8452A diode array spectrophotometer. The P-450 used for this project had a P-450 to protein ratio of 5.4 nmoles/mg, which was deemed sufficient for these studies (SDS gel electrophoresis showed only minor impurities).

Adrenodoxin was purified by the method of Orme-Johnson\(^{(19)}\) as follows. The preparation is identical to that for SCC up to the stage of sonication. For adrenodoxin and adrenodoxin reductase (see below), the supernatant following sonication was used. This was fractionated by ammonium sulfate precipitation. The adrenodoxin is concentrated in the 60-95% saturation fraction. This precipitate is resolubilized in and dialyzed against Tris buffer and then concentrated on a column of DE-52 (DEAE cellulose) eluted with high salt buffer. The proteins are then separated by size on a sephadex G-50 gel filtration column. Column fractions were assayed spectrophotometrically; all fractions with A414/A280 ratios
greater than 0.25 were pooled and frozen at -20 C until use. A complete spectrum showed peaks at 258, 320, 414 and 454nm which matched published spectra.

Adrenodoxin reductase was purified by the method of Sugiyama(25). The method is identical to that for adrenodoxin up to the stage of ammonium sulfate precipitation. Here, adrenodoxin reductase is concentrated in the 30-60% saturation fraction. The precipitate is resuspended in buffer, dialyzed and applied to an affinity column constructed of adrenodoxin covalently linked to sepharose. The bound adrenodoxin reductase is washed free of contaminating proteins and then eluted with high salt. Fractions with low 280/450nm ratios are pooled and then dialyzed against low salt buffer. Again, full spectra were identical to published standards.

Measurement of SCC Activity and Inhibition

P-450 catalysis of pregnenolone formation from cholesterol was assayed by the method of Takikawa with modifications by Nagahisa.(13, 26) Typically assay constituents were 2 µM adrenodoxin, 0.1 µM Adrenodoxin reductase, 1 unit/mL catalase, 1 unit/mL glucose-6-phosphate dehydrogenase, 10 mM magnesium chloride, 32 µM NADPH, 3 mM glucose-6-phosphate, 50 mM MOPS, 0.2% Tween 20, 100 µM cholesterol, and 20,000 cpm 7-[3H]-cholesterol. The assay mixture was pH 7.2. For SCC specific activity determination the P-450 content was varied from 0 to 0.25 µM. Incubations were carried out at 37 C for 15 minutes. At 15 minutes the reaction was halted by the addition of 1 N HCl and [14C]-pregnenolone was added as an internal standard. The mixture was passed through a pipette column of C-18 reverse phase gel (from Sep-Pac cartridges, Waters Co.), washed with distilled water and the steroids were eluted with methanol. The methanol was evaporated to dryness and the steroids were chromatographed by analytical TLC versus authentic standards. The pregnenolone bands were isolated, eluted with ethyl acetate and counted by scintillation using a Beckman LS-2800 liquid scintillation counter, all measurements were corrected for detector efficiency, sample quench and background, and monitored for chemiluminescence.

The specific activity of the protein used in these studies was measured as 8.6 nmoles pregnenolone formed per nmole P-450 per minute. It was desirable for the P-450 steroid labeling studies to have as little other protein in the incubation mixture as possible. Adrenodoxin reductase, glucose-6-phosphate dehydrogenase and catalase are only used in small amounts. Adrenodoxin, on the other hand, is used in excess to insure a maximal rate of turnover since the delivery of electrons to the P-450 by adrenodoxin is the rate limiting
step in the reaction. A study of the adrenodoxin dependence of the turnover rate showed that anything less than a two-fold excess of adrenodoxin resulted in a reduced turnover rate under our conditions.

For inhibition studies the cholesterol was replaced by inhibitor in the assay mixture and the entire assay mixture was made three times more concentrated. The SCC/adrenodoxin ratio was also increased to 0.5 (ranges from 0 to 0.125 were used in the activity assays). The purpose of this modification was to expedite the further processing of the inhibitor-labeled SCC.

Glucose-6-phosphate dehydrogenase, glucose-6-phosphate, cholesterol, MOPS, NADPH and catalase were purchased from Sigma. Cholesterol was recrystallized two times from methanol/ethanol prior to use. Radioactive cholesterol and pregnenolone were purchased from New England Nuclear and purified by analytical thin-layer chromatography versus authentic standards before use. Inhibitors were prepared as described below.

Synthesis and Characterization of Inhibitors

All three inhibitors used in this study were synthesized by the same method using different alkynes (Scheme 4):\(^{12,13}\)

To a stirred solution of alkyne (6 equiv.) in freshly distilled tetrahydrofuran under nitrogen at -78 °C was added dropwise a solution of n-butyl lithium in hexanes (5 equiv.). The solution was stirred 10 min, followed by addition of pregnenolone (1 equiv.). in dry tetrahydrofuran. The reaction was allowed to warm to room temperature and was monitored by TLC. The reaction was generally complete after 40 minutes. The reaction was quenched with water, extracted with ethyl acetate, dried over sodium sulfate and evaporated to dryness. The crude product was recrystallized from ethyl acetate and hexanes. \(^1\)H-NMR was used to prove structure and purity.

Larger excesses of alkyne and butyllithium were used when synthesizing the radioactive inhibitors, but it is critical that an excess of alkyne is used over the butyllithium, and sufficient time is allowed for the reaction between the two, otherwise residual butyllithium can react with the pregnenolone leading to side products. The reaction is otherwise the same except the crude product was purified by analytical TLC.

20-(1,5-hexadiynyl)-5-pregnen-3β,20α-diol (5, 20-HDY) was synthesized by me. NMR matched that reported by Nagahisa.\(^{13}\)

Two batches of tritium labeled 20-HDY were used in this study. The first was synthesized by Nagahisa in 1983 and repurified by me using TLC. Specific activity was determined using a microgram balance and scintillation counting to be 5.3 nCi/nmol. Later
a second batch was synthesized by me having a specific activity of 800 nCi/nmol, this was
diluted with cold inhibitor as needed for labeling studies.

20-(1-hexynyl)-5-pregnen-3α,20α-diol (3, 20-HY) was synthesized by E. Miao in
1989. NMR of that material showed it to be pure and undegraded.

4-[14C]-20-HY was synthesized by me and had a specific activity of 56 nCi/nmol.
20-(4-methyl-1-pentynyl)-5-pregnen-3β,20α-diol (4, 20-MPY) was synthesized
by S. Joardar in 1989. NMR of that material indicated it to be pure and undegraded. No
isotope-labeled version of this compound was used in these studies.

The alkynes (1,5-hexadiyne, 1-hexyne and 4-methyl-1-pentyne) were supplied by
Lancaster Synthesis Ltd. 7-[3H]-pregnenolone and 4-[14C]-pregnenolone were purchased
by New England Nuclear. n-butyllithium was purchased from Aldrich.

Measurement of Covalent Attachment of Inhibitor to Protein

In order to show covalent attachment of inactivators to the protein three types of
experiments were performed:

1) Dialysis. Enzyme incubated with inhibitors was dialyzed against cholesterol-
containing buffer in a micro-dialysis cell. The cell was constructed from of a 500μL
Eppendorf tube, in the cap of which had been bored a 5mm diameter hole. A small square
of dialysis tubing was placed over the mouth of the tube and the bored cap was closed over
it. The tube was then placed in a styrofoam block floating inverted in the dialysis buffer.
A pasteur pipette bent into a U-shape was used to remove any air bubbles obstructing the
dialysis fluid from the dialysis membrane. After several changes of buffer the residual
activity was measured by the turnover assay described above versus non-
inhibited(dialyzed) and inhibited(non-dialyzed) and non-inhibited(non-dialyzed) controls.

