Protein Chemistry, Peptide Mapping, and Preliminary Structural Characterization of *Saccharomyces cerevisiae* Sterol C-24 Methyltransferase Expressed in *Escherichia coli*

By

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A THESIS

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ABSTRACT

At this time, no 3-dimensional structure of the Sterol C-24 Methyltransferase (SMT) enzyme has been discovered. Having such a representation of this enzyme, especially with a bound inactivator, will illustrate what contact amino acids and motifs are necessary for catalysis of a methyl transfer from S-adenosyl- L-methionine (AdoMet) to the C-24 position on the preferred substrate of zymosterol in the ergosterol biosynthetic pathway.

In order to achieve this goal, protein chemistry techniques of Sodium Dodecyl Sulfate - Polyacrylamide Gel Electrophoresis (SDS-PAGE), Q-sepharose anion-exchange, and 26/60 Sephacryl^{TM} S-300 gel permeation chromatography were used to generate pure SMT for structure determination by our colleague Dr. David W. Christianson at the University of Pennsylvania. Quantification methods involving Bradford protein assay, **U**ltra**v**iolet (UV) absorbance at 280 nm, activity assays with [*methyl* - $^{3}H_3$] AdoMet, and Western blot analysis were developed to track the amount of total protein and active SMT from the yeast Wild Type (WT) and Y81W mutant throughout the purification process. Peptide mapping, using the mechanism-based inactivator [3-^{3}H]26,27 dehydrozymosterol (DHZ), was also developed to independently locate motifs in the primary sequence associated with the active center.

The main experimental findings are as follows: (1) From 5 g of *Escherichia coli* (*E. coli)* BL21 (DE3) host cell pellet harboring the pET23a(+) plasmid with the Y81W mutation was obtained 5 mg Fast Protein Liquid Chromatography (FPLC) pure recombinant Y81W mutant SMT-DHZ-AdoMet complex. (2) From a partial tryptic

digest of a SMT-DHZ-AdoMet complex was obtained 9.1 mg of DHZ bound peptide. The identity of the peptide-DHZ complex was not resolved. (3) Using a sample of FPLC pure Y81W mutant SMT complexed with DHZ was attempted X-ray diffraction. Poor resolution crystals were obtained and did not diffract well. The results are interpreted to imply the approaches developed herein to purify SMT can be applied to further structural determination.

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LIST OF ABBREVIATIONS

A_{280}	Absorbance at 280 nm
ACN	Acetonitrile
AdoHcy	S-Adenosyl-L-homocysteine
AdoMet	S-Adenosyl-L-methionine
AMBIC	Ammonium bicarbonate
BSA	Bovine Serum Albumin
Da	Daltons
ddH_2O	Double distilled water
DHZ	26, 27 Dehydrozymosterol
FPLC	Fast Protein Liquid Chromatography
GC/MS	Gas Chromatography/Mass Spectroscopy
GC	Gas Chromatography
H_2O_2	Hydrogen Peroxide
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	High Performance Liquid Chromatography
HPLC-RC	High Performance Liquid Chromatography – Radioactivity Count
IPTG	Isopropyl-1-β-D-Thiogalactoside
kDa	kiloDalton
$KHPO_4$	Potassium Phosphate
LB	Luria Bertani
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization – Time of Flight
N_2	Nitrogen
OD	Optical Density
RP-HPLC	Reverse Phase – High Performance Liquid Chromatography
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
TEMED	N,N,N',N'-Tetra-methyl-ethylenediamine
Tris	Tris [Hydroxymethyl] aminomethane

TFA	Trifluoroacetic Acid
UV	Ultraviolet
Vis	Visible
Y81W	Yeast mutant with Tyrosine replaced by Tryptophan at residue 81
SMT	Sterol Methyltransferase

CHAPTER I
INTRODUCTION

1.1 GENERAL OVERVIEW OF STEROLS

Sterol production in all organisms is necessary for the continuation of life. Sterol characteristics and the roles they play in cells of various species are highly researched. Recognizing how sterols vary across different species and how they operate in the cell allows researchers to discover new means to target harmful microbes, understand how genetic defects in these biosynthetic pathways can harm the human body, and how foreign sterols could provide health benefits.

Sterols are a major component in the cell membrane that aid in the fluidity and structure of the phospholipid bilayer. In the human body, cholesterol is the only sterol that is inserted across the phospholipid bilayer of the cell. This sterol differs in fungi (ergosterol), plants (sitosterol), and in prokaryotes where a pentacyclic molecule, hopanoid, serves as the membrane insert. Research into how sterols achieve this function and how they are synthesized have produced numerous antifungal agents that target sterols. Polyene drugs actually react to the ergosterol inserted into the membrane and form channels that create unbalance permeability selectivity for Mg^{2+} and K^{+}, which can be toxic for the fungal cell. Other drugs such as allylamine and azoles target enzymes along the path of sterol biosynthesis. Sterols also play a role as a "sparking" or signal molecule within the cell that promotes certain functions and operations the cell needs in its life cycle. Unbalanced cholesterol biosynthesis gives rise to abnormal amounts of cholesterol that can cause either, in high amounts, premature heart disease or, in low production, birth defects such as in the Smith-Lemli-Opitz syndrome[1]. Similar

deleterious effects have been observed in insects that contained an unusable sterol, in fungi with a hindered ergosterol pathway, or in plants with genetic engineering to damage phytosterol synthesis.[2] A recent, beneficial discovery has linked the introduction of phytosterols (sterols produced in plants) into a person's diet can decrease levels of plasma and LDL-cholesterol.[3] Sterols are known as isoprenoid derivatives that derive from a five-carbon compound called isoprene. These derivatives also include vitamins D and A_1, steroid hormones, and carotenoid pigments.[4] As mentioned above, prokaryotes form the pentacyclic hopaniod for their cell membrane insert. In this pathway, the starting substrate is squalene, also in the eukaryotic pathway, coils left-handedly to form (+)-tetrahymanol in the absence of oxygen or anaerobically.[2] This product can then be directly introduced to the prokaryotic cell membrane. On the other hand, eukaryotes proceed from squalene, aerobically, to squalene-2,3 (S)-epoxide through oxidation and left-handed cyclization (Figure 1.11). For this reason, hopaniods are believed to be the sterol precursors because the world was void of oxygen during the earliest stages of development of life.[5]

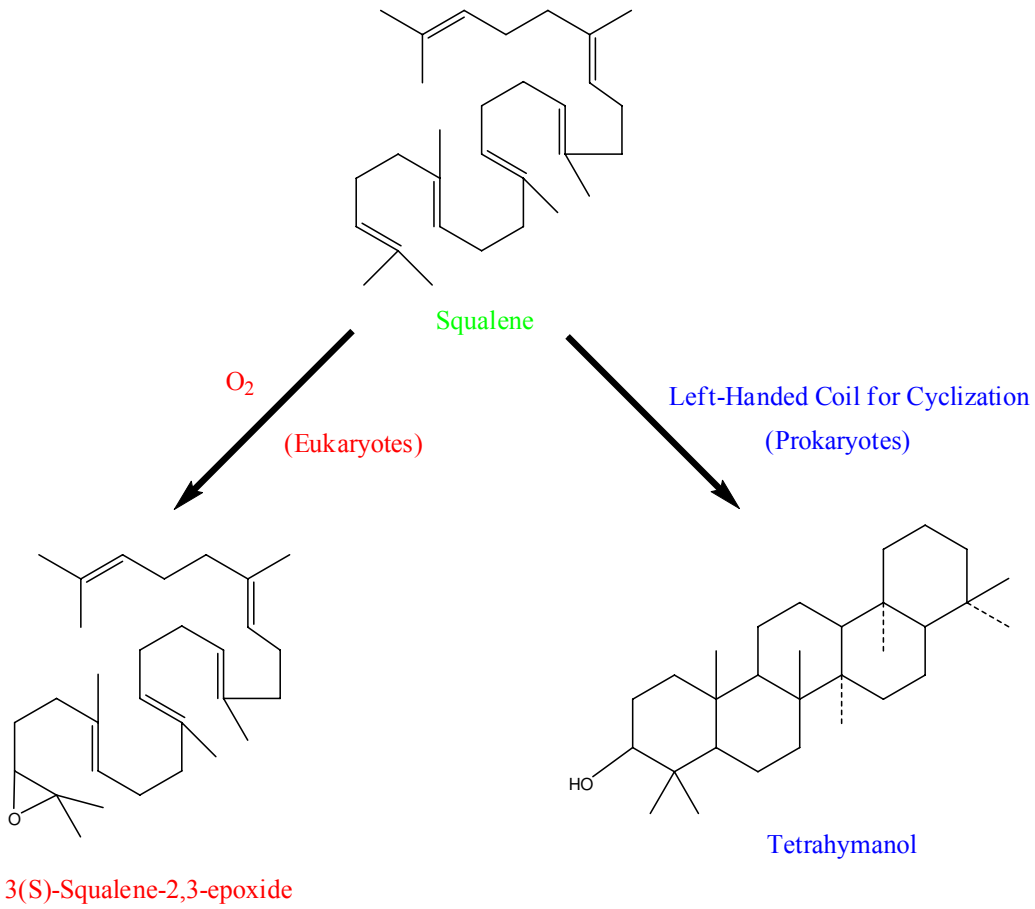


Figure 1.1.1. Evolutionary split between prokaryotes and eukaryotes in the biological pathway for their respective membrane sterols. Tetrahymanol (right) can be directly inserted into the prokaryotic phospholipid bilayer. 3(S)-Squalene-2,3-epoxide is forced to undergo further modifications according to the species' sterol production.

