An Abstract of the Thesis of

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2,5-dihydroxyacetanilide epoxidase from *Streptomyces* LL-C10037 (DHAE I) catalyzes the epoxidation of hydroquinone **4**, to form epoxyquinone **7**. DHAE I has been purified to homogeneity, with an overall purification factor greater than 23,000. N-terminal and internal amino acid sequences have been obtained from the purified enzyme so that the epoxidase gene may be cloned by using reverse genetics.

The epoxidation reaction of DHAE I requires only molecular oxygen and no added cofactors. The detailed chemical mechanism has been elucidated using isotopically labeled oxygen. DHAE I is a dioxygenase belonging to a new and unrecognized class of dioxygenases that are described as "hydroquinone epoxidizing dioxygenases."

DHAE I and another epoxidizing enzyme which acts on the same substrate but producing the enantiomeric epoxide, DHAE II from *Streptomyces* MPP 3051, have had their active sites examined for structural latitude. DHAE II shows more flexibility toward substrate analogs than DHAE I. However, both enzymes are very specific for the correct substrate. From these studies, crude three dimensional models of the two active sites have been constructed.

Purification of 2,5-dihydroxyacetanilide Epoxidase and Mechanism of Hydroquinone Epoxidases

by

Marc J. Kirchmeier

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Marc J. Kirchmeier, Author

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Table of Contents

Table of Contents (Continued)

List of Figures

List of Tables

List of Schemes

List of Schemes (Continued)

List of Abbreviations

ABQ:	acetamido-1,4-benzoquinone
AEBQ:	2-acetamido-5,6-epoxy-1,4-benzoquinone
CFE:	cell-free extract
DEAE:	diethylaminoethyl
DHA:	2,5-dihydroxyacetanilide
DHAE I:	2,5-dihydroxyacetanilide epoxidase from *S.* LL-C10037
DHAE II:	2,5-dihydroxyacetanilide epoxidase from *S.* MPP-3051
FPLC:	fast protein liquid chromatography
HPLC:	high performance liquid chromatography
IMAC:	immobilized metal affinity chromatography
MW:	molecular weight
NNM:	nanaomycin
PCC:	pyridinium chlorochromate
PVPP:	polyvinylpolypyrrolidone
QMA:	quaternary methylamine

Purification of 2,5-Dihydroxyacetanilide Epoxidase and the Mechanism of Hydroquinone Epoxidases

Chapter 1

Introduction

Introduction

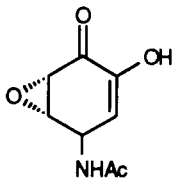
Many microorganisms (e.g. bacteria and fungi) have the ability to inhibit the growth of other organisms by producing antibacterial compounds. As a result, large numbers of these metabolites (natural products) exhibiting great structural diversity have been identified. These bioactive compounds can be clinically valuable antibiotics, chemotherapeutic agents or other useful pharmacological agents. In addition, many of these natural products are very important agrochemicals. Because of their tremendous importance, natural products and the organisms producing them have been studied in great detail. Over the last half century, organic chemists and biochemists have elucidated many complex biosynthetic pathways by which these organisms produce their natural products. These studies have not only allowed us to understand the nature of biological organic synthesis, but they have also given us insight into potential modification of these biosynthetic pathways.

With a deeper understanding of the particular chemistry utilized by these organisms we can exploit these synthetic capabilities. Enzymes involved in secondary metabolism which are normally present in low quantities can be over-expressed using the tools of molecular biology facilitating their isolation and subsequent study. In addition, over-expressed enzymes may serve as efficient catalysts to carry out *in vitro* synthesis.[1-3] The use of enzymes to conduct organic synthesis is gaining in popularity and is becoming more important as environmental issues surrounding chemical waste become a public concern.[4] Therefore, it is important to increase our knowledge of biological transformations and to increase the number of these over-expressed enzymes available for the synthetic organic chemist.

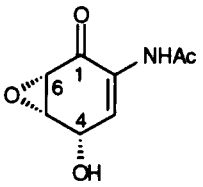
It is often the case that a particular natural product will possess great activity in an *in-vitro* assay yet show toxic side effects when administered in clinical trials. The ability to modify molecular structure may alleviate undesirable side effects. Hybrid natural products have traditionally been produced by total synthesis and semi-synthesis or by directed biosynthesis in the producing organism.[5] For example, the outcome of a biosynthetic pathway can be altered by feeding analogs of intermediates found in the pathway. In many cases, the organism will incorporate these analogs into the pathway affording new natural products. More recently, as a result of molecular biology, the construction of modified biosynthetic pathways which produce new hybrid metabolites has been undertaken.[6] This is accomplished by rearranging the chemical capabilities of the organism, (i.e., by rearranging the existing genes) and/or adding foreign genes.[7-9]

Previous Work on LL-C10037α

The anticancer antibiotic LL-C10037α was isolated in 1984 from fermentation filtrates of Streptomyces LL-C10037.[10] Its structure was subsequently revised to the epoxyquinol, **1**, on the basis of X-ray diffraction analysis and excitation circular dichroism studies.[11] By feeding specifically labeled acetate and glucose, Gould and Whittle determined **1** was derived via the shikimic acid pathway.[12]



1st assigned structure of LL-C10037α



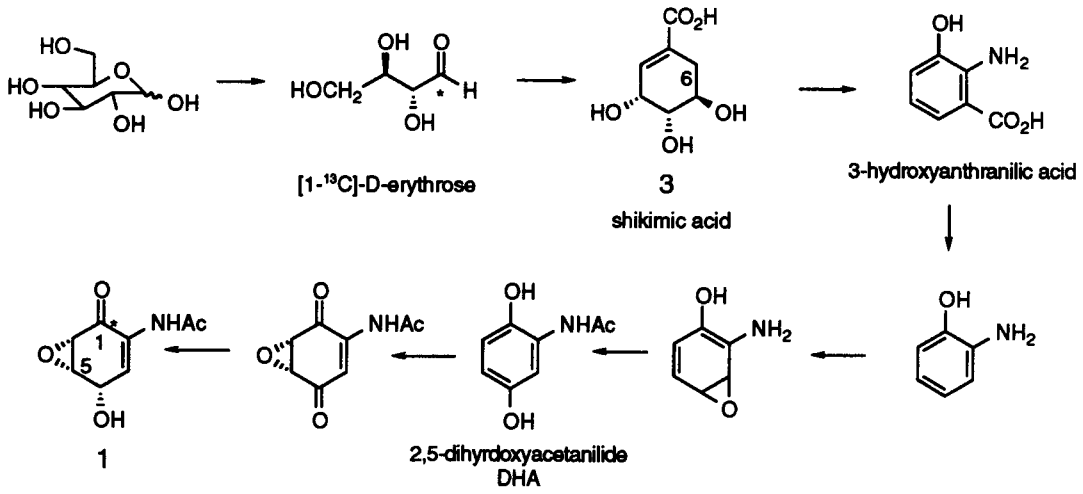
1

Revised Structure of LL-C10037α

The correct orientation of the shikimic acid precursor relative to **1** was elucidated by feeding [1-^{13}C]-D-erythrose, which specifically labeled **1** at C-1 rather than at C-5, (Scheme 1). Therefore, it followed that the amide nitrogen of **1** was introduced at C-6 of shikimic acid, **3**, rather then at C-2. The latter finding helped identify 3-hydroxyanthranilic acid as a key intermediate. By carrying out the fermentation under an atmosphere of $^{18}O_2$, the oxygen atoms at the oxirane and the secondary alcohol C-4 were identified as being derived from molecular oxygen. The oxygen at C-1 remained unlabeled by the $^{18}O_2$ atmosphere, indicating that this oxygen was retained from the precursor. An alternative explanation for the lack of ^{18}O label at C-1 was that **1** can undergo exchange of the oxygen at C-1 with the oxygen of water. The steps beyond 3-hydroxyanthranilic acid were elucidated by feeding specifically labeled intermediates in whole-cell and cell-free systems, (Scheme 1).[13]

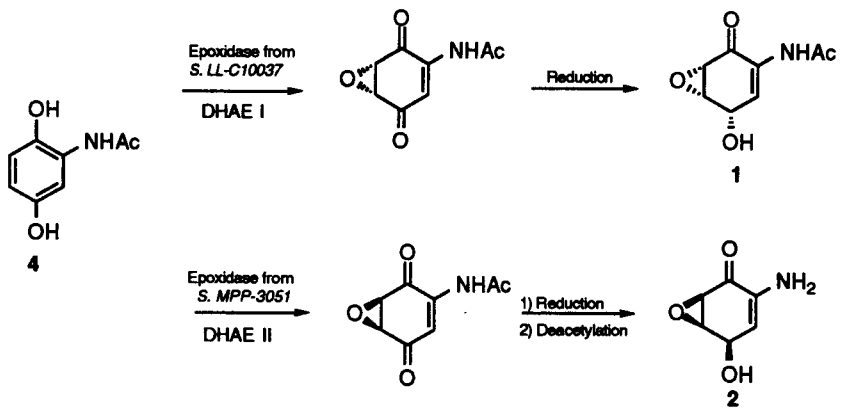
While Gould et al. were examining biosynthesis of LL-C10037α, Box and coworkers from Beecham Pharmaceuticals reported the isolation and structure elucidation of a very closely related antibiotic metabolite MM14201, **2**.[14] MM14201 was isolated from another *Streptomyces* species, MPP-3051, and was the deacetyl mirror image of **1**. Enantiomeric natural products are quite rare in nature and in only a few instances have the complementary enzymes been isolated and shown to utilize the same achiral substrate (see discussion in ref. 15).

Scheme 1
Biosynthesis of LL-C10037α, **1**



The MMP-3051 organism was obtained from Beecham Pharmaceuticals and the enzymology responsible for this enantiomeric relationship investigated. Shen and Gould were able to demonstrate the epoxidases from cell-free extracts of both organisms acted upon the same achiral substrate, 2,5-dihydroxyacetanilide (DHA), **4**, (Scheme 2).[16] Both epoxidases were purified to near homogeneity and characterization commenced.[15]

Scheme 2
Final steps in the biosynthesis of **1** and **2**



General Overview of Oxygenases

This thesis will focus on the further characterization of the two aforementioned epoxidase enzymes (DHAE I & II, Scheme 2). Since the two reactions appear, at least on the surface, to add one oxygen atom to their respective products, the reactions were assumed to be carried out by monooxygenases. However, the involvement of a dioxygenase mechanism could not be ruled out as a possibility. Therefore, a brief discussion of both oxygenase enzyme types is included here.

Monooxygenases

Monooxygenases react with a substrate by adding molecular oxygen. One atom of the oxygen molecule is introduced into the substrate, while the second atom forms water. Monooxygenases are further broken down into two classes.[17] Those which require an externally added reducing cofactor (NADPH, NADH, L-ascorbic acid or tetrahydrobiopterin) to reduce the second atom of oxygen to water are referred to as external monooxygenases (Scheme 3). Members of the second class, the so-called internal monooxygenases, have the ability to split hydrogen from the substrate itself.

Scheme 3
Overall reactions of external and internal monooxygenases

External Monooxygenases:

$$S + O_2 + XH_2 \longrightarrow SO + H_2O + X$$

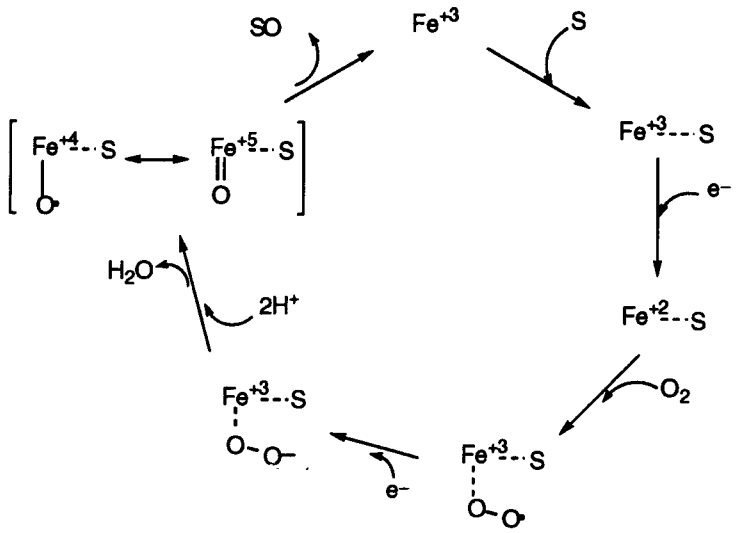
Internal Monooxygenases:

$$SH_2 + O_2 \longrightarrow SO + H_2O$$

S, SH_2: substrate XH_2: reduced cofactor
SO: oxidized substrate X: oxidized cofactor

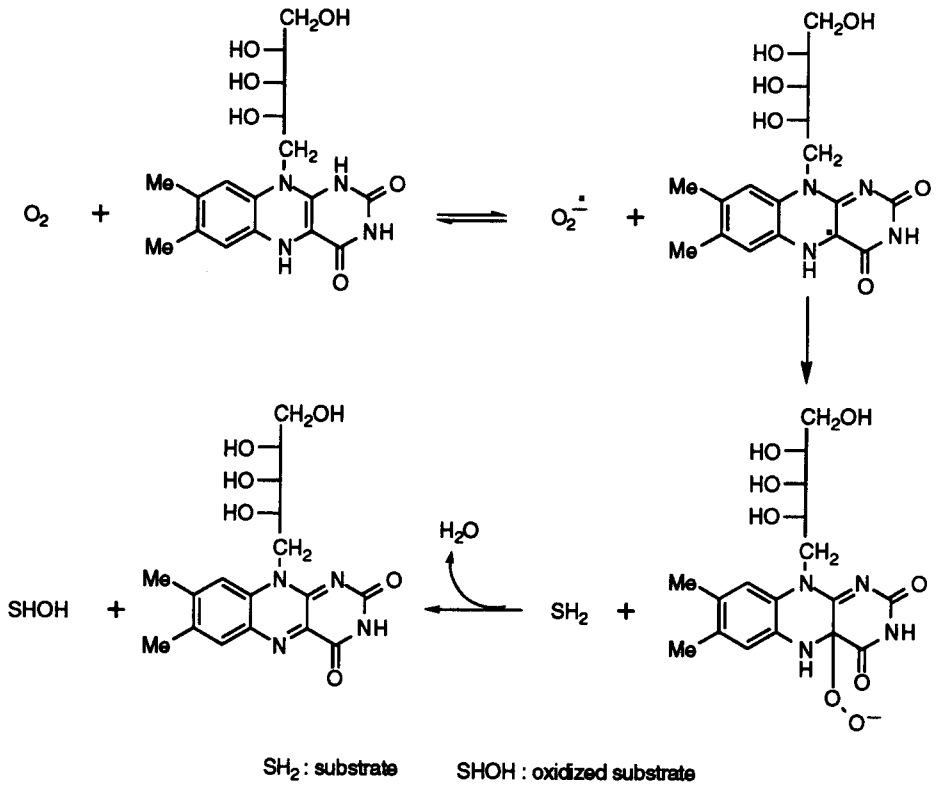
One of the most complex and ubiquitous of all the monooxygenase systems are the cytochrome P450-dependent monooxygenases.[18] The catalytic cycle begins with the binding of substrate (S) to the iron while it is in a ferric state (Scheme 4). The iron is then reduced by the action of another enzyme (flavoprotein NADPH-cytochrome P450 reductase). The ferrous enzyme quickly binds oxygen and the resulting oxygenated complex then accepts a second electron from ferredoxins or cytochrome b5, depending on the source of the monooxygenase. Water is lost from the iron-oxygen complex to yield what is believed to be an iron +5 species (perferryl), which then adds oxygen to the substrate giving back the P450 enzyme in a ferric state.

Scheme 4
Catalytic cycle for cytochrome P450-dependent monooxygenases



The second most common monooxygenases are the flavin-monooxygenases. These are the so-called internal monooxygenases because the flavin cofactor is very tightly bound to the enzyme (Scheme 3).[17,19] In some cases it is actually covalently bound. These oxygenases usually require a second enzyme to interact with them in order reduce the flavin cofactor and restore their oxidizing ability. To start this catalytic cycle molecular oxygen accepts an electron from the reduced flavin (Scheme 5). The resulting superoxide anion and semiquione combine to form the hydroperoxide. Upon reaction with the substrate O-O bond fission occurs giving the oxidized substrate and a molecule of water. The cofactor is then regenerated by the action of another enzyme (flavin-reductases).

Scheme 5
Oxidation by flavin-dependent monooxygenases



In addition to the above mentioned cytochrome P450-dependent and flavin dependent monooxygenases, there are pterin-dependent and ascorbate-dependent copper containing monooxygenases. These latter two, although worthy of mention, occur much less often and will not be described in further detail.[17]

Dioxygenases

Dioxygenases represent a much smaller group of enzymes than the monooxygenases. They are defined as enzymes that catalyze the incorporation of two atoms of molecular oxygen into the substrate or substrates.[17,20] Intramolecular dioxygenases are the most common type. In this reaction, one substrate receives two atoms of oxygen (Scheme 6). The other type are the intermolecular dioxygenases, which can involve either one atom each of the oxygen molecule incorporated into two different molecules of the same substrate or one atom each of the oxygen molecule incorporated into two different substrate molecules (Scheme 6).

Scheme 6
Overall reactions of intra- and intermolecular dioxygenases

Intramolecular dioxygenases

$$S + O_2 \dashrightarrow SO_2$$

Intermolecular dioxygenases

$$2\,S + O_2 \dashrightarrow 2\,SO$$

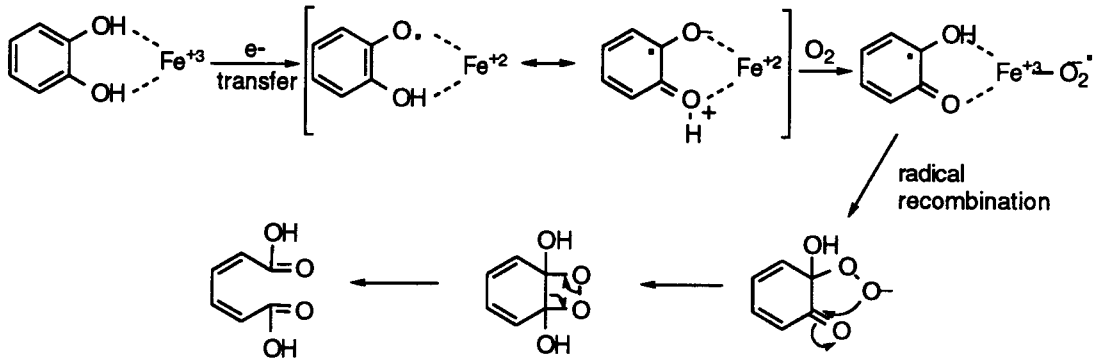
$$S + S' + O_2 \dashrightarrow SO + S'O$$

S & S': substrate SO_2: oxidized substrate
SO: oxidized substrate

Like the monooxygenase family of enzymes, there is more than one class of dioxygenase. Five classes have been identified: nonheme iron-containing, heme-containing, copper-containing, flavin-dependent and, α-ketoglutarate-requiring.[21] The first class, the nonheme iron-containing dioxygenases, represents the largest class. These are the intramolecular dioxygenases that act to cleave aromatic and heteroaromatic rings. Catechol 1,2-dioxygenase (pyrocatechase) is one of the best known members of this group. The mechanism is believed to involve the initial formation of a phenolic radical (Scheme 7).[17,21] The Fe^{+2} is then oxidized by oxygen to give the reactive oxygen species which combines with the phenolic radical forming the peroxy intermediate. The peroxy adduct rearranges to the dioxetane which undergoes a retro 2+2 cycloaddition giving the product.

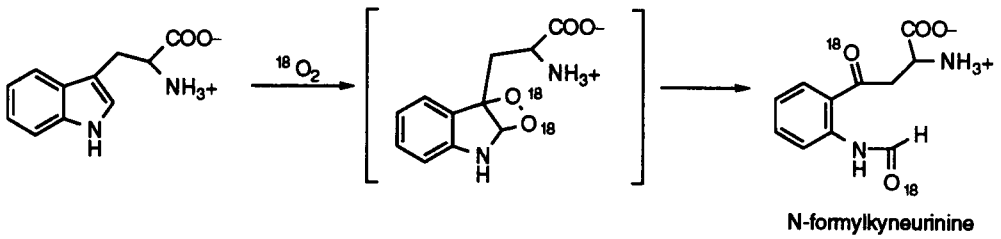
Scheme 7
Overall reaction of pyrocatechase a nonheme iron-containing dioxygenase

Proposed mechanism of pyrocatechase



Iron heme-containing dioxygenases have been found only sparingly in nature in comparison to the nonheme iron-containing dioxygenases.[21] One important representative of this class is tryptophan 2,3-dioxygenase which functions physiologically in the break-down of the indole ring forming N-formylkyneurinine. Tracer studies with pseudomonad tryptophan 2,3-dioxygenase in the presence of $^{18}O_2$ have demonstrated that both oxygens of molecular oxygen are retained in the carbonyls which where originally the 2 and 3 carbons of the indole ring (Scheme 8). The cyclic endoperoxide (dioxetane) has been proposed as an important intermediate in the mechanism of this enzyme.

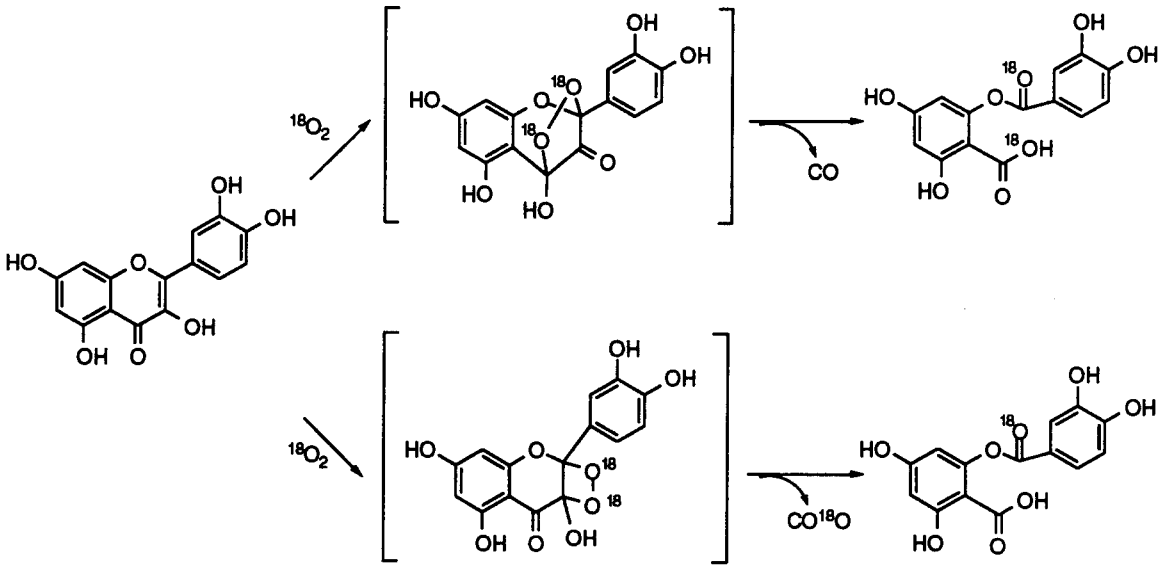
Scheme 8
Reaction of tryptophan 2,3-dioxygenase an iron heme-containing dioxygenase



N-formylkyneurinine

In addition to the iron-containing dioxygenases, copper has also been found to be an important cofactor in a couple dioxygenases (copper is utilized much more often in the monooxygenase family). A representative from this class is quercetin 2,3-dioxygenase which is an inducible extracellular enzyme from *Aspergillus flavus*. This enzyme forms two distinct intermediates which give different labeling patterns when $^{18}O_2$ is used as a tracer (Scheme 9).[22] The top intermediate of Scheme 9 looses carbon monoxide leaving both labeled oxygens in the product. However, the lower intermediate (dioxetane) undergoes a decarboxylation and looses the labeled oxygen to the carbon dioxide. This copper-containing dioxygenase could be considered both an intra- and intermolecular dioxygenase, making it a very interesting example of the diversity seen in these oxygenases. It also demonstrates the pitfalls one can fall in when attempting to classify this family of enzymes.

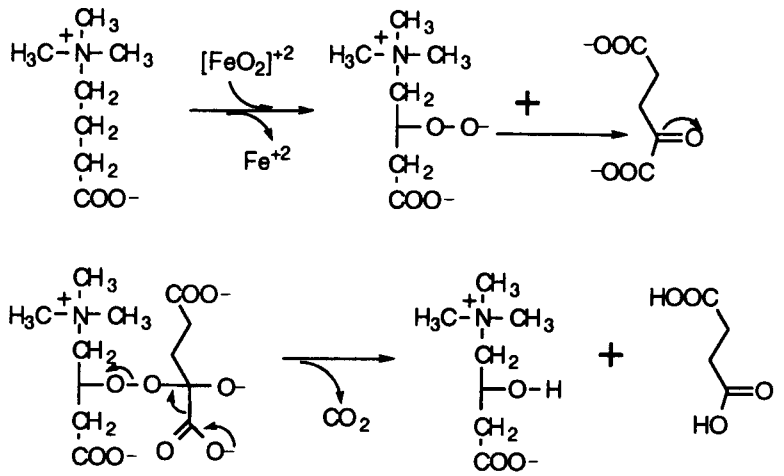
Scheme 9
Reaction of quercetin 2,3-dioxygenase a copper-dependent dioxygenase



The last class of dioxygenases discussed in this brief introduction are the α-ketoglutarate-requiring dioxygenases. These reactions are the so-called intermolecular dioxygenases. They are known to require iron and a reducing cofactor (usually ascorbic acid) in addition to the α-ketoglutarate.[17] One example of this class is γ-butyrobetaine 2-oxoglutarate dioxygenase (Scheme 10).[22] In the mechanism proposed by Lindstedt, the substrate is first converted into an active peroxy anion. The peroxide of the substrate then attacks the carbonyl carbon of α-ketoglutarate. The glutarate potion of the conjugate then decarboxylates forming the three products.

Scheme 10

Reaction of γ-butyrobetaine 2-oxoglutarate, an α-ketoglutarate-requiring enzyme



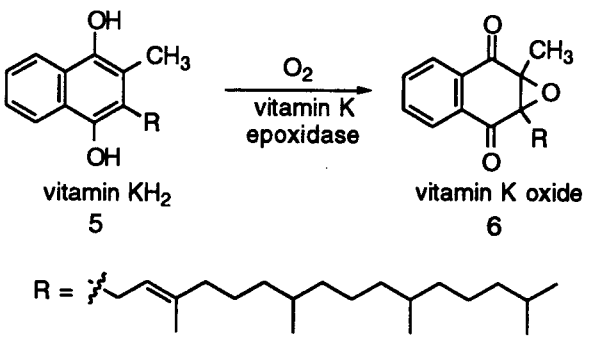
Unusual Characteristics of DHAE I and DHAE II

The enzymes, dihydroxyacetanilide epoxidase I (DHAE-I) and DHAE-II from *Streptomyces* strains LL-C10037 and MPP-3051, respectively, were initially suspected of belonging to the monooxygenase family of iron and copper metalloenzymes. However, there are some unusual features which distinguish them from that class of enzymes. DHAE-I and -II bioassays showed neither flavin nor cytochrome P_{450} dependence. In addition, they were unaffected by adding Fe^{+2} or Cu^{+2}, but, surprisingly, were positively effected by the addition of Co^{+2}, Ni^{+2} and, Mn^{+2}. When the DHAE I apoenzyme was generated by adding the chelating agent, 1,10-phenathroline, activity could only be restored with these latter three ions.[15]

Only three other hydroquinone epoxidases have been partially characterized (see Chapter 3). Like DHAE I and DHAE II, these investigations have demonstrated the cofactor independence of this type of epoxidation reaction One enzyme of particular interest to human health is vitamin K epoxidase which is integrally involved in blood clotting.

The mammalian epoxidase carries out the epoxidation of vitamin K hydroquinone (vitamin KH_2), **5**, to the vitamin K oxide, **6**, (Scheme 11).[23] This enzyme, once thought to be a monooxygenase, has been re-examined using isotopic labeling. The results indicate the addition of both oxygen atoms from molecular oxygen into the vitamin K epoxyquinone, **6**.[24,25] Mammalian vitamin K epoxidase has been re-classified as a dioxygenase.

Scheme 11
Overall reaction of vitamin K epoxidase



The substrates and products of vitamin K epoxidase and epoxidases DHAE I & II possess common structural features. In addition, the vitamin K epoxidase from mammalian cell lines shares a couple of the same "unusual" characteristics found in the two epoxidases. Vitamin K epoxidase is also cofactor independent. The substrate vitamin K hydroquinone itself serves as the reducing equivalent. In addition, neither iron or copper ions have, as of yet, been identified as being necessary for activity.

These epoxidases may signify a new class of oxygenases. Because DHAE I & II come from a bacterial source, they are easier to obtain then enzymes from higher organisms and therefore may serve as a paradigm for the study of this new class of "hydroquinone epoxidizing" oxygenases. The metabolism of oxygen plays such an essential role in biological systems, so further study of the mechanism for forming this oxirane functionality may provide information for understanding oxygen activation in other enzyme mediated oxidations.