2) Hydroxyapatite chromatography. After incubations with radioactive inhibitor the
protein mixture was diluted with low-salt, detergent containing buffer and applied to small
(pasteur pipette) columns of hydroxyapatite equilibrated in that buffer. When the non-
bound inhibitor ceased eluting from the column with low-salt washes (scintillation counting
returned to baseline) the protein was eluted with high-salt buffer. The residual radioactivity
in the protein fraction was measured by scintillation.

3) Acetone/HCl protein precipitation. (A similar procedure has been used to
prepare apo-P-450cam free of heme and steroid\textsuperscript{(13, 30)}. An aliquot of SCC-inhibitor
incubation was quenched into a tube containing 1 mL of ice-cold acetone/HCl (500:1),
vortexed, and placed on ice for 15 min. The resulting precipitate was centrifuged and the
supernatant decanted. This was repeated until the supernatant showed no residual
radioactivity (usually 2-3 washes). Sometimes the resulting pellets were unable to be
resuspended by vortexing, so they were sonicated in a sonication bath. The final pellet was
dissolved in 6 M Guanidine/HCl buffer (pH 7.0) and counted by scintillation. Nagahisa
reported the protein recovery in this technique to be 78\%.\textsuperscript{(13)}

Cleavage of Inhibitor-labeled SCC into two Fragments, \( \Phi_1 \) and \( \Phi_2 \), by
Trypsinolysis and Separation of these Fragments for Analysis

The following procedures are modifications of those reported by Chaschin.\textsuperscript{(1, 6, 7)}
Trypsin was added directly to the enzyme inhibition incubations after they had cooled from
37 C to 25 C. A 1:30 trypsin:SCC molar ratio was used, and the mixture was allowed to
react 15-20 minutes with occasional swirling of the reaction tube. The reaction was then
quenched by the addition of 2\( \mu \)L trypsin inhibitor on agarose beads for every 1\( \mu \)g of
trypsin used. The trypsinized mixture is centrifuged to remove the trypsin-trypsin
inhibitor-sepharose complex and the supernatant is diluted 4-fold with low-salt, detergent-
containing buffer and applied to a pipette column of hydroxyapatite. The column is washed
free of excess inhibitor with low salt buffer and then the proteins are eluted with high-salt
buffer. SDS-PAGE of an aliquot of the mixture at this time shows nearly quantitative
conversion of the SCC to two fragments at approximately 27 and 22 kilodaltons (\( \Phi_1 \) and
\( \Phi_2 \), respectively). The protein solution is adjusted to 1 M NaCl and 1 mM EDTA and then
applied to a column of "activated" thiopropylsepharose 6B at a flow rate of 1 mL/min.
After washing with high-salt buffer and then low-salt buffer, the \( \Phi_1 \) fragment is eluted from
the column using guanidine buffer (50 mM potassium phosphate, 0.1 mM EDTA, 7 M
guanidine-HCl, pH 7.2) and then the remaining bound $\phi_2$ fragment is released using the same buffer containing 0.1 M $\beta$-mercaptoethanol. SDS-PAGE shows only small cross-contamination of the two fragments by each other. (This technique is based on the convenient circumstance that SCC possesses only one surface cysteine which serves to crosslink the protein to the thiopropyl sepharose. The guanidine unfolds the protein, releasing fragment $\phi_1$, which is not covalently attached to the column support.)

Trypsin, trypsin inhibitor-sepharose and activated thiopropyl sepharose were purchased from Sigma. SDS-PAGE was performed using the Phast system from Pharmacia.

**Results**

**Inactivation by Various Alkyne-Containing Steroids**

Nagahisa reports a ~60% loss of residual activity when the inhibitor 20-HY (3) is incubated with SCC ([SCC]=1.3 $\mu$M, [20-HY]=18 $\mu$M) under turnover conditions for 1 hour compared with SCC incubated for 1 hour without added steroid. Under the conditions used here ([SCC]=4 $\mu$M, [20-HY]=100 $\mu$M) for 2 hours compared to SCC incubated for 2 hours in the presence of cholesterol followed by dialysis and then turnover assay, only a 39% loss of activity is observed. The inhibitor 20-MPY (4) results in a 50% loss of activity under these conditions. However, this experiment was only done once, and it was noted at the time that the recovery of steroid from the incubations was quite low, probably due to excessive warming of the extracts while evaporating the methanol, and thus the reliability of these numbers is questionable.

Since the 20-MPY inhibitor had not been evaluated before, we performed the following experiment to prove that the inactivation of the enzyme was turnover dependent and covalent. Pre-incubation was carried out with cholesterol or with the inhibitor and each was divided into two incubations, one with NADPH the four ancillary enzymes of the turnover mixture and one without these. All of these were incubated for 2 hours, dialyzed against cholesterol-containing buffer and assayed for residual activity. The results showed clearly (see Table 1) that the inhibition by 20-MPY is dependent on the turnover of the enzyme. The somewhat lowered activity of the incubation with inhibitor but without the electron-supplying system (NADPH, etc.) suggests that some inhibitor remained bound to the enzyme even after dialysis and was able to subsequently inhibit the enzyme. In stark
contrast to the earlier results, these results suggest an 89% loss of activity due to this inhibitor (compared to 50% earlier, see previous paragraph).

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The 86% labeling of SCC by the diyne inhibitor 20-HDY (5) after 2 hours (10-fold excess of inhibitor over enzyme) reported by Nagahisa in his thesis was confirmed by repetition of his experiment. Here, a 46-fold excess of inhibitor was used in a two-hour incubation. After the incubation the mixture was subjected to the acetone-HCl precipitation experiment (see above) and the resulting guanidine extract indicated roughly 95% labeling of the protein. (This experiment was carried out using the sample of tritium-labeled 20-HDY synthesized in 1983 which was repurified and whose specific activity was measured manually. Thus the accuracy of this determination depends on the accuracy with which that number was determined, which is estimated at only 20%.) In a separate experiment it was confirmed that this labeling is dependent on turnover; incubations lacking NADPH in the turnover mixture resulted in no label incorporated into the protein phase as tested by the acetone-HCl precipitation experiment.

In contrast to this result, incubation with ¹⁴C-labeled 20-HY (3), the linear side-chain monoyne inhibitor, yielded only 9.6% protein labeling.

**Localization of the Site of Labeling to one of the Two Tryptic Fragments, φ₁ or φ₂**

The experiments to localize the site of labeling on the protein to either the φ₁ or φ₂ tryptic fragments proved to be considerably less straightforward that was initially projected. Two methods were used to estimate the amount of radioactivity in each of these fragments; thiopropyl-sepharose covalent chromatography and SDS gel electrophoresis. In these experiments a freshly synthesized sample of tritium-labeled 20-HDY (5) was used. Unexpectedly, the radioactivity incorporated into SCC was about 10-fold lower using this
batch of inhibitor. Several possibilities present themselves to account for this discrepancy. The most likely is a factor-of-ten error in the amount of cold carrier that was added during the synthesis. Other possibilities are that some component of the turnover mixture had degraded or that the inhibitor is contaminated with another compound which inhibits but does not covalently label the enzyme. We pressed on with these experiments because the amount of label that was incorporated into the protein was sufficient to answer the relevant questions. Considering these circumstances, these experiments should be reconfirmed by future researchers.