There are four distinct regions on the final sterol product that make it unique and complementary for membrane insertion: i. Equatorial 3-hydroxyl group, ii. Planar tetracyclic ring system also known as the nucleus, iii. Right-handed C-20 R configuration, iv. C8- or C10-side chain (Figure 1.12). These four domains possess strategically positioned chiral carbons that contribute to the polarity and shape of the sterol, producing an alternating all trans-anti stereochemistry of the ring system. The equatorial 3-hydroxyl group acts as the polar head group, and the side chain functions as a non-polar tail. The nucleus undergoes various changes as the sterol is modified from 4, 4 dimethyl to 4-desmethyl. This change can even alter another domain by changing the tilt of the 3-hydroxyl group. Throughout the metabolism of sterols, double bonds are introduced, removed, and/or shifted to achieve the final product that can be inserted into a membrane. These modifications generate an amphipathic molecule that is essentially planar and has a length equivalent to the span of a phospholipid bilayer (Figure 1.13). All of these specific features that make sterol insertion into membranes ideal result in a specific stereochemistry of the molecule. In theory, the fungal membrane insert ergosterol has 256 stereoisomers, while the plant insert sitosterol has 512 possibilities of stereochemistry. However, in vivo, only two stereoisomers are produced for each respective membrane sterol. The diastereoisomeric pairs detected are ergosterol versus 24-epiergosterol in fungi and sitosterol versus clionasterol.[6]

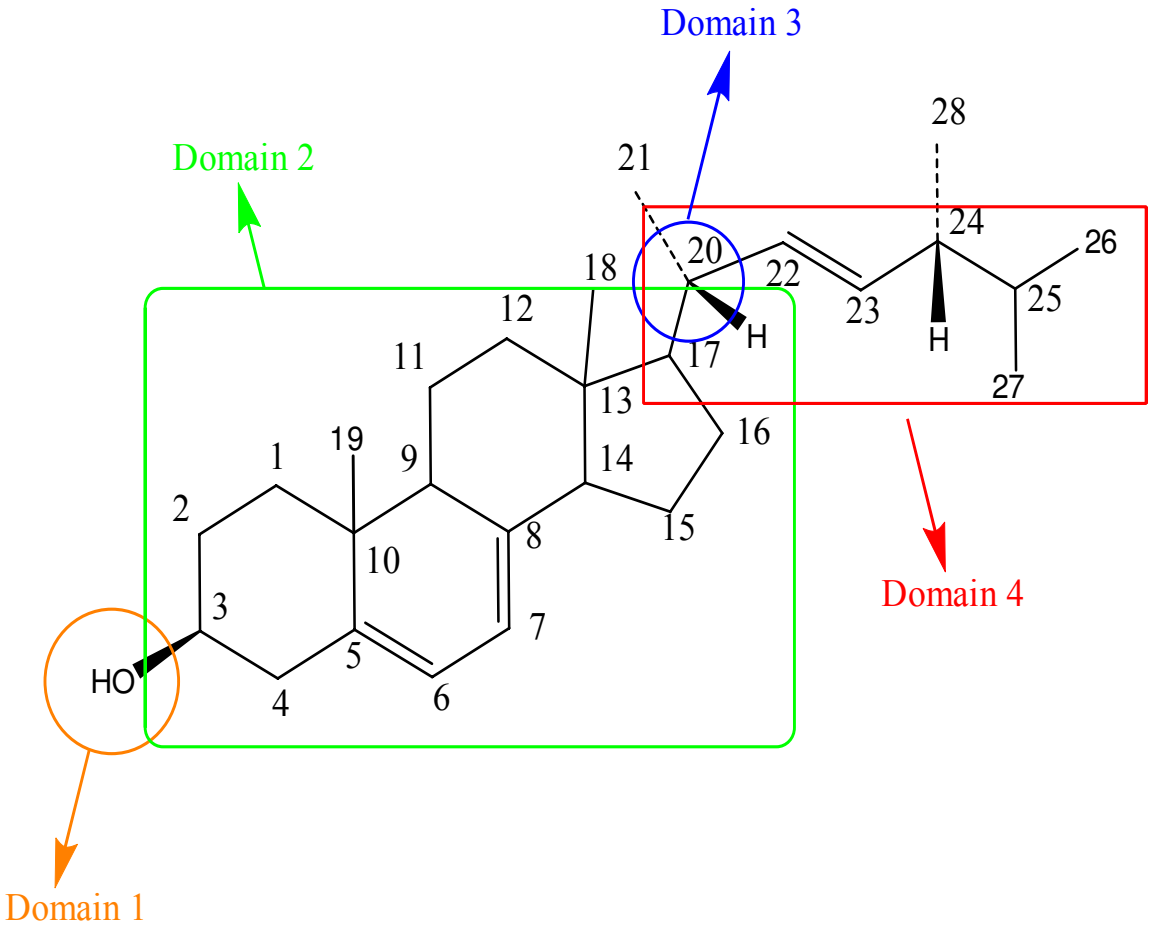


Figure 1.1.2. Diagram of the four regions of importance for membrane insertion, as illustrated on ergosterol (fungal sterol). Domain one is the 3β-Hydroxyl group; Domain 2 is the planar tetracyclic ring system also known as the nucleus; Domain 3 is the right-handed C-20 R configuration; Domain 4 is the C8- or C10-side chain.