The use of DHAE I and DHAE II as Catalysts for Carrying out Organic Synthesis and for the Production of Fine Chemicals (7 and 8)

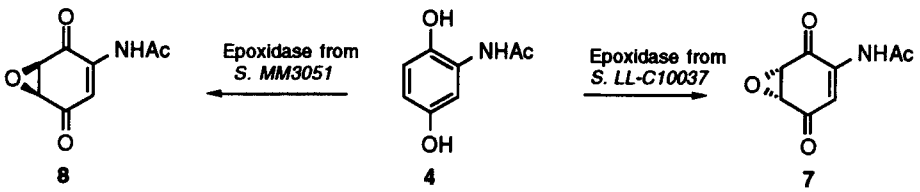
Biotechnology is beginning to play a major role in the synthesis of enantiomerically discrete natural products. Whole-cell microbial fermentations and cell-free enzymatic transformations are being utilized to carry out industrial scale production of chiral synthons.[1-3,7] In general, these biological transformations are more sterioselective and more environmentally benign then their non-biological counter-part. For this reason, there is a major search going on for enzymatic systems that will produce complex molecules on an industrial scale.

While whole-cell microbial fermentations allow for much diversity in the types of reactions available to the synthetic chemist, the product yields can suffer due to continued metabolism of the desired products and complications experienced when extracting the desired product. Furthermore, the typical synthetic organic chemist may not have the biochemical background or laboratory equipment it takes to grow and maintain the organism which carries out the desired bio-organic transformation. Because of the problems associated with whole-cell bio-transformations, the use of purified enzymes has gained in popularity. These pure enzymes or partially-purified enzymes can be utilized much like any chemical reagent is used to carry out organic synthesis. Furthermore, they can be used on both small and large scales.

The use of enzymes to produce biologically active compounds both in academic and industrial settings is experiencing a boom at this time. Most of the bio-transformations can be summarized into three types of reactions: 1) those that involve the kinetic resolution of racemates, 2) asymmetric hydrolysis reactions of esters, 3) reduction reactions of diasteriotopic substrates.[1] A fourth reaction type, which is much less exploited, is oxidation reactions. The problem with oxidative bio-transformations, and the reason they are used less than other types of reactions, is that most oxidative enzymes require organic cofactors. These cofactors are expensive, unstable, and complicate the reaction process. There are ways to keep the cofactor cost down, which involves using only catalytic amounts of the cofactor and an additional enzyme which recycles the cofactor back to its useful form. However, most traditional synthetic organic chemists will avoid using a bio-organic reaction when the reaction is complicated by the requirement of multiple enzymes working together in a cycle.

The four electron oxidation reactions of DHAE I and II do not require the addition of cofactors (Scheme 12).[15] Moreover, the natural products of this pair of epoxidases (**7** and **8**) are commercially valuable in their own right or as starting materials for more complex synthesis. Therefore, DHAE I and DHAE II make excellent candidates to be used as catalysts (reagents) for carrying out organic synthesis.

Scheme 12
Enzymatic production of epoxyquinone fine chemicals

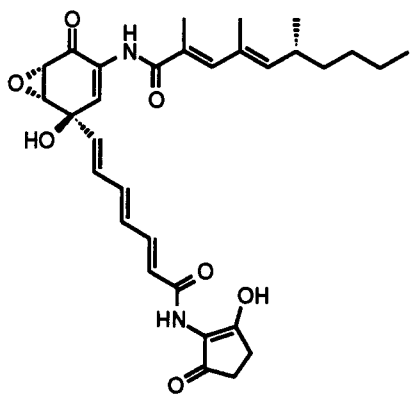


8 **4** **7**

When considering the development of DHAE I and II as catalysts to carry out organic synthesis, it is not enough to suggest their use based solely on the fact that neither requires an organic cofactor. This cofactor independence is an important consideration, however, there must also be a need for this type of epoxidation chemistry. This bio-transformation must fill a niche which conventional organic synthesis is unable to achieve.

The importance of this chemistry is clearly demonstrated by the recent interest in the total synthesis of the epoxyquinol skeleton.[26-29] In the last three years, three total syntheses of the epoxyquinol natural product, LL-C10037α, **1**, have been reported. Another synthesis, within this same time-frame, reports the total synthesis of bromoxone, a natural product with the same epoxyquinol backbone as **1**, but having a bromine substituted for the N-acetyl side chain. These four total syntheses are relatively short when compared to other synthetic projects appearing in the literature (7 and 9 steps for racemic **1**, 13 steps for optically pure **1**, and 8 steps to bromoxone). However, when compared to the possibility of producing **1** via enzyme biosynthesis using the enzymes from *S.* LL-C10037 they appear lengthy and inefficient.

Much of this synthetic interest is a direct result of recent research which has demonstrated that manumycin, a member of the epoxyquinol family of natural products, is a very potent and selective inhibitor of ras farnesyltransferase.[30,31] The ras oncogene is involved in over 30% of all human cancers, which has led to an intense search for selective inhibitors of Ras p21 processing.



Manumycin A

Cloning Strategy

Because DHAE I and II have such interesting and unique characteristics (unusual metal requirements, neither flavin nor cytochrome P450 dependent oxygenases), we wanted to obtain large quantities of each enzyme. Having substantial quantities of each enzyme would help facilitate their physical and mechanistic characterization, in addition to making possible the commercial scale synthesis of the products, **7** and **8**. Since these two epoxidases are expressed at very low levels, as with most other enzymes responsible for the production of secondary metabolites, we need to rely on the tools of molecular biology to allow the cloning and over-expression of the pair.

In order to begin the cloning process, the genomic DNA encoding the desired protein must be identified. Today, there are a several techniques for identifying the gene of interest.[32-34] Since the enzymes DHAE I & II were both nearly isolated, we chose to approach the identification of their genes through reverse genetics.

In this technique, one epoxidase is isolated to homogeneity and partially sequenced (N-terminal or internal). From the partial peptide sequence, the genetic code or codon preferences for streptomyces is utilized to identify the corresponding DNA sequence that would be responsible for encoding this partial amino acid sequence. The DNA sequence is chemically synthesized and radioactively labeled. The radioactive DNA is then used to probe membranes containing isolated pieces of genomic DNA for the gene of interest. The probe constructed from the partial sequence of DHAE I should also be used to probe for the gene responsible for DHAE II.

After identifying the epoxidase genes, it will then be possible to obtain large amounts of these enzymes by introducing this DNA into appropriate expression vectors. Today many recombinant organisms are available to serve as hosts for the over-expression of the heterologous DNA. It is not uncommon to obtain the product from the cloned gene as five percent or more of the total protein mass. Obtaining the two epoxidases in these quantities should allow for much easier isolations. In addition, the complete primary amino acid sequences of the enzymes can be determined from the recombinant DNA clones. This information, coupled with comparisons to previously characterized proteins, can be used to gain tremendous insight into the structural and functional features of the proteins.

Purpose of the Present Study

Preliminary enzymatic studies on DHAE I and II revealed many interesting features about the pair of epoxidases. To extend these studies, the initial purpose of the subsequent research had three goals.

The first objective was to improve purification the epoxidase from *S. LL-C10037*. The previous purification (not yielding pure epoxidase) utilized seven steps and took over a month to process. Using the pure enzyme, N-terminal and internal amino acid sequences would be obtained in an effort to begin the cloning of the structural genes.

A second objective was to investigate the detailed chemistry utilized by these epoxidases. New evidence had suggested that this family of hydroquinone epoxidases, once thought to be monooxygenases, may actually be dioxygenases.[24,25] This work probed this question for DHAE I and DHAE II.

As a final objective, the active sites of DHAE I and DHAE II would be examined. To probe the active site structural latitude of these epoxidases, a series of substrate analogs bulkier than **4** (replacement of the n-acetyl group with propyl, benzoyl, and *p*-nitrobenzoyl functionality) would be synthesized. These substrate analogs may act as alternative substrates or maybe as inhibitors. Demonstration of active site inhibition with these simple analogs will direct future work toward more elaborate syntheses of mechanism-based suicide inhibitors.

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Chapter 2

Isolation and Amino Acid Sequence of 2,5-Dihydroxyacetanilide Epoxidase I (DHAE I)

Marc J. Kirchmeier

Introduction

Only a small percentage of the thousands of enzymes that have been isolated are involved in secondary metabolism. A much smaller fraction have been cloned and over-expressed. Much of this imbalance is due to the importance of understanding the enzymatic transformations in primary metabolism since malfunction of these pathways causes many human disease states. A second reason is the difficulty isolating enzymes responsible for the production of secondary metabolites. These enzymes are expressed at very low levels and are only expressed in a very narrow window in an organism's lifetime. The enzymes are inherently unstable and very susceptible to protease hydrolysis.[1] Furthermore, they often must be isolated from cells containing large amounts of secondary metabolites which can denature and inactivate enzymes. It is, however, becoming less difficult to isolate the enzymes involved in secondary metabolism since protein chromatographic technology is improving.

The goal of this component of research was to isolate a homogeneous epoxidase (DHAE I) and obtain N-terminal and internal amino acid sequence information. This sequence information would be used to construct the DNA probes necessary for cloning DNA responsible for DHAE I and DHAE II.

Previous Isolation Studies

Despite the problems associated with isolating the enzymes of secondary metabolism, Gould and Shen have reported the isolation, to near homogeneity, of 2,5-dihydroxyacetanilide epoxidase I (DHAE I).[2] Their isolation protocol took five weeks to process and involved six chromatographic steps starting from an ammonium sulfate pelleted epoxidase: 1) S-200 size exclusion, 2) Quaternary Methyl Amine (QMA) anion exchange, 3) high resolution phenyl hydrophobic interaction chromatogaphy (phenyl HIC), 4) high resolution diethylaminoethyl (DEAE) anion exchange in a phosphate buffer system, 5) high resolution 300 SW size exclusion, and 6) high resolution DEAE in a Tris buffer system. Using this procedure they were able to isolate 112 μg of near homogeneous DHAE I from 180 grams of cells. The final preparation showed eight bands by silver stained SDS PAGE and unfortunately, the band corresponding to DHAE I was never unambiguously identified. Therefore, the amino acid sequence could not be determined.

Although the epoxidase enzyme was not purified to homogeneity, an enormous amount of information was gathered. A great deal was learned about stabilizing the epoxidase activity. Initially, the cell free extract was prepared in a 10 mM potassium phosphate buffer, pH 7.0. In this buffer the activity was completely lost in 24 hours at 4 °C. With the addition of 0.2 mM EDTA , 20% glycerol, and an increase to 50 mM potassium phosphate, 87% of the activity was retained at 4 °C after 48 hours. Furthermore, the enzyme, in this EDTA / glycerol phosphate buffer solution, could be stored for several months at -80 °C without significant loss of activity. These early investigations revealed useful information about the chromatographic behavior of DHAE I on anion exchange and phenyl hydrophobic interaction resins, as well as the enzyme's stability during ammonium sulfate precipitation.

After carefully reviewing these preliminary isolation procedures, it was decided that an improved protocol was needed in order to get a pure enzyme. Although the ammonium sulfate precipitation was utilized to concentrate the activity, no significant increase in specific activity (1.2 fold purification) was found and 40% of the total activity units from the cell free extract (CFE) were lost. The S-200 size exclusion column was selected because the resuspended ammonium sulfate pellet could be added to the column without requiring a prior desalting step. However, this step, when fully optimized, only gave a 2.2 fold purification with an additional 20% loss in total units. The combination of the ammonium sulfate precipitation followed by size exclusion chromatography only gave a 3.8 fold purification from CFE and 60% of the total units were gone. Therefore, the first two steps (ammonium sulfate precipitation and S-200 size exclusion chromatography) in the purification needed to be replaced with a procedure that would purify while also retaining activity.

New Isolation Procedures

Included in this section is a summary of the protocol (outlined in Table 1) which was utilized to reproduceably isolate homogeneous DHAE I.

DE-52 Anion Exchange

A single DE-52 anion exchange column was more productive than the combination of the ammonium sulfate precipitation and S-200 size exclusion column in obtaining a homogeneous enzyme (Table 1). After some practice, this anion exchange step gave a six fold purification and, surprisingly, also gave an increase in the total units of activity.

This increase in activity, from CFE to recovered DE-52 fractions, was likely due to the removal of interfering molecules which inhibited the DHAE I reaction. The recovered DE-52 protein fractions were then concentrated using centriPrep 30s (30,000 molecular weight cut off, Amicon Inc.). In addition to protein concentration, the use of these Amicon filters also functioned as a size exclusion step because all the proteins less than 30 kD were removed.

Table 1
Current isolation scheme utilized to obtain homogeneous DHAE I

Stage of Purification	Vol (mL)	Protein[a] (mg)	U[b]	U%	Specific Activity	Fold Purification[c]
Cell free extract	600	1,012	93.6	100%	0.0935	0
DE-52	128	274	93.6	100%	0.561	6
IMAC	40	63	66.0	70%	1.68	18
C-4 or 5 HIC	30	9	52.4	56%	20.2	216
Phenyl HIC	4	0.107	36.5	39%	202	2160
DEAE	2	0.006	21.0	23%	2,150	23000
BioRad 491 cell	NA	NA	NA	NA	NA	NA

a. Protein concentration was determined by Bradford's method (BioRad protein assay, see experimental)
b. Percent recovery is defined as the total units recovered from a particular isolation step divided by the total units in the CFE. One unit of activity is defined as the production of 1 µmol epoxyquinone / min
c. Fold purification is defined as the specific activity of recovered DHAE I divided by the specific activity of DHAE I in the CFE. Specific activity is defined as units of activity / mg protein

Immobilized Metal Affinity Chromatography (IMAC)

Since the preparation starts with so much cellular mass (typically around 250 grams of cells), 63 mg of crude protein still remained after the DE-52 anion exchange column. Therefore, a large preparative-sized column was sought as the next step. The ideal matrix should be commercially available in bulk quantities, have a large binding capacity and possess high flow rates. Immobilized Metal Affinity Chromatography (IMAC) met all of these criteria so it became the next sucessful purification step.[3,4]

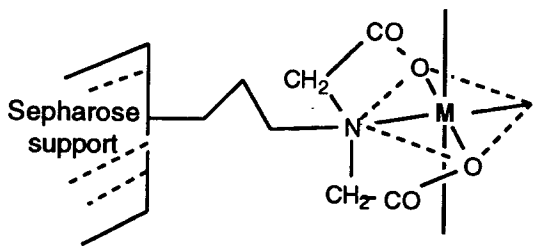
IMAC is relatively new, so a brief description of how it separates proteins follows. This is a technique for separating proteins based on their ability to bind metal ions which have been immobilized on a stationary phase. Pharmacia sells a chelating sepharose backbone which has tridentate iminodiacetic acid ligands attached to hydrocarbon arms (Figure 1). These ligands have the ability to chelate various metal ions from three sides which leaves three open chelation sites on the metal where the proteins can bind. The sites are occupied by water prior to being displaced by the protein. The proteins are partitioned depending on the number of surface cysteine, histidine, and tryptophan residues which coordinate to the chelated metal.

Another reason for seeking an IMAC affinity matrix for the purification of DHAE I stems from the epoxidase's probable need for metal ions in the active site.[5] With this in mind, the epoxidase should have some means of obtaining the required metal and also distinguishing the correct metal from all the other possibilities. Therefore, passing a semi-pure enzyme preparation or apoenzyme preparation through individual columns charged with different metals should reveal which metal ion is bound best by the enzyme. This may help confirm the metal ion requirements of DHAE I in addition to giving the desired purification.

However, surprising results have been obtained. A recent review considers comparative studies on metalloproteins where carboxypeptidase, a zinc containing enzyme, and apocarboxypeptidase were found to have no affinity for immobilized zinc.[3] Yet both were retained on a copper charged column. These observations serve as a caution that the bound metal may not be part of the enzyme or relevant to its mechanism.

Figure 1

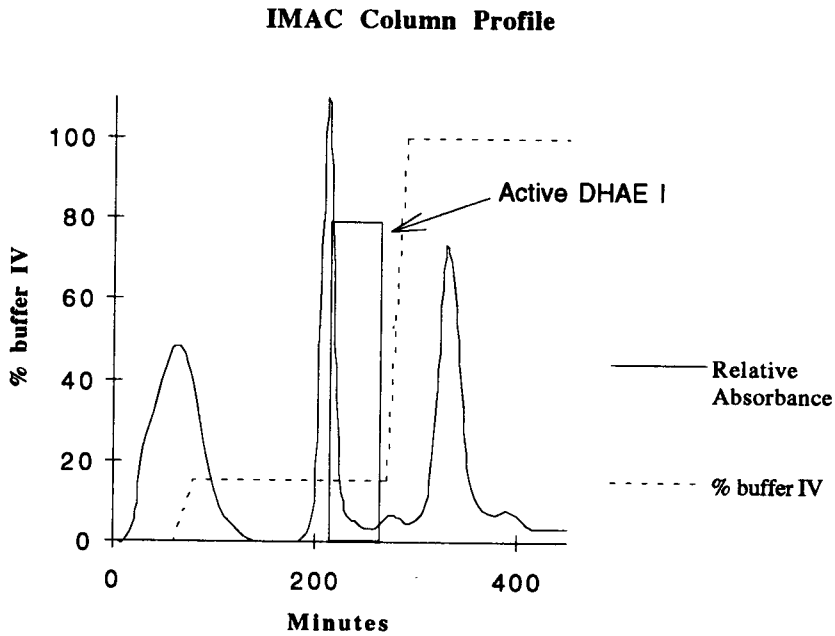
Schematic illustration of surface bond metal chelate.



In order to find an immobilized metal ion that would bind DHAE I, a series of small individual columns was prepared, each with a different metal ion. The following metal ions were examined: Ni^{+2}, Fe^{+3}, Ca^{+2}, Mn^{+2}, Co^{+2}, and Cu^{+2}. Binding was observed with only two of the immobilized ions, Ni^{+2} and Cu^{+2}. Immobilized Cu^{+2} bound DHAE I tightly while Ni^{+2} only bound weakly. With both columns, DHAE I could be eluted with an imidazole gradient. Thus, Cu^{+2} ion was selected for scale up to the preparative sized column.

The active fractions from the DE-52 anion exchange column were pooled and concentrated. The entire preparation was loaded onto the Cu^{+2} IMAC column and the adsorbed protein was eluted from the matrix with a gradient of imidazole. After optimizing the gradient conditions (Figure 2), 70% of the activity units were recovered and an overall 18 fold purification was achieved.

Figure 2
Typical copper IMAC profile of DHAE I preparation. The box designates active epoxidase (see experimental section for more details).

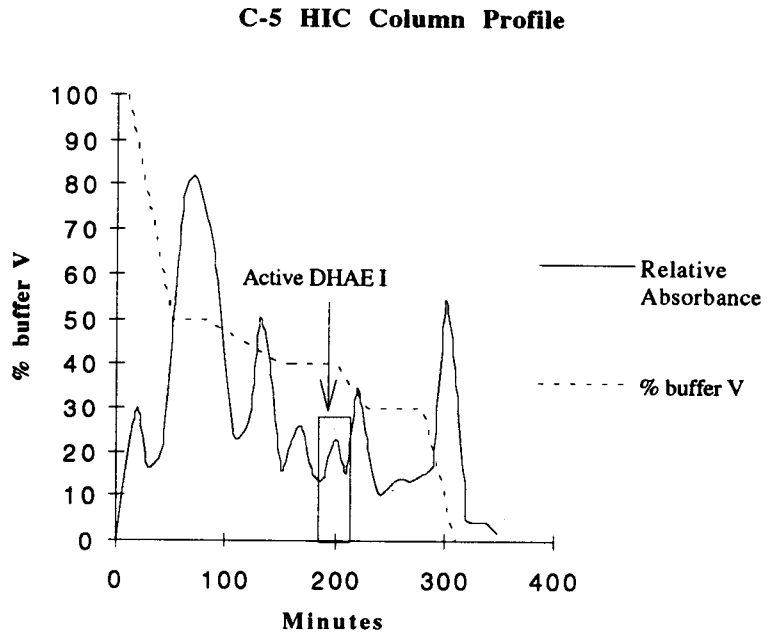


Low Resolution *n*-pentyl Hydophobic Interaction Chromatography (HIC)

Straight chain HIC was utilized next because it offers a different mode of separation than the previous two columns. It is also commercially available in bulk quantities and is fairly inexpensive. The choice of carbon length, C-2 to C-12, was made by utilizing a test kit (Sigma) consisting of small columns with resins of each chain length. C-2 and C-3 HIC matrices did not bind while C-6 to C-12 bound too tightly. The optimum chain length for retention of the epoxidase was estimated to be C-4 or C-5.

Since a bulk C-5 agarose column was already available, it was tried first. The column was loaded in high salt ($(NH_4)_2SO_4$) to maximize the hydrophobic interaction. DHAE I was then eluted by lowering the salt concentration. After optimizing this step, a 12 fold purification from the IMAC fractions was achieved (Figure 3). Overall this gave a 216 fold purification from the CFE. Only 14% of the total activity was lost during this step leaving 56% of the overall activity remaining after the three columns (DE-52, IMAC, C-5 HIC). The active fractions from this step were run on SDS PAGE and visualized by silver staining. Butyl (C-4) HIC was later found to give the same results.

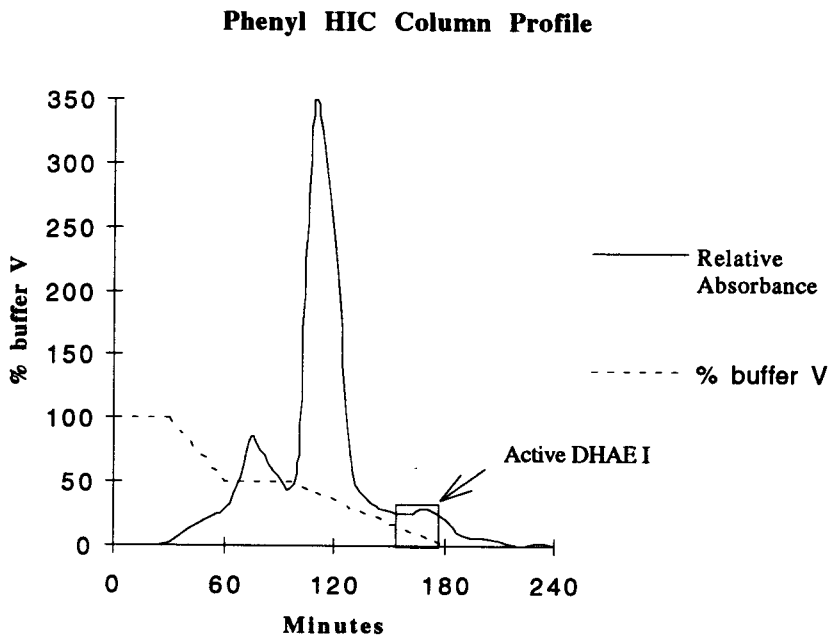
Figure 3
Typical C-5 HIC column profile. The box designates active epoxidase (see experimental section for more details).



High Resolution Phenyl Hydrophobic Interaction Chromatography

At this stage, the protein quantity was reduced to less than 5 milligrams. This was a good point in the purification scheme to return to an earlier purification protocol and use the high resolution phenyl HIC Fast Protein Liquid Chromatography (FPLC) column. This was an ideal choice of columns to follow the C-5 HIC since the enzyme was already in $(NH_4)_2SO_4$. The addition of more salt to bring the C-5 HIC fractions back to 1 M was all that was necessary; no buffer exchange or concentration step was required. Because the protein concentration was so low, the entire sample could be loaded onto the column in one step. After optimizing the gradient conditions, a consistent 10 fold purification was achieved in this single chromatographic step and only 17% of the activity was lost (Figure 4). Overall, these four chromatographic steps (DE-52, IMAC, C-5 HIC, phenyl HIC) accomplished a 2160 fold purification with 39% of the activity remaining from the CFE. The active fractions from this step were run on SDS PAGE and visualized by silver staining. The decision of which fractions to carry to the next step was based on both the SDS PAGE gel and the enzyme activity.

Figure 4
Typical phenyl HIC profile of DHAE I preparation. The box designates active epoxidase (see experimental section for more details).

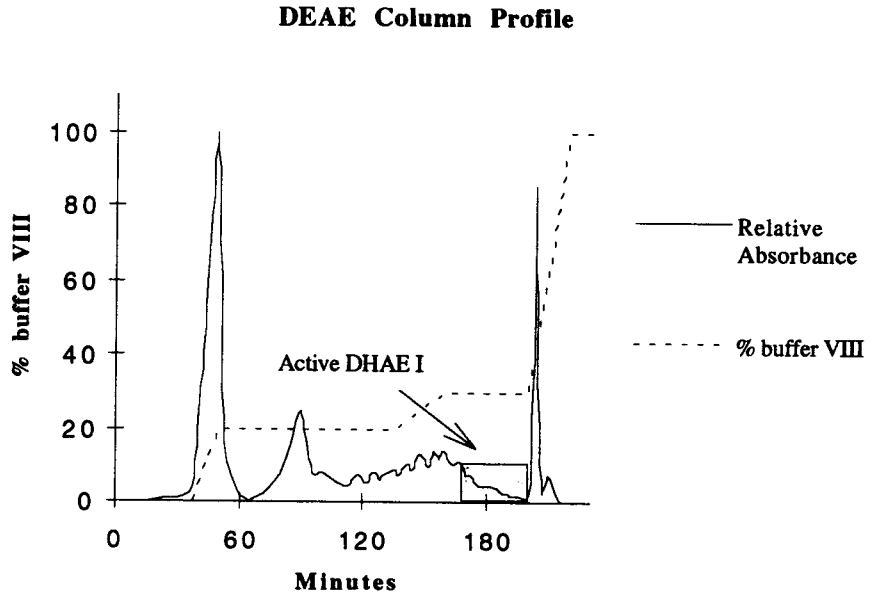


High Resolution Diethylaminoethyl-Cellulose (DEAE-Cellulose)

At this point, the use of a high resolution DEAE-cellulose FPLC column was evaluated as a means of final purification. In the earlier attempts at purification, 50 mM potassium phosphate, pH 7.0 and 50 mM Tris·HCl, pH 7.5 buffers were utilized to give, at best, a respective 4 and 1.4 fold increase in purification. When these columns were run, the pI had been estimated to be 6.5. Since then, the pI has been more accurately determined to be 4.6 by using the RF3 isoelectric focusing unit available through the Center for Gene Research and Biotechnology (OSU). Therefore, by changing buffers and lowering the concentration of the buffering component, it should be possible to "fine tune" this anion exchange step. Choosing a histidine solution which buffers best at pH 6 would still leave DHAE I negatively charged. This pH, however, will be lower than the pI of many of the interfering proteins. This would facilitate the removal of contaminating proteins because they would become positively charged and hence, would no longer adhere to the positively charged matrix.

The combined active fractions from the phenyl HIC were concentrated and the buffer was exchanged with the new 20 mM histidine buffer. The protein was loaded onto the DEAE-cellulose column and DHAE I was eluted with a linear gradient of 1 M NaCl in histidine buffer. Once the ideal gradient conditions were found, this column consistantly gave a 10.6 fold purification and only 16% loss of activity (Figure 5). Overall, from the five chromatographic steps (DE-52, IMAC, C-5 HIC, phenyl HIC, DEAE-cellulose), a 23,000 fold purification with 23% of the activity remaining from CFE was accomplished. However, SDS PAGE of the active fractions showed that, even after these five steps, the preparation was not pure DHAE I. In fact, at least five distinct bands were visible on the silver stained gel.

Figure 5
Typical DEAE 8HR profile of DHAE I preparation. The box designates active epoxidase (see experimental section for more details).



Native electrophoresis

Other column matrices and column schemes were attempted without obtaining any better purification. Therefore an alternative approach to column chromatography was investigated. Preparative native electrophoresis was utilized as a final step in the purification of DHAE I.

From previous work with the preparative isoelectric focusing unit (RF3, Rainin) it was demonstrated that DHAE I activity is sensitive to low ionic strength and/or electric currents. Nonetheless, epoxidase activity was detected in fractions collected from the RF3 experiments, and this was encouraging enough to examine preparative native electrophoresis, which also requires low ionic strength and electric currents. (Since the overall objective for running the native electrophoresis cell was to obtain pure DHAE I for amino acid sequencing, it would not matter if only 5% of the activity remained. It only had to be active enough to detect).

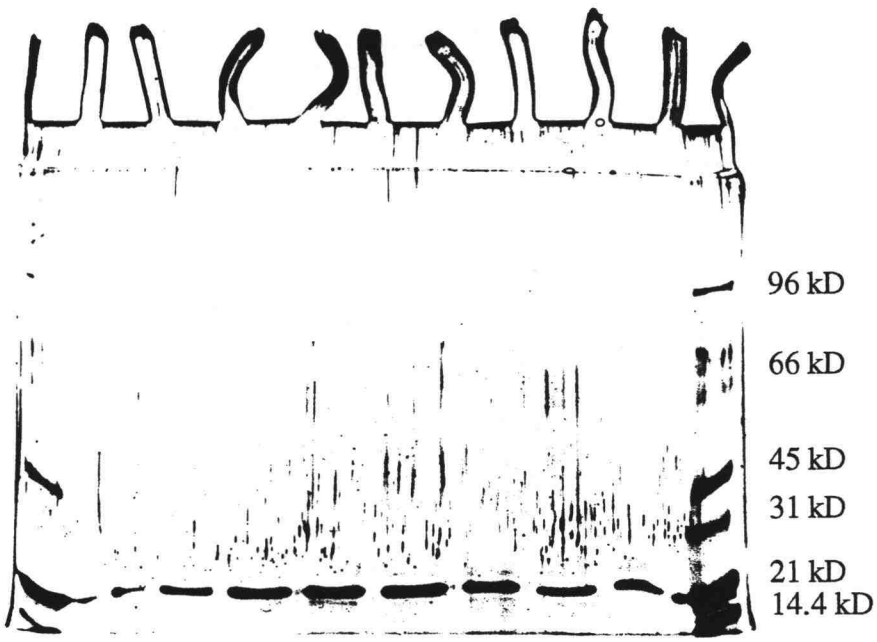
To test the feasibility of preparative native electrophoresis, we explored whether active protein could be recovered from a smaller analytical electrophoresis cell (MiniProteanII Cell, BioRad) run under nondenaturing (native) conditions. Part of the active DEAE fractions were run under these native conditions using a buffer system devised by Jovin.[6] After electrophoresis in this analytical gel, it was possible to excise an "active" section of gel which, when subjected to denaturing conditions (i.e. SDS PAGE), ran as a single band at 21 kD.