SCC was incubated with 20-HDY (5) and the resulting mixture was applied to a hydroxyapatite column after 10 times dilution with low salt buffer. The excess steroid was washed off, and the protein was eluted with high salt buffer. As a control, this sample was applied to thiopropyl sepharose without trypsinolysis. The solution was diluted with high salt thiopropyl sepharose buffer and applied to the column at a low flow rate. Nevertheless, approximately one-third of the radioactivity flowed through the column unbound. The possibilities for explanation of this unbound fraction include: lability of the steroid adduct, chemical modification of the lone surface cysteine of some of the protein molecules, a sub-population of protein which is abnormally folded such that the surface cysteine is buried or shielded from reaction, disulfide formation between SCC molecules resulting in an inaccessible disulfide, the flow rate was too high for the disulfide exchange rate or residual aggregation of SCC molecules even in 1M salt. Lack of sufficient thiopropyl sepharose was ruled out as a possibility. Elution with guanidine released another 30% of the total radioactivity while elution with β-mercaptoethanol released 37%. Since the sample was not trypsinized the radioactivity released in the guanidine fraction must result from either steroid which had been labilized but not yet released from the enzyme active site, or the aggregation of unbound SCC molecules with bound SCC molecules. When the same experiment was repeated with sample which had been trypsinized (as described above), the same distribution of label in the three fractions was observed. The only way this is possible (besides the failure of the trypsinolysis to cleave the protein at all), is if the label is localized to the φ2 fragment, the one which is released in the β-mercaptoethanol fraction. It had been originally planned to use the thiopropylsepharose technique as a purification step to remove nearly half of the SCC molecule prior to exhaustive proteolysis and HPLC purification of the labeled fragment (see below). The results discussed above were discouraging because of the partitioning of the label into three different fractions. Additional study is required on the cause of this inefficiency.
In the SDS-PAGE experiments it was found that it was extremely difficult to count any tritium from a slice of polyacrylamide in scintillation cocktail. After several trials it was found that boiling the gel slices in 3.33 N NaOH followed by boiling in an SDS and β-mercaptoethanol solution and neutralization of the solution with HCl, that a small amount of the radioactivity could be labilized (~15%). Thus, SCC was incubated with 20-HDY (5), trypsinized, rinsed free of excess steroid by hydroxyapatite chromatography, concentrated to a small volume using a mini-centrifugal membrane concentrator cell (Ultrafree-MC from Waters), mixed with Laemmli buffer concentrate and boiled briefly. This solution was loaded onto a Phast-system mini gel and the proteins were separated by electrophoresis. The molecular weights were marked by pre-stained standards which had been previously calibrated with the 27 and 22 kilodalton tryptic fragments. Without staining of the gel, regions of the protein-loaded lanes corresponding to the two fragments were excised and subjected to the extraction treatment outlined above. Results indicate that, after subtracting a background radioactivity level on the gel, that the radioactivity in fragment φ2 (22 kDa) is greater than that in fragment φ1 (27 kDa) by a factor of roughly 6 to 18. This is in qualitative agreement with the results from thipropylsepharose chromatography.

Although both of these methods are far from decisive, they both tend towards the conclusion that the label is solely associated with the φ2 fragment. This fragment has been identified by sequencing to be the C-terminal portion of the molecule, the site of the heme-binding region and, according to modeling based on the P-450cam structure by Vijayakumar(28) and Joardar,(9) the location of helix I which Joardar predicts to be the likely site of alkynyl inhibitor covalent labeling (see above).

**Experiments towards the Identification of the Precise Location of the Steroid Adduct**

The possible lability of the steroid adduct continued to be a concern throughout these experiments. Various conditions were assayed for their ability to labilize the adduct, but none could be pinpointed as particularly detrimental. Nevertheless, there seemed to be a constant, slow decline in the amount of radioactivity associated with the protein fraction with time and experimental manipulations. This point also requires further study.

In order to break the protein into pieces of moderate size the endoproteinase (Glu-C, Protease Type XXXV) from *Staphylococcus aureus* V8 has been used by Chashchin and coworkers. This treatment results in the formation of 18 peptide fragments from fragment φ2 (and another 24 from fragment φ1 if it has not been separated first). This
reference suggests that prior maleylation of the lysine groups of the peptide improves the production of non-overlapping peptides by the endoproteinase by unfolding the protein and exposing the entire peptide to digestion.

An initial experiment on \(^{3}H\)-steroid-labeled \(\phi_2\) fragment purified by thiopropyl sepharose used the maleylation conditions suggested by Butler and Hartley.\(^{(3)}\) This required the concentration of the guanidine and \(\beta\)-mercaptoethanol-containing solution using a centrifugal membrane concentrator. This concentrate was diluted with carbonate buffer and maleic anhydride was added. The reaction mixture was again concentrated in preparation for proteolysis. Counting of an aliquot of this solution, however, indicated that less than 10% of the original radioactivity remained in the concentrate. About half of the radioactivity was found in the final concentration filtrate. The remaining 40% was unaccounted for, although the first filtrate was not examined. Explanations include cleavage of the label by the \(\beta\)-mercaptoethanol, or adsorption of the hydrophobic steroid-peptide conjugate to the concentration membrane. Previous control experiments had shown that the maleylation conditions did not promote label cleavage, and during the SDS-PAGE experiments (see above), it was found that brief boiling with \(\beta\)-mercaptoethanol-containing solutions similarly had no effect. This seems to be the last major hurdle to overcome before the proteolysis and HPLC separation of the label-containing peptide can be accomplished.

In order to optimize the conditions for the maleylation of SCC several spectroscopic experiments were carried out. The focus of these experiments was to find conditions which were suitable for maleylation, proteolysis with the endoproteinase and HPLC and which would not cause precipitation of the protein. In this way it was hoped to minimize the number of manipulations required prior to the HPLC analysis. To monitor the progress of the maleylation the Soret band of the heme in P-450 at 418 nm was monitored. Buffers containing 250 mM potassium phosphate, 0.25% cholate and various concentrations of acetonitrile were found to be particularly effective. The endoproteinase is effective up to 10% acetonitrile without a decrease in activity. 250 mM phosphate is sufficient to buffer the maleic acid formed by the hydrolysis of maleic anhydride, in this way there is no need to change buffers from carbonate to phosphate when moving from the maleylation to the proteolysis. As the maleylation progresses the Soret band shifts towards shorter wavelengths indicating that the heme is being exposed to a less hydrophobic environment. Increasing the amount of acetonitrile in the solution accentuates this shift and speeds up the transition. To further assay whether the heme pocket of the protein has been completely unfolded, the circular dichroism (CD) spectrum of the protein was measured during the course of the reaction (using a Jasco J-500C spectrophotometer). Prior to addition of the
maleic anhydride the CD spectrum of P-450\textsubscript{scC} in 250mM phosphate and 0.25% cholate (no acetonitrile, so that the reaction will be slow enough to monitor) was measured between 330 and 440 nm. Two peaks, one at \( \sim 346 \) nm, another at \( \sim 410 \) nm were observed. The molar ellipticity calculated from the more intense band at 410 nm was found to be 58,300. After addition of the maleic anhydride the peak begins to disappear, and is undetectable by one hour. Thus, after maleic anhydride treatment the heme is not only in a less hydrophobic environment (shifted to shorter wavelengths), but is in an achiral environment as well, \textit{i.e.} it has been extruded into the bulk solution.