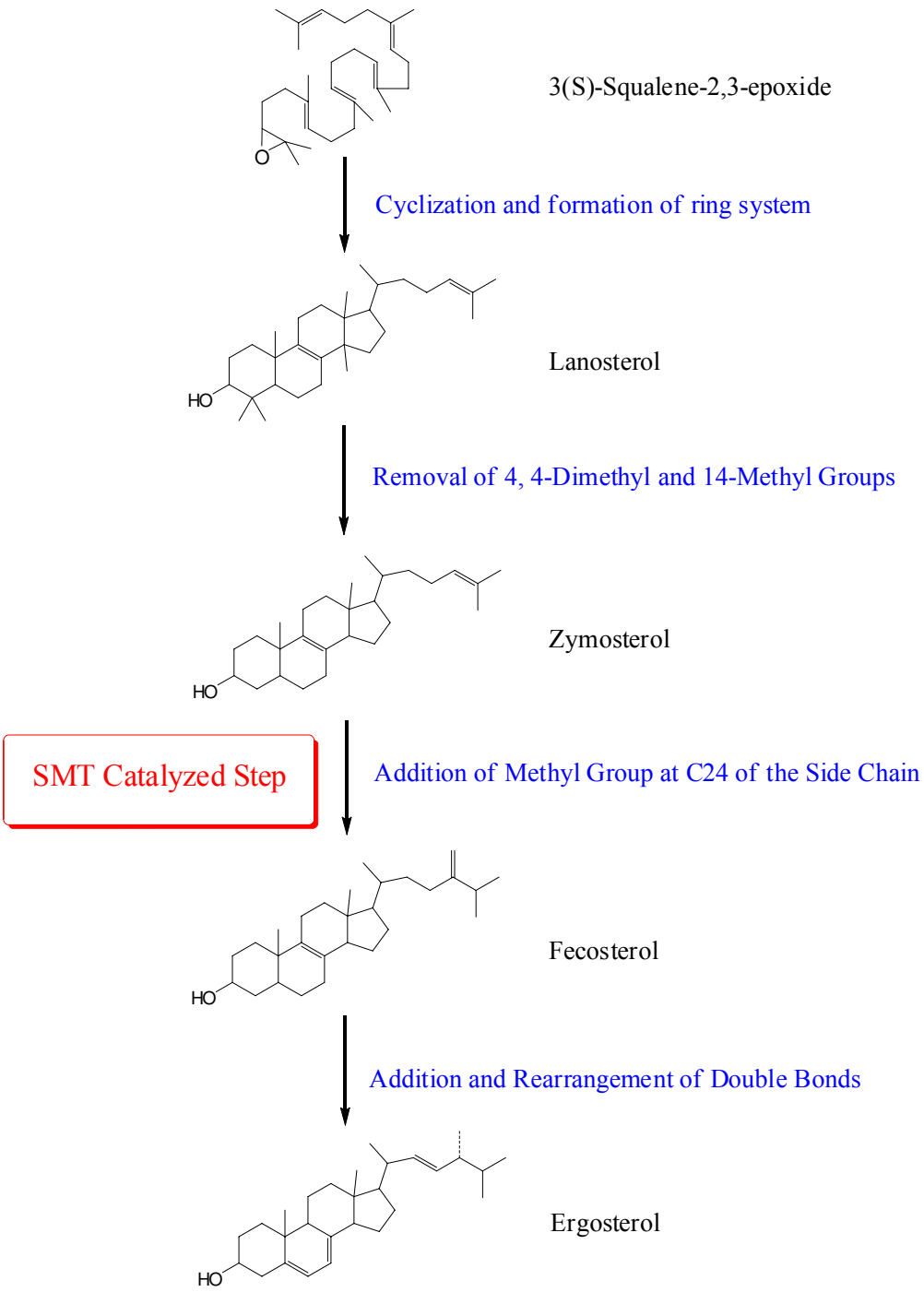


Figure 1.1.3. Ergosterol biosynthetic pathway in fungi showing all the necessary steps for the production of a membrane inserted sterol. All of these steps create stereochemical properties ideal for membrane insertion.

1.2 STEROL METHYLTRANSFERASE ENZYME

Another transition in sterol production is the alkylation step that occurs in phytosterols and fungal sterols at the C24 in the fourth mentioned region of the sterol, the side chain. In fungi, the final product ergosterol has the addition of a 24β-methyl group while, in plants, the membrane-inserted phytosterol has a 24β-ethyl group attached to the C24 position. Since this alkylation is not found in either prokaryotes or eukaryotes, it is suggested that this evolutionary modification occurred in eukaryotes after the transformation of cholesterol from hopaniods[7]. However, some researchers have reason to believe that the SMT enzyme evolved from prokaryotes since blue-green algae are shown to produce small amounts of unique phytosterols. One conclusion that is not in debate is the importance of this alkylation step to the role that fungal and phytosterols play as membrane inserts. Examining the mechanism of the sterol methyltransferase enzyme that performs this step can lead to a better understanding of stereochemical benefits that it might bestow on the sterol. In yeast, the sterol methyltransferase (SMT) enzyme is often given the designation of *Erg*6p in correspondence to the gene nomenclature (*ERG*6) The SMT enzyme (accession number NP_013706) produced by the yeast species *S.* cerevisiae natively acts on the zymosterol intermediate in the ergosterol pathway. SMT catalyzes the transfer of a methyl group, donated by S-Adenosyl-L-Methionine (AdoMet), to the C-24 of the side chain of zymosterol creating the monol fecosterol and byproduct S-adenosyl-homocysteine (Figure 1.21, 1.22). Fecosterol is further modified into the final product ergosterol. This C-methylation reaction is a significant slow step in the phytosterol pathway[8]. This assessment suggests SMT enzymes to be under regulation by substrates such as ATP and/or differential

protein trafficking[8, 9]. The enzymology of SMT and the reaction pathways leading to the formation of C-24 alkyl sterols have been comprehensively diagramed.[8] Information gathered from the purification and characterization of numerous SMTs from species ranging from plants and fungi to protozoa show them to possess similar properties[9, 10, 11, 12]. These features include a p*I* value that ranges from 5 to 8, a pH optimum within one unit of 7, a turnover number of approximately 0.01 s^{-1}, and a native molecular mass between 160 and 172 kDa (all as tetramers). Even though these similarities exist across SMTs from various species, differences such as how the SMT interacts with substrates are present. For example, yeast SMT produces a single product by concerted action, while all other SMTs that have been studied can synthesize products by a step-wise mechanism by means of the production of cations at C-24 and C-25[13]. Also, the kinetic mechanism of the yeast SMT enzyme functions in a random bi bi method where either the sterol or AdoMet can bind to the enzyme in any order and without the presence of the other. In contrast, a plant SMT enzyme from soybean operates in an ordered mechanism with AdoMet binding first.[9]

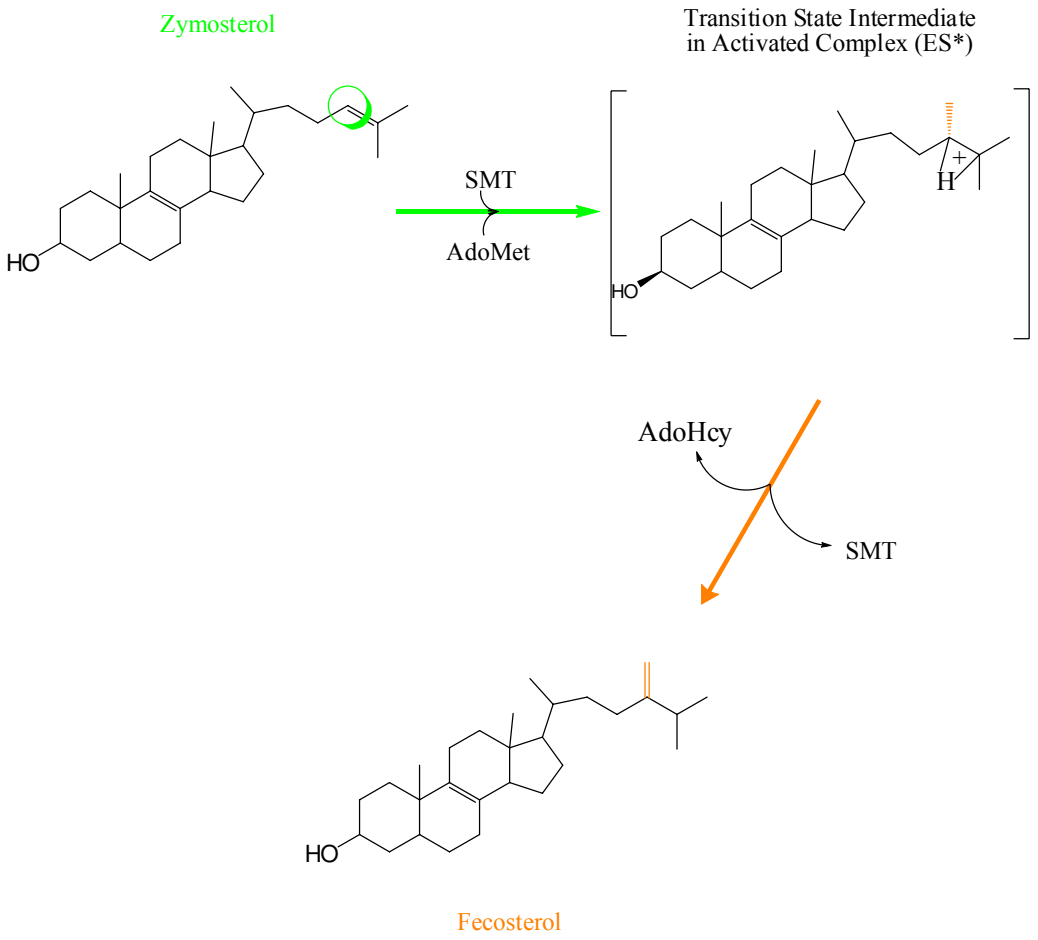
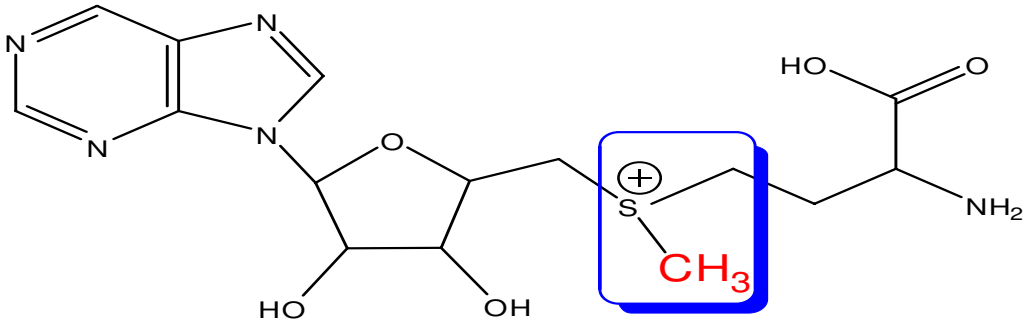
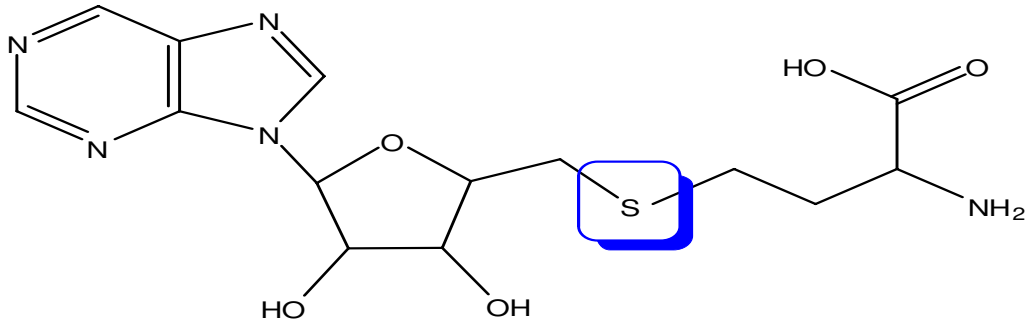