After seeing this single 21 kD band, we looked back upon the SDS PAGE gels run on fractions from phenyl sepharose and DEAE columns (at least six gels from six different preparations). In those gels, a 21 kD band became visible as the epoxidase activity appeared and the band disappeared as the activity also vanished. Furthermore, since it is highly unlikely that two proteins will have the same migration distances when subjected to both these electrophoresis methods, we believe this 21 kD SDS PAGE band is the epoxidase (DHAE I). This analytical native electrophoresis experiment represents the first time the epoxidase was isolated to homogeneity.

After laying the ground work, a preparative electrophoresis cell (Prep. 491, BioRad) was utilized. DHAE I was isolated again as an active single 21 kD band, but on a preparative scale so that N-terminal sequencing could commence (Figure 6).

This electrophoresis step worked very well for the purpose of generating homogeneous enzyme for amino acid sequencing. However, the total activity recovered was very low (It was very difficult to accuratly quantitate this activity but it was less than 5 %). Thus an alternative purification would have to be developed if both purity and higher activity were required.

Figure 6
Silver stained SDS PAGE gel of active Prep. 491 cell fractions. The far right and left lanes are molecular weight markers the eight inside lanes are highly concentrated "epoxidase active" Prep. 491 cell fractions.



Alternative Columns and Strategies

This section of the chapter contains suggestions and summarizes alternative efforts which did not work for the objective of obtaining pure DHAE I. However, much was learned from these alternative approaches and, therefore, these strategies are included to assist the continuing efforts on the study of this enzyme.

Quaternary Methyl Amine Anion Exchange Chromatography (QMA)

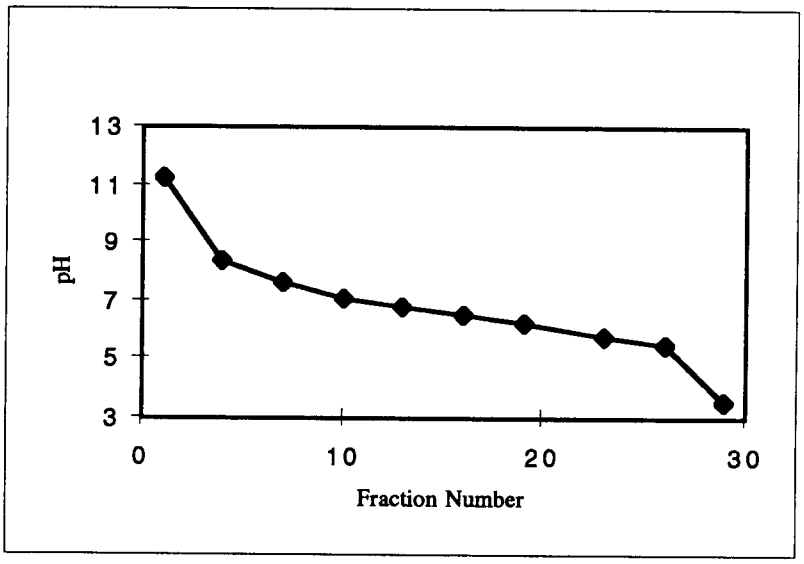
Since there was too much protein following the IMAC column to go onto the smaller high resolution columns without doing multiple runs, one more bulk step was sought. QMA anion exchange chromatography (Accell, Whatman) was utilized quite successfully when used after the S-200 column (ammonium sulfate, S-200). A 12.8 fold purification was achieved and only 20% of the activity was lost.

However, when the same QMA anion exchange column followed the DE-52 and IMAC columns, only a 2.5 fold purification and again a 20% loss of activity was achieved. An additional anion exchange column, QMA, did not improve on the DE-52 anion exchange results. Clearly, the choice for the third bulk resin would have to utilize a different chemistry to achieve protein purification.

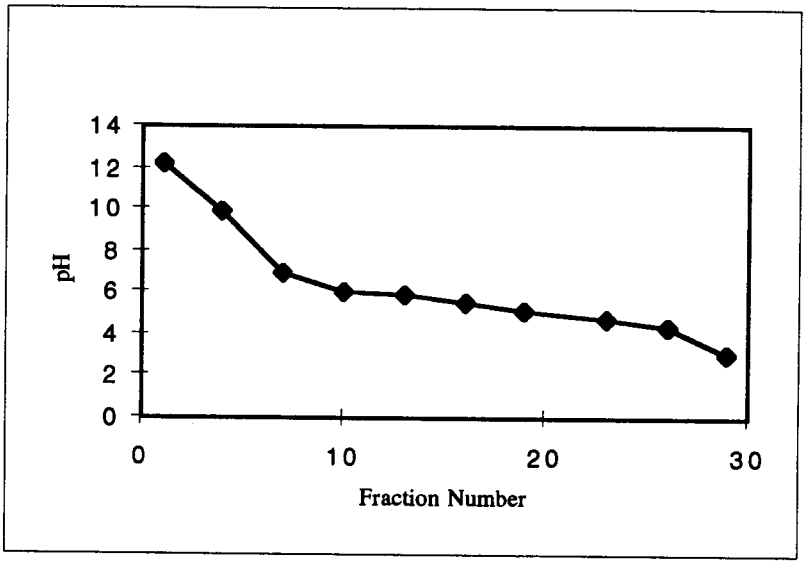
Preparative Isoelectric Focusing

The next purification step proposed to follow the IMAC column was preparative isoelectric focusing. This technique can be a very effective purification step.[7] The availability of a Rainin Preparative RF3 isoelectric focusing unit through the OSU Center for Gene Research and Biotechnology made it possible to try this technique on DHAE I. An ampholyte range was selected based on an estimated pI of 6.5. This estimate was obtained from the binding properties of DHAE I to DE-52 anion exchange resin over a defined pH range. The first attempt at isoelectric focusing used ampholytes with a linear range from pH 5 to pH 8. Unfortunately, the low ionic strength required for this step, coupled with the harsh electronic fields, failed to allow DHAE I to survive intact. Activity was detectable, but less than 5% of the total units remained (pI $\approx$ 4.7) (Figure 7, Top). In addition, precipitation of the protein in the focusing cell was a problem. In the next attempt, a lower pH and narrower range of ampholytes (pH 4 to 6.5) was used. Again the activity was barely detectable, however, the sample did focus cleanly and a more accurate pI value of 4.5 was obtained (Figure 7, Bottom). The active fractions were subjected to SDS PAGE and viewed by silver staining. Due to the numerous (at least ten) distinct bands present and, because of their low activity, the isolation of this preparation was terminated at this stage in the purification. This technique was not effective. However, if an acceptable method of removing the carrier ampholytes is discovered, it could become useful as a final purification routine.

Figure 7
Graph of pH profile from RF3 with Pharmalyte 5 - 8 ampholytes.
Fractions 26 through 28 were active.



Graph of pH profile from RF3 with Pharmalyte 4 - 6.5 ampholytes.
Fractions 20 through 25 were active.



Low Resolution Phenyl Hydrophobic Interaction Chromatography (HIC)

During the earlier purification efforts, a bulk phenyl Sepharose hydrophobic interaction chromatography (HIC) column was utilized and found to be an ineffective method for separating the DHAE I. After reviewing the particular buffers and overall procedures, it was decided to reinvestigate this HIC resin. The column used initially was very long and narrow, creating low flow rates. In addition, the column was run in 20% glycerol due to its ability to prevent inactivation of the epoxidase. This might become useful should one: 1) use less resin 2) use a wider column with a shorter bed of resin 3) avoid high levels of glycerol whilie loading the protein and 4) try to elute bound DHAE I from the matrix with glycerol if lowering the salt concentration does not work.

DHAE I was found to be partially stable without glycerol, so a larger column with a shorter bed height was used. The column was initially loaded with a high ammonium sulfate concentration then washed with a step gradient (see experimental) to lower the salt concentration. DHAE I eluted when all the of ammonium sulfate was absent from the elution buffer. More DHAE I eluted from the column when it was further washed with a 10% glycerol buffer. This column gave a 20 fold purification with a loss of 30% of the total activity. This purification was encouraging, however the loss of activity was much higher than desired, so an alternative HIC resin was sought.

S-200 Size Exclusion Chromatography

The use of low resolution Sigma S-200 size exclusion chromatography was ineffective when utilized in the manner described in the Previous Isolation Studies section. However, a high resolution, prepackaged, S-200 column (Pharmacia) was found to be an effective step when used later in the purification.

N-terminal and Internal Amino Acid Sequencing

The goal of this section of the chapter was to obtain N-terminal and internal amino acid sequence information from purified DHAE I.

N-terminal Sequence

Half of the homogeneous epoxidase recovered from the Prep. 491 electrophoresis cell was concentrated and subjected to SDS PAGE. DHAE I was then removed from the gel by electroblotting onto a PVDF membrane (Millipore, Immobilon-P^{QS}).

The bound epoxidase was excised from the rest of the Immobilon membrane and submitted for N-terminal sequencing to the OSU Center for Gene Research and Biotechnology (CGRB). Unfortunately, there was not enough protein present to obtain sequence. Based on UV absorbance of known standards, the CGRB estimated that less than a picomole of peptide was present. To ensure that most of the DHAE I had transferred to the immobilon membrane during the electroblotting step, the SDS PAGE gel was silver stained. No DHAE I remained on the gel. The remaining half of the homogeneous epoxidase (not subjected to SDS PAGE) was submitted for analysis as a concentrated liquid. Again no sequencing results could be obtained. The concentration of DHAE I was too low for adequate detection of the derivatized amino acids.

Following the same purification protocol, a similar quantity of newly purified DHAE I was submitted for N-terminal sequencing at the Center for Protein and Nucleic Acid Research (PAN) at Stanford University. The N-terminal sequence was obtained, (Figure 8) on their more sensitive equipment. The corresponding nucleic acid codons, based on the *Streptomyces* codon preferences,[8] are also shown. (*Streptomyces* codon preferences are slightly different from other organisms because their DNA has a high G + C content.)

Figure 8
The N-terminal amino acid sequence of DHAE I. Also included is the corresponding codon preferences and their percent usage (based on a compilation of data from 27 *Streptomycete* genes or open reading frames for which a translational start and stop codon have been identified)[7].

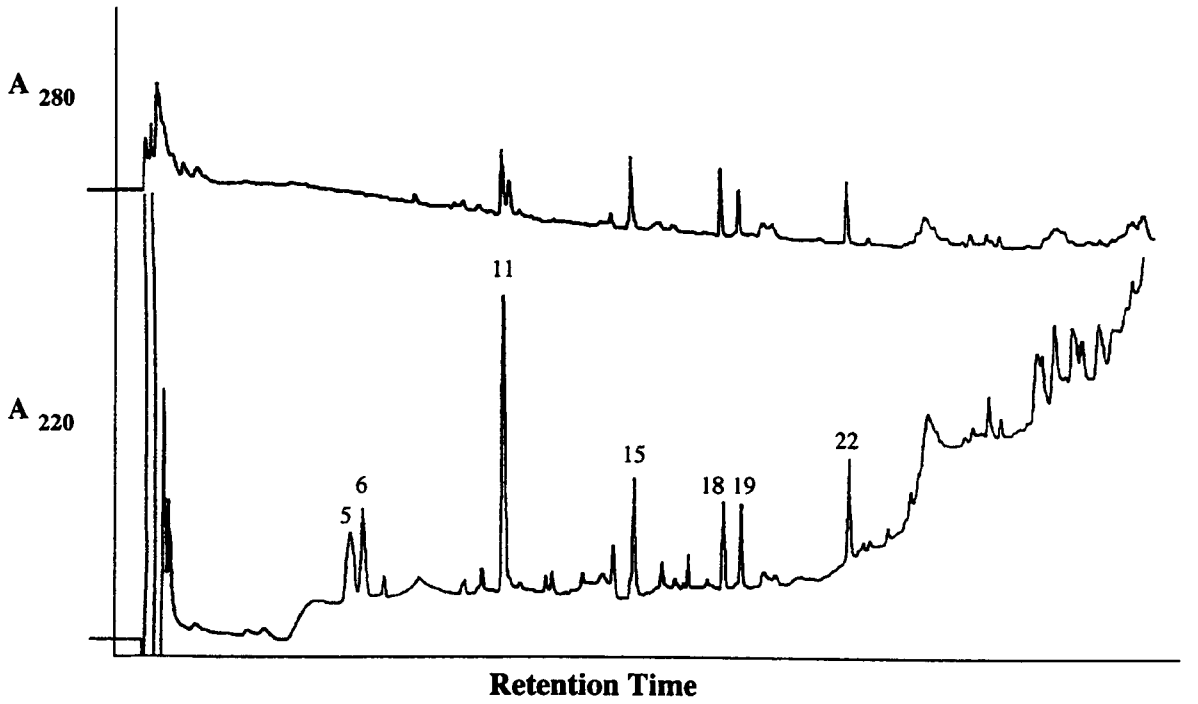
#	Amino Acid	Codon#1	Codon#2	Codon#3
1	-	-	-	-
2	Lys	AAG(91.2)	AAA(8.8)	-
3	Val	GTC(56.2)	GTG(37.9)	-
4	-	-	-	-
5	Leu	CTG(55.8)	CTC(37.3)	-
6	Ile	ATC(91.5)	ATA(4.6)	-
7	Thr	ACC(72.8)	ACG(21.7)	-
8	Gly	GGC(62.8)	GGG(19.2)	GGA(10.1)
9	Ala	GCC(60.0)	GCG(32.1)	-
10	Ser	TCC(37.4)	AGC(27.5)	TCG(25.3)
11	Ser	TCC(37.4)	AGC(27.5)	TCG(25.3)
12	Gly	GGC(62.8)	GGG(19.2)	GGA(10.1)
13	Phe	TTC(96.7)	TTT(3.3)	-
14	Gly	GGC(62.8)	GGG(19.2)	GGA(10.1)
15	Arg	CGC(39.9)	CGG(35.8)	CGT(9.3)
16	Ala	GCC(60.0)	GCG(32.1)	-
17	Ile	ATC(91.5)	ATA(4.6)	-
18	Ala	GCC(60.0)	GCG(32.1)	-
19	Glu(Gln)	GAG(78.0)	GAA(22.0)	(CAG(91.4))
20	Ala	GCC(60.0)	GCG(32.1)	-
21	Ala	GCC(60.0)	GCG(32.1)	-
22	Leu	CTG(55.8)	CTC(37.3)	-
23	Ala	GCC(60.0)	GCG(32.1)	-

Internal Sequence

With the N-terminal sequence in hand, attention was focused on obtaining internal sequence information for designing another oligonucleotide. With the oligomer from the N-terminal sequence and an oligo from the internal sequence, the polymerase chain reaction (PCR) can be used to amplify *S.* LL-C10037 genomic DNA. The PCR product can then be radioactively tagged and used to probe genomic libraries of both *S.* MPP-3051 and *S.* LL-C10037. The identification of the epoxidase genes in the two organisms is the first step to cloning and over-expressing of the epoxidases.

The internal sequence was obtained by first digesting the immobilized epoxidase on the Immobilon membrane with trypsin. Modified trypsin (Promega) was used to avoid self-digestion which gives rise to interfering peptides. The digested fragments were extracted and separated by C-18 reverse phase HPLC (Vydac, C-18) (Figure 9). The isolated peptides were collected and dried onto polybrene discs which were sequenced directly by PAN at Stanford University.

Figure 9
The HPLC chromatogram of DHAE I peptides obtained from tryptic digestion. The numbers correspond to fractions which were collected. Fractions 11, 15, 18, and 19 were sequenced.



Experimental

General Conditions

NMR spectra were recorded using either a Bruker 300 MHz or 400 MHz NMR. Low-resolution mass specta were taken on a Varian MAT CH-7 spectrometer. High-resolution mass spectra were taken on a Kratos MS 50 TC spectrometer. Water was purified with a MilliQ System, Millipore Corp. Activity assays were analysed by HPLC, performed on a Waters 600E HPLC instrument (UK-6 injector) with either a Waters 996 photodiodarray detector and a Dell Pentium computer housing Waters Millinium software or a Linear UVIS 200 detector with an HP 3396A integrator. Reverse phase C_{18} (Econosphere, 5 mm, 250 X 4.6 mm, Alltech Assoc.) columns were used for activity assay analyses. Tryptic digestions were analyzed using a Waters 600A pump with a UK-6 injector (equipped with a 500 μL loop), a Linear UVIS 200 detector with an HP 3396A integrator, and a Vydac C18 reverse phase column (218TP54). Bacterial fermentations were carried out in a gyrotary incubator (Lab-Line incubator shaker).

Cell disruption was performed with a sonicator (Model W-225R, Heat Systems-Ultrasonic, Inc.). Eppendorf tubes were centrifuged in a Biofuge, (Baxter Inc). Refrigerated centrifugations were done in an IEC B-20a centrifuge. Each isolation step was examined at by SDS PAGE and each (or every other) fraction collected was checked for epoxidase activity . Selection of which fractions to be carried to the the next step was based on the SDS PAGE, the specific activity and the column UV chromatogram. All purification steps were performed at 4 °C. All concentration steps and buffer exchanges were carried out using Amicon Centriprep 30 and 10 filters. All buffers were prepared from molecular biology grade reagents. Epoxidase activity was monitored by following the consumption of **4** and the production of **7** simultaneously by HPLC, (Scheme 4). Fractions from each step were stored at -80 °C.

Buffers

(I) 50 mM KH_2PO_4, 10 % glycerol, 1 % soluble polyvinylpyrrolidone (PVP-10), 0.1 mM EDTA, pH 7.0

(II) 50 mM KH_2PO_4, 10 % glycerol, pH 7.0

(III) 100 mM KH_2PO_4, 50 mM NaCl, 20% glycerol, pH 6.5

(IV) 100 mM KH_2PO_4, 50 mM NaCl, 83.3 mM imidazole, 20% glycerol, pH 6.5

(V) 100m M KH_2PO_4, 1.2 M $(NH_4)_2SO_4$, 5% glycerol, pH 6.5

(VI) 100 mM KH_2PO_4, 5% glycerol, pH 6.5

(VII) 20 mM histidine, 20% glycerol, pH 6.0

(VIII) 20 mM histidine, 20% glycerol, 1 M KCl, pH 6.0

(IX) 50 mM KH_2PO_4, 20% glycerol, 0.2 mM EDTA, pH 7.0.

Standard Enzyme Activity Assay

An assay based on differential UV absorbance of the substrate **4** verses the product **7** was not feasible because there was no suitable wavelength where the two compounds differed greatly enough in their absorbances. Therefore, the epoxidase assay was based on the separation of **4** from **7** using C18 HPLC with single wavelength UV detection at 225 nm. Although this HPLC based assay was slow to process, it had the advantages of being extremely sensitive, simple to prepare, and reproducible.

To insure success, it was very important to prepare the substrate stock solutions under oxygen free conditions at 0 °C . The hydroquinone rapidly oxidized to the quinone if the solution was exposed to oxygen or air. As a solid, **4** was redox inactive toward air. It was important to have sodium dithionite present as well as using the deoxygenated water (sparged with N_2, 0 °C) while making a stock solution of **4**.

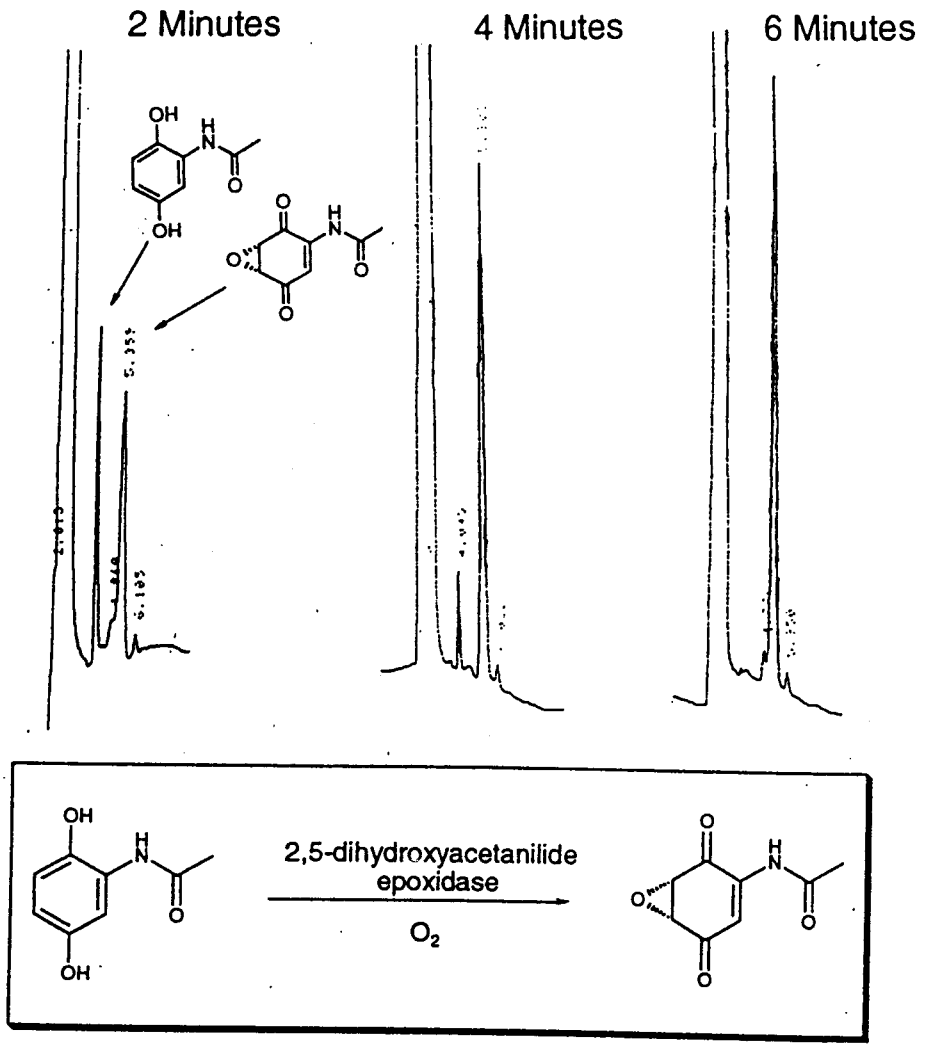
Sodium dithionite (≈ 20% by weight) was present in the substrate stock if dithionite saturated ethyl acetate was used during the work-up of the synthetic **4**. If **4** was mixed without the reducing agent (i.e. from a recrystallized sample), a portion of it (≈10% as seen by HPLC chromatogram) would be oxidized to the quinone. To insure uniform stock stability, 25 mL of a substrate stock was prepared and divided into 25 one dram vials. Prechilled (ice bath, 0 °C) vials were flushed with argon, capped, and placed back on ice prior to addition of the substrate solution. The vials were again flushed with argon after being filled. These vials were stored for up to six months at -80 °C without detectable quinone formation.

A typical assay composition consisted of 100 µL MilliQ H_2O, 100 µL 0.2 mM Co^{+2} solution (from $CoCl_2$), 50 µL 1 M K_2HPO_4, pH 6.5, 25 µL 2 mM dihydroxyacetanilide, and 125 µL enzyme preparation. This was incubated for 3 to 10 minutes whereupon 100 µL terminating solution (CH_3CN / H_2O / TFA: 66 / 27 / 7) was added to stop the reaction and precipitate the proteins. If activity was too high or too low the amounts of enzyme preparation and MilliQ H_2O could be adjusted to give the desired turnover. The running time, typically 3 to 5 minutes, could also be varied if only small changes in turnover were needed.

For convenience and to save time "assay starters" were prepared. Large quantities of 1.5 mL Eppendorf tubes individually containing MilliQ H_2O and phosphate buffer were prepared and stored at -20 °C. These solutions, which contain half of the assay contents, could be removed from the freezer whenever a typical assay needed to be performed. The assay starters where allowed to equilibrate at 30 °C (heat block) for at least 2 minutes prior to continuing with the assay.

Typical assay Protocol: After preparing enzyme and terminating solution, 25 µL of 0 °C, 2 mM dihydroxyactanalide was added to an assay starter. Promptly after substrate addition, the ice cold epoxidase preparation was added and the assay was allowed to incubate (30 °C) for a fixed time before 100 µL terminating solution was added. For the control, terminating solution was added prior to the epoxidase preparation. Once terminated, the assays were placed on ice. If quantitation could not take place within a few hours, the assays were frozen at -20 °C. The assays were centrifuged (Biofuge, Baxter Inc.) at 13,000 rpm for 3 minutes to remove precipitated proteins, then analyzed by HPLC (H_2O / CH_3CN : 85 / 15, Alltech Econosphere C_{18} column), . Typically 5 µL of each assay was injected and the following elution profile was observed: substrate dihydroxyacetanilide, 3.5 - 5.5 minutes; product epoxyquinone, 4.5 - 8.0 minutes; and acetamidobenzoquione, 6.0 - 10.0 minutes, (Figure 10).

Figure 10
HPLC chromatograms from three DHAE I activity assays run with increasing incubation time periods. At 2 minutes **4** and **7** are approximately equal, at 4 minutes the substrate **4** is nearly gone and at 6 minutes only product,**7**, is present.

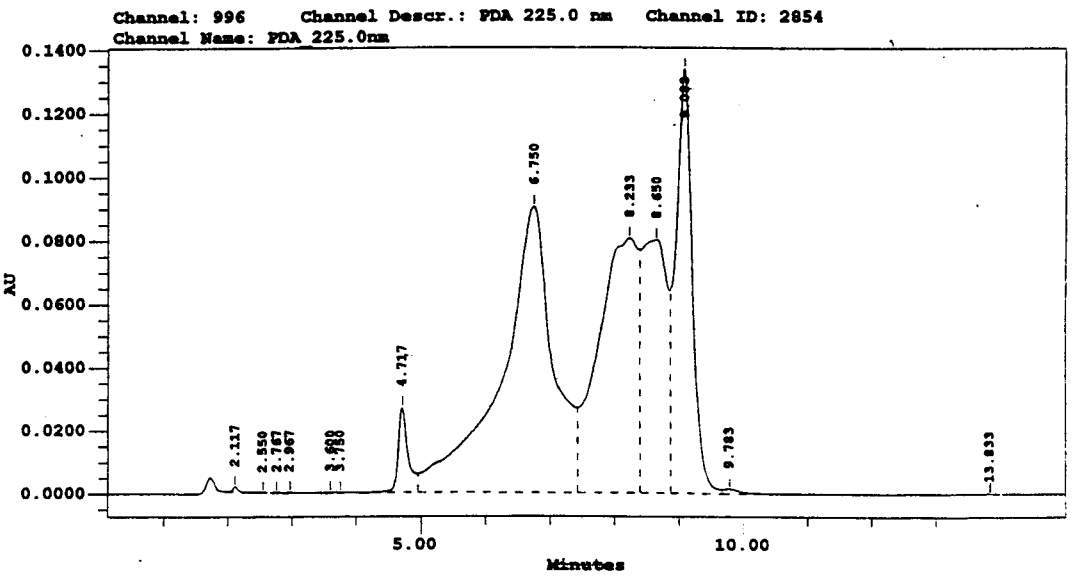


Although this assay was for the most part reproducible, nonenzymatic oxidation of the substrate was a problem when injecting assays at temperatures above 27 °C. This problem depended on ambient temperature, and was worse in the summer months. Use of the photodiodarray detector, revealed the source of the interference since the full UV spectrum of all the peaks was available: the substrate oxidized as it was running through the column. Quinone would elute for 2 or more minutes, often covering the product peak as well as preventing accurate quanititaion of the substrate peak (Figure 11).

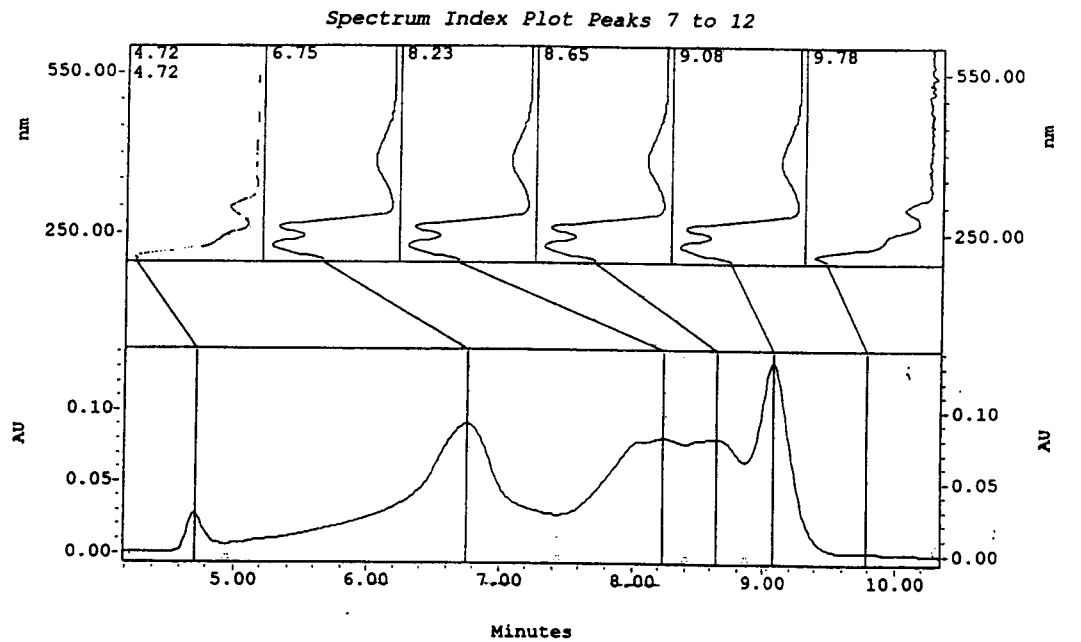
Figure 11

A, is an HPLC chromatogram of an assay injected at 27 °C. Substrate, **4**, elutes at 4.7 minutes followed by a broad elution of quinone starting at 5 minutes and ending at 9.6 minutes. **B**, the same assay from 4 to 10 minutes. The top of the box contains the U.V spectrum from 200 nm to 600 nm of each peak. UV peaks at 6.75, 8.23, 8.65, and 9.08 are characteristic of acetamidobenzoquione spectrum.