**Discussion: Future Studies**

Interspersed throughout the text of this chapter have been inserted notes as to where certain experiments are in need of repetition or elaboration. The nature of the problem with the poor labeling by the freshly synthesized diynyl inhibitor is most bothersome and must be resolved before any of the conclusions discussed here can be found anything more than tentative. The most conservative course here is probably warranted, these experiments should be repeated with proteins of the highest purity available and all radio-labeled inhibitors should be purified by HPLC methods. A technique that was never applied to these experiments, but should have been, is the addition of cold carrier inhibitor after the conclusion of every radio-incubation to assist in the stripping of non-covalently bound inhibitor. A thorough kinetic analysis of the three inhibitors should be carried out under identical conditions to those reported earlier for comparison, and the observation that the mono-yne inhibitors do not label the protein efficiently needs to be confirmed and correlated with their inactivation rates to determine a partition ratio (number of inactivation events over number of covalent attachment events). The possible covalent attachment of these inhibitors to the heme group needs to be investigated.

The observation that the monoyne inhibitors do not result in covalent protein labeling while the diynes do is cause for speculation. The possibility exists that the modification of the protein may be mediated by an intramolecular activation of the terminal alkyne by the P-450-activated internal (disubstituted) alkyne of the diyne. All evidence from the literature suggests that terminal alkynes and alkenes cause modification of the heme group by N-alkylation, while internal alkynes and alkenes result in isolable oxidized products without covalent attachment.\textsuperscript{(20-22, 24)} Intramolecular chemistry requires that the diynyl side-chain be bound in the active site in a recurved orientation indicating a transoid torsion about the 3'-4' single bond in the side chain. This conformation and several
Scheme 5: a) a conformation of the inactivator 20-HDY (5) bringing the two alkyne groups in close proximity. b) Some possible reaction pathways involving covalent labelling of the enzyme, the iron-ligated enols of the products are shown, each has a corresponding unsaturated ketone form. Other pathways based on radical or carbene mechanisms are possible, as well. c) The two proposed enediyne inhibitors discussed in the text.
possibilities for the relevant chemistry are shown in Scheme 5a and b. Through these mechanisms the chemically reactive species is moved several angstroms away from the iron atom towards, presumably, the active site residues of the protein. The question of the conformation of the bound side chain of cholesterol derivatives in the active site of SCC should be answerable via the synthesis of inhibitors with conformationally restricted groups such as, for instance, cis- and trans- enediynes (Scheme 5c, (9) and (8), respectively). One would expect that the trans enediyne would have a stronger binding interaction if this type of intramolecular mechanism is in effect. In addition, these enediyne inhibitors may prove to be excellent mechanism-based inhibitors.
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CHAPTER 4

THE DISCOVERY AND SIGNIFICANCE OF CALPROTECTIN IN RENAL STONES ASSOCIATED WITH URINARY TRACT INFECTION
Preface

In the spring of 1991 William Orme-Johnson ("Bill"), a man well known for his infectious smile, began to frown. At first one might have mistaken it for consternation, perhaps, or concern about some private matter, but soon there was no mistaking it (even his graduate students noticed), Bill was not well. Sitting at his place at the head of the conference table, the frown became a teeth-clenched grimace, and that was promptly topped with glazed eyes and a bead or two of sweat. The day's speaker spoke nervously on. Later, so I am told (and by a fairly reliable source), Bill finds his way to the Medical Center and, bent over like a ski jumper and knees buckling, says, "I'm {gasp} fine, {groan} it's nothing, just..." To which the nurse on duty replies, without much emotion, "Kidney stone...call the ambulance."

In this way Professor Orme-Johnson found his way into the capable hands of one Dr. Steven Dretler, M.D., who, after much prodding, poking, irradiating and ultrasonic shocking declared him to be cured, for now, thank you very much. Ignoring, for the moment, the jokes by the Harvard Medical School residents when confronted with the immobilized MIT professor, the ever curious O.J. began to discuss the biochemistry of kidney stones with the equally curious doctor. The rest, as they say, is history.
Introduction

Kidney stones (renal calculi) are concretions of inorganic and organic salts that form in the renal pelvis. Having very much the appearance and consistency of stones or corals, the condition becomes symptomatic when they reach a size which precludes their exit from the kidney via the ureter. Blockage of the ureter by a kidney stone causes extreme pain and, if not passed into the bladder by the peristaltic (spasmodic) action of the surrounding musculature will result in interruption of the flow of urine from that kidney resulting in renal damage. If not treated these conditions would result in renal failure and death. A recent review of the disease and its treatment has been published by Coe, Parks and Asplin.\(^{(14)}\)

Urinary calculi have been afflicting mankind since recorded history and have been identified in 7000 year old mummies in the Sudan.\(^{(36)}\) Calculi occur in 2.7% of Americans, with the highest incidence in the 30-50 year age group, and results in the hospitalization of 1.6 individuals per thousand yearly.\(^{(39)}\) The chances of an individual having a recurrence of the disease are estimated to be as high as 67%. With over 250,000 patients in the United States requiring an interventional treatment for urinary lithiasis yearly and each treatment costing over seven thousand dollars, the minimum cost for interventional treatment is 1.7 billion dollars per year. This figure does not include the 100,000+ patients who have a stone episode which requires supportive treatment but no intervention nor does it take into account lost productive labor. The international incidence of stone disease, especially in underdeveloped countries, is estimated to be considerably higher.\(^{(18, 43)}\) However, in the third world countries, if medical care is available, the clinical stone episode often means an open operation, prolonged illness and, sometimes, death.

There are not sufficient financial resources worldwide to treat the disease. The only realistic possibility for the care of these patients is stone prevention. The present understanding of the processes responsible for stone formation is not sufficient for any type of reliable preventative regimen to be developed. For years, for instance, medicine has suggested that patients having suffered from a calcium-containing stone reduce the amount of calcium in their diet. Recently, however, a thorough study of stone patients has revealed that, among calcium oxalate stone sufferers, those with lower calcium intake had a slightly higher incidence of stone episodes.

The ionic species that make up kidney stones are common to normal urine: calcium, magnesium, phosphate, oxalate, ammonium, carbonate, uric acid and occasionally
cysteine. Often urine is supersaturated with respect to combinations of these salts, especially calcium oxalate which is normally four times more concentrated than its solubility.\(^{(13)}\) Kidney stones are classified by their mineralogical composition.\(^{(14)}\) The most common type (27\%) and the physically hardest are stones composed of pure calcium oxalate. Calcium phosphate is found in several crystal forms such as hydroxyapatite and brushite as well as amorphous phases and makes up 8\% of all stones. These two minerals are often found together in the same stone (33\%). Thus, calcium oxalate is found in 60\% of all stones. Somewhat rarer are stones consisting of uric acid in whole or in part and cysteine (4\% and 4.5\%, respectively).