Figure 1.2.1. Sterol Methyltransferase catalyzed reaction with zymosterol and (S)-adenosyl-L-methionine (methyl donor) to form the intermediate fecosterol and byproduct (S)-adenosyl-homocysteine. C24 is the position where the methyl group is added and is highlighted in green on zymosterol. The methyl group added at the C24 position is highlighted in orange on the activated complex (ES*) and fecosterol.



(S)-adenosyl-L-methionine



(S)-adenosyl-homocysteine

Figure 1.2.2. Molecular structures of methyl donor (S)-adenosyl-L-methionine and the byproduct of the SMT catalyzed reaction, (S)-adenosyl-homocysteine. The methyl group donated is highlighted in red on (S)-adenosyl-L-methionine.

1.3 STERIC-ELECTRIC PLUG (SEP) MODEL

The mode of the SMT interaction with its sterol substrate was proposed by Dr. WD Nes to be the steric-electric plug model[14]. This model actually incorporates all of the key, structural features of sterols in its mechanism of binding to the SMT enzyme. This conclusion was made after studies systematically varied the structure of the sterol by one feature at a time and assaying with the SMT enzyme. These studies discovered that with a *S. cerevisiae* SMT, productive binding required the sterol substrate to have a 3β-hydroxyl group, a planar nucleus, an intact side chain with the length of the native zymosterol, a 20-R-configuration, and a Δ^{24}- double bond[6] (Figure 1.31). These features are also important in becoming architectural components of membranes, mentioned before in this chapter. Work performed by Arigoni on the stereochemical course of the reaction advocated that the methyl transfer from SMT is a direct biomolecular transfer through a S_n2-like transition state led to the formation of steric-electric plug model for SMT catalysis[8, 15]. This deduction was also backed up by the knowledge of the substrate conformation to explain the stereochemistry of the C-methylation reaction[16, 17]. Outlined in the steric-electric plug model, C-methylation of the native substrate zymosterol by the yeast SMT is a bisubstrate reaction that progresses in a sequential and non-covalent nature. This reaction occurs with a 'β-face' methyl addition to the Δ^{24} –bond and deprotonation of C-28 that proceed to give rise to a nucleophilic rearrangement where H-24 migrates to C-25 on the *re*-face of the substrate double bond in combination with the initial ionization to afford the bound fecosterol. Also according to the SEP model, a catalytic cycle is formed whereby C-methylation of the Δ^{24} –sterol substrate gives rise to the parent cations of the various key structural types (Figure 1.32). In yeast, termination

of these ionic reactions by elimination of a proton generates the corresponding olefin of $\Delta^{24(28)}$ fecosterol. Sequential C-methylation of fecosterol was not considered possible because 24-ethyl sterols are not present in yeast, and studies with $\Delta^{24(28)}$ substrates show no productive binding[18, 19]. The rate-limiting step is believed to be from the conformational change of the sterol side chain and the enzyme through the course of making and breaking of chemical bonds to release the product, not the deprotonation of the C-28 methyl group.

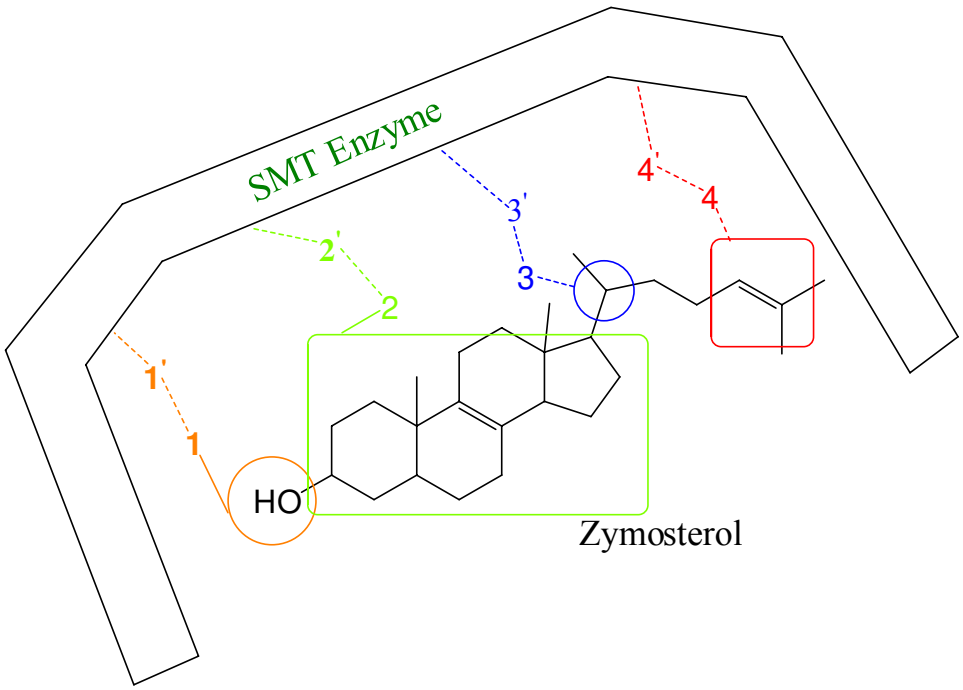


Figure 1.3.1. SMT Interaction with various domains on zymosterol. Each domain is highlighted with its own color: Orange - 3β-hydroxyl group, Green - a planar nucleus, Blue - a 20-R-configuration, and Red - a Δ^{24}- double bond. The coordination of this figure is not to imply that these exact locations of these interactions are known.