A



B



If a single wavelength detector was being used the identification of the problem was more difficult , therefore close attention must be payed to column temperature. To alleviate this problem, the acetonitrile and water eluents for the HPLC separation were cooled in an ice bath. In severe heat (above 29 °C), the column was also cooled by suspending it in a Styrofoam cooler filled with ice (the ice was not allowed to touch the column because severe back pressure would result).

Coomassie Blue Binding for Determination of Protein Concentration

Reference: This procedure was modified from the original protocol of Scopes.[11]

Reagent: 600 mg Coomassie Brilliant Blue G-250 (dye content 90%, Aldrich) was dissolved in 1 liter 2% perchloric acid and filtered to remove undissolved material. The absorbance at 465 nm was between 1.3 and 1.5. This solution is stable indefinitely.

Protocol: The original protocol has been modified to allow for a smaller sample size. A pair of matched 1.5 mL quartz cuvettes were used for this determination. Both cuvettes were washed with 30% ethanol followed by rinsing with MilliQ H_2O. The cuvettes were emptied (not dried) and to each cuvette deionized H_2O (800 µL) and Coomassie reagent (200 µL) was added. The two tubes were placed in the spectrophotometer (one in the sample cell and one in the reference cell). To allow for minor differences in cuvette thickness, the absorbance at 595 nm was set to zero. The cuvette in the reference cell was left while the cuvette in the sample cell was removed. This cuvette was washed with 70% ethanol, rinsed with MilliQ H_2O and shaken to remove excess water (not dried). The protein sample was added to the clean cuvette (usually less then 50 µL, but some experimentation with this quantity was necessary to keep the absorbance at 595 nm within the standard curve for BSA). MilliQ H_2O was then added to make a final volume of 800 µL. Coomassie reagent (200 µL) was added to the cuvette. This was shaken periodically for 5 minutes, whereupon it was placed back in the reference cell and its absorbance at 595 nm was measured. Protein qantitation was carried out by comparing the absorbance of the protein sample to a standard curve made from known quantities of bovine serum albumin (Sigma, lyophilized powder).

Standard Culture Conditions.

Streptomyces LL-C10037 cells were grown and harvested as earlier reported [2] except that the cells were collected and washed using a sheet of 100 µm nylon mesh.

Preparation of Cell-Free Extract

The washed *Streptomyces* LL-C10037 cells from a 6 liter, 96 hour fermentation (250 g, wet cell weight) were suspended in 400 mL buffer I (4 °C). To this suspension, polyvinyl polypyrrolidone (2.25 g) and washed XAD-4 anion exchange resin (2.25 g) were added. (These resins are added to remove small aromatic molecules which interfere with the HPLC based epoxidase activity assay.)[9] The cellular suspension was equally distributed into four beakers, and each portion was disrupted by sonication (maximum power, 90% duty, pulsed for 3 X 20 sec). Cell debris was removed by centrifugation (4 °C, 10,000 rpm, 20 min), and the supernatants were combined to afford a crude cell free extract (CFE), approximately 600 mL.

DE-52 Anion Exchange

The column was prepared and equilibrated at least 12 hours before preparation of cell free extract. 80 grams of bulk DE-52 resin (Whatman) was washed with H_2O, buffer II, 1M KCl in buffer II, and again with buffer II. The buffers were added to a 400 mL beaker containing the resin. This was mixed and allowed to settle whereupon the buffers were decanted. The washed resin was gravity packed into a large (75 X 6 cm) BioRad econo column. This was rinsed once with buffer I and left at least 12 hours at 4 °C. Cell free extract (≈ 500 mls) was carefully placed on the column and allowed to flow under gravity (≈ 2 hours). Once loaded, the resin was rinsed with buffer II (125 mL) and the following step gradient of KCl was used to elute the enzyme: 125 mL of each dissolved in buffer II, 50 mM KCl, 100 mM KCl, and 200 mM KCl. Four 32-mL fractions were taken once the 200 mM KCl eluate was begun; all showed epoxidase activity. These active fractions were stored at -80 °C.

Immobilized Metal Affinity Chromatography (IMAC)

Mini-Columns: In order to find an immobilzed metal that would bind DHAE I, a series of mini-columns were prepared (10 mL, 14 X 1 cm, BioRad econo columns) which contained 8 mL of each bed volume type. The void volume was estimated to be one third that of the bed volume. 20% ethanol (made with MilliQ H_2O to avoid metal ions) was used as a running eluent to help gravity pack the columns at room temp. Once packed the columns were transferred to the cold room (4 °C) where they were stored. As per manufacturer's recommendation, the beads were charged to 30 mmoles metal ion / mL bed volume. Individual metal ion solutions were prepared by adding 500 mg of the following reagents to 100 mL MilliQ H_2O: $Ni(NO_3)_2 \cdot 6H_2O$, $CuSO_4$, $MnCl_2 \cdot 4H_2O$, $CoCl_2$, $FeCl_3$, and $CaSO_4$.

Protocol: One column at a time was attached to the Waters FPLC pump so that a consistent gradient could be run for the various columns. Buffer III (50 mL) was passed through the column to wash and equilibrate it. Before the column was loaded, the buffer which remained above the resin bed was manually forced into the bed by removing the column cap and attaching a syringe full of air, then forcing the buffer through the column until the bed surface was visible. The epoxidase preparation (1 mL from the ammonium sulfate pelleting step followed by S-200 size exclusion step and concentration) was then added using a Pipetman P-1000 (Gilson) for consistence. The enzyme solution was forced onto the column with a syringe, as explained above, followed by the addition of buffer III (1 mL) which was also manually forced onto the column. Once the top of the bed was exposed, the column was filled with buffer III and the cap was fitted back onto the column. The column was run at 0.5 mL / min with buffer II from 0 - 20 min., then a linear gradient of buffer III to buffer IV from 20 - 60 min. The same epoxidase preparation and procedures were used for each metal column.

Preparative IMAC Cu^{+2} Column: Since the Cu^{+2} mini column was the only metal ion column which tightly bound DHAE I, it appeared to be the best candidate for selective desorption of the epoxidase. Therefore, a larger low pressure column was prepared in the same way the small trial columns were made (33 x 3 cm BioRad econo-column). It was important to wash the freshly packed and charged Cu^{+2} column with 500 mL of buffer IV prior to use. The Cu^{+2} would leach from the column during the first several runs if this imidazole washing was not carried out. Cu^{+2} has the ability to help oxidize the substrate, **4**, during the activity assays, making detection of activity difficult.

Active protein fractions from the DE-52 column were concentrated using Centriprep 30 filter units (from ≈130 mL to ≈30 mL). This was loaded onto the pre-equilibrated column through the injection loop of the FPLC (2.5 mL / min). The column was allowed to equilibrate (2.5 mL / min buffer III) until the protein preparation visibly entered the resin bed, whereupon the following linear gradient was started: 0 - 50 min. buffer III; 50 - 80 min. buffer III - 15 % buffer IV; 80 - 260 min. 15% buffer IV; and 260 - 290 min. 15% buffer IV - 100% buffer IV (Figure 2). When the linear gradient started, 12.5 mL fractions were collected. From every other fraction where DHAE I was known to elute (≈ 200 min, 10 mM imidazole), 300 µL was removed and placed in individually labeled 1.5 mL eppendorf tubes. The 12.5 mL fractions were placed immediately in the -80 °C freezer while the smaller 300 µL fractions were used to assay activity and determine the protein content. At this point, all the active fractions (see Fig. 2) were carried on to the next step (60 mL).

Hydrophobic Interaction Chromatography

In hydrophobic interaction chromatography the protein need not be loaded in a small volume. Therefore, this technique was a good choice to follow the Cu^{+2} IMAC column, since the recovered DHAE I from this step was very dilute.

Mini-Columns: In order to determine the optimum carbon chain length for retaining the epoxidase a series of hydrophobic agarose resins was used. The 2.5 mL prepackaged mini-columns ranging from 2 to 12 carbons were obtained from Sigma.

Protocol: All eight columns were stored at 4 °C in 20 % ethanol. The columns were washed first with 20 % ethanol (50 mL), then equilibrated in buffer V (25 mL). To each column, the enzyme sample (1 mL of ammonium sulfate pellet dissolved in buffer V (4.7 mg protein / mL)) was added. When the top of the frit became visible, buffer V (200 µL) was added so any proteins clinging to the inner walls were washed onto the column. When the frit was again visible, buffer V (3.8 mL) was added in 1 mL portions. The first 1.5 mL recovered after the addition of buffer V was discarded and the next 2 mL was collected from each column [fraction 1]. When the frit was again visible, buffer VI (4 mL) was added in 1 mL increments and three 2 mL fractions were collected from each column [fractions 2-4]. The resulting twenty-four fractions (four collected from each of the eight columns) were assayed for activity.

Pentyl Hydrophobic Interaction Chromatography

Once C-5 was determined to be the best chain length for this separation, a preparative column was packed (C-5 agarose from Sigma (short extender unit), column BioRad econo, 2.5 X 20 cm). The column was wet packed in buffer V at 27 °C and then was transferred into the cold room (4 °C).

Protocol: The column was first washed (gravity fed)with MilliQ H_2O (200 mL), 0.1N NaOH (100 mL) and, again with MilliQ H_2O (200 mL). After washing it was equilibrated with buffer V (200 mL) and allowed to drain until the top of the bed was visible. While the column was equilibrating the IMAC fractions were prepared. They were quickly thawed at 27 °C and once thawed, they were placed at 4 °C (never letting the temperature of the fractions get higher than 4 °C). The fractions were pooled, and brought to 1.2 M ammonium sulfate (≈ 60 mL). This was slowly and carefully added to the top of the column.

When all the enzyme had been added, the top of the column was washed by adding buffer V (2 mL) and allowing the column to drain until the top of the bed was again visible. The column was topped-off with buffer V and connected to the FPLC whereupon, the following linear gradient was started at 1.5 mL / min: 0 - 30 min. buffer V - 50% buffer VI; 30 - 70 min. 50% buffer VI; 70 - 150 min. 50% buffer VI - 60% buffer VI; 150 - 190 min. 60% buffer VI; 190 - 230 min. 60% buffer VI - 70% buffer VI; 230 - 270 min. 70% buffer VI; 270 - 310 min. 70% buffer VI - 100 buffer VI. Fractions were collected every 3 minutes (4.5 mL) and 300 µL of every other fraction where DHAE I elutes (≈ 200 min.) was taken for activity assays and protein quantitation. The fractions were stored at -80 °C. Active fractions (see Fig. 3) where evaluated for both specific activity and SDS PAGE cleanliness to decide which would be carried on to the next step of purification. Usually about seven fractions where carried-on.

Phenyl Hydrophobic Interaction Chromatography

Protocol: The phenyl column (Waters, Phenyl 5 PW column, for the AP1 glass-pack column holder, 1.5 X 10 cm) was stored in 20% ethanol at 4 °C and therefore, was first washed with deionized H_2O (50 mL), then equilibrated with buffer V (100 mL). Pooled active fractions from the C-5 hydrophobic column (≈ 30 mL) were brought to 1.2 M $(NH_4)_2SO_4$ prior to applying the sample to the phenyl hydrophobic interaction column. This sample (30 mL) was loaded through the FPLC injection port at 0.3 mL / min. This column has a high pressure cut-off of 450 psi so the loading must be closely monitored. Once the sample was loaded the following linear gradient was started (0.3 mL / min.): 0 - 20 min. buffer V; 20 - 60 min. buffer V - 50% buffer VI; 60 - 90 min. 50% buffer VI; 90 - 180 min. 50% buffer VI - 100% buffer VI. Fractions were taken every 5 minutes (1.5 mL) and 200 µL of every other fraction where DHAE I elutes (≈ 170 min.) was taken for activity assays and protein quantitation. The fractions were stored at -80 °C. Active fractions were evaluated for both specific activity and SDS PAGE cleanliness to decide which fractions would be carried on to the next step of purification. Usually about ten fractions where carried-on; see Fig. 4.

Diethylaminoethyl-cellulose (DEAE) Anion Exchange Chromatography

Protocol: This column (Waters, 8 HR, AP-1 glass pack column, 1.5 X 8.5 cm) was stored in 20% ethanol. The ethanol was washed out with the addition of 30 mL MilliQ H_2O and then the column was charged with chloride ions with buffer VIII (50 mL).

Finally, the column was equilibrated in buffer VII (50 mL) and ready for loading. The fractions from the phenyl HIC column (≈ 15 ml) were concentrated and the buffer was exchanged to buffer VII (2 hr., Centriprep 30). The 2 to 5 mL which remained after this buffer exchange was loaded through the FPLC injection port and the following linear gradient was started (1 mL / min.): 0 - 30 min. buffer VII; 30 - 50 min. buffer VII - 20% buffer VIII; 50 - 130 min. 20% buffer VIII; 130 - 160 min. 20% buffer VIII - 30% buffer VIII; 160 - 190 min. 30% buffer VIII; 190 - 220 min. 30% buffer VIII - 100% buffer VIII. Fractions were collected every 2 minutes (2 mL) and 200 µL of every other fraction where DHAE I elutes (≈ 180 min.), was taken for activity assays and protein quantitation. The fractions were stored at -80°C. Active fractions (see Fig. 5) where evaluated for both specific activity and SDS PAGE cleanliness to decide which would be carried on to the next step of purification. Usually about ten fractions where carried-on.

Quaternary Methyl Amine Anion Exchange Chromatography (QMA)

This column was first run as described by Shen (in a phosphate buffer, pH 7.0) and a consistent 3 fold purification was achieved.[2] However, after better defining the isoelectric point (pI 4.6) a histidine buffer, with a lower pH, was used and a consistent 11 fold purification was obtained. A 20% loss of total units was observed in both cases. This chromatographic step was omitted altogether after the first step in the new purification was changed to anion exchange chromatography instead of the original ammonium sulfate pelleting and size exclusion column. In the latter case, only 2.5 fold purification was achieved.

Protocol: A 6 cm bed of QMA (Whatman) was poured dry into a BioRad econo column of dimensions 30 X 2.5 cm. This was charged with buffer VIII (100 mL) and then equilibrated in buffer VII (100 mL). Active IMAC fractions were pooled, concentrated and buffer exchanged to buffer VII (≈ 30 mL). This was loaded through the FPLC injection port at 1 mL / min. Once loaded the following linear gradient was started: 0 - 30 min. 100% buffer VII; 30 - 60 min. 100% buffer VII - 20% buffer VIII; 60 - 130 min. 20% buffer VIII; 130 - 150 min. 20% buffer VIII - 30% buffer VIII; 150 - 180 min. 30% buffer VIII; 180 - 200 min. 30% buffer VIII - 100% buffer VIII. Fractions were taken every 10 min and 300 µL of every other fraction where DHAE I elutes (≈ 170 min.) was taken for activity assays and protein quantitation. The fractions were stored at -80 °C.

Phenyl Sepharose Hydrophobic Interaction Chromatography (Low Resolution)

This was another column that was omitted in the revised isolation scheme because this HIC matrix denatured DHAE I giving an unsatisfactory loss of total units of activity.

Protocol: A short plug of the phenyl sepharose HIC resin (15 mL, Sigma) in buffer VII was wet packed in a BioRad econo column (20 X 2.5 cm). Fractions from the IMAC column were thawed, pooled and brought to 1.2 M $(NH_4)_2SO_4$. This sample was loaded as described previously for the C-5 HIC column, paying particular attention to the bed surface (With only a 3 cm pack, the probability of drying out the resin was high.) Once loaded a step-wise gradient of the following buffers was used to elute the epoxidase: 25 mL rinse buffer VII, then 15 mL each of buffer VII with 75, 50, 25, and 0 mM $(NH_4)_2SO_4$, then buffer VII (15 mL) with 10% glycerol . Fractions of 3 mL each were collected throughout. The epoxidase was retained on the column at high salt concentration and eluted when $(NH_4)_2SO_4$ was absent, typically in fractions 21-25.

Preparative Isoelectric Focusing

The Rainin **R**ecycling **F**ree **F**low **F**ocusing (RF3) protein fractionating instrument was assembled according to the manufacturer's recommendation. Two ampholyte mixtures were purchased from Pharmacia; Pharmalyte 5 - 8 and 4 - 6.5.

Protocol: The chiller was turned on and allowed to equilibrate (1 hour). The peristaltic pump shoe was removed and the 30 tubes were well oiled with mineral oil. Since the unit was stored in 10% ethanol, the inlet connector was removed while at the same time the vent tube was closed, and the ethanol was pumped out. To remove the residual ethanol, the system, including vent tubes, was flushed (both directions) with MilliQ water. While the flow cell was being flushed, the electrolyte reservoirs, including the pump tubing, was also rinsed with MilliQ water. Once the system was cleared of the ethanol, the inlet connector was connected, the vent tube was closed, and the flow cell and reservoirs were filled with ampholyte solution (Pharmalyte 5 - 8). The electrolyte reservoirs were also filled with their respective electrolyte solutions (0.1 M NaOH (4 g / liter), 0.1 M phosphoric acid (5.5 mL 85% acid / liter)). Pre-focusing of ampholytes was started (1500 V, 200 mA, 100 W). This was allowed to focus for 2 hr. while the protein sample was prepared.

3.8 mL of active protein solution recovered from an Cu^{+2} IMAC column (ammonium sulfate pellet, S-200 size exclusion, IMAC) was Centricon concentrated and the buffer concentration lowered to less than 10 mM KH_2PO_4. A portion of this was saved for protein quantification and activity assays. The remaining 3.2 mL (5 mg protein / mL) was transported to the RF3 for loading.

Prior to loading, the voltage and current were lowered (1000 V, 100 mA). The 3.2 mL protein sample was loaded into the bubble trap (well # 16). After 15 minutes of focusing, precipitation was observed in lanes 25 - 28. After 1 hour, the voltage was lowered to 500 volts and the protein was allowed to focus for 30 min. longer. Fractions (30 fractions, 3 mL each) were collected at the end of the 30 min.. The apparatus was then cleaned by flushing with MilliQ water (as explained above) and prepared for storage by filling with 20% ethanol. Immediately following the fraction collection, the following fractions were buffered by the addition of 1 milliliter of 1 M KH_2PO_4, pH 6.5 and placed on ice: 2, 3, 5, 6, 8, 9, 11, 12, 14, 15, 17, 18, 20, 21, 23, 24, 26, 27, 29, 30. The fractions that didn't receive the buffer had their pH checked to make sure the focusing worked properly (Figure 3 top). After the pH check, these fractions were also buffered with 1 M KH_2PO_4, pH 6.5. Activity was found in fractions 26, 27, and 28 (the fractions which had precipitated, pI $\approx$ 4.7). This activity was barely detectable because the strong electric fields or the low ionic strength, required for this procedure, had denatured most of the enzyme. The ampholytes were also hard to completely remove making protein quantitation difficult.

This procedure was repeated with a less concentrated protein sample and a more narrow pH range ampholyte solution (Pharmalyte 4 - 6.5). The protein sample was the same purity as the previous one, however, it was only 0.17 mg / mL and less volume was loaded (1.9 mL). Again slight precipitation occurred during the focusing (tubes 22 and 23) however, DHAE I focused and the pI was determined to be 4.5, (Figure 3, bottom).

Sephacryl S-200 Size Exclusion Chromatography

This column (Sigma, exclusion limit of 250 K, 30 x 60 cm) was not used in purification which yielded pure DHAE I. It was used in the first step of the previous epoxidase purification (not giving pure epoxidase), because the ammonium sulfate pellet could be dissolved in a minimum amount of buffer and could be added to the column directly without desalting or dialysis. The column was omitted from the new scheme (which produced homogeneous DHAE I) because other chromatographic columns were found to yield better results.

Protocol: The 50-70% ammonium sulfate pellet was dissolved in a minimum amount of buffer IX, usually 5 to 10 mL. This was loaded onto the column through the injection port (0.5 mL / min.) of the FPLC (The column was run upside-down so solvent flows upward). Fractions were collected (10 mL), DHAE I did not elute at reproducible times so most fractions throughout the run were checked for activity.

One significant problem with using this column for dissolved ammonium sulfate pellets was injecting such a crude preparation through the injection port. These heterogeneous crude preparations caused pressure build-ups. Such increases in pressure shut off the flow since the pressure maximum for this Sephacryl matrix was 100 psi. This problem was decreased somewhat by prefiltering through 4.5 μm microfilters (Whatman).

Native Electrophoresis

The following recipes were used to prepare the Jovin buffers[5] for the analytical cell PAGE (Analytical Gel 10%, BIORAD MiniProteinII system).

	Resolving	Stacking
H_2O	4.0 mL	3.0 mL
4 X Gel Buffer	2.5 mL	1.25 mL
Acrylamide/BIS (30 / 0.8)	3.3 mL	625 μL
DTT (100 mM)	100 μL	----
Ammonium persulfate (10%)	75 μL	35 μL
TEMED	5 μL	4 μL

Buffers	Reservoirs		
	upper	lower	4X Stack / Resolving gel Buffer
TES (g)	10.07	----	----
1 N HCl (mL)	----	50	21.6
BIS TRIS (g)	23.7	13.1	10.3
Final Volume (mL)	1000	1000	100
pH	7.25	5.9	6.61

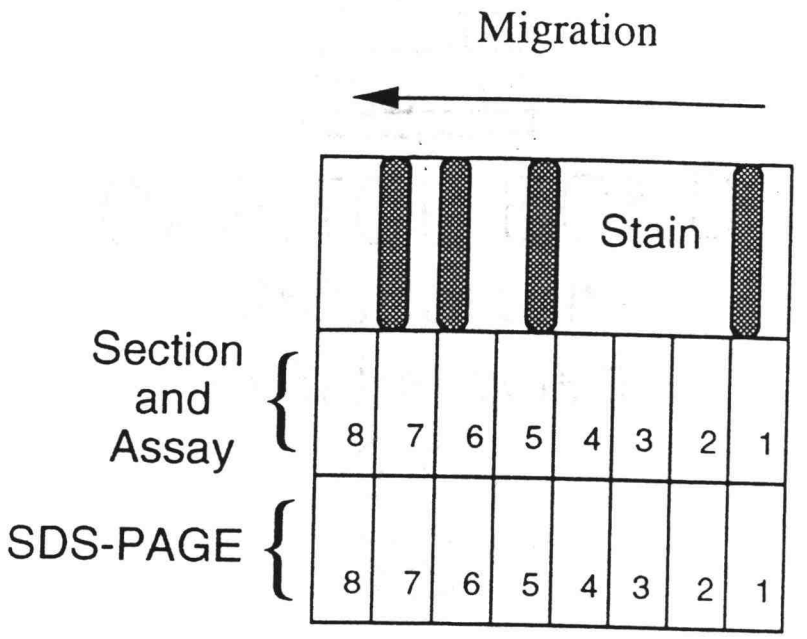
Sample Loading Buffer	
4X Gel Buffer	5 mL
H_2O	11 mL
Glycerol	4 mL
DTT	30 mg
Bromophenol Blue	Trace

Analytical (BioRad MiniProtean II)

Protocol: A 10% polyacrylamide gel was poured using the reagents and quantities given above (see SDS PAGE below). This gel utilized the preparative, one lane, comb so the proteins would migrate as single bands the entire width of the gel. The cell was assembled at 4 °C and placed in an ice bath, being careful to prevent the electrical leads from touching the water in the ice bath. The cell chambers were filled (1 X stack / resolving gel buffer with 0.1 mM thioglycolate (11 mg / L)) and the gel was pre-run for 30 min. at 20 mA. While this was running, a single active fraction from a DEAE column was concentrated and its buffer exchanged into sample loading buffer. The thioglycolate-containing buffer was removed from the chambers.

The chambers were rinsed and filled with the upper and lower Jovin buffers. The protein was loaded (500 µL) and electrophoresis was started at constant current (25 mA, 0.9 V). After 1 hour the voltage was set to 30 V (6 mA). After 2 hours, the voltage was increased to 100 V (16 mA). This was allowed to run for 12 hours until the dye front reached the bottom of the gel, then the electrophoresis was stopped. The gel was carefully placed on a wet glass surface where it was cut into sections, (Figure 12). Each of the sixteen sections was placed into individual Eppendorf tubes (1.5 mL). Eight of the smaller sections (from the center 1/3 of the gel) were broken into smaller pieces using a glass stirring rod and assayed for activity utilizing the standard assay conditions. The other eight individual sections were subjected to SDS PAGE (one section per lane) and visualized by silver staining. The active sections from the Jovin gel were then correlated with their corresponding lane on the SDS PAGE gel.

Figure 12
Diagram showing the method for slicing the Jovin gel. The gel was first sectioned, perpendicular to the protein bands, into three equal parts. One section (1/3 of gel) was silver stained while each of the other two sections were further sliced parallel to the protein bands into eight smaller sections.



Prep. 491 electrophoresis cell

The following recipes were used to prepare the Jovin buffers[5] for preparative cell.

Buffers	Reservoirs		
	upper	lower	4X Stack / Resolving Gel Buffer
TES (g)	40.28	----	----
1 N HCl (mL)	----	200	108
BIS TRIS (g)	94.8	52.4	51.5
Final Volume (mL)	4000	4000	500
pH	7.25	5.9	6.61

Sample Loading Buffer		Elution Buffer	
4X Gel Buffer	5 mL	Bis-Tris	23.6 g
H_2O	11 mL	10% Glycerol	100 mL
Glycerol	4 mL	Final Volume	1000 mL
DTT	30 mg	pH	7.0
Bromophenol Blue	Trace		

	Resolving	Stacking
H_2O	20 mL	5.9 mL
4X Gel Buffer	12.5 mL	2.5 mL
Acrylamide/BIS (30-0.8)	16.5 mL	1.25 µL
DTT (100 mM)	500 µL	100 µL
APS (10%)	375 µL	70 µL
TEMED	25 µL	8 µL

Protocol: Using the small gel assembly (the 491 has two sizes): To assemble the membranes properly and pour a gel in this system, it was best to follow the manufacturer's instructions. With this preparative system, unlike the analytical BioRad MiniproteanII system, the gel contents were degassed (≈ 2 min.) under vacuum prior to addition of APS and TEMED. In addition, the resolving gel, once freshly poured, was overlaid with H_2O saturated 2-butanol. It was then left overnight before the stacking gel was poured. The stacking gel was only degassed slightly (30 sec. to 1 min.) to keep it from polymerizing too quickly. The stacking gel was also overlaid with 2-butanol immediately after being poured (let it stand at least 2 hours).

The resolving gel was poured to a height of 9.5 cm and the stacking gel to a height of 1.5 cm for a total height of 11 cm.

Once the gel was poured, the manufacturer's instructions were again used to assemble the cell. The whole apparatus was then transferred to the cold room (4 °C). Once assembled, the top of the gel was rinsed with Jovin upper reservoir buffer. Then the upper buffer (250 mL) was prepared with 5 mg thioglycolate (the thioglycolate must be added just prior to use). This thioglycolate / TES buffer was then added to the upper chamber and the lower buffer was placed in the lower chamber. The cell was then allowed to run for 45 min. at 45 mA. (This step is carried out to rid the gel of any residual free radicals that may be present.) After the thioglycolate / TES run the upper chamber was washed out with Jovin upper reservoir buffer and then filled with this same buffer.

The active protein sample from DEAE 8HR (previously equilibrated in Jovin loading buffer and concentrated to 5 mL) was then loaded onto the stacking gel. The power was set at constant voltage 10 watts and the UV detector and recorder were both started. The elution buffer flow rate was then manipulated to give approximately 1 mL / min. The eluent was collected as one batch fraction until the dye front emerged. Then individual fractions were collected (4 mL). Activity was difficult to detect in the individual fractions, so the 150 µL fractions were combined (600 µL) (#1-4, 5-8, 9-13, 15-18, 19-22, 23-26, 27-31, 33-36, 37-40, and 41-44), then each of the combinations was concentrated to 125 µL. Activity was detected in the concentrated pooled samples 27-31, 33-36, and 37-40. In addition, these fractions ran as a single 21 kD band by SDS PAGE (silver stained).

Tryptic Digestion of DHAE I

This procedure was modified from the original protocol of Fernandez, *et al.*.[10]
The following recipe was used to prepare the Towbin buffer:

Trizma Base	3.03g	25 mM
Glycine	14.41g	25 mM
Methanol	400 mL	20%
Final Vol.	-----	2000 mL

No pH adjustment necessary (should be 8.3)
Digestion Buffer: 1% RTX-100 (hydrogenated Triton X-100) / 10 % acetonitrile / 100 mM Tris-HCl, pH 8.0
Ponceau S Dye: 0.2% in 1% acetic acid
Trypsin: Promega Modified Trypsin was purchased as a lyophilized powder. Modified trypsin was selected because it has been alkylated which prevents self-hydrolysis. This modification eliminates the possibility of sequencing a peptide which comes from trypsin itself. A stock was prepared by dissolving 20 µg of powder in 40 µL of the resuspension buffer provided by Promega (stored at -20 °C). This stock (10 µL) was then diluted with resuspension buffer (90 µL) to give a final solution of 0.05 µg / µL.
HPLC column: Vydac C18 reverse phase column (218TP54), 250 mm length, 4.6 mm inner diameter, 5 µm particle size.

SDS PAGE and Electroblotting

Fractions containing homogeneous DHAE I, collected from the Prep 491 cell (26 mL) were concentrated (0.6 mL) using Centricon 10s (Amicon). This retenate was further concentrated (200 µL) using Microcon 10s (Amicon).