The other major classification of stones is called "infection stones." These consist of struvite (magnesium ammonium phosphate) often mixed with either hydroxyapatite or carbonate apatite, and account for 23.5\% of all stones.\(^{(23)}\) Infection stones are so named because they are very often associated with infections of the urinary tract. Although infection stones account for only about one-fifth of all kidney stones, they are responsible for a disproportionate amount of the number of clinical procedures performed, and these procedures are generally characterized by a greater number of complications and the associated mortality and morbidity of affected patients. The reasons for this discrepancy are two-fold. First, infection stones are often quite large and display a staghorn (multi-lobed) morphology that fills the convoluted interior space of the renal pelvis. This makes the treatment and removal of these stones a painstaking process that often requires multiple techniques to break up and remove the stone fragments from all of the lobes of the pelvis. This is accompanied by a greater rate of damage to the renal epithelium due to the close fit between the available space and the stone. Second, since the infection stones often harbor colonies of the bacteria which initially caused the condition,\(^{(44)}\) if any fragments of the stone remain behind the stone will generally reform and require additional treatments.\(^{(21)}\) Antibiotic treatment of infected stones is sometimes ineffective because the microorganisms are so well shielded within the structure of the stone.\(^{(9, 19)}\) Furthermore, electron microscopy studies have indicated that the bacteria themselves may be the source of much struvite stone material in the form of a "biofilm" of exopolysaccharide and other constituents which may further protect the microbial colonies.\(^{(33)}\)

The final and rarest type of stone are called "matrix" stones and are characterized by their softness and very low mineral content compared to other stones. Although this type of stone has been only occasionally studied, it has been proposed that matrix stones are very poorly mineralized infection stones since they tend to have similar shapes and internal structure as other stone types and tend to be associated with struvite and hydroxyapatite.\(^{(5)}\)
A question that comes to mind when one considers the composition of urine is not, why do stones form, but rather what keeps them from forming with much greater frequency? Like blood, which is supersaturated with respect to calcium phosphate, urine is a concentrated solution because of the great selective advantage that is gained by organisms by minimizing the need for water. Mammals can go far longer without food than water. Recognizing this need for water conservation, and thus the benefit of giving up as little water as possible in the passage of wastes, we would expect that, over the course of evolution, mechanisms will have evolved to specifically handle the possibility of stone formation in these concentrated urines since such a condition leads to death.

It is reasonable to hypothesize then, that some factor or factors must be released in the urine which inhibit the formation of stones. These factors should be small molecules or proteins which have the ability to sequester calculogenic salts as soluble complexes and thus reduce the free concentration of these salts to below saturation, or alternatively, the ability to coat the surfaces of forming crystals in such a way that the growth and aggregation processes are slowed to such an extent that these micro-crystals can be passed in the urine without incident. It has been long known that urine concentrates have just this property when assayed for crystal growth under controlled conditions. Several proteins have been identified in the last ten years which posses calcium oxalate crystal growth inhibition activity. These include nephrocalcin, uropontin, "uronic acid-rich protein" (UAP) and Tamm-Horsfall mucoprotein. It seems quite clear that the management of supersaturated salt solutions in humans is multifactorial.

Considering the existence of these various protective mechanisms, kidney stone formation can be considered to be, at least in some cases, a disorder resulting from the degradation of one or more of these systems. This might be due to genetic factors (stone disease does seem to run in some families), secondary to other disease or metabolic disorder, or due to the interference of some factor with the protective system, something like a compound in the diet or natural product of a microbe. Thus stone formation would be due to either the lack of some factor in the urine, or perhaps caused by the presence of a mutant or altered form of the factor. In the case of calcium oxalate stones, for instance, nephrocalcin from stone-former urine has been found to be deficient in the modified amino acid gamma-carboxyglutamate which is believed to be responsible for calcium ion binding at the surface of growing crystals. A deficiency in the production of an unaltered factor, or a metabolic condition causing the salts in question to be excreted in amounts too voluminous to effectively inhibit would result in the factors bound at the crystal surface to be included in the growing structure of the stone.
Another class of explanation for stone formation encompasses the possibility of crystallization *promoters* in the urine. Clearly, crystallization promoters will not be proteins selected for this property, but those which posses it as an unfortunate side effect of some other function. For instance, a protein which is released into the urine to fight a urinary tract infection might also have the unfortunate property of promoting certain types of crystal growth (this possibility will be discussed further below in regard to the experimental work reported in this chapter). Additionally, the mutation or other modification of a crystallization inhibitor might result in the generation of a crystallization promoter, especially considering the likelihood that any functional inhibitor would posses the property of binding to the surface of growing crystals. The dimerization of such an inhibitor, for instance, might promote the aggregation of crystals to which it bound.

For these reasons, some researchers have followed a program in parallel to the investigations of urinary proteins possessing crystal growth inhibition properties. In these explorations, the stones themselves are the source of protein or other factors potentially responsible for the stone's formation and growth. The difficulty in this type of work is the tenacious insolubility of these samples and techniques for the extraction of these materials vary considerably.

All kidney stones contain some proportion of protein. This material was first observed in 1684 by Anton von Hyde who showed that after all of the crystalline components of a stone had been dissolved, the gross structure of the stone remained unchanged. This material was later termed the "matrix" and was first hypothesized to be composed of protein in 1884 by Meckel von Hemsbach. Generally this matrix protein accounts for only 2-5% of the dry weight of a stone, but there is slightly more in struvite stones and matrix stones average 65% matrix.

Studies have identified proteins that are selectively concentrated in the matrix of stones relative to the amounts found in urine, and that the type of protein found in the matrix is dependent on the mineralogical type of the stone. This should not be surprising considering the common biochemical technique of adsorption of proteins onto hydroxyapatite in order to perform chromatography.

We report below our initial isolation and identification, by N-terminal peptide sequencing and immunochemical methods, of the anti-microbial, calcium-binding protein known as calprotectin from a single matrix stone and three struvite infection stones (of the three that we have investigated thus far). The protein is known by several names and in several contexts. Although recent convention has assigned the name calprotectin , it is
perhaps most widely known in medicine as the cystic fibrosis-associated antigen (CF Ag). In other contexts it has been known as the leukocyte antigen L1, calgranulin and migration inhibitory factor-related protein (MRP). This protein is found to be elevated in the serum of cystic fibrosis homozygotes and obligate heterozygotes, in the macrophages of rheumatoid arthritis sufferers, in the epidermal scales of psoriasis patients, associated with neoplastic skin diseases, inflammatory disorders and microbial infections as well as in virtually all circulating blood neutrophils and monocytes in healthy humans. The protein appears to be released at the time of granulocyte death and thus is concentrated at sites of inflammation and infection where neutrophil turnover is high.

The functional protein is believed to be a hetero-dimer that binds two calcium ions per subunit, although there have been reports of alternative subunit ratios. Of the two subunits, the smaller one corresponds to the protein we have N-terminally sequenced. The larger subunit has been reported to be N-terminally blocked. Monoclonal antibodies have been raised to this protein, and the gene coding for it has been identified in a cDNA library from human chronic myeloid leukemia (CML) cells. The primary site of expression of this protein appears to be in the granulocytes. Evidence has been gathered showing calgranulin to be an inhibitor of some protein kinases as well as an antimicrobial agent when released by neutrophils at a site of tissue infection. Significantly, calgranulin has been shown to comprise approximately 45% of the cytosolic protein in these neutrophils.