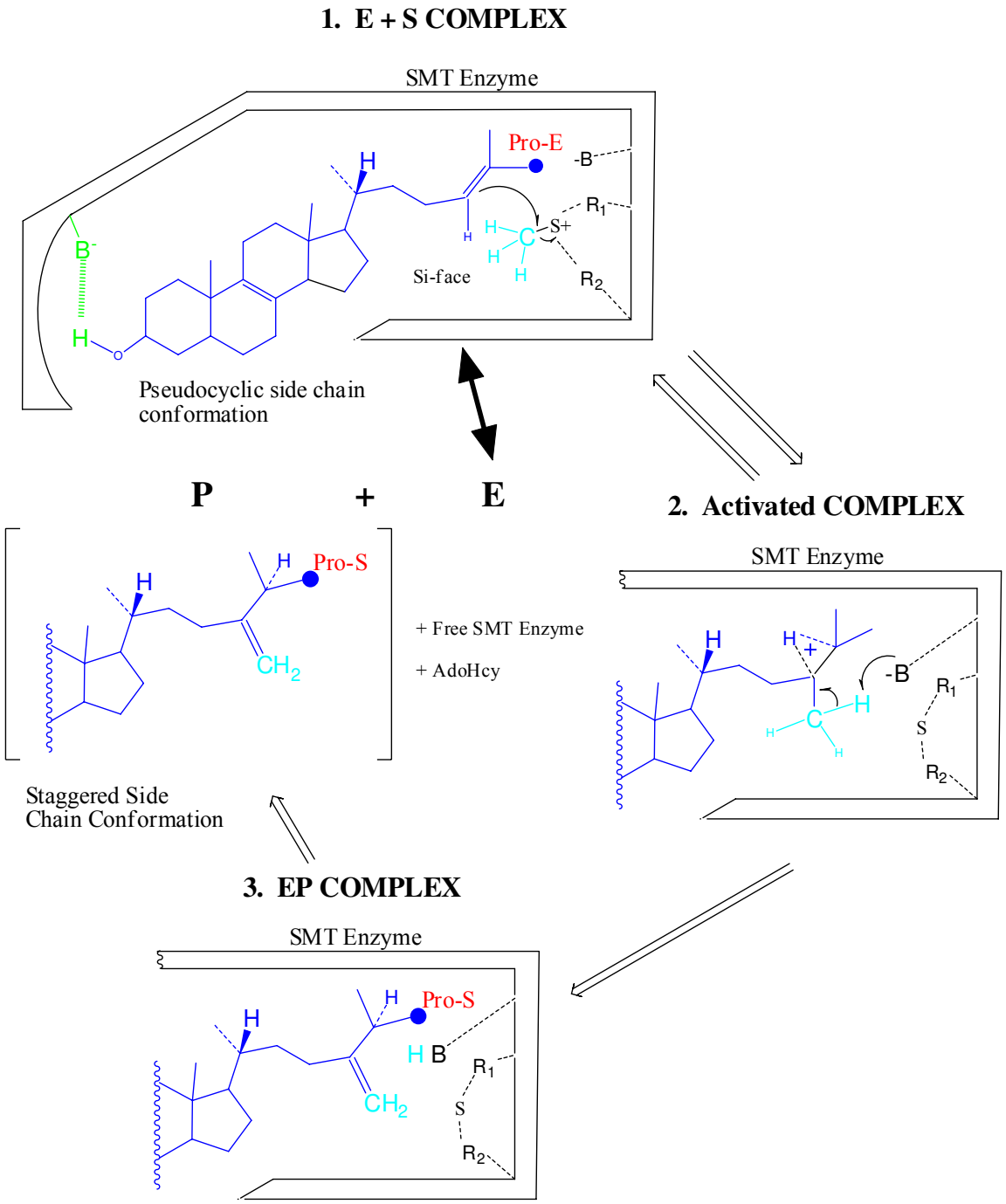


Figure 1.3.2. The steric-electric plug model representing the SMT catalyzed C-methylation reaction. Zymosterol and the resulting fecosterol side chain are in blue. The methyl group being transferred from AdoMet to zymosterol is in torques. The following notations are used: E – enzyme, S – substrate, P – product, and B – presumed basic amino acids of the SMT that interact with the sterol substrate.

1.4 FUNCTIONAL DOMAINS OF STEROL METHYLTRANSFERASE

To signify the role of the AdoMet cofactor in the sterol C-methylation reaction, the SMT enzyme is often labeled AdoMet-dependent Sterol Methyltransferase. However, in the superfamily of AdoMet-dependent Methyltransferase, the SMT enzyme was found to be a unique group based upon primary structure analysis. The first SMT gene (ERG6) was cloned from *S. cerevisiae*[20]. Later, two distinct SMT isoforms, SMT1 and SMT2, were discovered through the work done by Benveniste and coworkers who cloned and expressed *A. thaliana* SMT cDNA in *S. cerevisiae* (*erg*6 strain). These isoforms were characterized base upon their different substrate specificity.[21] SMT1 will bind to $\Delta^{24(25)}$-sterols such as cycloartenol (plant) and zymosterol (fungi), and SMT2 will bind to a $\Delta^{24(28)}$-sterol like 24(28)-methylenelophenol. These SMT2 isoforms possess a hydrophobic domain approximately 25 amino acids long near the N-terminal position that is absent in SMT1 isoform[22]. This variation in the SMT2 amino acid composition could be evidence of a divergent evolutionary channeling between the two isoforms. The majority of known SMTs have an amino acid length of 360 to 383 residues. By studying the primary sequence of SMTs from a variety of species, ranging from plants and fungi, three highly conserved motifs, referred as Region I, II, and III, have been discovered (Figure 1.41). These motifs are arranged sequentially and separated in analogous distances along the primary sequence. Region 1 in the Erg6p gene expression enzyme spans 11 residues from Tyrosine[81] to Phenylalanine[91] and is unique to the SMT enzyme subclass of AdoMet-dependent methyltransferases. Region 1 has been characterized as the binding site for the sterol side chain by studies with the mechanism-based inactivator [3-^{3}H] dehydrozymosterol (26, 27 DHZ). In the Erg6p SMT, Region II starts at

Leucine[124] and terminates at Proline[132] and appears in all AdoMet-dependent methyltransferase. This motif is conserved because it is the binding site for AdoMet as proven through [*methyl*-$^{3}H_3$] AdoMet photoaffinity labeling studies[23]. Region III, in Erg6p, stretches from Tyrosine[192] to Proline[201] and is also unique to the SMT subclass. The derived amino acid sequence of Erg6p or the *S. cerevisiae* SMT enzyme has a sizeable similarity (<35%) to more than 40 other SMT sequences available in the GenBank®. However, comparing only the sequences of these domains, the conservation of residues increases to between 84 and 93% similarity to that of the Erg6p[24] (Figure 1.42).

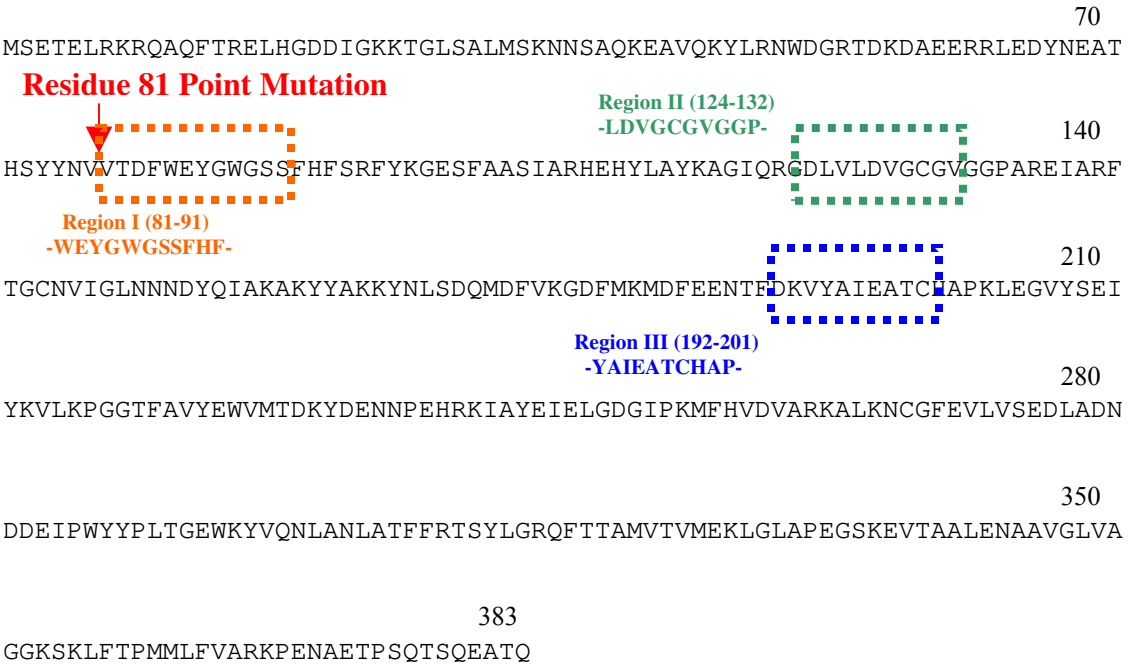


Figure 1.4.1. Amino Acid Map of Y81W Mutant Sterol Methyltransferase 1 (Wild Type Accession # - NP_013706). This enzyme has a theoretical molecular weight of 43.45 kDa. Note that this sequence has implemented a point mutation of tryptophan at position 81. The wild type enzyme has a tyrosine at this position.

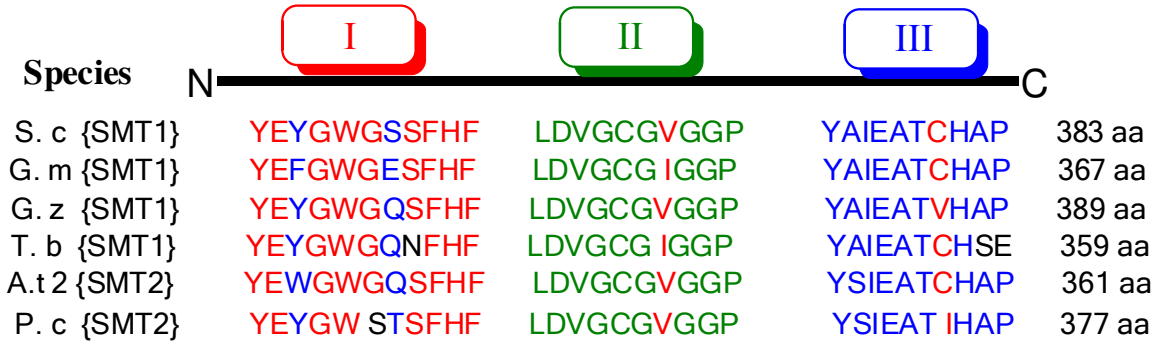


Figure 1.4.2. Amino Acid Map of Regions I, II, and III of wild type sterol methyltransferases (SMT) from various species of yeast and plants. Identical residues conserved in the primary structure are highlighted in their corresponding colors. The SMT studied is the top species (*S. cerevisiae)* with an Y81W point mutation.