600 µL of MilliQ H_2O was used to rinse the retenate which was then again concentrated to 125 µL. The concentrated DHAE I was subjected to standard SDS PAGE using 5 well combs (see procedure below). Two gels were loaded with 65 µL each and run as usual. Following the electrophoresis the gels were placed in chilled (4 °C) Towbin buffer for 15 minutes. The Genie electroblotting apparatus was assembled following the instructions in the Genie manual. Starting from a Towbin buffer filled lower tray, the following pieces were stacked taking care to not trap bubbles: two bubble screens, cathode plate (not platinized), scotch-brite pad, 3 mm chromatographic paper (Whatman), SDS PAGE gels, Immobilon P membrane (prepared by placing for 3 seconds in methanol and 2 minutes in MilliQ H_2O and then placing into Towbin buffer before use), 3 mm chromatographic paper, 3 Scotch-brite pads, ribbed bubble screen with ribs upward, platinized anode. This was compressed using the plastic anode cover and this whole assembly was carefully slid into the tray holder. The tray holder was placed in a vertical running position. After connecting the cathode and anode wires the apparatus was electrophoresed at 20 volts for 50 minutes at room temperature (27 °C). Following electroblotting, the apparatus was disassembled and the PVDF membranes were placed in MilliQ H_2O for 30 seconds. They were then transferred to the Ponceau S staining solution for 1 minute and then destained in MilliQ H_2O for 1 minute. The membranes were allowed to air dry (1 hour) prior to being stored in fresh aluminum foil (4 °C).

Using a new razor blade, the DHAE I bands were excised from the membranes, and placed in individual 1.5 mL Eppendorf tubes. The membranes were washed and destained as follows: rinsed 3 times with MilliQ H_2O (10 seconds each), destained with 1 mL of 200 µM NaOH / 20 % CH_3CN (1.5 minutes), again rinsed with MilliQ H_2O (10 seconds). The membranes were then treated for 30 minutes with 0.2 % PVP-40 in methanol (27 °C) in order to block the remaining non-specific protein binding sites. The excess PVP-40 was rinsed from the membrane by washing 6 times with 1 mL of MilliQ H_2O. The membranes were then cut into smaller pieces (1 mm) using a fresh razor blade and returned to the same Eppendorf tube. Digestion buffer (60 µL) and Promega trypsin (15 µL of 0.05 mg / mL) were added and the tube was incubated for 24 hours at 35-37 °C. The digestion mixture was sonicated for 5 minutes (Branson 2200 sonicator) followed by 5 minutes of centifugation at 1700 rpm (Baxter Microfuge). The supernatant was removed and more of the digestion buffer (50 µL) was added to the pellet. This was sonicated and centifuged again as described previously and then the second supernatent was added to the first. 0.1% TFA in 10 mM hexafluoroisopropanol (50µL) was added followed again by the same sonication and centrifugation steps. This third supernatant was pooled with the other supernatant for a total volume of 270 µL.

Isolation of DHAE I Fragments

The solution containing the DHAE I peptides was then injected onto an HPLC system to separate the individual peptides (Waters 600A pump, U6K injector , Vydac C18 reverse phase column (218TP54), 500 µL injection loop). The following buffers and linear gradient were used for this separation: Eluent A: MilliQ H_2O with 0.1 % TFA, Eluent B: Acetonitrile with 0.085% TFA, flow rate of 0.5 mL / min., 0 - 63 min. 98 % A - 70 % A; 63 - 95 min. 70 % A - 40 % A; 95 - 105 min. 40% A - 20% A; 105 - 117 min. 20 % A; 117 - 147 min. 20 % A - 98% A. The chromatogram was monitored using two ultraviolet / visible detectors (Kratos Analytical Spectroflow 757 at 220 nm and Linear UVIS 200 at 280 nm) both of which were connected to integrators (Hewlett Packard HP3396A). Fractions were collected in 5 mL test tubes based on the elution profiles seen in the two chromatograms (Figure 22). The isolated peptides were then dried onto polybrene discs 30 µL at a time with N_2 gas slowly blowing it dry. Peptides 15 and 22 were then sequenced directly from the discs (PAN at Stanford University). Dr. Roy E. LaFever proceeded with the subsequent cloning and overexpression work.

SDS PAGE

This technique is a modification of the original procedures of Laemmli.[12] Mini-Gel electrophoresis apparatus: Mini ProteanII electrophoresis cell, power source model 1000 / 500, both from BioRad.

Resolving Gel:	1 Gel	2 Gels	
40% Acrylamide	2.43 mL	3.65 mL	
2% Bis	1.35 mL	2.03 mL	4X Running Buf.
4X Running Gel Buffer	2.50 mL	3.75 mL	375 mM Tris
10% SDS	0.10 mL	0.15 mL	pH = 8.8
H_2O	3.57 mL	5.35 mL	
Ammonium persulfate (10%)	0.10 mL	0.15 mL	
TEMED	10 µL	15 µL	

Stacking Gel:	1 Gel	2 Gels	
40% Acrylamide	0.75 mL	1.25 mL	
2% Bis	0.36 mL	0.06 mL	4X Stacking Buf.
4X Stacking Gel Buffer	1.50 mL	2.50 mL	125 mM Tris
10% SDS	3.30 mL	5.50 mL	pH = 6.8
H2O	0.06 mL	0.10 mL	
Ammonium persulfate (10%)	35 mL	100 mL	
TEMED	3.5 mL	10 mL	

Sample loading buffer: 1 g SDS, 2 mL glycerol, 2 mL Bromophenol Blue (0.1 % aqueous solution weight / volume), 1.25 mL 1 M Tris buffer pH 6.8, 2 mL β-mercaptoethanol, diluted with $H_2O$to a final volume of 10 mL (stored at -20 °C).

Molecular weight standards and preparation: The standards (low molecular weight) were purchased from BioRad.

Phosphorylase B ----------97,400
Bovine serum albumin-------66,200
Chicken egg albumin---------45,000
Carbonic anhydrase----------31,000
Soybean trypsin inhibitor----21,500
Lysozyme----------14,400

A stock solution was prepared by adding 4 µL standards to 40 µL sample buffer (in a 1.5 mL eppendorf tube). This was boiled for 4 minutes and stored at -20 °C.

Coomassie Staining:

Coomassie staining and fixation solution: 1 g Coomassie Brilliant Blue R-250, 450 mL methanol, 100 mL acetic acid diluted to 1 liter with MilliQ H_2O.

Destaining solution: 70 mLs acetic acid diluted to 1 liter with MilliQ H_2O.

The gels were placed into the staining solution for about 12 hours. When the staining was complete, the gel were destained by placing it in the destaining solution (usually ≈24 hours or until the background became visibly clear).

Silver Staining:

This technique is a modification of the original procedures of Blum *et al.*.[13]

Fix	50% methanol, 12% acetic acid	> 1 hour
	37% HCOH (250 mL / 500 mL)	
Wash	50% Ethanol	3 X 20 min.
Pretreat	$Na_2S_2O_3$ * 5 H_2O (100 mg / 500 mL)	1 min.
Rinse	MilliQ water	3 X 20 sec.
Impregnate	$AgNO_3$ (30 g / 500 mL)	20 min.
	37% HCOH (375 mL / 500 mL)	
Rinse	MilliQ water	2 X 20 sec.
Develop	Na_2CO_3 (30 g / 500 mL)	personal preference (2 - 10 min.)
	37% HCOH (250 mL / 500 mL)	
	$Na_2S_2O_3$ * $5H_2O$ (2 mg / 500 mL or 10 mL)	
Stop	50% methanol, 12% acetic acid	10 min.
Wash	50% methanol	> 20 min.

Protocol: Two gels were always prepared, even when only one was needed. The electrophoesis cell requires that two gels be installed to function properly. Pre-poured gels (BioRad) were also stocked, in the event that one of the gels would leak when the cell was assembled. Prior to pouring the gels, the glass plates were washed with ethanol making sure that no residual acrylamide was present. The plates were assembled as described in the MiniProteanII manual. The resolving gels were poured first by combining all the above reagents excluding the ammonium persulfate. These reagents were mixed by slowly swirling the beaker and then the ammonium persulfate was added. The gels were poured, being careful not to introduce air bubbles, and leaving 2 cm for the stacking gel. Once poured they were overlaid with MilliQ water, tapped to make a uniform surface and, left to polymerize. One hour later, the water overlay was removed, a comb was fitted between the two glass plates, and the stacking gels were poured just as the resolving gels. The stacking gels were left to polymerize (1 hour). The combs were removed from both gels and the electrophoresis cell was assembled. Again, for placement of the gels it was best to follow the manufacturer's illustructions. Once assembled, electrophoresis buffer was added to the top chamber (just slightly over the top of the gel about 1 cm) and allowed to sit for at least 15 minutes to see if the top chamber was going to leak. If no leakage or at least very slow leakage was observed, the bottom chamber was then filled with the same buffer.

The protein samples (recommended concentration 0.1 to 1 mg / mL) were prepared as follows: 20 µL of each protein sample was pipeted into a separate 1.5 mL eppendorf tube. Then sample buffer (20 µL) was added to each tube. The tubes were boiled for 4 minutes and allowed to cool. Once cool, the tubes were centrifuged for 20 sec. in order to get the sample back to the bottom of the tube. These protein samples were then loaded onto the gel (under the chemical hood to avoid breathing the β-mercaptoethanol). Each protein sample (5 to 20 µL, depending on the concentration) was carefully added to a single well. The prepared low molecular weight standards (2 to 10 µL) were loaded in convenient lanes to make the gel most readable. It was best to load at least two lanes with two different protein concentrations. When the lanes had all been loaded, the top of the apparatus was put in place and the power was set at a constant 250 volts and with no more than 15 milliamps of current. The gels took 1 to 2 hours and were stopped when the dye front reached the bottom of the plates. When electrophoresis was completed, the gel was carefully removed from the glass plates and placed in either a fix solution for silver staining or directly in the staining solution for coomassie staining.

Drying the gels: The gels were dried using a procedure which sandwiches the gels between two layers of transparent membrane. The Bio-Gel-Wrap was purchased from BioDesign Inc. of New York. The Bio-Gel-Frame was not purchased but made in house out of two pieces of hard polyurethane. The following protocol was carried out for each gel. Two pieces of Bio-Gel-Wrap 1 cm larger (on each side) than the polyurathane frame were cut. Then the top of the gel, where the wells are, was removed using a razor blade so that the gel was flat on top. The gel was rinsed with water (This rinse should be avoided for a silver stained gel). One piece of the Bio-Gel-Wrap was wet with either MilliQ water for a Coomassie stained gel or wash solution for a silver stained gel for 5 seconds or until the wrap turns opaque. The wrap was carefully placed onto the premoistened bottom part of the frame (The bottom part has not been cut out in the center) avoiding trapped air bubbles. To avoid air bubbles, it was best to lay one end down and slowly lower the rest of the wrap; this technique pushes air bubbles out as the wrap is lowered. The rinsed gel was placed onto the moist Bio-Gel-Wrap with the same procedure as described for avoiding air bubbles (i.e. put one end down first). The second piece of Bio-Gel-Wrap was then wet with the appropriate solution (mentioned above) and carefully placed over the gel avoiding air bubbles. The top part of the frame was put in place and this whole assembly was pulled tight while the clamps were attached. If the gel was needed immediately, a 60 watt light bulb was then placed 9 inches from the gel so it could dry quickly. After 3 hours the gel was dry and the light was turned off. The gel was removed and the Bio-Gel-Wrap was trimmed.

If the gel was not needed right away, it was left at room temperature to dry. It was best to leave the gel in the frame for 72 hours or longer because it will curl at the corners if removed too early. Once removed and trimmed, the gel was taped to a piece of typing paper to keep it from curling at the corners.

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Chapter 3

Mechanism of Hydroquinone Epoxidation and Active Site Studies of DHAE I and II

Marc J. Kirchmeier

Introduction

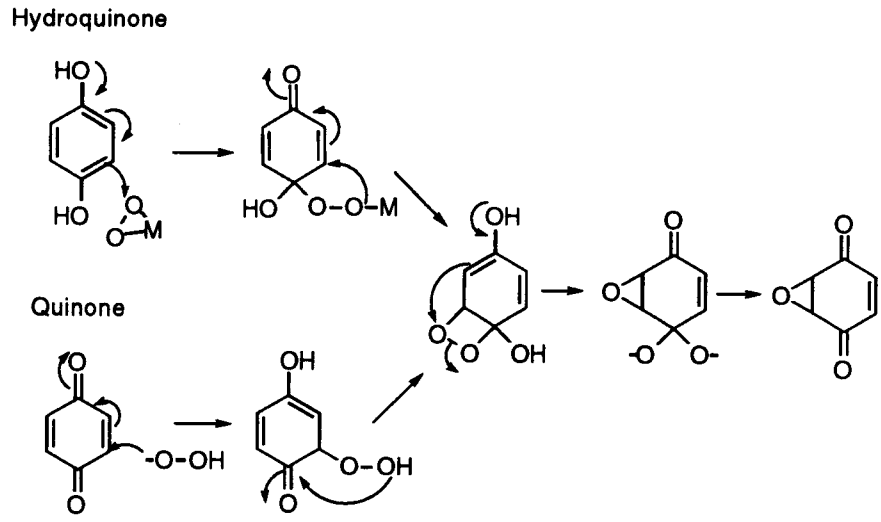
Numerous epoxyquinone and epoxysemiquione natural products have been isolated (see discussion ref. 1, pg 4-9). However, very few details about the mechanism of this epoxide formation are known. In all cases studied, it has been established that the oxirane oxygen is derived from molecular oxygen.[2] Most researchers speculated that a cytochrome P_{450} monooxygenase enzyme is responsible for the formation of the oxirane and did not pursue further mechanistic studies of the epoxidation step because cytochrome P_{450} monooxygenase enzymes are so ubiquitous.[3]

What is the Substrate for This Type of Epoxidation Reaction ?

Among the investigators who have studied these transformations, there have been conflicting reports about the nature of the substrate (quinone or hydroquinone). However, three recent biosynthetic studies and two mechanistic studies have confirmed that the hydroquinone, not the quinone, is the substrate.[4-7] There is an additional study were it is likely a hydroquinone is being epoxidized, however this study was incomplete since the hydroquinone was never tested as a substrate (see nanaomycin discussion below).[8] These results are perhaps part of a common mechanistic theme for epoxyquinone formation and further research will be necessary to more fully expose all the mechanistic details of this epoxidation reaction.

It is important to establish the correct substrate since nucleophilic attack of hydrogen peroxide on the quinone or electrophilic attack of the oxygen on the hydroquinone will lead to the same intermediate in the pathway (Scheme 13). The nature of the activated oxygen in these enzymes would be completely different. In order to react with the quinone, a nucleophilic oxygen species, probably at a peroxide oxidation state, would be required. An electrophilic oxo species, on the other hand, would be required for the interaction with the hydroquinone substrate.

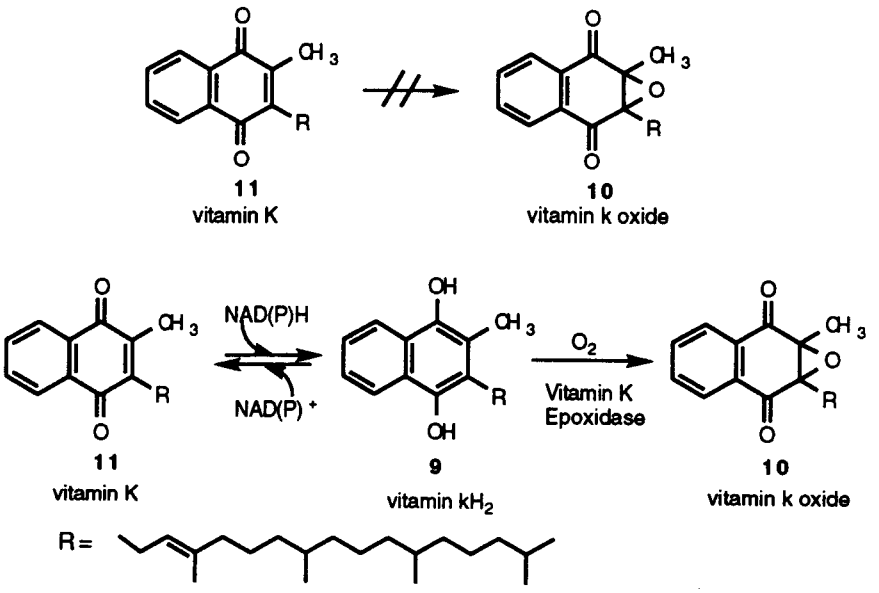
Scheme 13
Two possible substrates (hydroquinone and quinone) for the epoxidation reaction and their possible chemical pathways to the same epoxyquinone product.



Vitamin K epoxidase:

Suttie and coworkers prepared postmitochondrial supernatants that catalyzed the conversion of dihydrovitamin K, **9**, to vitamin K oxide, **10**, from vitamin K deficient rat livers, (Scheme 14).[5] When the quinone **11** was incubated with the microsomal preparation, no epoxidation occured unless either NADH or NAD(P)H was also supplied. If chemically reduced hydroquinone **9** was incubated with the extract, the epoxidation did not require the additon of the reduced cofactor. The identity of hydroquinone **9** as the epoxidase substrate was further confirmed by a competitive epoxidation of labeled quinone **11** and hydroquinone **9**. ^{3}H labeled **9** and ^{14}C labeled **11** were incubated in either the presence or absence of the reduced cofactor NADH. When NADH was omitted, only ^{3}H could be detected in **10**. When the cofactor was supplied to the reaction, both ^{3}H and ^{14}C were detected in **10**. Although it was not determined if NAD(P)H or NADH can chemically reduce **11** to **10**, it has been reported that this reduction can be catalyzed by an NAD(P)H dependent dehydrogenase.[5] Dihydrovitamin K epoxidase or as it is now called, dihydrovitamin K-dependent γ-glutamyl carboxylase, showed no inhibition from cytochrome P_{450} inhibitors, establishing that this oxygenase is not a P_{450} enzyme.[5]

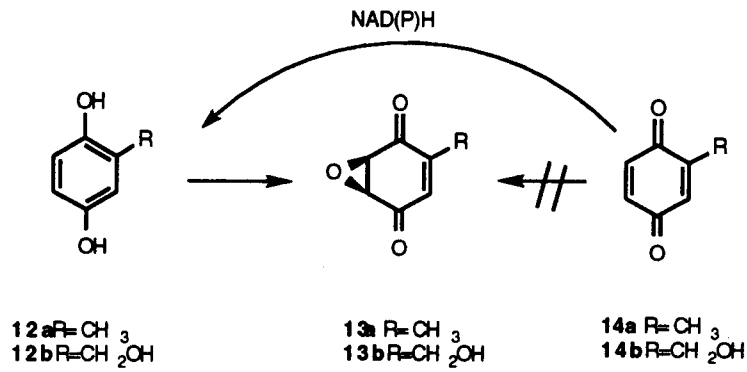
Scheme 14
The formation of vitamin K oxide, **10**, via vitamin K epoxidase does not depend on added NAD(P)H cofactor.



Phyllostine

In 1989, Priest and Light prepared a particulate cell-free extract from cultures of *Penicillium patulum* that had the ability to convert hydroquinones **12a** and **12b** into quinones **13a** and **13b** (phyllostine) respectively (Scheme 15).[6] The quinones **14a** and **14b** were not substrates unless NAD(P)H was supplied, suggesting that the quinone is first reduced to the hydroquinone prior to epoxidation. In addition, a quinone dehydrogenase converting **14a** to **12a** and **14b** to **12b**, was also found in the particulate cell-free extract. The hydroquinone epoxidase showed no inhibition by KCN or CO apparently ruling out the involvement of a cytochrome P_{450} system.

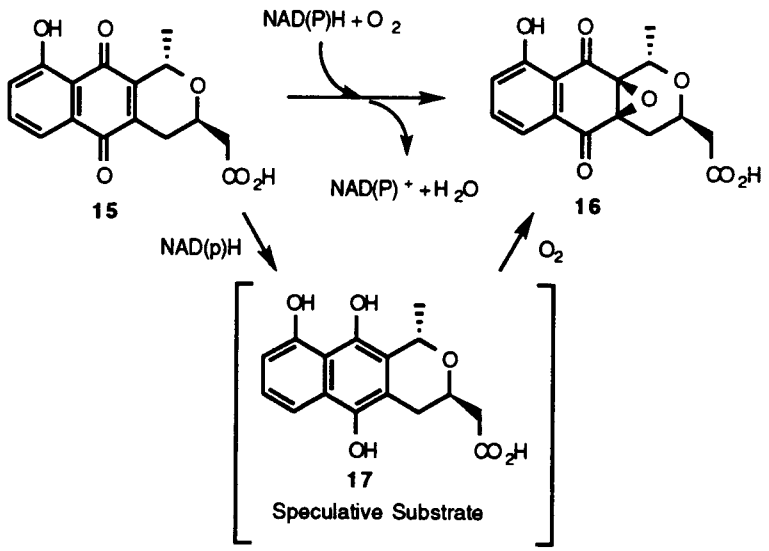
Scheme 15
The enzymatic formation of **13a** and **13b** utilized the hydroquinones **12a** and **12b** as substrates. Quinones **14a** and **14b** were not substrates for this epoxidation reaction.



Nanaomycins

Omura and coworkers discovered another hydroquinone epoxidase while investigating the biosynthesis of nanaomycins, a class of antifungal and antimycoplasmal antibiotics from *Streptomyces rosa* var. *notoensis* OS-3966.[7] The group found monoxygenase activity in a 70-90% $(NH_4)_2SO_4$ saturated pellet that could epoxidize NNM-A, **15**, to NNM-E, **16**, only when NAD(P)H was present, (Scheme 16). NNM-A hydroquinone, **17**, was never tested nor was it determined if NAD(P)H could chemically reduce **15** to **17**. In light of the work presented previously, one might speculate that the NAD(P)H is reducing **15** to **17**, either chemically or enzymatically, and it is actually the hydroquinone that is the true substrate. These studies did not state whether cytochrome P_{450} inhibitors affected the reaction. No further attempts at purification could be found in the literature.

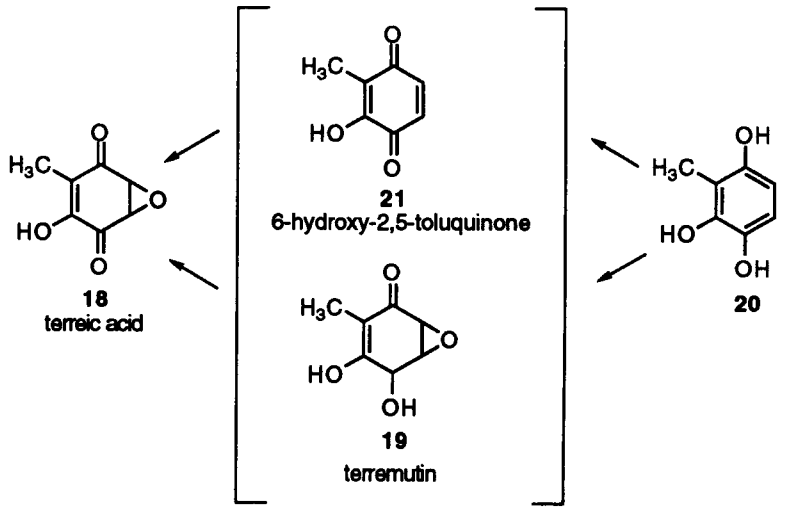
Scheme 16
The epoxidation of **15** to form **16** is most likely going through the hydroquinone, **17**, as shown.



Terreic acid

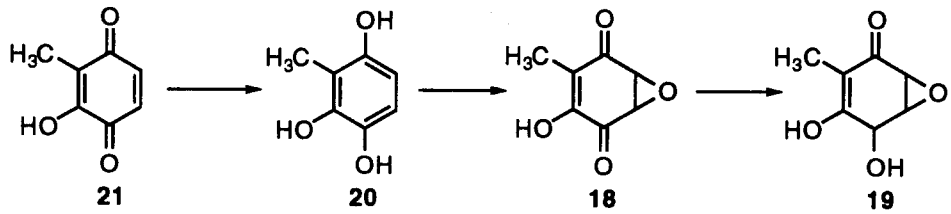
One other system worth mentioning involves the biosynthesis of terreic acid, **18**, in *Aspergillus terreus*. It was established that the epoxide oxygen was derived from molecular oxygen.[8] In addition, a related metabolite terremutin, **19**, was isolated from an *A. terreus* mutant. Read and coworkers proposed that the hydroquinone **20** was the precursor to either 6-hydroxy-2,5-toluquinone, **21** or **19**, and that one of these two compounds, **21** or **19**, was the immediate precursor to **18**, (Scheme 17).[8]

Scheme 17
Proposed route to terreic acid.



In light of the more recent work presented here, a more likely proposal might be as in Scheme 18 where **18** serves as the immediate precursor to **19**. The conversion of **20** to **18** is clearly supported by literature precedence, and support for the reduction of **18** to **19** is found in the biosynthesis of **1,** (Scheme 2, pg. 3).

Scheme 18
Revised route to terreic acid, **18**.

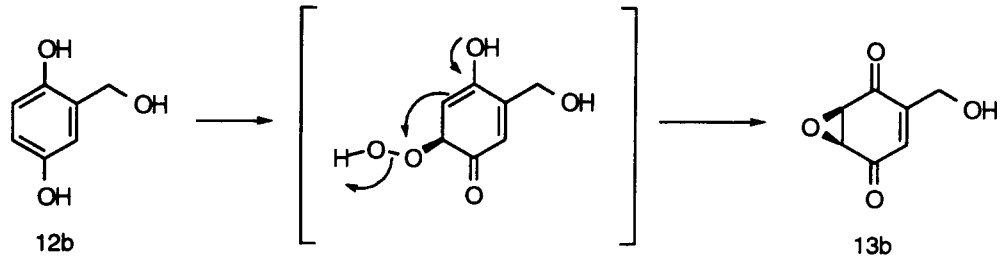


Detailed Mechanistic Chemistry

Hydroquinone Epoxidases as Monooxygenases

In the formation of Phyllostine **13b**, Priest and Light have proposed a monooxygenase mechanism (Scheme 19).[6] They speculated this mechanism would involve the hydroperoxy intermediate shown in scheme 19.

Scheme 19
Monooxygenase mechanism proposed for phyllostine formation

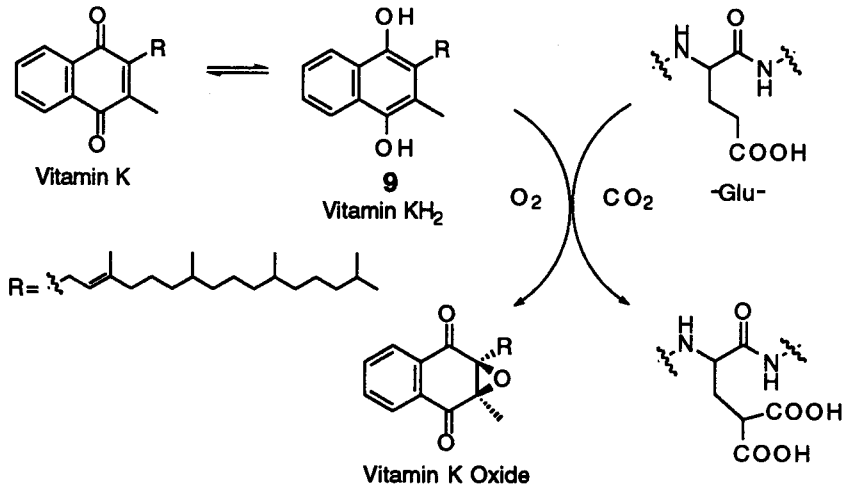


Hydroquinone Epoxidases as Intramolecular Dioxygenases

Dihydrovitamin K epoxidase, also known as dihydrovitamin K dependent γ-glutamyl carboxylase, is involved in a coupled reaction which forms the γ-carboxyglutamyl residues in thrombin, (Scheme 20). Once the bidentate γ-carboxyglutamyl residues are formed, the carboxylated proteins chelate Ca^{+2} and interact with platelet membrane surfaces, an event that is essential for the formation of blood clots.[9] Dihydrovitamin K, **9**, is first oxidized yielding intermediate **22** (see complete mechanism, Scheme 21). Acting as a strong base, **22** abstracts the γ-methylene protons from glutamic acid residues and the reaction is completed by the attack of the glutamic acid carbanion on CO_2, (Schemes 20 & 21).

Scheme 20

Overall reaction of dihydrovitamin K-dependent γ-glutamyl carboxylase.