The properties of the protein are strongly affected by the local calcium concentration. Calprotectin has been reported to associate strongly with the cell membrane pancreatic elastase and the keratinocyte cytoskeleton. Further, the C-terminal section of both subunits of calprotectin have strong homology with small proteins known as neutrophil-immobilizing factors. It has been suggested that calprotectin may also posses the ability to direct neutrophils to sites where the protein is bound. These properties suggest that calprotectin has distinct binding properties which may explain its association with matrix and struvite stones and lead us to an understanding of its function in the urinary tract and possible role in renal lithogenesis.
The Identification of Calprotectin in Renal Stones

Studies on a "Matrix" stone

In October of 1991 an unusual case came to the attention of Dr. Steven Dretler in his practice at the Department of Urology, Massachusetts General Hospital. This patient was suffering from a "large-volume, amorphous renal concretion," a matrix stone. Because of the soft and adherent nature of this material, uroscopic techniques for the physical removal of the material were unsuccessful as would have been lithotripsy. The large volume of the stone, present in only one kidney, but nearly occluding passage of urine from that one, required the intervention of major surgery to remove the material from the renal pelvis. Unfortunately, even after this procedure, the material began to form again and eventually it was necessary to remove the patient's kidney entirely. This condition was rare both in the fact that it involved a matrix stone and in the excessive quantity of material that was formed.

During the treatment of the patient, however, thanks to the scientific relationship that had developed between Dr. Dretler and Professor Orme-Johnson, samples of the matrix material that had been passed in the urine and that which had been surgically removed were sent to our labs at M.I.T. for analysis. Our initial goal was to identify conditions which might be applied in vivo to promote the dissolution of this material. Our rudimentary analysis of the problem was based on the observation that matrix stones possess a larger proportion of protein than other, harder, stones. Thus, we hoped, with this particularly soft and apparently fast-growing sample, that basic biochemical knowledge about the solubilization of proteins could be applied to some good effect. The protein-protein interactions thus interrupted, the diffuse inorganic phase of the material could be rinsed from the renal pelvis by irrigation.

The matrix material was prepared as follows. The samples were centrifuged and the supernatants were decanted away. The pellets were washed twice with distilled water and lyophilized to dryness in order to get accurate weights of the material.

In order to determine what mechanisms were responsible for the insolubility of the protein phase of the matrix material we first tried harsh conditions for solubilization, looking for unambiguous results which we could then modify into a milder regime once we knew what principles to apply. The various reasons for protein insolubility that we hypothesized were: 1) disulfide-based crosslinking, 2) aggregation due to hydrophobic effects, 3) aggregation mediated by the chelation of cations and 4) aggregation by unfolding and deranged refolding. To attempt to interfere with these mechanisms the solutions listed
in Table 1 were used with the accompanying results. 1 mL of each solution was added to 10 mg of dried matrix stone material and was warmed to 65° C for one hour. The resulting solutions were clarified by centrifugation and the resulting supernatants assayed for total protein by the Bradford dye-binding method (BioRad kit). It is quite apparent that none of these methods was particularly successful in solubilizing large amounts of protein, in fact, visual inspection of the pellets after these experiments showed no change in appearance at all. The best method was the combination of urea (to unfold the protein), β-mercaptoethanol (to break disulfides), EDTA (to remove cations) and acetic acid.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Protein (µg/mL)</th>
<th>% of total weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.6µM EDTA pH 4.5</td>
<td>16</td>
<td>0.02</td>
</tr>
<tr>
<td>0.5 M EDTA pH 8.0</td>
<td>54</td>
<td>0.05</td>
</tr>
<tr>
<td>0.2 M β-mercaptoethanol (βME)</td>
<td>31</td>
<td>0.03</td>
</tr>
<tr>
<td>10M Urea</td>
<td>171</td>
<td>0.17</td>
</tr>
<tr>
<td>10% acetic acid</td>
<td>127</td>
<td>0.13</td>
</tr>
<tr>
<td>50% acetic acid</td>
<td>148</td>
<td>0.14</td>
</tr>
<tr>
<td>0.1% SDS, 0.2 M βME</td>
<td>76</td>
<td>0.08</td>
</tr>
<tr>
<td>0.2 M βME, 6.6µM EDTA</td>
<td>32</td>
<td>0.03</td>
</tr>
<tr>
<td>0.2 M βME, 8 M urea</td>
<td>154</td>
<td>0.15</td>
</tr>
<tr>
<td>6.6µM EDTA, 8.7M urea</td>
<td>146</td>
<td>0.15</td>
</tr>
<tr>
<td>10% acetic acid, 0.1M EDTA</td>
<td>60</td>
<td>0.06</td>
</tr>
<tr>
<td>10% acetic acid, 18M urea</td>
<td>152</td>
<td>0.15</td>
</tr>
<tr>
<td>6.6µM EDTA, 0.2 M βME, 6.7M Urea</td>
<td>215</td>
<td>0.22</td>
</tr>
<tr>
<td>0.1M EDTA, 0.2 M βME, 10M Urea,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10% acetic acid</td>
<td>239</td>
<td>0.24</td>
</tr>
</tbody>
</table>

The relative ineffectiveness of these treatments suggested that our assumptions about the protein content of this material might be unfounded. To test this assumption the solids were incubated with proteinase K (Sigma) in buffer containing 100 mM NaCl, 10 mM Tris (pH 8), 25 mM EDTA and 0.5% SDS at 50° C overnight. After this treatment centrifugation yielded only trace insoluble material. Thus it appears that in the absence of protein, the matrix material would be solubilized. What then is holding the proteins together and causing their insolubility? One possibility is crosslinking via transamidation, sugar linkages or other covalent modification.
Figure 1: SDS-PAGE of proteins extracted from struvite and matrix stone samples using the Phast electrophoresis system on a homogeneous 20% acrylamide gel and stained with coomassie blue (Pharmacia). Molecular weight markers are Bio-Rad prestained SDS-PAGE standards, low range; reported molecular weights are approximate due to shifts caused by the attached dye molecules. Bands a have been identified as the small subunit of calprotectin by N-terminal peptide sequencing. Band b of lane 4 (matrix sample) has similarly been identified as lysozyme.
The protein that had been solubilized was further analyzed by electrophoresis in search of clues concerning the composition of the matrix material. The protein-containing supernatant from the EDTA-βME-urea extraction was concentrated by trichloroacetic acid precipitation and separated by SDS polyacrylamide gel electrophoresis using the Phast mini-electrophoresis system (Pharmacia). Protein bands were visualized with silver nitrate stain. The most prominent bands on the gel by far were a triplet of low-molecular weight proteins (apparent molecular weights 6-16 kilodaltons, see Figure 1, lane 4). These proteins were not evident in concentrates of the urine passed with the matrix stone material; that is they were essentially insoluble in urine under the conditions existing in this patient. Although the protein assays indicated otherwise, it was subsequently found that the SDS buffer used to dissolve the protein precipitates prior to electrophoresis was quite effective in solubilizing the proteins directly from the matrix material. This provided a one-step procedure for electrophoresis of these proteins. The reason for the discrepancy between the protein assay and the evidence of the stained gels has not yet been determined.