1.5 RESEARCH AIMS

Questions dealing with how yeast SMT more precisely interacts with its substrate zymosterol in it reaction pathway to fecosterol need to be answered before medical applications against the SMT enzyme can be used to fight fungal infections. Such infections as *Candida albicans* that causes candidiasis in immune deficient patients and *Pneumocystis carinii* that causes lung infection and eventual pneumonia make fungi an objective of much research. The yeast SMT expressed in fungi contains a significant amount of conserved amino acid composition with other SMTs and undergoes a simple reaction mechanism. Because of these desirable traits and that the enzyme does not exist in the human body, the SMT enzyme is an ideal target for rational drug design. Nes and colleagues have performed previous work on the cloning of the S. cerevisiae SMT1 in Escherichia coli BL21 (DE3) host cells to perform detail analysis on the contribution of the highly conserved motifs to the enzymology of the SMT. They have achieved some success by purifying the SMT enzyme to homogeneity and performing kinetic analysis to understand the mechanism and employing techniques that can explore the three-dimensional structure of the SMT. Also by using site-directed mutagenesis, mutants of the yeast SMT have been helpful in understanding the importance and role of the conserved regions. One particular mutant of interest is the Y81W mutant. As mentioned in a previous section, the 81st position is the starting amino acid in Region I. This domain has already been established as the binding site for the sterol side chain, so therefore, a valuable target for investigation. Previous studies suggest that the stabilization of the cationic intermediates formed during the activated complex (ES*) was aided by the π-electric density contributed by aromatic amino acids such as tyrosine at position 81. This

π-electric density was increased by substituting tryptophan with tyrosine and thus increasing the catalytic efficiency of this mutant SMT enzyme[24]. Furthermore, preliminary work done with the Y81W mutant SMT enzyme and the mechanism-base inactivator 26, 27 DHZ has indicated that two enzyme bound products may be generated. It is believed that the mutation at Residue 81, changing tyrosine, with a sterically large OH group, to tryptophan, has changed the active site in such a way that the transition state intermediate carbocation is longer lived and allowed for reaction channeling to proceed to another diol (term used to signify the existence of two hydroxyl groups located on the 3β and side chain positions on the sterol product). These results are compared to studies with the WT SMT enzyme and 26, 27 DHZ where only one diol is produced in our limits of detection (Figure 1.52). For this unusual formation of products, the Y81W mutant SMT enzyme and 26, 27 DHZ were used in the verification of these results and in the investigation into the three-dimensional representation of the interaction between the enzyme and substrate by means of X-ray Diffraction analysis. Specifics aims of this study are as followed:

1. The concept of reaction channeling to different diol productions in the Y81W mutant SMT enzyme compared to its WT counterpart will be reexamined using a tryptic digest assay of the [3-^{3}H] 26, 27 DHZ complex with both enzymes and saponification studies. Reverse Phase-High Performance Liquid Chromatography (RP-HPLC) will be utilized to observe the chromatographic properties of the peptide fragments bound to the 26, 27 DHZ substrate and the saponified sterol products.

2. The purification protocol for *S. cerevisiae* WT and Y81W mutant SMT1 enzymes will be refined and standardized for future studies by adding a larger NaCl gradient in the early Q-sepharose chromatography stages and employing a Gel-Permeation step before introducing the protein to the Mono-Q column. Both columns will be coupled to a Fast Protein Liquid Chromatography system in attempt to increase the total yield of pure SMT sample for study.

3. The Y81W mutant SMT enzyme will be complexed with 26, 27 DHZ. This covalently bound complex will be purified to homogeneity, accordance with the said above protocol, and sent to the University of Pennsylvania for X-ray Diffraction analysis.

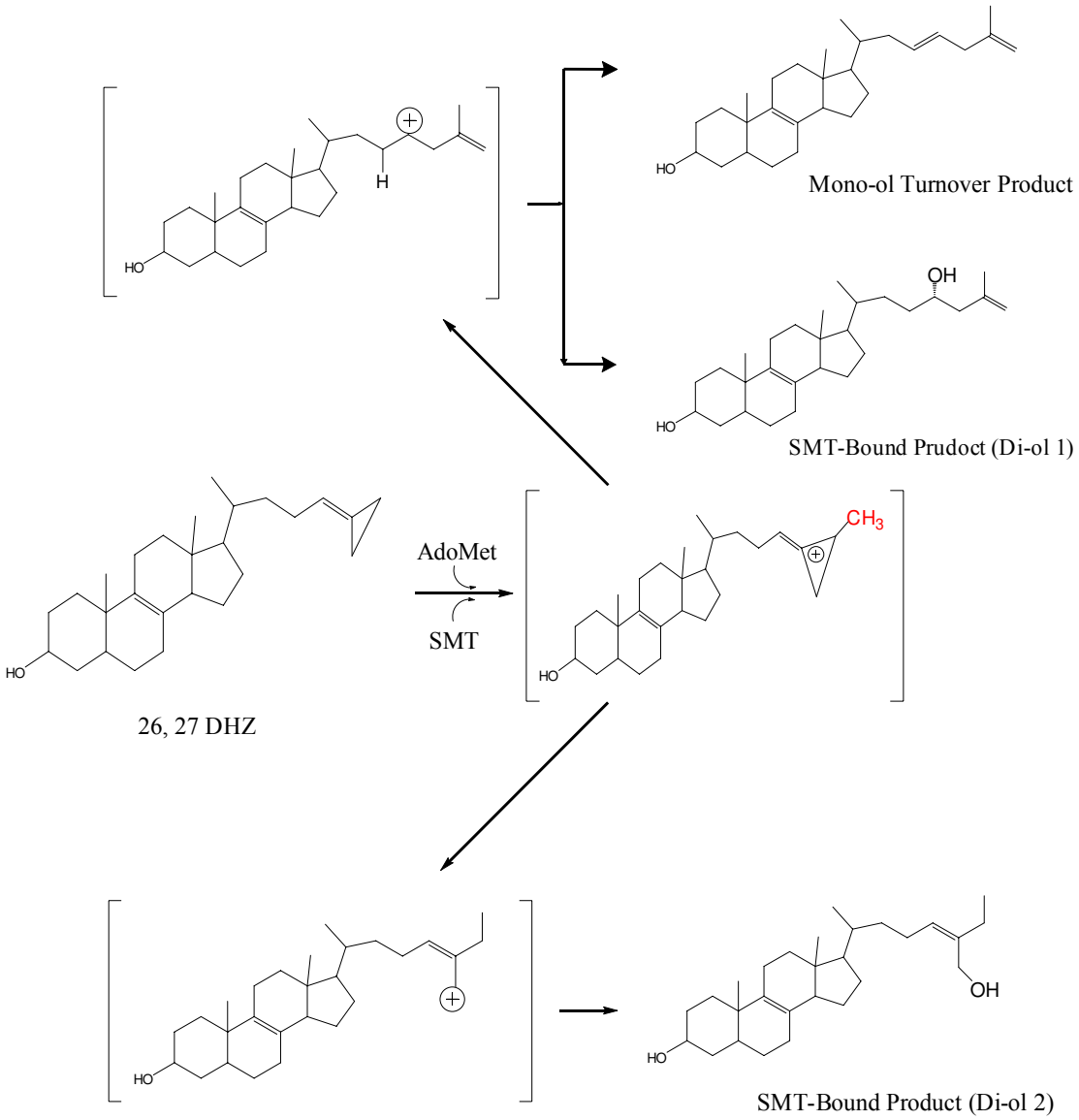


Figure 1.5.2. Diagram depicting the reaction SMT has with 26, 27 DHZ, and the reaction channeling that occurs to possibly produce two different diol products.