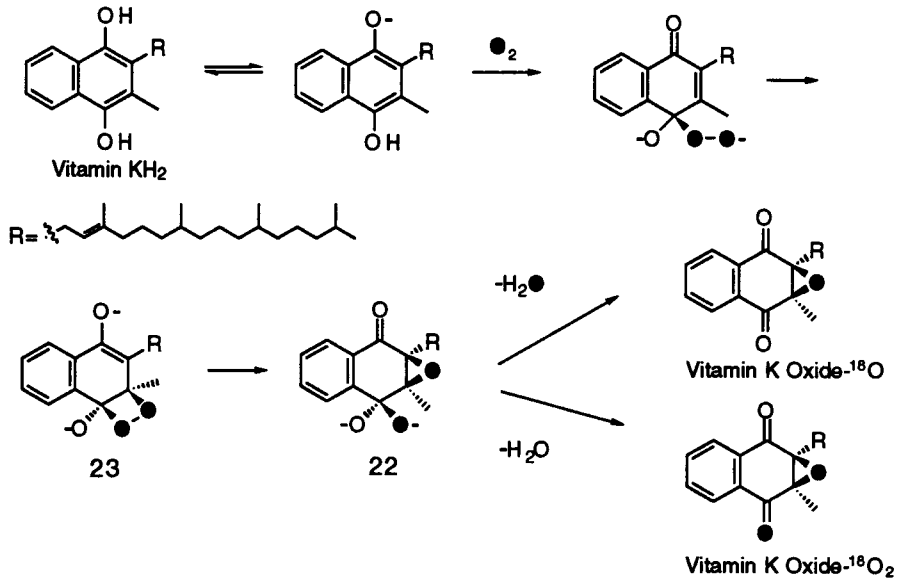
Recently Dowd and Ham have developed a model system which mimics the epoxidation reaction of dihydrovitamin K hydroquinone.[10] Thermodynamic analysis of this model reaction suggests that an intramolecular dioxygen addition, by way of the dioxetane intermediate **23** leading to hydrate **22**, will provide the base strength required for the abstraction of the γ-methylene proton in glutamic acid, (Schemes 20 and 21). The proposed mechanism suggests that two oxygen atoms will incorporate into Vitamin K oxide. In an earlier experiment, Sadowski *et al.* unequivocally demonstrated that the epoxide oxygen of the vitamin K oxide, **11**, was derived from molecular oxygen.[5] In these studies, the vitamin K epoxidase reaction was carried out under an atmosphere of 99% $^{18}O_2$ and the epoxide product was analyzed by EI-MS. By re-analyzing Sadowski's data, Dowd found that the ion peak at 470 (M+4) peak was enhanced 4-fold above natural abundance indicating that not only is the epoxide oxygen derived from O_2, but the ketone oxygen is as well.

Soon after Dowd had communicated his newly found evidence, Walsh *et al.* published the confirming experiment that vitamin K-dependent γ-glutamyl carboxylase is a dioxygenase.[11] Immediately after Walsh's group had reported their labeling study, Dowd *et al.* confirmed their report by carring out a parallel $^{18}O_2$ enrichment study.[12]

The key features of Dowd's mechanism include the formation of dioxetane intermediate **23** leading to the *gem*-diol hydrate intermediate **22**. Intermediate **22** loses either H_2O or $H_2{}^{18}O$, yielding vitamin K oxide-^{18}O or vitamin K oxide-$^{18}O_2$, respectively. The latter products were obtained in a 4:1 ratio of ^{18}O:$^{18}O_2$.[12]

Scheme 21

Mechanism of dihydrovitamin K-dependent γ-glutamyl carboxylase. $^{18}O_2$ was utilized to elucidate this mechanism.

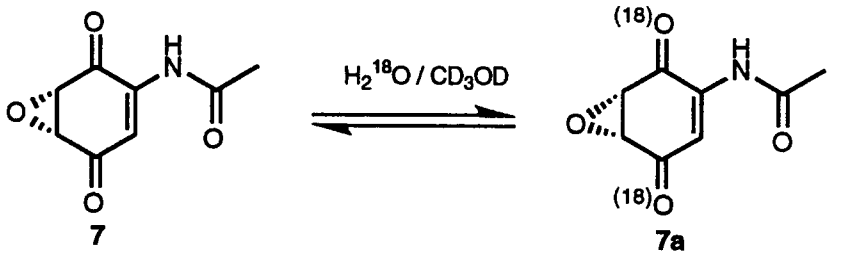


This mechanistic work carried out on Dowd's model system and dihydrovitamin K-dependent γ-glutamyl carboxylase itself is very intriguing. Besides the obvious structural similarities of the substrates (Vitamin KH_2, **9**, and 2,5-dihydroxyacetanilide, **4**), dihydrovitamin K-dependent γ-glutamyl carboxylase shares some characteristics with DHAE I. Both enzymes are cofactor independent and neither epoxidase is a cytochrome P_{450} enzyme. In addition, there has been no metal identified as being necessary for the activity of dihydrovitamin K-dependent γ-glutamyl carboxylase.

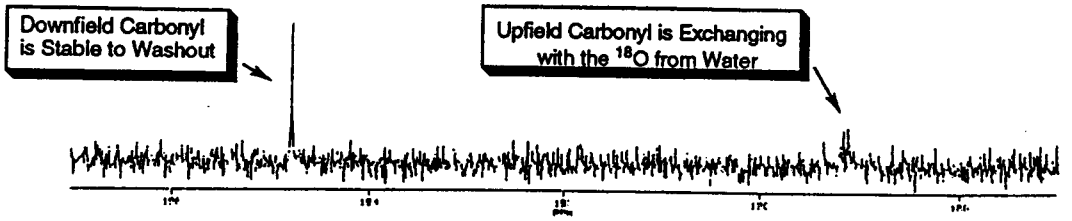
Prior to labeling studies of DHAE I with $^{18}O_2$, to determine if DHAE I conforms to a monooxygenase (Priest and Light[6]) or dioxygenase (Dowd[12]) mechanism, the possibility of an interfering carbonyl exchange in the product **7** was investigated. Pure **7** was mixed with $H_2{}^{18}O$ and examined over time using ^{13}C NMR, (Scheme 22). The upfield quinone carbonyl resonance gave rise to a second isotope-shifted peak.[13] However, the quinone carbonyl resonances of **7** had not yet been assigned, so the regiospecificity of this exchange was not able to be assigned either.

Scheme 22

Above is the experiment to test the exchange of the carbonyl oxygens with ^{18}O. Below is the ^{13}C NMR results of the experiment.

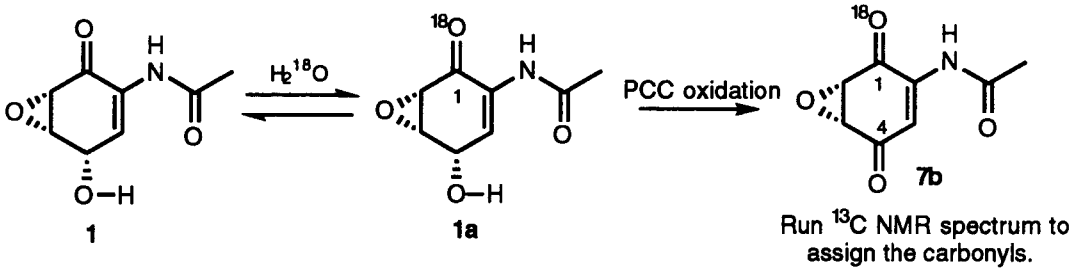


7 **7a**



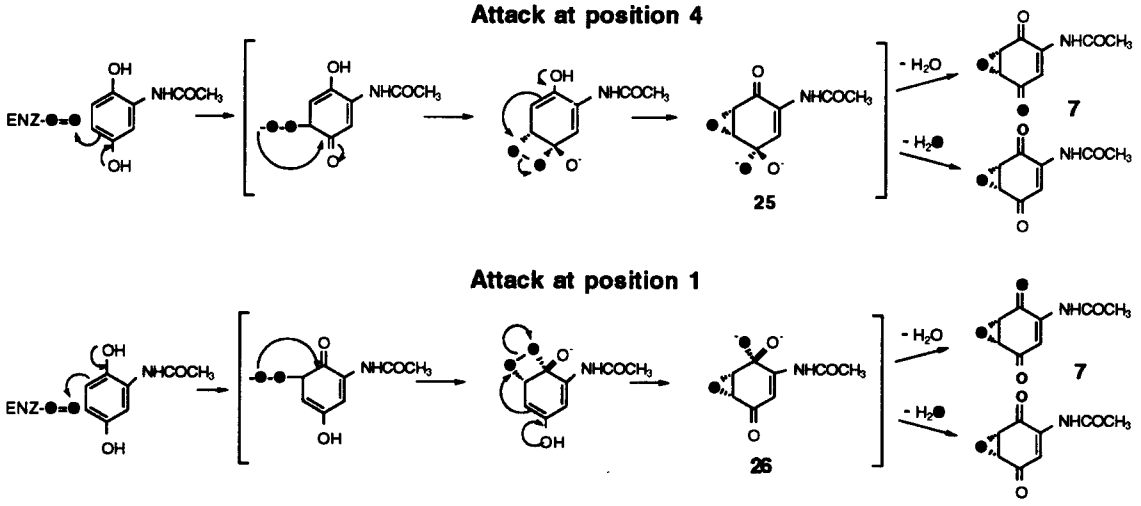
To assign the quinone carbonyl resonances, isotopically labeled **7** was synthesized. This was acheived by allowing the carbonyl at C-1 of **1** to exchange with $H_2{}^{18}O$, (Scheme 23). **1a** was then oxidized with pyridinium chlorochromate (PCC) to yield **7b** labeled exclusively at C-1 with ^{18}O. The ^{13}C NMR spectrum revealed an isotope shift for the carbonyl resonance at δ 194 ppm.[13] Therefore, the chemical shift assignments for C-1 and C-4 were δ 194 ppm and δ 188 ppm, respectively. Thus, it is the carbonyl at C-4 of **7** that undergoes rapid exchange with water.

Scheme 23
Labeling experiment to assign the ^{13}C NMR carbonyl resonances of 7.



If Preist and Lights monooxygenase mechanism holds true for DHAE I, only the epoxide oxygen would be labeled. However, if the Dowd mechanism holds true for DHAE I, then two regiospecific possibilities for the oxygen incorporation would exist. The results of incorporation studies would depend on the regiospecificity of the initial addition of dioxygen as well as the loss of water from the two diastereotopic oxygens in the hydrates **25** and **26**, (Scheme 24).

Scheme 24
Two possible regiospecific outcomes of Dowd's mechanism when applied to DHAE I.

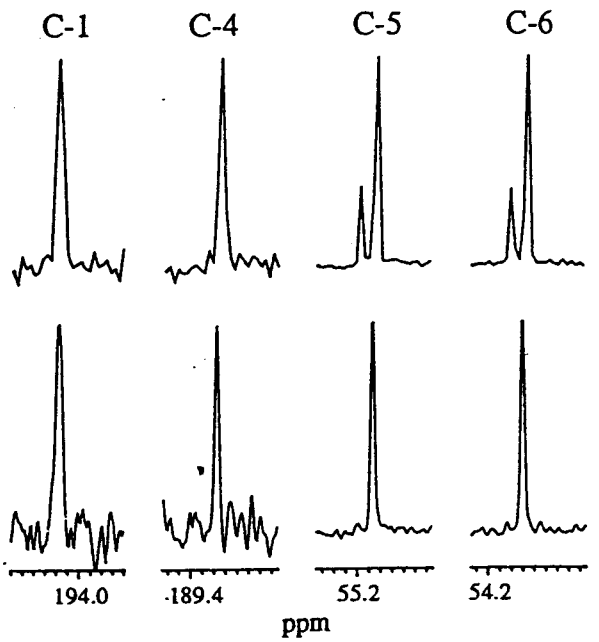


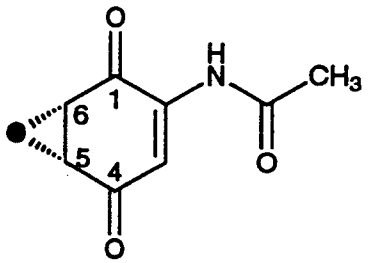
If the DHAE I epoxidation reaction is carried out under an atmosphere of $^{18}O_2$, and if the initial attack occurs at C-1 of **7** rather than at C-4, the label will be stable to water exchange during the enzyme incubation and subsequent work-up. However, if the alternate regiospecificity is correct and the initial attack takes place at C-4, the label could be lost due to exchange with water (see Schemes 22 and 23).

The epoxidation reaction was carried out under an atmosphere of $^{18}O_2$. The product **7c** was isolated and analyzed by mass spectrometry and by ^{13}C NMR, (Figure 13). Addition of 20% unlabeled **7** revealed the original sample was approximately 100% labeled at oxygen through the appearance of two new downfield signals at C-5 and C-6. No enrichment was found at either of the carbonyl carbons (C-1 & C-4). This result supports a monooxygenase mechanism. On the other hand, a dioxygenase mechanism may still be operative, since the second label might be washed out or lost during a stereospecific dehydration. (If the attack of dioxygen is taking place at C-1, there must be a stereospecific loss of the diastereotopic ^{18}O at C-1 of hydrate **26**. Otherwise, this position is stable to exchange. It should also be pointed out that addition of ^{18}O at C-4 may have occurred but the label had washed out).

Figure 13

The bottom four ^{13}C NMR resonances represent **7c** without the addition of unlabeled **7**. Each resonance is a single peak. The top set of resonances represent the same sample of **7c** but with the addition of ≈ 20 % unlabeled **7**. The oxygen at the oxirane carbons (C-5 & C-6) is clearly 100 % enriched with ^{18}O while no ^{18}O is found at either of the carbonyl carbons.

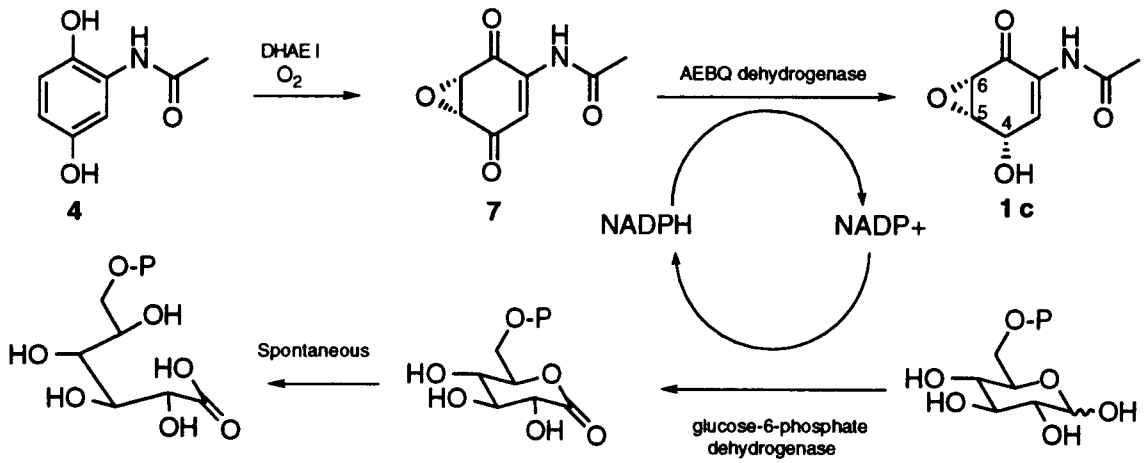




7c

The possibility that the ^{18}O label was lost by exchange with water from C-4 was investigated by enzymatically reducing the carbonyl at C-4 of **7** as it was produced. The NAD(P)H dependent enzyme 2-acetomido-5,6-epoxy-1,4-benzoquinone (AEBQ) dehydrogenase is the next committed step, beyond the epoxidation step, in the biosynthesis of **1** (Scheme 1, pg. 3). This enzyme activity was isolated and partially purified from a cell-free extract of *Streptomyces* LL-C10037. Conditions for coupling the two enzymes (DHAE I / AEBQ dehydrogenase) were established. Since the C-4 carbonyl exchange of **7** was rapid, it was very important to not allow **7** to accumulate. Optimal coupling of the two enzymes showed only substrate **4** and product **1** in the HPLC separation of the reaction. In addition, a cofactor regeneration system based on glucose-6-phosphate dehydrogenase was used to generate the NAD(P)H *in-situ* (Scheme 25). Based on the amount of **4** remaining and the extent of **1** produced in the reaction, substrate, **4** was added at varying intervals. Over a four hour period, 50 milligrams of **1c** was produced.

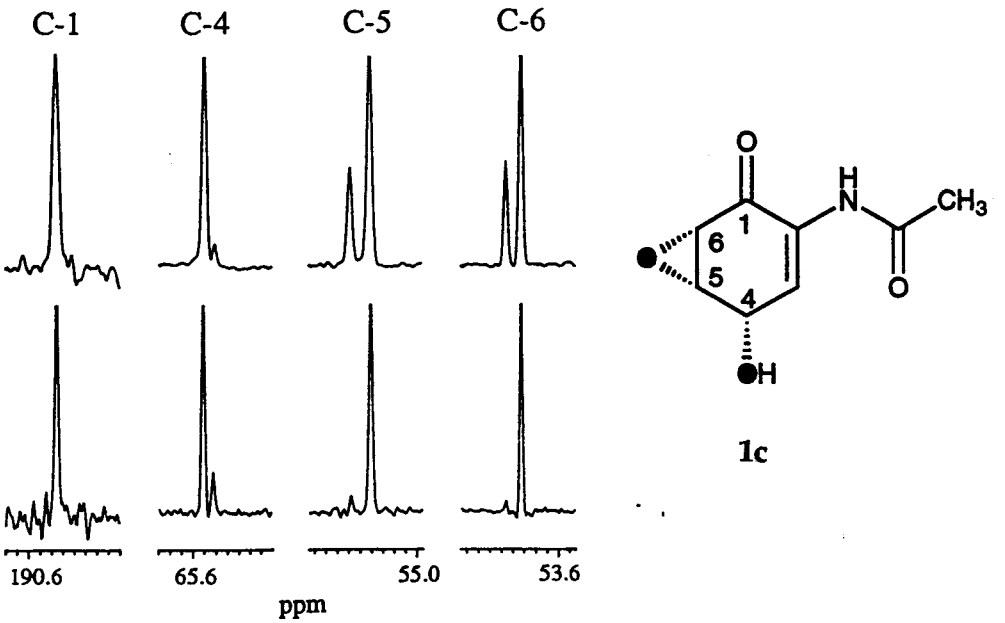
Scheme 25
Coupled reaction sequence utilized to trap ^{18}O at C-4 of 7.



The ^{13}C NMR data from the reaction in Scheme 25 is shown in Figure 14. Two isotopically labeled oxygen atoms were incorporated into **1**; one at the oxirane position and a second that was subsequently trapped at C-4 by the reduction. Authentic unlabeled **1** was again used to spike the NMR sample to confirm that the isotope shifts were real. The presence of two oxygens in **1** is consistant with the intramolecular dioxygen mechanism proposed by Dowd for dihydrovitamin K-dependent γ-glutamyl carboxylase.

Figure 14

The bottom four ^{13}C NMR resonances represent labeled **1c** isolated from the $^{18}O_2$ labeling study, (Scheme24). The top four resonances represent the same sample of **1c** with the addition of ≈ 30% unlabeled **1**. The oxygen at C-4 is ≈ 20% enriched with ^{18}O. In addition, the oxygen at the oxirane (C-5 & C-6) is almost completely enriched.



Initially, DHAE I was suspected of belonging to the monooxygenase family of iron and copper metallo epoxidizing enzymes. However, Gould and Shen found some unusual features which distinguished DHAE I from this class of enzymes. DHAE I bioassays showed neither flavin nor cytochrome P-450 dependence. In addition, the epoxidase was unaffected by adding Fe^{+2} or Cu^{+2}, but-surprisingly-were positively effected by addition of Co^{+2}, Ni^{+2}, and Mn^{+2}. Activity was restored to DHAE-I apoenzyme only with these latter three ions.[2]

We have now demonstrated that DHAE I is an intramolecular dioxygenase, consistant with the dihydrovitamin K epoxidase. Since, numerous epoxyquinones and epoxyquinols are known in nature, we believe these represent a previously unrecognized family of "hydroquinone dioxygenase (epoxidizing)" enzymes.

Biomimetic Hydroquinone Epoxidation Reaction

One of the exciting features of studying the bioorganic transformations involved in forming complex natural products is the discovery of new reaction types. Epoxidation reactions are not new to organic chemistry. In fact, there are many reagents to carry out the epoxidation of double bonds.[14] Epoxidations reactions are common practice in the synthesis of complex molecules. However, the epoxidation of hydroquinones, via the pathways described earlier in this chapter, represents new chemistry.

Whenever new chemistry is discovered in nature, it is important to investigate the possibility of doing the same reaction without the use of the enzyme. These biomimetic reactions can serve as models for studying the detailed chemistry occurring during the reaction processes.

Ham and Dowd have recently reported a non-enzymatic model for the vitamin K-dependent γ-carboxylase reaction, (Scheme 26).[15] It was the design and execution of this biomimetic reaction of vitamin K dependent γ-carboxylase which finally led Dowd to propose today's accepted mechanism for the enzyme itself. In designing the model, α-naphthoxide, **27**, was used in place of dihydrovitamin K. Because **27** only has one hydroxyl group, the course of the oxidation reaction could be mapped. Since vitamin K-dependent γ-glutamyl carboxylase catalyzes the formation of a new carbon-carbon bond between carbon dioxide and glutamate residues, an intramolecular (Dieckmann) condensation reaction was employed. This was a very clever aspect in the design, since the intramolecular condensation mimics the capacity of enzyme to hold the two substrates in the correct orientation and in close proximity to one another.

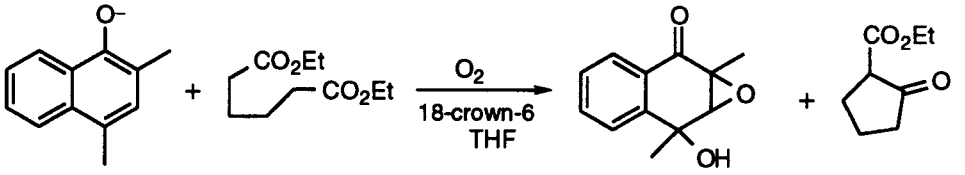
The weakly basic naphthoxide anion does not possess the base strength to extract a proton from the α-methylene next to the carbonyl and facilitate the Dieckman condensation of diethyl adipate. However, the oxidized intermediate, **28**, has enhanced base strength and abstracts the proton thus allowing for the condensation to occur.

Nature has used an oxidation reaction to enhance base strength of vitamin K in order to carry out an acid / base reaction. This is indeed new and fascinating chemistry. The model introduced a novel basicity enhancement reaction which was the key to understanding how vitamin K functions in the carboxylation reaction. This model demonstrates the power of what can be learned from breaking down a biological reaction into its chemical components.

Scheme 26

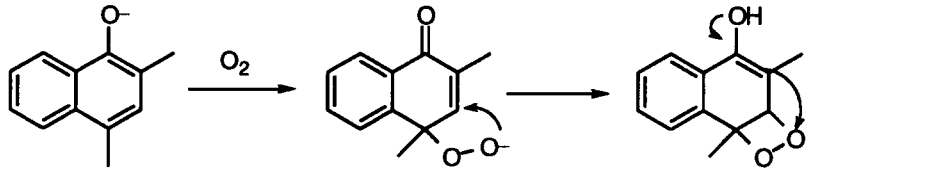
Non-enzymatic model for the vitamin K-dependent γ-carboxylase reaction.

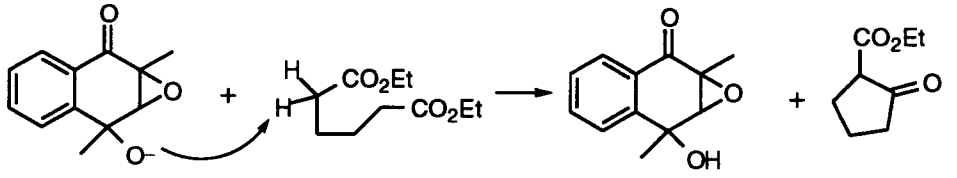
Overall Reaction



27

Detailed Explanation



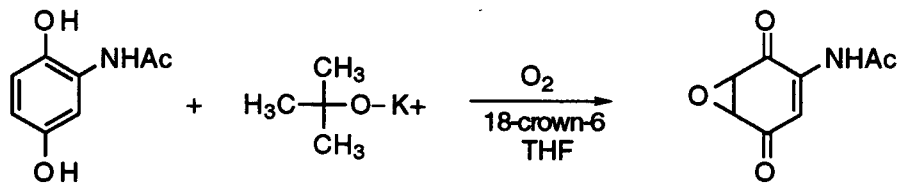


28

The epoxidation reaction of DHAE I and II is not as complicated as the reaction of vitamin K-dependent γ-carboxylase since no coupled carboxylation reaction is involved. To remove one of the phenolic protons from the hydroquinone, **4**, the strong base potassium tert-butoxide was used in the presence of 18-crown-6, (Scheme 27). The DHAE biomimetic reaction produced epoxyquinone. However, the yield was low in these reactions (less than 10% epoxyquinone was recovered).

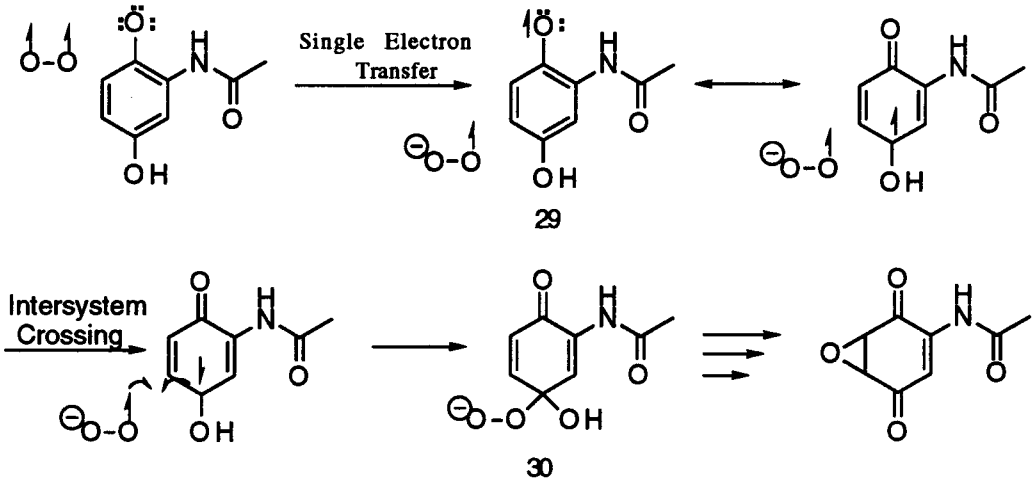
Scheme 27

Non-enzymatic model for the epoxidation of DHA (Biomimetic reaction of DHAE I & II)



Based on known reactivity of triplet oxygen[16] we speculate that the DHA anion, **29**, is transferring a single electron to triplet oxygen (Scheme 28). The resulting DHA radical then rearranges and the unpaired electron undergoes a spin flip (intersystem crossing). The singlet oxygen and the DHA radical then combine to form the peroxide intermediate, **30**. The peroxide then rearranges and dehydrates (as in Scheme 24 above) to form a mixture of epoxyquinones, **7** and **8**.

Scheme 28
Proposed mechanism for the biomimetic reaction of DHAE I & II.



Active Site Studies

Introduction

Upon understanding the mechanism by which DHAE I and II catalyze their respective epoxidation reactions, one starts gaining an appreciation for the chemical makeup of these two active sites. The active sites of DHAE I and II both contain two substrates, dioxygen (O_2) and DHA, **4**. Each active site is thought to contain a base, to start the reaction, and both have many other amino acid side groups which aid in recognition and polarization of the substrate pair.

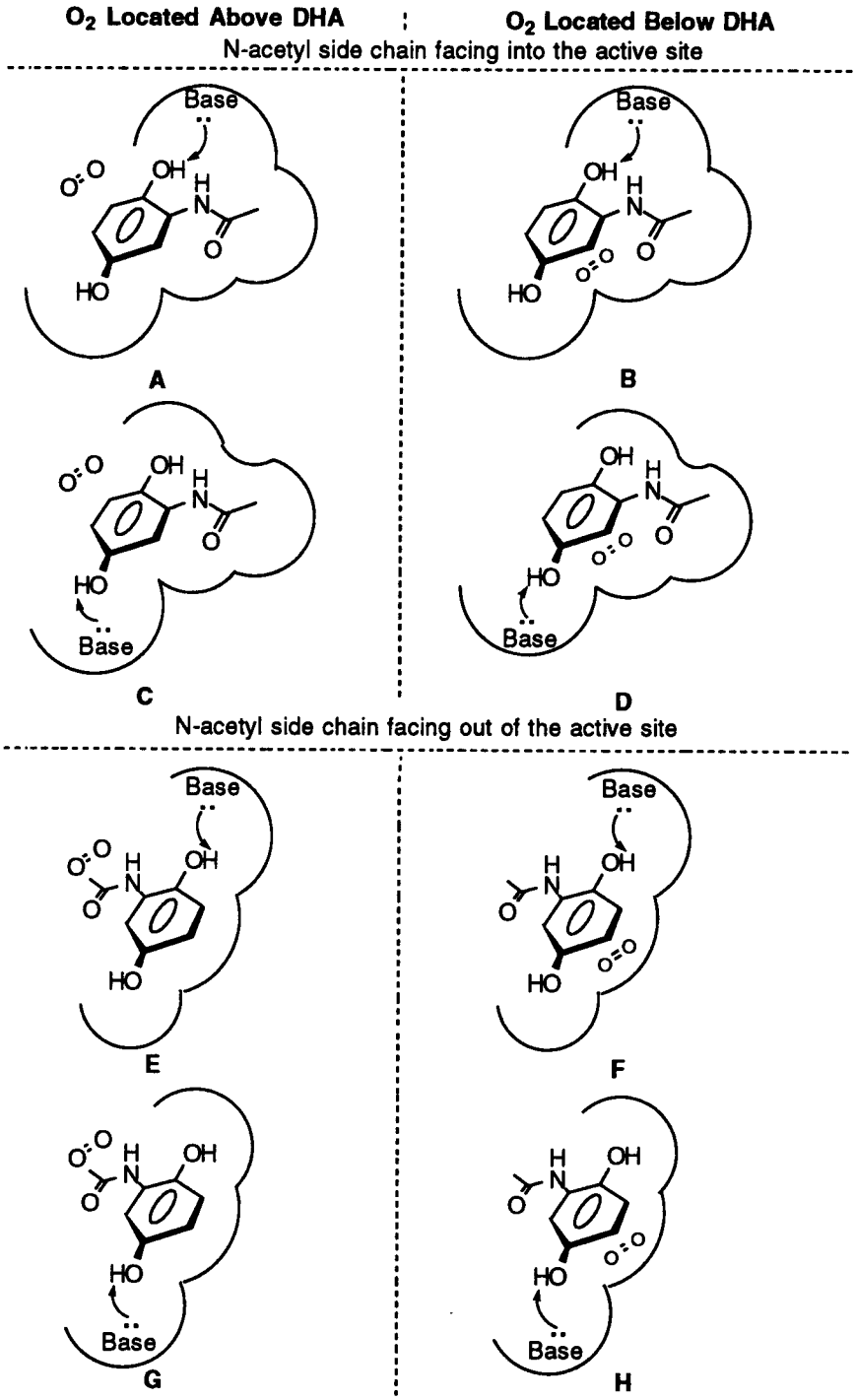
Since the DHA ring is planar, it is possible to construct models for the geometric relationships of the active site amino acids for DHAE I and DHAE II. For each epoxidase, there are eight possibilities for the orientation the active site base with the two substrates, **4** and O_2.