In order to identify these three proteins by amino acid sequencing, a sample of matrix solid was solubilized in SDS, subjected to electrophoresis, electroblotted onto ProBlott membranes and visualized with Coomassie blue stain. The triplet of protein bands were excised and subjected to Edman degradation to determine their N-terminal sequences (analysis performed by the MIT Biopolymers Lab, via standard techniques.) The degradation failed to yield sequence information with the protein in the first (~16 kDa) band, most likely due to N-terminal blockage of that protein. The second (middle) band yielded an N-terminal amino acid sequence identical to that of human lysozyme except for one leucine residue (amino acid #2) which is reported to be a valine in the human protein and isoleucine in the baboon protein (Figure 2a). These three amino acids are very similar in terms of hydrophobicity as well as the nucleotides which code for them and probably represent a site of allelic variation. This assignment is curious in that human lysozyme has a molecular weight of 18.5 kDa and this band has an apparent molecular weight of around 10 kDa. The reason for this anomalous electrophoretic behavior has not yet been determined.

The third (~6 kDa) protein yielded an eighteen amino acid sequence that was exactly matched by two sequences in the Nbrf database of protein sequences. The first match was to the protein known as the cystic fibrosis antigen (CFAg), a peptide isolated from the blood of cystic fibrosis patients and obligate heterozygotes, although this is not the (as of yet unidentified) product of the defective gene in cystic fibrosis. The second match is to a protein known as MRP-8 or MIF-related protein (MIF=migration inhibitory factor). A search of the relevant literature (see introduction) shows that these two proteins have been
proven to be one in the same. Furthermore, this protein has been known be a number of other names including leukocyte antigen L1-light chain, p8, calgranulin B and, as it is now most commonly called, calprotectin. This protein is reported to consist of 93 amino acids and have an apparent molecular weight on SDS-PAGE of 8 kDa which is consistent with

![Edman degradation N-terminal amino acid sequences of two proteins](image)

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Amino Acid Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Lysozyme</td>
<td>Lys-Val-Phe-Glu-Arg-Cys-Glu-Leu-Ala-Arg-Thr-Leu-Lys-Arg-Leu</td>
</tr>
<tr>
<td>Baboon Lysozyme</td>
<td>Lys-Ile-Phe-Glu-Arg-Cys-Glu-Leu-Ala-Arg-Thr-Leu-Lys-Arg-Leu</td>
</tr>
<tr>
<td>C. F. Antigen</td>
<td>Met-Leu-Glu-Leu-Glu-Lys-Ala-Leu-Asn-Ser-Ile-Ile-Asp-Val-Tyr-His-Lys</td>
</tr>
<tr>
<td>CaBP MRP-8</td>
<td>Met-Leu-Glu-Leu-Glu-Lys-Ala-Leu-Asn-Ser-Ile-Ile-Asp-Val-Tyr-His-Lys</td>
</tr>
</tbody>
</table>

**Figure 2:** Edman degradation N-terminal amino acid sequences of two proteins extracted from a matrix stone and matching sequences extracted from the Nbrf database of protein sequences. a) 10 kDa band. xxx stands for unclear assignments, residues in bold show mismatches. b) 6 kDa band. C. F. Antigen = cystic fibrosis antigen, CaBP MRP-8 = calcium binding-protein migration inhibitory factor related protein.

our rough estimates. The protein is almost always associated with a second subunit of ~14 kDa (MRP-14, L1-heavy chain, p14, calgranulin A) which is reported to be N-terminally blocked and thus resistant to Edman degradation. This is consistent with our observation that the largest band of the triplet, ~16 kDa, is also N-terminally blocked and suggests that this is the larger subunit of calprotectin.

Commercially available monoclonal antibodies to calgranulin (DAKO, Carpenteeria California) were used in an ELISA (Enzyme-Linked ImmunoSorbant Assay) to confirm the identification of calprotectin in these solids. The assay was performed as follows:

A lyophilized powder of matrix material was added to a plastic vial and blocked with bovine serum albumin (BSA) in phosphate-buffered saline (PBS). The primary antibody (mouse anti-calprotectin, 50:1 diluted) was allowed to bind, followed by the
secondary antibody, biotinylated goat anti-mouse (cross-reactivity with human serum eliminated by affinity chromatography, purchased from Pierce). Then a complex of avidin and biotinylated horseradish peroxidase was allowed to bind to the biotinylated antibody (Pierce ABC peroxidase kit). Each of these steps was followed by several washes with BSA in PBS. The amount of calgranulin originally on the surface of the matrix powder was measured by reacting the reagent ortho-phenylenediamine (OPD) with the bound peroxidase and hydrogen peroxide. The enzyme catalyzes the conversion of the weakly colored OPD into a strongly colored product. The amount of colored product produced was measured spectrophotometrically (A_492).

The ELISA clearly shows a positive test versus stringent controls (Table 2). When the lyophilized matrix powder is serially diluted with an inert carrier (ground glass) calgranulin can still be detected in one part matrix powder per 100,000. When the primary antibody is substituted with a blank wash (or extra secondary antibody) there is a low background response. In the absence of any matrix material (but with the primary antibody) there is almost no response at all. This study confirms our identification of calprotectin as a component of these samples.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>1° Antibody</th>
<th>Response (A_492)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Matrix powder</td>
<td></td>
<td>Mouse anti-calprotectin</td>
<td>&gt;3</td>
</tr>
<tr>
<td>&quot;</td>
<td>1:10</td>
<td>&quot;</td>
<td>&gt;3</td>
</tr>
<tr>
<td>&quot;</td>
<td>1:100</td>
<td>&quot;</td>
<td>&gt;3</td>
</tr>
<tr>
<td>&quot;</td>
<td>1:1000</td>
<td>&quot;</td>
<td>~3</td>
</tr>
<tr>
<td>&quot;</td>
<td>1:10,000</td>
<td>&quot;</td>
<td>2.6</td>
</tr>
<tr>
<td>&quot;</td>
<td>1:100,000</td>
<td>&quot;</td>
<td>2.2</td>
</tr>
<tr>
<td>&quot;</td>
<td>1:1000</td>
<td>None</td>
<td>1.7</td>
</tr>
<tr>
<td>Ground Glass</td>
<td></td>
<td>Mouse anti-calprotectin</td>
<td>0.4</td>
</tr>
</tbody>
</table>

The Identification of Calprotectin in Struvite Infection Stones

The notable association of calprotectin with the immune system and especially antimicrobial defenses (see Background and Significance section, above) as well as our observation of lysozyme (a granulocyte protein) in the matrix stone material suggested that calprotectin might be released into the urinary tract as a common defense against urinary
tract infections. An inquiry into the medical history of the patient who supplied the matrix material confirmed that the patient had suffered from recurrent *Candida* infection of the urinary tract at various times prior to the collection of our samples. Thus, calprotectin might be expected to be associated with "infection" stones.

Infection stones are composed of struvite (magnesium ammonium phosphate) and carbonate-apatite which are believed to be undersaturated in normal urine.\(^{(23)}\) In this case urinary tract infection by urea-splitting bacteria have been widely recognized as a primary cause of supersaturation. The splitting of urea into ammonium and bicarbonate by the enzyme urease in these organisms results in the alkalization of the urine which promotes both the formation of carbonate from bicarbonate and the relatively insoluble $\text{PO}_4^{3-}$ from $\text{HPO}_4^{2-}$.\(^{(24)}\) This rationale has gained general support although notable exceptions exist. The largest correlation of struvite/carbonate-apatite stones and bacterial infection is only 77%, and in 20% of these stones no urea-splitting organisms could be cultured.\(^{(9)}\) *Escherichia coli*, which does not express urease, has been reported to promote infection stone formation,\(^{(9)}\) and quite often urinary tract infections with urea-splitting organisms do not result in infection stone formation. These results suggest that the formation of infection stones is a complex process and that, like the metabolic stones, somatic proteins may play an important role.