CHAPTER II
MATERIALS AND METHODS

2.1 GENERAL METHODS

*2.1.1 SDS-PAGE Analysis***.** 12% and 20% polyacrylamide gels were made for certain analysis throughout this research project. First step is to place a 1 mm spacer plate and short plate together in the casting frame. This casting frame was clamped into a casting stand (2 casting frames per casting stand). To make four 12% gels, 5 mL of Tris-HCl (18.2 g/100mL) at a pH of 8.8, 6.70 mL of ddH_2O, 0.2 mL of 10 % (w/v) SDS (10 g/100mL), 8.0 mL of 30% (w/v) Acrylamide (30 g acrylamide and 0.8 g Bisacrylamide/100mL) were combined, vortexed for thirty seconds, and degassed. To this volume, 200 μL of 10% (w/v) ammonium persulfate (APS) (0.1 g/1mL) and 20 μL of TEMED was added and briefly vortexed. This volume was transferred by 1.0 mL plastic pipette to the space created by the spacer plate and short plate until the space was filled three quarters up the short plate. This transfer process would be repeated to three more casting frames. On top of the bottom layer, 0.5 mL of the top layer of water-saturated butanol was placed to disrupt the formation of any bubbles. After the bottom layer was allowed to harden (~45 min), the top or stacking layer was made. 6.10 mL of ddH_2O, 2.5 mL of Tris-HCl (6g/100mL) at a pH of 6.8, 0.1 mL of 10% (w/v) SDS solution, and 1.3 mL of 30% (w/v) Acrylamide were mixed together and degassed. To this volume, 100 μL of 10% (w/v) APS and 20 μL of TEMED were added and briefly vortexed. The butanol layer was removed via a long syringe, and this top layer solution was place on the hardened bottom layer adhering to the same method stated above. However, this time, a comb was inserted into the same space to form ten wells when the

top layer hardened. After the top layer hardened (~30 min), the comb was removed, and the entire gel with the plates was wrapped in a damp paper towel, sealed in a sandwich bag, and placed in a 4°C refrigerator. The 20% gel was made in the same manner, but the components were altered for the increased polyacrylamide composition. 12% and 18% (polyacrylamide, Tris-HCl, 50 μL, 10 wells) gels were also bought through Bio-Rad. To perform a SDS-PAGE, the gel must be placed into the electrode assembly. The short plate must be facing inwards, and the gel clamped. If only one gel is being used, a buffer dam must be placed on the opposite side. The electrode assembly with the gels was placed in the tank, and the tank was filled with Running Buffer (3 g/L Tris-HCl, 14.4 g/L glycine, 1 g/L SDS; pH 8.3). The space between the gels was completely filled, and the space outside the gels was filled until the volume reached 5 mm down the top of the gel plates. The protein sample being examined must be combined with the loading buffer (1 mL Tris-HCl, pH 6.8, 0.8 mL glycerol, 1.6 mL 10% (w/v) SDS, 0.4 mL β-mercapto-ethanol, 0.4 mL 0.05% (w/v) Electrophoresis Purity Reagent Bromophenol Blue, Bio-Rad, and 3.8 mL ddH_2O). Solutions high in protein concentration are diluted more with the loading buffer, i.e. 1:4 ratio of protein solution to loading buffer. After the combination is achieved, the SDS-PAGE sample, held in a 1.5 mL Eppendorf tube, was heated at 90°C for 20 minutes. 40 μL of each sample tested was loaded onto their respective well. A SDS-PAGE molecular weight standard was also placed in a well, as a frame of comparison. After all wells were loaded, the lid was placed onto top the lid with the electrodes attached to the corresponding wires. The other end of the wires was attached to a Bio-Rad Power Pac power supply unit. The unit was set at 150 V, and allowed to run until the Bromophenol Blue stain line reached just above the bottom of the

gel (~1 hr). All SDS-PAGE equipment used in the above method belonged to the Bio-Rad Mini-PROTEAN® 3 Electrophoresis Cell. The gel was taken from inside the plates and placed in a Tupperware® container filled with 50 mL of staining solution (40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) ddH_2O, and 250 mg/100mL Coomassie® Brilliant Blue R-250, Bio-Rad). The gel was allowed to stain for 45 minutes. The gel was transferred to another Tupperware® container with 50 mL of destaining solution (40% (v/v) methanol, 10% (v/v) acetic acid, 50% (v/v) ddH_2O) and allowed to destain until the desired color contrast was achieved. The gels were either stored in a Tupperware® container filled with 50 mL of ddH_2O or dried between cellophane sheets (preferable for longer durations). Kaleidoscope and Unstained Polypeptide, Unstained Low Range, and Prestained Broad Range SDS-PAGE Molecular Weight Standards were all obtained through Bio-Rad®. For Kaleidoscope and prestained standards, 5 μL could be directly loaded onto a single well. For unstained standards, 2 μL of standard, 18 μL of ddH_2O, and 20 μL of loading buffer were mixed in a 1.5 mL Eppendorf tube. This volume could be used for two wells (20 μL per well).

*2.1.2 Bradford Quantification Method***.** Fractions in the early phases of purification underwent a Bradford Assay to determine the concentration of the protein. First, BSA solutions of known concentrations were tested to give an equation that the optical density (OD) of unknown samples could use to extrapolate their respective concentrations. All of these known concentrations of BSA, along with any analyzed unknown sample, were put through the following method. 25 μL portions of the sample and 1 M NaOH (filtered) each were placed in a 1.5 mL Eppendorf tube. Combining 25 μL each of the protein's

buffer and 1 M NaOH created a blank solution. The solutions were vortexed for thirty seconds and put aside for 20 minutes. During this time, according to how many samples are being tested, a volume of Bradford Reagent was formulated by combining 20% (v/v) Bio-Rad Protein Assay Dye Reagent Concentrate and 80% (v/v) ddH_2O. After 20 minutes, the 950 μL of Bradford Reagent was added to each assay or tube. The solutions were vortexed and allowed to sit for 10 minutes. With a spectrophotometer set at 595 nm, the blank was first used to tare the system. Each sample required at least three readings. The average was taken from the readings and placed into the equation created by the known concentrations of BSA and their associated OD readings. This equation was acquired using Microsoft® Excel software. The equation was in the form of y = mx + b where y is the absorbance reading at 595 nm, m is the slope of the trend line of the points, calculated by the software, x is the concentration of the protein solution, and b is the y-intercept, calculated by the software. The Bradford method can use plastic cells to obtain data and does not require a quartz cell. All work done using a spectrophotometer was done on a Beckman Coulter DU® 530 UV/Vis Spectrophotometer.

2.1.3 A_{280} Quantification Method. For pure or 85% pure protein solutions, measuring the protein concentration was achieved by measuring the absorbance of the solution at 280 nm. This absorbance was then placed into the Beer Lambert's Law Equation {$A_{280} = (\varepsilon)(C)(L)$, where A_{280} is the absorbance at 280 nm, ε is the extinction coefficient for WT and mutant yeast SMT enzymes (58,370 M^{-1} cm^{-1} or 1.36 $(mg/mL)^{-1}$ cm^{-1}), and L is the path length of the cell in the spectrophotometer (1 cm)}. ε was calculated by the equation: $\varepsilon = \Sigma$ {n (tyr) x 1280 + n (Trp) x 5690 + n (Cys) x 120}. It could also be

verified if the primary sequence was known which can be determined through Swissprot Protein ExPASy Server or on NCBI databases[24]. All work done using a spectrophotometer was done on a Beckman Coulter DU® 530 UV/Vis Spectrophotometer. A Nanodrop ND-1000 Spectrophotometer was also used to detect A_{280} of a pure Y81W sample. A 2 μL aliquot of the previously purified Y81W mutant SMT enzyme was placed on the instrument, and the software from the spectrophotometer gave the concentration in mg/mL.

*2.1.4 Production of a Standard Curve of Cholesterol Standards***.** Concentrations of 0.25, 0.5, and 0.75 mg/mL of cholesterol were made by appropriately diluting, with ethanol, portions of a stock solution of 1mg/mL of cholesterol in ethanol. One μL of each concentration, including the stock solution, was loaded onto the GC. By graphing the integral of the resulting data versus the known concentration of the cholesterol standards and obtaining a standard equation, the mass of an unknown sample of 26, 27 DHZ was more precisely determined.

2.1.5 Concentration and Filtration of Samples. All concentrating during the purification process was done either by a 50 mL or 10 mL Amicon® ultrafiltration system. The nominal molecular weight limit (NMWL) of the filter used in this system was 10,000 kDa and had a diameter of 44.5 mm in the 50 mL system and 25 mm in the 10 mL system. The buffer transfer between Qa+D to 25 mM HEPES with 0.2% B-D-G was performed by the Macrosep® Centrifugal Device, developed by PALL Life Science, with a filter size of 100 kDa. The separation of the tryptic-digested samples was performed

using a Millipore® Centricon Centrifugal Filter Device with a YM-10 (10 kDa) Molecular Weight membrane filter size. The vacuum filtration device used in filtering buffers was made by VWR and used a nylon, 0.22 micron, and 47 mm diameter filter made by Magna.