First, the dioxygen can be positioned above or below the plane of the ring (Figure 11). In this figure, the left side of the slashed line represents O_2 positioned above A, C, E, G, and on the right side of the slashed line the O_2 is located below the DHA plane B, D, F, H. Secondly, the base can abstract the hydroxyl proton from carbon-1 or carbon-4, (Carbon-1 abstraction in A, B, E, F and Carbon-4 abstraction in C, D, G, H, Figure 3). Lastly, the acetamide side chain at carbon-2 will face into or out of the active site (into active site A - D and outside of active site E - H).

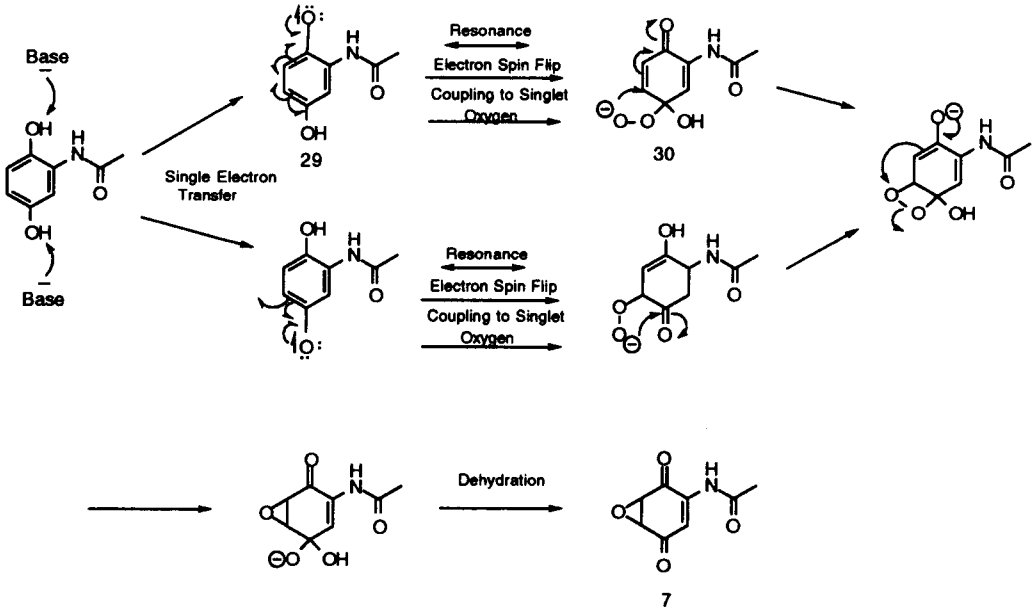
From an earlier discussion about the possible outcomes for the $^{18}O_2$ labeling of the DHAE I product, **7**, (Scheme 24), one might speculate that the proton abstraction is taking place from the hydroxyl of carbon-4 of **4** since the regiochemical outcome of this $^{18}O_2$ addition is now known. Carbon-4 of **7** retains a portion of one isotopic oxygen from labeled dioxygen while the second oxygen is found in the epoxide.[18] However, knowing the regiochemistry for this addition of dioxygen into the product does not necessarily indicate the regiochemistry of the proton abstraction. Abstraction of the hydroxyl proton on carbon-1 can lead to the same intermediate as that found for the abstraction of the hydroxyl proton on carbon-4 (Scheme 29).

The active sites of these two epoxidases produce enantiomeric products. Therefore, the sites have opposite symmetry with respect to their enzyme-substrate complexes. This asymmetry could result from controlling the orientation of the substrate with the dioxygen being delivered from the same side, (A and E, B and F, C and G, D and H, Figure 15). Alternatively, the substrate could be bound in the same orientation and the dioxygen could be delivered from the opposite sides, (A and B, C and D, E and F, G and H, Figure 15).

Figure 15
Model for understanding the active site geometry's of DHAE I and DHAE II.



Scheme 29
The active site base can abstract either hydrogen of DHA and get the same product.



Outside of the terpene family, enantiomeric natural products are rare in nature. Having a detailed understanding of the DHAE I and DHAE II systems may provide fundamental information to help understand not only the evolutionary relationships between these two epoxidases but also may help in understanding the formation of other nonterpene enantiomeric natural products.[2] Therefore, it is important to investigate the factors controlling the selective formation of this enantiomeric pair of natural products (**7** and **8**). Also, these two enzymes can serve as a paradigm for understanding other hydroquinone epoxidases. Since hydroquinone epoxidases represent a large new class of oxygenases it is essential to gain as much information as possible from these two enzymes.

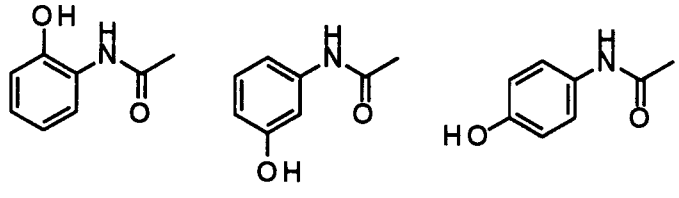
The best approach toward elucidating the orientation of active site amino acids would involve individually crystallizing the two epoxidases with and without their substrate, **4**, to detrermine their three dimensional structureby X-ray diffraction. Unfortunately, these enzymes are only available in very small quantities, so another approach must be taken . An alternative approach, which doesn't require large quantities, involves the use of substrate analogs. Well designed substrate analog studies are an effective tool for the interpretation of three dimensional information found in enzyme active sites.[18]

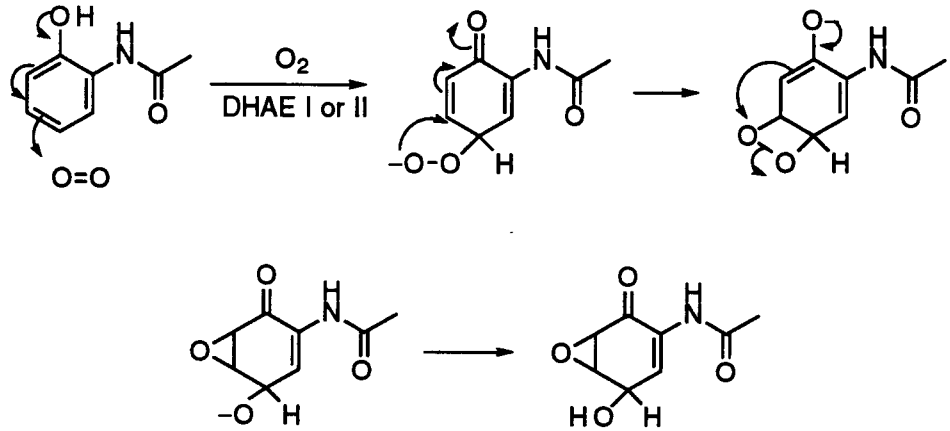
Acetamidophenol Analogs

To investigate the regiochemistry of the proton abstraction and other geometric constraints of the active sites in DHAE I & II, three acetamidophenols were examined: 2-acetamidophenol **31**, 3-acetamidophenol **32**, 4-acetamidophenol **33**, (Scheme 30, top). Analogs **31** and **32** are like the substrate however, they lack a second hydroxyl group found on the ring of DHA. Both are expected to fit into the active sites of the two epoxidases. However, only one of the pair should have the correct orientation to undergo proton abstraction, thus starting the epoxidase reaction sequence. In this case, the analog may act as an alternative substrate and a new product will be formed, (Scheme 30, bottom). Alternatively, the analogs can act as competitive inhibitors by blocking the active sites of the two epoxidases. Analog **33** was used to examine the flexibility of the active sites. Competitive inhibition of either epoxidase in the presence of **33** would demonstrate looseness in that active site specificity.

Scheme 30

Three acetamidophenol substrate analogs are shown above. Below is the possible outcome of the epoxidase reaction on the 2-acetamidophenol. It is assumed that the 3-acetamidophenol would undergo a similar reaction if the orientation of the active site base was reversed.



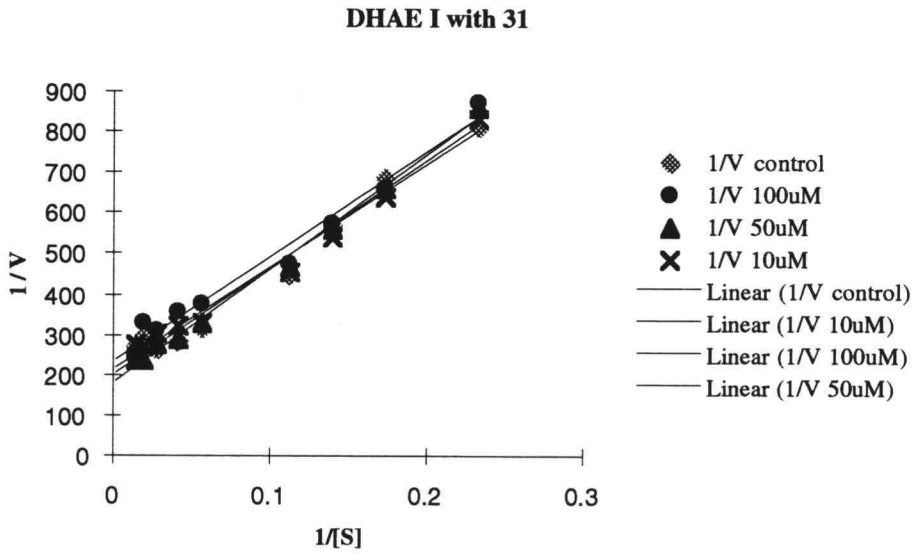


The three analogs were also assayed as alternative substrates with DHAE I by individually adding each analog to an active enzyme preparation (DHAE II was never tested for this ability). The assays were allowed to incubate over 10 times longer than it took for the same amount of protein to convert the actual substrate, **4**, into the product **7**. (It took three minutes for the control assay to convert the **4** to **7**. So the same molar amount of each analog was left to incubate for thirty minutes.) Surprisingly, no new compounds were detected when each of these assays were injected under the standard HPLC conditions.

The three analogs were then assayed for their ability to inhibit the standard epoxidase reactions of DHAE I and II. Assay conditions were selected which allowed for the measurement of the initial velocity over a wide range of substrate concentrations (4.3, 5.8, 7.2, 9, 18, 24.4, 35.9, 53.2, 71.9 μM). Once the conditions were established, each analog was individually assayed at three different concentrations with each of the two epoxidases: 10 μM, 50 μM, and 100 μM . At each of the three concentrations of analog, nine different concentrations of substrate were assayed, giving a total of 36 assays for one analog with one epoxidase. For each of the 36 assays, the percent production of **7** or **8** was determined from HPLC analysis. This data was then analyzed using a computer software program (Scientist For Experimental Data Fitting Version 2.0, Micromath Scientific Software, Salt Lake City, Utah) which fits the data to mathematical equations representative of competitive, non-competitive, and uncompetitive inhibition. Kinetic parameters were obtained from the equation which best fit the data. From the computer analysis and Lineweaver-Burk reciprocal plots (1/V vs 1/[S]), (Figure 16), the type of inhibition was determined for each analog with each epoxidase, (Table 2).[19]

Figure 16
Lineweaver-Burk plots of acetamidophenol inhibitors with DHAE I and DHAE II. Each plot represents a set of 36 assays. Each inhibitor concentration (100 μM, 50 μM, 10 μM, and control) represents a single least squares linear regression line on the plot. For DHAE I with **32** the 10 μM inhibitor line is lacking because of experimental error. A. (DHAE I with **31**, B. DHAE I with **32**, C. DHAE I with **33**, D. DHAE II with **31**, E. DHAE II with **32**, F. DHAE II with **33**)

A



B

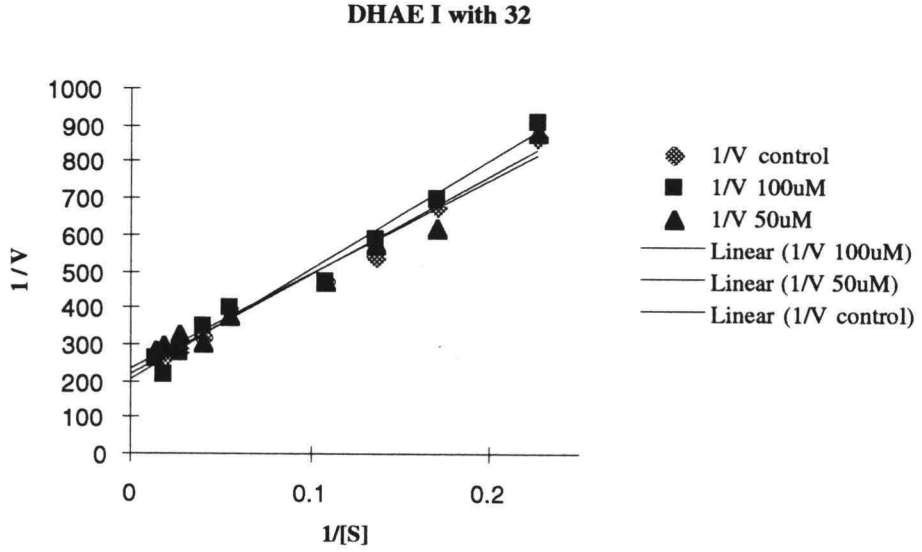
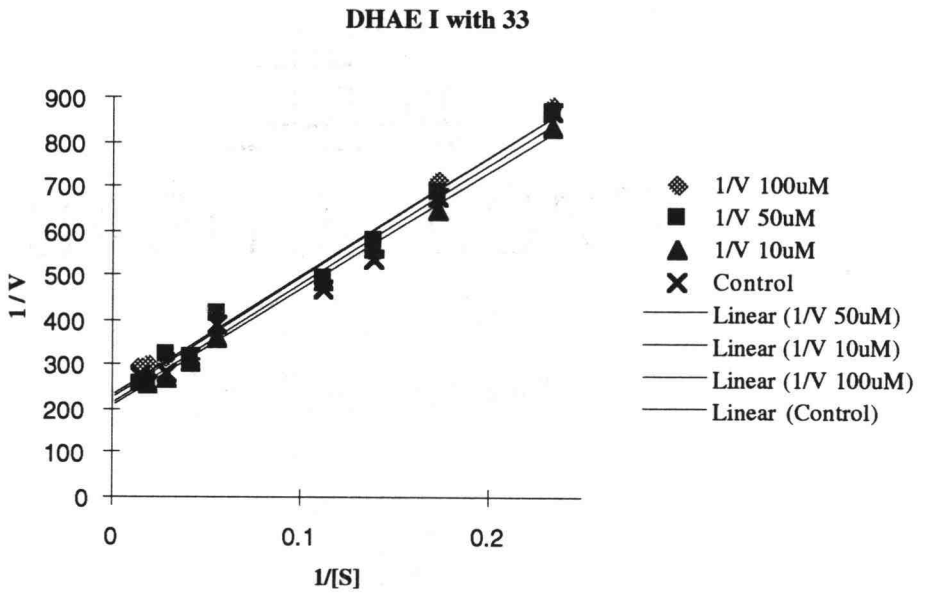


Figure 16 (Continued)

C



D

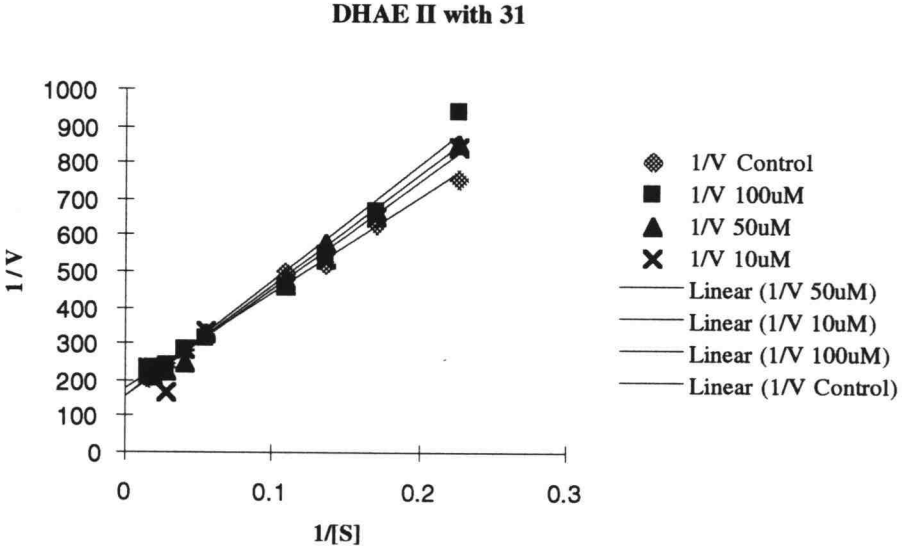
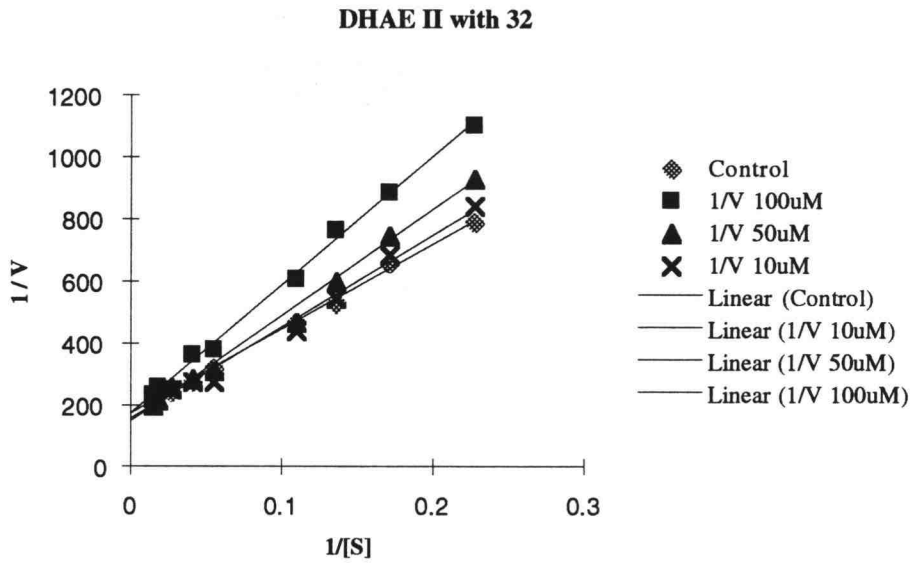


Figure 16 (Continued)

E



F

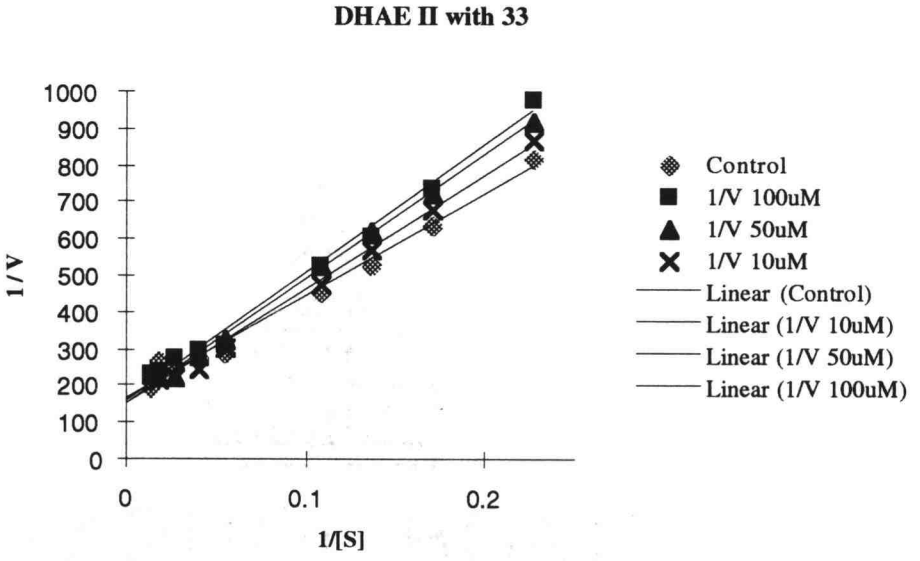


Table 2
Kinetic parameters from the individual sets of Reactions. (Units: Vmax = μmoles **7** or **8** produced / minute, Km = μM, Ki = μM)

DHA Epoxidase From *S.* LL-C10037 (DHAE I)

Analog	Type of Inhibition	Vmax	Km	Ki
31	Competitive	0.0046±.0004	11.8±1.82	103±8326
32	Competitive	0.0042±.0004	10.0±1.8	1348±1854
33	No Inhibition Observed	0.0043±.0004	10.7±1.8	--

DHA Epoxidase From *S.* MPP 3051 (DHAE II)

Analog	Type of Inhibition	Vmax	Km	Ki
31	Competitive	0.0058±.0004	14.86±3.2	1208±2059
32	Competitive	0.0062±.0004	18.06±3.2	505±158
33	Competitive	0.0058±.0004	15.94±3.2	979±544

It can be seen from the data presented in Table 2, that the individual kinetic parameters of Vmax and Km match very closely between the three sets of runs for each epoxidase. For DHAE I the Vmax deviation is ± .0004 μmoles **7** produced / minute and Km deviation is ± 1.8 μM. For DHAE II the Vmax deviation is ± .0004 μmoles **8** produced / minute and Km deviation is ± 3.2 μM). Because of this internal consistency, one can safely compare and contrast the individual analog runs.

In general, the three analogs did not bind as tightly as predicted from the close similarity of their structures with that of DHA, **4**. The best inhibitor / epoxidase combination was the 3-isomer, **32** with DHAE II, but even this inhibitor was very weak (apparent Ki is 28 times higher then the Km). Clearly, all three functional groups of DHA (*p*-hydroxyls and an N-acetyl side chain) are important for the binding to be efficient. Nonetheless, inhibition was seen in all cases except the 4-isomer, **33**, with DHAE I. All of the inhibition was competitive. Therefore, the analogs (except the 4-isomer, **33** with DHAE I) are binding to the active site of each.

Overall, the Ki values for DHAE I are higher than the Ki's for DHAE II. In the case of **33** with DHAE I, no inhibition was observed. Therefore, DHAE I has a tighter fit with the substrate than DHAE II. This would suggest that DHAE I is less able to accept alternative substrates than DHAE II.

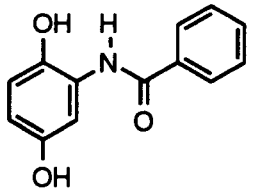
Both enzymes recognized the 3-isomer, **32**, over the 2- and 4-isomers, **31** and **33**, respectively. This preference suggests that the hydroxyl at carbon-4 of DHA makes a stronger hydrogen bond with amino acids in the active sites than the bonding of either the hydroxyl proton of carbon-1 or the nitrogen and carbonyl of the N-acetyl side chain .

When we compare the behavior of the two epoxidases with the 4-isomer, **33**, we get an idea about the flexibility or rigidity of the active sites. In DHAE I, this pocket is extremely rigid since **33** did not inhibit the reaction. On the other hand, the flexibility or looseness of fit in DHAE II can be seen by the certitude that **33** is a competitive inhibitor. In fact, **33** is a better inhibitor than the 2-isomer, **31**.

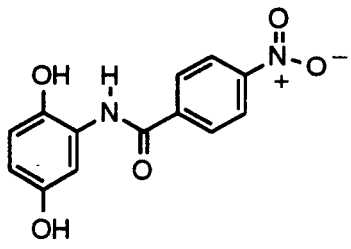
With the kinetic parameters for these inhibitors now established, it is not so surprising that no alternative products were formed when they were assayed as substrates. The assays were not run long enough to compensate for the poor binding of the analogs. Moreover, the analogs were never tried as alternative substrates with DHAE II. Therefore, these analogs need to be tested as alternative substrates with both epoxidases. The assays must be incubated at least 50 times longer than a control experiment which completely converts **4** to **7**. This may require two or three separate additions of enzyme to the same assay since this incubation time is so long (three hours or more) and the epoxidases are not stable for extended times at 30 °C.

Extensions of the N-acetyl side chain

The next pair of inhibitors designed to investigate the active site flexibility's of the two epoxidases were bulkier than **4**. The N-acetyl side chain was replaced with benzoyl, **34**, and *p*-nitrobenzoyl, **35**, side chains. These analogs were potential alternative substrates or inhibitors of the two epoxidase reactions. Their behavior with DHAE I and DHAE II was used to explore the presence of active site pockets for N-acetyl group.



34



35

Figure 17
Lineweaver-Burk plots of N-acetyl chain extension inhibitors with DHAE I and DHAE II. Each plot represents a set of 36 assays. Each inhibitor concentration (100 μM, 50 μM, 10 μM, and control) represents a single least squares linear regression line on the plot. In the plot of DHAE I with **34** only two lines are present since complete inhibition was observed beyond 10 μM inhibitor. This was also observed for DHAE II with **35** at 100 μM inhibitor. (A. DHAE I with **34**, B. DHAE I with **35**, C. DHAE II with **34**, D. DHAE II with **35**)

A

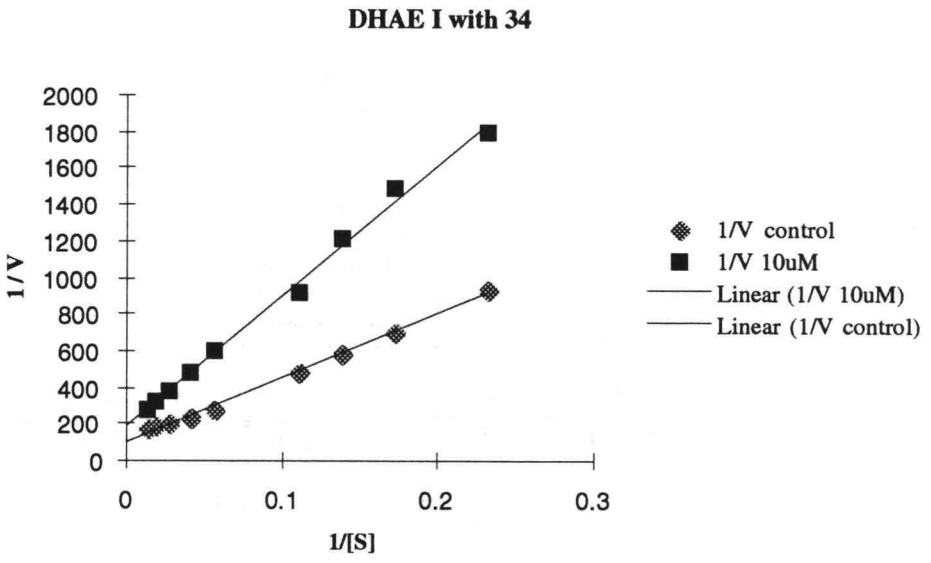
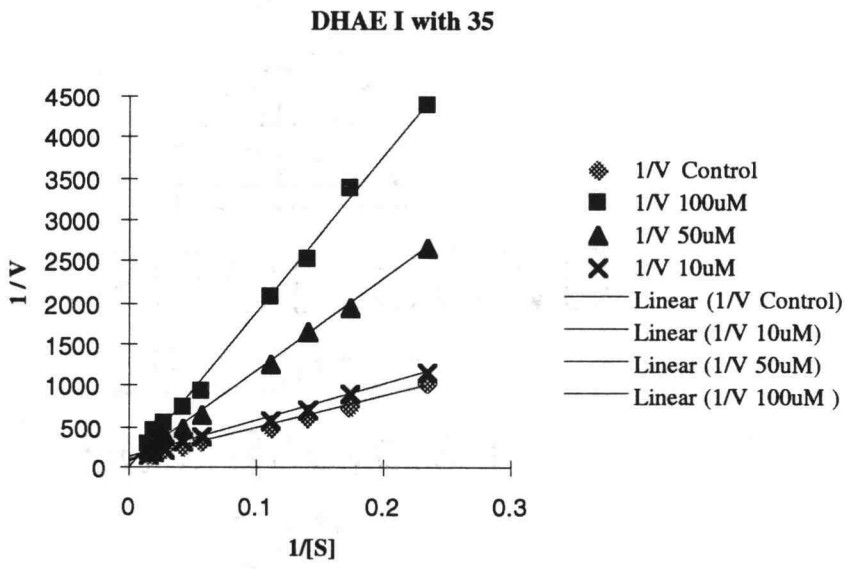


Figure 17 (Continued)

B



C

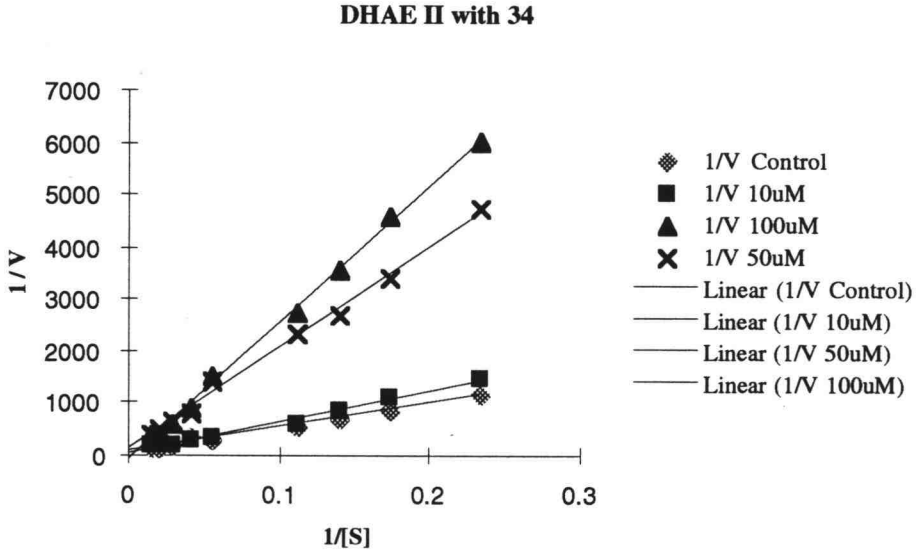


Figure 17 (Continued)

D

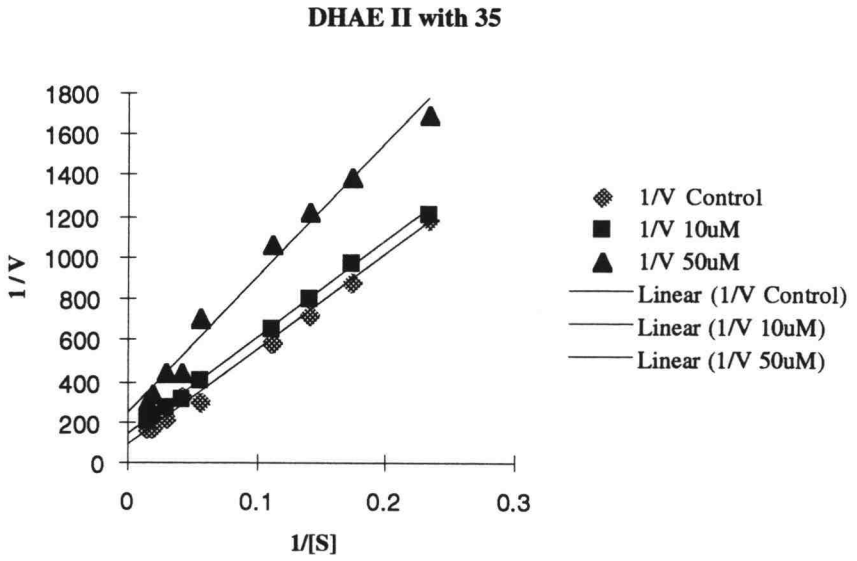


Table 3
Kinetic parameters from the individual sets of reactions. (Vmax = μmoles **7** or **8** produced / minute, Km = μM, Ki = μM)

DHA Epoxidase From *S.* LL-C10037 (DHAE I)

Analog	Type of Inhibition	Vmax	Km	Ki
34	Competitive	0.0080±.0023	24±13	22±10
35	Competitive	0.0092±.0023	35±13	32±2

DHA Epoxidase From *S.* MPP 3051 (DHAE II)

Analog	Type of Inhibition	Vmax	Km	Ki
34	Competitive	0.022±.011	149±47	27±6.4
35	Competitive	0.011±.011	106±47	2±28

First of all, the differences seen in the Vmax and Km values from this table, as compared with Table 2, reflects the use of 10% ethanol in these assays. The ethanol was necessary to keep these bulkier and more hydrophobic compounds in solution. This solvent use affected DHAE II more than it affected DHAE I. For DHAE II, the Vmax was approximately six times greater in the presence of ethanol than without the solvent, whereas, DHAE I was less than 2 times greater with ethanol. These ratios were the same when comparing the increase in their Km's (DHAE II required about 6 times the concentration to reach 1/2 Vmax and DHAE I was just under 2 times as much as the unsolvated assays). The deviation within these values was also higher in these solvated assays (for DHAE I Vmax deviation was ± 0.0023 μmoles **7** produced / minute and Km deviation was ± 13 μM. For DHAE II the Vmax deviation was ± .011 μmoles **8** produced / minute and Km deviation was ± 47 μM).