Samples of three struvite stones from three different patients (thanks to Dr. Dretler and the Massachusetts General Hospital Kidney Stone Center) were analyzed by the techniques described above. The results were quite similar (but not identical) to those observed for the matrix stone, except only two low molecular-weight bands were observed (Figure 1, lane 3). The same pattern of bands was observed in all three samples. In addition, we considered the possibility of surface contamination of the stones with white blood cells. All stones were thoroughly washed with distilled water prior to crushing, and one stone was carefully dissected into "core" and "surface" fractions, both samples showed the same amounts of protein bands (Figure 1, lanes 2 and 3).

N-terminal sequencing of all three stone samples shows the upper band to be N-terminally blocked, and the lower one to be the lower molecular weight subunit of calprotectin. The sequence of the low-molecular weight subunit of calprotectin was found to be identical in all three samples. Unlike the matrix stone, no lysozyme was observed. Also differing from the matrix stone sample we were able to completely dissolve the struvite stones (including the protein matrix) in our urea, EDTA and $\beta$-mercaptoethanol solutions. Because of this, we are able to estimate from the optical density of the stained bands, that approximately 40% of the protein content of these stones consists of calprotectin. The slight difference in mobility of the calprotectin band (compare bands 3
and 4 of Figure 1) is presently unexplained but may indicate differences in calcium ion content of the sample solutions. Presently we have no sequence information for the other prominent bands observed in these gels, perhaps due to N-terminal blockage.

EDTA, β-mercaptoethanol and urea extracts of these struvite stones were serially diluted and blotted onto nitrocellulose paper which was then probed by the ELISA in much the same way as described above except the insoluble peroxidase-activated dye 4-chloro-1-naphthol (4-CN) was used instead of OPD. The stained nitrocellulose blots were quantitated by scanning densitometry. The response of this assay under these conditions is shown in Figure 4. In the absence of a pure standard of calgranulin we cannot calibrate this assay, although this will be remedied in the near future (see below).

One calcium oxalate monohydrate stone was demineralized by dialysis versus several changes of 0.025M EDTA over the course of two weeks. The remaining solids were further extracted by dialysis versus 10% acetic acid and then by boiling with the EDTA, β-mercaptoethanol and urea solutions used for the struvite samples above. Concentrates of all of these extracts were blotted onto nitrocellulose and assayed by the ELISA method but showed no response above background.

![Figure 4: Standard curve showing linear response range of ELISA for calprotectin by dot-blot and densitometry with 4-chloro-1-naphthol peroxidase stain.](image-url)
Proposals for Continued Research

Having identified calprotectin as a component of a single matrix stone and three struvite "infection" stones (of three tested), and having found calgranulin to comprise approximately 40% of the protein content of these struvite stones, the following experiments are proposed to further investigate the clinical relevance of this protein to disorders of the urinary tract. As discussed above, calprotectin has been found to be an effective bacteriocide and fungicide at physiological concentrations in human serum versus Candida species, Cryptococcus neoformans, Escherichia coli, Klebsiella species, Staphlococcus aureus, and Staphlococcus epidermis.\textsuperscript{(42)} It is believed that the protein, which comprises up to half of the cytosolic protein of granulocytes but lacks signal sequences for export, is released at the time of granulocyte lysis. Considering the range of inflammatory and infectious conditions with which calprotectin has been associated, it seems likely that this protein finds its way into the renal pelvis as a specific response to irritation due to the early stages of stone formation at the renal tubules, or as a response to urinary tract infection. The possibility must also be considered, however, that a protein such as this which has a high circulating concentration in the serum and is relatively low-molecular weight may be cleared from the serum at a relatively constant rate by filtration through the renal glomerulae.

Considering the inflammatory or constant filtration modes of calprotectin release into the renal pelvis, the possibility exists that calprotectin may yet be found as a component of the matrix of non-infection stones such as calcium oxalate, calcium phosphate or other varieties. The reported (above) investigation of a single calcium oxalate monohydrate stone showed no calprotectin, but presently we are only able to detect down to about 1/500 of the amount of calprotectin found in a standard sample of struvite after extraction with EDTA, β-mercaptoethanol and urea. This is in contrast to the 100,000-fold dilution that was achieved when the solid matrix stone was assayed without extraction. Very likely the harsh conditions used for the extraction have so denatured or otherwise altered the protein that it is no longer efficiently recognized by the antibody. Alteration of the extraction techniques, and use of spectroscopic detection in microtiter plates instead of the rather crude densitometric quantitation should significantly increase the sensitivity of this assay. It is expected that stones not associated with urinary tract infections would have significantly less calprotectin (if any), so this added sensitivity is crucial.

The association of urinary calprotectin levels with urinary tract infection must be investigated. Using ELISA techniques the urine of patients suffering from various infection will be compared with normal controls. The effect of calprotectin on several
common bacteria and fungal species has been investigated in growing cultures under laboratory conditions. We will extend these investigations to include species common to the urinary tract in standard culture and growing in urine (it has been reported that the antimicrobial activity of calprotectin is strongly dependent on the nature of the culture medium\(^{42}\)). These results will be correlated with the observed levels of calprotectin in infected urines. The antimicrobial effect of calprotectin has been found to be reversible by added zinc\(^{41}\) and this has prompted the speculation that the protein acts by sequestering zinc from the growing microbes. Why, in the case of struvite stone-associated infection, is calprotectin ineffective? The status of trace zinc in infected urines will be investigated as well as other possibilities for the mechanism of antimicrobial action for calprotectin.

In order to determine whether calprotectin is relevant to the process of renal lithogenesis, purified calprotectin (either recombinant, or from human blood granulocytes) will be added to urines supersaturated with respect to the various common stone forming salts. Many studies have been published detailing the effects, inhibitory and promotive, of various urinary proteins, and these techniques will be applied to our future work. We will focus in particular on the process of struvite formation in infected urines.

Often stones are found to be mixtures of different mineral types. Our investigations of primarily struvite stones will be extended to stones with varying amounts of struvite to determine the correlation between struvite deposition in particular and calprotectin incorporation into growing stones.

Finally, we will revisit the original matrix stone sample. The persistent insolubility of this material continues to be perplexing and, in this way, may be a relevant model for the equally insoluble matrix of the non-infection stones. The possibility of intermolecular covalent linkage as the basis for insolubility will be investigated by limited proteolysis and other chemical digestion methods. Proteolytic fragments will be sequenced in order to identify some of the component proteins of this material.
References


When I think back on all the crap I learned in Grad school,
It's a wonder I'm still sane at all,
Though the excess of education hasn't hurt me none,
I can read the writing on the cell:

Cytochrome,
They give us those deep red colors,
Give us the greens of summer,
Makes you think all the world's made of heme, oh yeah.
I got an NIH grant,
I'd love to take a spectrograph,
Mamma don't take my cytochrome away.

If you took all the hours I've spent in my lab,
Working without windows day and night,
I know they'd never match my sweet appreciation,
For the healing powers of real sunlight.

Cytochromes,
The give hydroxylations,
Give us respiration,
Makes you think all the world's made of heme, oh yeah.
I've got a touch of frost bite,
From summers in the cold room, So mamma don't take my cytochrome away!