Secondly, these two compounds were assayed as possible substrates for DHAE I and shown not to be substrates for the enzyme. Again, as with the acetamidophenol analogs, these analogs where never tested with DHAE II. However, during the inhibition studies with DHAE II no epoxyquinone products corresponding to any of the analogs was observed in the HPLC traces. Furthermore, the HPLC peak corresponding to the inhibitors was very constant, supporting the idea that these analogs are not alternative substrates DHAE II.

The analogs were very good inhibitors with both enzymes. The Ki's were approximately equal to the Km for DHAE I and with DHAE II they were even better inhibitors, being 4.5 times less than the Km for **34** and 60 times less for **35**. For both these epoxidases a strong stabilizing interaction for phenyl ring system exists. However, if the analog was able to fit into the active site in the same orientation as the true substrate, **4**, an epoxyquinone product would be expected. Given that neither of these analogs produced an alternative product in these assays, the enlarged side chains are most likely located outside of the active site pocket where they are being stabilized by a strong hydrophobic pi to pi interaction (with either tyrosine, phenylalanine, histidine, or tryptophan).

Experimental

General Conditions

NMR spectra were recorded using either a Bruker 300 MHz or 400 MHz NMR spectrometer. Low-resolution mass spectra were taken on a Varian MAT CH-7 spectrometer. High-resolution mass spectra were taken on a Kratos MS 50 TC spectrometer. Water was purified with a MilliQ System, Millipore Corp. Activity assays were analyzed by HPLC, performed on a Waters 600E HPLC instrument (UK-6 injector) with either a Waters 996 photodiodarray detector and a Dell Pentium computer housing Waters Millennium software or a Linear UVIS 200 detector with an HP 3396A integrator. Reverse phase C_{18} (Econospere, 5 mm, 250 X 4.6 mm, Alltech Assoc.) columns were used for these assays. Bacterial fermentations were carried out in a gyrotary incubator (Lab-Line incubator shaker). Cell disruption was performed with a sonicator (Model W-225R, Heat Systems-Ultrasonic, Inc.). Eppendorf tubes were centrifuged in a Microfuge, (Baxter) refrigerated centrifugations were done in an IEC B-20a centrifuge.

Chemicals

$^{18}O_2$ (95-98%), $H_2{}^{18}O$ (50%), and $H_2{}^{18}O$ (98%) were purchased from Cambridge Isotope Laboratories, Andover, MA. G6PDH and all other chemicals (unless otherwise stated in the text) was purchased from Sigma, St. Louis, MO. DE-52 anion exchange resin was purchased from Whatman (prior to use, the resin was washed with ethanol and rinsed with MilliQ H_2O).

Standard Culture Conditions.

Streptomyces LL-C10037 cells were grown and harvested as earlier reported [2] except that the cells were collected and washed using a sheet of 50 μm nylon mesh.

Streptomyces MPP 3051: Cells were grown using a modification of the culture conditions.[2] Spores were maintained in sterile soil at 4 °C. A loop full of this material was used to inoculate 50 mL of seed medium containing 3% glucose, 1% bacteriological peptone (Oxoid Ltd), 0.2% $KH_2PO_4{\cdot}3H_2O$, 0.1% NaCl prepared in MilliQ water, adjusted to pH 7.0. The seed medium was incubated at 28 °C on a gyrotory shaker for 72 hours (250 mL erlenmeyer flasks closed with foam plugs). This seed medium was used to inoculate (5% by volume) the following production broths.

The production broths, 200 mL in 2 liter erlenmeyer flasks, contained 1% glucose, 1% $NaNO_3$, 0.1% Pharmamedia (cotton seed flour), 0.5% $CaCO_3$ prepared in MilliQ water, adjusted to pH 7.0. Production broths were incubated 28 °C for 24 hours in a gyrotory shaker. Cells were harvested and washed as reported above for *Streptomyces* LL-C10037.

Preparation of Cell-Free Extract

Streptomyces LL-C10037: The CFE was prepared as reported in the isolation chapter pg. 43. The following buffers were used in the purification and DHAE I and AEBQDH I. buffer I: 50 mM potassium phosphate, 10% glycerol, 10 g/liter soluble polyvinyl pyrrolidone-10,000 MW, pH 6.5. buffer II: 50 mM potassium phosphate, 10% glycerol, pH 6.5. buffer III: buffer II with 50 mM KCl. buffer IV: Buffer II with 100 mM KCl. buffer V: buffer II with 200 mM KCl. buffer VI: buffer II with 300 mM KCl.

Streptomyces MPP 3051: The CFE from this organism was prepared as reported for *Streptomyces* LL-C10037 except there was only 30 grams of cells at the start. All the buffer quantities were adjusted (proportionately) to reflect this lower quantity of cells.

DE-52 Anion-Exchange Column For DHAE I and AEBQDH I

Prior to use, DE-52 resin (80 g) was washed sequentially with 0.2 liters of the following: MilliQ H_2O, 1M KCl, and buffer I. The washed resin in buffer I (2:1) was gravity packed in a 10 X 100 cm BioRad Econo column. Freshly prepared cell free extract (600 ml, from 200g of*Streptomyces* LL-C10037) was added to the column. The resin was washed sequentially with 0.125 liters of the following: buffer II, buffer III, and buffer IV. DHAE I was eluted with buffer V (125 ml) followed by elution of AEBQDH I with the addition of buffer VI (125 ml). The two enzymes were stored up to 8 wks, at -80°C, without significant loss of activity.

Determination of water exchangeability in **7**.

In an NMR tube, **7** (25 mg) was dissolved in MeOH-*d4*, and the ^{13}C NMR (300 MHz) spectrum was obtained. 200 ml of $H_2{}^{18}O$ (50%) was added and the ^{13}C NMR (300 MHz) spectrum was obtained every 30 min. for 4 hrs.

^{18}O Exchange of **1** to **1a** and PCC Oxidation of **1a** to **7b**

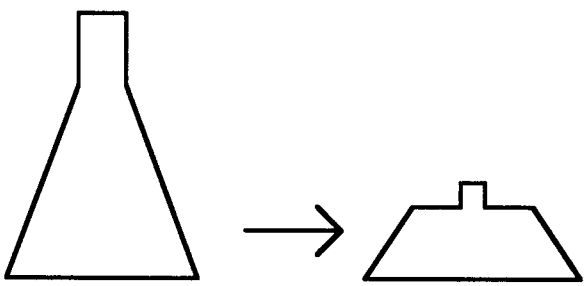
To dry **1** (35 mg, 0.2 mmol), 60 ml $H_2{}^{18}0$ (98%) and 100 ml of MeOH were added. The mixture was stirred for 24 hours (25°C), and then was concentrated *in vacuo* .

The residue was dissolved in dry CH_2Cl_2 (25 ml) and NaOAc (16.0 mg, 0.2 mmole) and PCC (82 mg, 0.37 mmole) were added. The resulting solution was briefly sonicated and allowed to stir for 1.5 hrs (25°C). The rusty brown reaction mixture was passed through a 1" plug of 50:50 Florisil:Celite packed in a 2 ml pasteur pipet. The CH_2Cl_2 fractions containing the product were combined and concentrated *in vacuo* to provide a yellow solid, **7b**, (16.6 mg, 48 % yield, 20% ^{18}O enriched by ^{13}C NMR)

Oxidation of **4** to **7c** with DHAE I.

Because of the high cost of $^{18}O_2$ and the small volume of $^{18}O_2$ actually needed for the labeling experiment, a new flask was designed. An Erlenmeyer flask was modified by removing the top half, generating a new constriction, and refitting with a ground glass 24/40 joint. This modification afforded a flask with less head space while retaining the same amount of surface area (Figure 18).

Figure 18
Diagram of modified Erlenmeyer flask used in $^{18}O_2$ labeling experiments to reduce excess air space.



To this flask DE-52 purified DHAE I (100 ml, activity 1.04 X 10^{-4} µmole **7** produced/min ·µl, 4°C) was diluted 1:4 with O_2-free buffer II (the buffer was sparged with helium at 4°C for 1 hr). A rubber septum was fitted over the flask and a vacuum was generated using a high vacuum pump. The enzyme mixture was allowed to come to 25°C, *in vacuo* (20 min). $^{18}O_2$ (1 l) was added and the flask was placed in a rotory shaker (25 rpm, 30°C). **4** (20 mg) was added as a solution (20 ml of 1 mg/ml O_2 free H_2O) and a 400 µl aliquot was taken for a zero time starting point. In 15 minutes **4** had been completely converted (HPLC analysis) and more **4** (20 mg) was added. The reaction was checked periodically and more **4** was added as needed. In 2.5 hours 140 mg of **4** had been converted to **3c** and the reaction was stopped.

KH_2PO_4 was added to bring the pH to 4.7 and the solution was brought to 50 % $(NH_4)_2SO_4$ saturation. Ethyl acetate was used to extract **7c** (5 X 150 ml). The EtOAc extract was dried over anhydrous $MgSO_4$ and concentrated *in vacuo* affording a burgundy colored oil. 40 % of the crude **7c** was further purified by flash silica gel chromatography (40% EtOAc/hexanes), yielding **7** as a yellow solid (50 mg). The ^{13}C NMR spectrum is seen in Fig. 13; and mass spectrometry of **7c** showed 98% incorporation of one ^{18}O atom.

Conversion of **4** to **1c** with DHAE I / AEBQDH / G6PDH.

The same modified flask used in the conversion of **4** to **7c** was used in this procedure (Figure 18). DE-52 purified AEBQDH (20 ml, 2.06 X 10^{-5} µmol **1** produced/min · µl), DE-52 purified DHAE I (50 ml, 1.04 X 10^{-4} µmole **7** produced/min ·µl,), and O_2-free buffer II (300 ml) were placed in the flask (4°C). The flask was brought to 25°C *in vacuo* and $^{18}O_2$ (400 ml) was added. G6PDH (23.96 units in buffer II), G-6-P (145.72 mg), and $NADP^+$ (91.7 mg) were added and the flask was placed in a shaker (25 rpm, 30°C). After five minutes, **4** was added to start the reaction. Immediately after addition of **4**, a zero time aliquot (400 µl) was removed. The reaction was monitored by HPLC and more G-6-P, $NADP^+$, and **4** were added as needed. After four hours, 30 mg of **4** had been converted to **1c** and the reaction was stopped. Work-up yielded 24 mg of **1c**. The ^{13}C NMR spectrum is seen in Fig. 14; mass spectrometry showed 84.3% incorporation of one ^{18}O and 14.5% incorporation of 2 ^{18}O atoms.

Chemical Oxidation of DHA

In a flame-dried round bottom flask (100 mL) containing argon, 2,5-dihydroxyacetanilide, **4**, (0.83 mg, 4.97 mmole) and newly distilled THF (50 mL) were added. Under argon, this was stirred using a magnetic stir bar until **4** was completely dissolved. 18-crown-6 was added (1.5 g, 6 mmole) and the contents turned golden tan. Potassium *tert*-butoxide (10 mL of a 1 M solution in THF, 10 mmole, Aldrich) was added dropwise (under argon). A black precipitate formed with each drop but would slowly disappear as the mixture was stirred. Upon complete addition of the alkoxide the reaction was dark olive green. This was allowed to stir (1 h), whereupon a steady stream of O_2 was bubbled into the mixture using a long needle connected to an O_2 tank. The O_2 was bubbled into the reaction for 1.5 hours. The reaction was quenched and worked-up by adding saturated ammonium chloride (100 mL) and extracting with EtOAc / THF (50 / 50) 3 X 50 mL. The extracts were dried over anhydrous Mg_2SO_4 and the product was collected *in vacuo*.

A small portion of the crude product was dissolved in H_2O and analyzed by HPLC (Waters 600E HPLC instrument with Waters 996 photodiodarray detector and a Dell Pentium computer housing Waters Millennium software. Reverse phase C_{18} (Econospere, 5 mm, 250 X 4.6 mm, Alltech Assoc.) column 15 % aqueous-acetonitrile). The epoxyquinone peak was easily detectable from its retention time (6.7 minutes) and its distinctive U.V. spectrum. However, there were many other products. The yield was estimated to be less than 5 % based on a U.V. standard curve for **7**.

Analogs 31-35 as Substrates

The analogs were individually added to active preparations of DHAE I. The assays were allowed to incubate 10 times (30 min) as long as it took for the a same amount of protein to convert the actual substrate, **4**, into products **7** or **8** (3 min).

Kinetic Assays for Type of Inhibition of Analogs 31-34

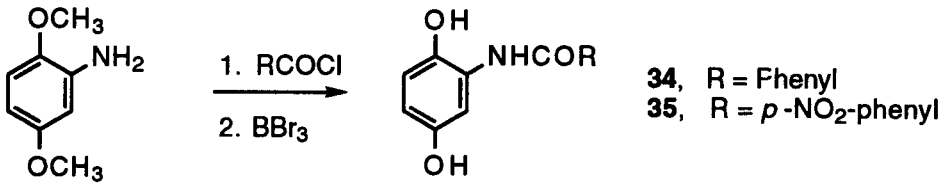
For each inhibitor and enzyme, the assays (36 total) were run on the same day and as close together in time as possible. The same two enzyme preparations (one for DHAE I and one for DHAE II) were used for all the studies. DHAE I was recovered from a C-5 HIC column (DE-52, Cu+2 IMAC, C-5 HIC). The specific activity was 1.93×10^{-4} mmole **7** produced / min · mg protein. DHAE II was recovered from a DE-52 column. The buffer was exchanged to remove the ammonium sulfate. The specific activity was 2×10^{-5} mmole **8** produced / minute · mg protein. Below is the actual table used for preparing the 2-acetamidophenol assays. For each analog with each enzyme, a similar table was constructed. For **34** and **35**, 10% ethanol was included in the assays. Since the analogs were dissolved in ethanol, varying amounts of ethanol were added to each assay to keep the total consistent.

	Control	**100 μM**	**50 μM**	**10 μM**
0.1 mM 2-acetamidophenol	0.0 μL	154.4 μL	77.21 μL	15.44 μL
MilliQ H_2O	200 μL	45.6 μL	122.79 μL	184.56 μL
1 M KH_2PO_4, pH 6.5	100 μL	100 μL	100 μL	100 μL
MilliQ H_2O	600 μL	600 μL	600 μL	600 μL
Enzyme	100 μL	100 μL	100 μL	100 μL
2.9 mM substrate	X	X	X	X
MilliQ H_2O	Y	Y	Y	Y
Terminating Solution	300 μL	300 μL	300 μL	300 μL

Substrate concentrations	73.6 μM	54.5 μM	36.8 μM	25.0 μM	18.4 μM
X =	50 μL	37 μL	25 μL	17 μL	12.5 μL
Y =	150 μL	163 μL	175 μL	183 μL	187.5 μL

Substrate concentrations	9.2 μM	7.36 μM	5.89 μM	4.42 μM
X =	6.25 μL	5 μL	4 μL	3 μL
Y =	193.75 μL	195 μL	196 μL	197 μL

Synthesis of Analogs



34, R = Phenyl
35, R = *p*-NO_2-phenyl

2,5-dihydroxybenzanilide, **34**

2,5-dimethoxyaniline (1.5 g, 9.7 mmol) was dissolved in dry CH_2Cl_2 (30 mL) in a dried 100-mL round-bottomed flask which was then flushed with Ar and cooled in an ice bath to 0 °C. While this mixture was being stirred, newly distilled Et_3N (2.7 mL, 20 mmol) was added, followed by the dropwise addition of benzoyl chloride (2.32 mL, 20 mmol). After 1 h, the stirred reaction was warmed to room temperature and stirred for an additional 2 h. The reaction mixture was quenched with water (20 mL) and the CH_2Cl_2 was washed two more times with 50% NaCl saturated water (20 mL each) to remove the triethylammonium chloride salts.

The organic layer was dried over Na_2SO_4 and concentrated *in vacuo* to give nearly pure 2,5-dimethoxybenzanilide as a greyish white solid (1.79g, 72% yield, m.p. 81-84 °C). 2,5-dimethoxybenzanilide (1.79 g, 6.9 mmol) was placed in a 100 mL round-bottomed and dried under high vacuum (24 h) to remove any residual water prior to the next deprotection step. The round-bottomed flask containing the methylated compound was removed from high vacuum and fitted with a septum. The vessel was flushed with Ar and dry CH_2Cl_2 (60 mL) was added. With stirring, the reaction contents were cooled in a dry ice / acetone bath (-78 °C) and 1M BBr_3 in CH_2Cl_2 (20 mL, 20 mmol) was added dropwise (smoke emitted with each drop and the reaction turned yellow). Once all the BBr_3 was added the stirred reaction was slowly brought to room temperature where it was allowed to continue stirring for an additional 20 h. The milky orange reaction mixture was quenched by pouring over an ice bath (100 mL) which contained sodium dithionite (2 g) and was previously sparged with He. **51** was then extracted with sodium dithionite saturated ethyl acetate. The ethyl acetate extract was dried over $MgSO_4$ and **34** was recovered *in vacuo*. The greyish-cream colored solid was dissolved in EtOAc while leaving the sodium dithionite as a solid. Decanting the solution from the solid reducing agent followed by concentration *in vacuo* gave **34** as small white crystals (1.42 g, 89% yield): m.p. 184-192 °C; ^1H NMR (acetone-d6, 300 MHz) δ 9.3 (2H, bs), δ 8.4 (1H, s), δ 8.0 (1H, dd), δ 7.5 (4H, m), δ 7.3 (1H, d), δ 6.8 (1H, d), δ 6.5 (1H, dd); ^{13}C NMR (acetone-d6, 300 MHz) δ 167.8, 151.9, 142.0, 135.1, 132.4, 129.4, 129.4, 128.1, 128.1, 127.8, 117.3, 112.6, 109.3; high resolution MS. calc. 229.0739 found 229.0738.

2,5-dihydroxy-*p*-nitrobenzanilide, **35**

35 was synthesized using the same procedures as described for the preparation of **34** except for the following difference. **35** was recrystallized from MeOH / EtOAc to give yellow-orange crystals (96% yield): m.p. 222 °C sublimed; ^1H NMR (acetone-d6, 300 MHz) δ 9.4 (2H, bs), δ 9.1 (1H, s), δ 8.3 (1H, dd), δ 8.2 (1H, dd), δ 7.5 (1H, d), δ 6.8 (1H, d), δ 6.6 (1H, dd); ^{13}C NMR (acetone-d6, 300 MHz) δ 164.5, 151.2, 150.5, 141.5, 140.8, 129.62, 129.62, 127.3, 124.3, 124.3, 117.9, 112.9, 109.6; high resolution MS. calc. 2274.0590 found 274.0591.

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Chapter 4

Conclusion

Marc J. Kirchmeier

What has this Work Accomplished?

DHAE I has been purified to homogeneity. The N-terminal and parts of its internal amino acid sequences have been obtained. Obtaining this sequence information was essential for beginning the cloning of the epoxidase gene.

DHAE I was suspected of belonging to the monooxygenase family of iron and copper metalloenzymes. However, Gould and Shen found some unusual features which distinguished DHAE I from this class of enzymes.[1,2] We have now demonstrated that DHAE I is an intramolecular dioxygenase, consistent with the dihydrovitamin K epoxidase. The fact that the same type of chemistry is observed in the production phyllostine, **13b**, LL-C10037α, **1**, MM14201, **2**, and vitamin K oxide, **10**, (and most likely the nanaomycins and terreic acid), tempts one to speculate that this is a general mechanism in the formation of other epoxyquinones as well. Since numerous epoxyquinones and epoxyquinols are known in nature, we believe these epoxidases represent a previously unrecognized family of "hydroquinone dioxygenase (epoxidizing)" enzymes.
With the experimental procedures for dealing with carbonyl oxygen exchange and the identification of carbonyl ^{13}C NMR resonances worked out, this research has laid the foundation for the investigation of other hydroquinone epoxidizing enzymes found in natural product biosynthesis.

Finally, assay conditions were established which allow the behavior of DHAE I and DHAE II toward various substrate analogs to be assessed. Studies with simple substrate analogs have defined some important features of the active sites. The carbon 4-hydroxyl of DHA has been identified as having the strongest interaction with the active sites of both DHAE I and DHAE II. It was also demonstrated that both active sites behave in a similar manner toward analogs with aromatic rings substituted for the acetyl side chain. It was not yet clear however, whether the stabilizing interaction occurs as a result of the analog binding with the aromatic substituent positioned in the same hydrophobic space which would normally be occupied by the acetyl side chain or whether the aromatic ring is aligned in an alternative manner.

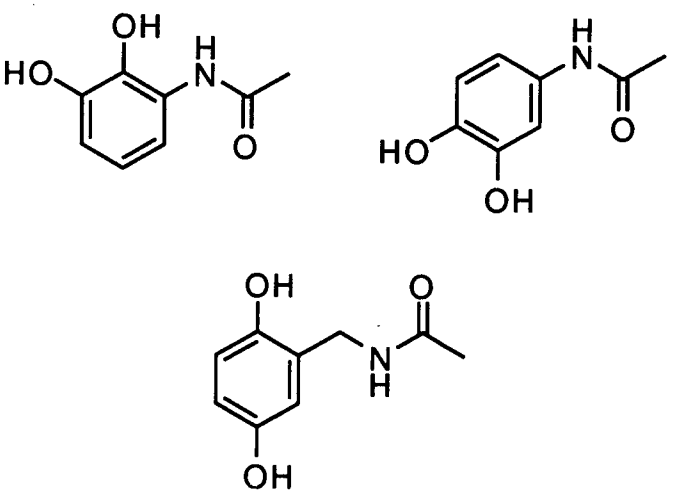
What are the Areas of Future Study?

An area which needs addressing, is the location of the active site base of both DHAE I and II. This regiospecificity could be addressed by revisiting the acetamidophenol analogs.

In the preliminary studies, the three analogs were also assayed as alternative substrates with DHAE I by individually adding each analog to an active preparation DHAE I (DHAE II was never tested for this ability). However, these experiments were not run long enough to reflect the poor binding of the analogs. Therefore, these analogs (at least **31** and **32**) need to be re-tested as alternative substrates with both epoxidases, and the assays must be incubated at least 50 times longer than a control experiment which completely converts **4** to **7**. This may require two or three separate additions of enzyme to the same assay since this incubation time is so long (three hours or more) and the epoxidases are not stable for extended periods of time at 30 °C. If a product was observed with either the 2- or 3-acetamidophenol,(**31** and **32**, respectively) this would be a strong indication that the base was activating at that site (See Scheme 30, pg. 84).

Now that reliable conditions for studying the kinetic behavior of theses epoxidases with analogs have been established, there are many analogs which should be examined. For example, 4-hydroxyphenol (1,4-benzhydroquinone) would be expected to be a very good inhibitor based on what was learned from the behavior of both DHAE I and DHAE II with **31** and **32**. Figure 19 shows three analogs which are currently available in the laboratory. The top two analogs of Figure 19 were designed as chelation inhibitors for probing the possibility and location of active site metals.[3] The results of subsequent analog studies can be used as a guide for the construction of more elaborate analogs. All of these studies can help us gain a more clear model of the active sites of these epoxidases.

Figure 19
Substrate analogs which are currently available in the laboratory.



Suicide inhibitors may be synthesized with radioactive tracers and used to help identify active site amino acids and the overall location of the active site within the peptide chain. A protocol for digesting the purified epoxidases has been established and an HPLC chromatogram of the separated trypsin-digested DHAE I fragments is available (Figure 9, page 38) to assist in this type of experiment.

In addition to determining the location of any active site base(s), amino acid inhibitors can be utilized to identify the specific amino acid(s).[4,5,6] Sulfhydryl directing (cysteine) and histidine directing agents would be the best place to start for DHAE I and II since these side chains are the best candidates for the active site base. To distinguish whether inactivation by the inhibitor was associated with an active site residue, the assay would be carried out both in the presence of and absence of the substrate. If the substrate was able to block the inhibition, this would be a strong indication that the inhibitor was acting on an active site residue.

One of the intriguing questions which can be answered once the DHAE I gene is cloned and overexpressed is the metal needs (or non-metal needs) of the active enzyme. Is there a necessary metal? And if so, is it catalytically active or is it a metal required to maintain structural integrity?

Recently, dihydrovitamin K dependent γ-glutamyl carboxylase has been purified from bovine liver microsomes[7] and both the human and the bovine cDNAs have been cloned.[8] The enzyme is a single polypeptide of molecular weight 94,000. The C-terminal portion of the carboxylase shares a 19% sequence homology over 198 amino acids with a soybean lipoxygenase that converts polyunsaturated fatty acids to the corresponding monoperoxy fatty acids in the presence of O_2. The lipoxygenase utilizes an essential non-heme Fe^{+2} to carry out the activation of molecular oxygen. The implication that the lipoxygenase and carboxylase share a common mechanism for O_2 activation based on the observed sequence homology remains to be seen and it will be interesting to see if the carboxylase does in fact contain Fe^{+2} as a redox-active transition metal.

DHAE I is either a pentamer or hexamer of homogeneous 21kd subunits and is soluble. Clearly, DHAE I and dihydrovitamin K epoxidase are very different proteins with very conserved chemistry. Moreover, no DNA sequence homology was found between either the lipoxygenase or the vitamin K epoxidase and the 300 bp PCR product which was amplified from our DHAE I based oligomers.[9] However as the genetics of DHAE I progresses, the entire DNA sequence responsible for DHAE I will become available and regions of homology will most likely be identified.

The DHAE I apoenzyme, prepared from 1,10-phenanthroline chelation, was only reactivated by the addition of Ni^{+2}, Co^{+2}, and Mn^{+2}. If any or all of these metals is actually responsible for the activation of molecular oxygen, this epoxidase will serve as a paradigm for the study of this dioxygen activation by these metals. The activation of molecular oxygen plays such an essential role in nature that further study of this epoxidase mechanism should be carried out.

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