*2.1.6 Preparation of Buffers***.** Qa buffer used in the purification of Y81W mutant SMT enzyme consisted of 25 mM (5.96 g/L) HEPES, 2 mM (0.41 g/L) $MgCl_2$, 1 mM (0.37 g/L) EDTA, 2 mM (0.14 mL/L) β-mercampto-ethanol, and 5% (by volume) glycerol with the pH adjusted to 7.5. Qb buffer contains an additional 1 M NaCl. A stock solution of Qa buffer with the concentration five times that described above was produced first. From this solution, 200 mL were taken and added to 800 mL of ddH_20 to make the final volume for Qa buffer. 58.44 g of NaCl, per one liter, was added, at this time, to produce Qb buffer. Qa or Qb buffer with detergent (Qa+D or Qb+D, respectively) contained 0.4% (by volume) Polyoxyethylene-10-tridecyl ether detergent. A stock solution of 10% (by volume) Polyoxyethylene-10-tridecyl ether detergent in Qa buffer was first made. A 40 mL portion of the stock detergent solution was added per one liter of Qa or Qb buffer to make 0.4% (by volume) detergent solution. The NaCl gradient used in the purification of the SMT enzyme was made by combining Qa+D and Qb+D buffers at different concentrations. 10% NaCl, 15% NaCl, 16.5% NaCl, and 18% NaCl, 20% NaCl, and 22.5% NaCl Qa+D solutions were produced. For an example, the 10% NaCl Qa+D solution was made by mixing 90 mL of Qa buffer with 10 mL of Qb buffer. The other concentrations were made in this same manner. A Tris buffer was created for the purification of the Wild Type and Y81W mutant SMT enzymes for the Chemical Affinity

Labeling studies. This Tris buffer consisted of 50 mM (6.06 g/L) Tris-HCl, 2 mM (0.41 g/L) $MgCl_2$, 1 mM (0.37 g/L) EDTA, 2 mM (0.14 mL/L) β-mercampto-ethanol, and 5% (by volume) glycerol with the pH adjusted to 7.5. A Tris buffer with 1 M NaCl was made, along with both buffers in 0.4% (by volume) Polyoxyethylene-10-tridecyl ether detergent (+D). A NaCl gradient was also made from the Tris+D buffers as described in the HEPES buffer methods. A 50 mM (3.96 g/L) ammonium bicarbonate (AMBIC) buffer was prepared for the tryptic digestion in the Chemical Affinity Labeling studies. To produce this solution, a stock 500 mM solution was made. From this stock solution, 100 mL was taken and mixed well with 900 mL of ddH_2O. For the buffer comparative analysis, a 0.1 M potassium phosphate ($KHPO_4$) buffer was made by first developing 1 M stock solutions of dibasic (K_2HPO_4, 45.64 g in 200 mL) and monobasic (KH_2PO_4, 27.22 g in 200 mL) potassium phosphate. By combining 83.4 mL of 1 M dibasic, 16.6 mL of 1 M monobasic, and 900 mL of ddH_2O, a 0.1 M potassium phosphate buffer with a pH of 7.5 was created. Before the purification of the yeast antibody could be started, 300 mL of Rabbit Application buffer and 100 mL of regeneration buffer was made according to the protocol developed by Bio-Rad®. A portion of 1.3 g of rabbit buffer solids (given by the kit) was dissolved in 300 mL ddH_20. Before final volume was achieved, pH was adjusted to 8.0 using 6 M HCl or 6 M NaOH. The regeneration buffer was made by dissolving 12.2 g of regeneration solids into 100 mL of ddH_20. No pH adjustment was necessary for the regeneration buffer. Both buffers were then filtered through the vacuum filtration device. The purified Y81W mutant SMT and 26, 27 DHZ complex was transferred in a 25 mM HEPES buffer with 0.2% octyl-β-D-glucopyranoside (B-D-G) to the University of Pennsylvania for crystallization and X-ray diffraction analysis. This buffer was

created by combining 0.298 g B-D-G and 0.2979 g HEPES in a to 40 mL of ddH_2O. The pH was adjusted to 7.5, and the volume corrected to 50 mL. It was then vacuum filtered.

*2.1.7 Preparation of Stock Solutions***.** The ampicillin stock solution used in growing cell cultures was produced by dissolving 2.5 g of ampicillin salt, purchased from Fischer Bioreagents®, into 50 mL of ddH_20. The resulting concentration of the ampicillin stock solution is 50 mg/mL. The isopropyl-β-D-thiogalactopyranoside (IPTG) stock solution used in inducing the production of the SMT enzymes was made by dissolving 4.766 g of IPTG, purchased from Research Product International Corp., into 50 mL of ddH_20. The final concentration of the IPTG stock solution was 400 mM. Dissolved in 50 mL of ddH_20, the glucose solution to be added during growth of the Y81W mutant *S. cerevisiae* yeast cells for X-ray diffraction analysis contained 12 g of glucose, 0.74 g of $MgSO_4$, and .015 g of Vitamin B1 or thymine. The amino acid solution, also needed during the growth of the Y81W mutant *S. cerevisiae* yeast cells for X-ray diffraction analysis, was made of 100 mg each of lysine, threonine, and phenylalanine and 50 mg each of leucine, isoleucine, and seleno-methionine dissolved in 50 mL of ddH_20. The composition of both solutions was intended for one liter of M9 medium. Both solutions were separately vacuumed filtered before introduction to the M9 medium. Phosphate buffered Saline (PBS) was produced to transfer the Y81W mutant SMT enzyme from the 12% acrylamide SDS-PAGE gel to the nitrocellulose membrane. First, combining 80 g NaCl, 2.0 g KCl, 14.4 g of Na_2HPO_4, and 2.4 g of KH_2PO_4 in 800 mL of ddH_2O created a 10x PBS stock solution, and the pH was adjusted to 7.4. The volume was then corrected to 1 L. 100 mL of the stock solution was then combined with 900 mL of ddH_2O and the pH

maintained at 7.4. The western blot studies needed many different solutions. A 2 L PBST (0.1% Tween 20) solution was created by combining 200 mL of 10xPBS stock solution, 8 mL of polyoxyethylene sorbitan monolaurate (Tween 20) made by Sigma®, and 1782 mL of ddH_20. Mixing together 10 mL of DMSO and 40 mL of PBST produced a 20% DMSO/PBST wash. A 3% blocker solution was made by mixing 0.9 g of powdered blocker given by the Bio-Rad Amplified Opti-4CN™ Substrate kit to 30 mL of PBST. The blocker powder was added in small quantities over a period of thirty minutes and on top of a magnetic stirrer and heater set at 50°C. The blocker solution stirred for an additional 30 minutes until all the powder was dissolved. An antibody dilution buffer was made to dilute the primary and secondary antibodies, as well as diluting streptavidin-HRP. Adding 0.75 g of Bovine Serum Albumin (BSA) per 75 mL of PBST and mixing thoroughly made this antibody dilution buffer (1% BSA in PBST). The primary or SMT antibody was diluted by a factor of 1:200, GAx-HRP secondary antibody by a factor of 1:3000, and the streptavidin-HRP by factor of 1:1000. Combining 20 mL of 2x Amplification diluent, 10 mL of 4x BAR, and 10 mL of ddH_20 made the Bio-Rad Amplification Reagent. The Opti-4CN substrate solution used for detection of the antibody-SMT complex was made by mixing together 2 mL of Opti-4CN diluent concentrate, 18 mL of ddH_20, and 0.4 mL of Opti-4CN substrate. The GAx-HRP (Goat Anti- Rabbit IgG bound to horse radish peroxidase (HRP)) secondary antibody, streptavidin-HRP, 2x Amplification diluent, 4x BAR, Opti-4CN diluent concentrate, and the Opti-4CN substrate were all given by the Bio-Rad Opti-4CN™ Substrate kit. In complexing the Y81W mutant SMT enzyme and 26, 27 DHZ and radioactivity assays, 5% Tween 80 is needed to dissolve 26, 27 DHZ, a hydrophobic sterol, into the

hydrophilic buffer containing the enzyme. 2.5 mL of polyoxyethylene sorbitan mono-oleate (Tween 80) made by Sigma® was vortexed with 47.5 mL of ethanol thoroughly.

*2.1.8 Preparation of Non-radiated and [methyl - $^3H_3$] AdoMet***.** Non-radiated (cold) AdoMet was prepared as a 6 mM or 0.6 mM stock solution in 0.001 N H_2SO_4 as 1 mL aliquots and stored at -20°C. The AdoMet was corrected to 100% pure as compared to 83% purity in commercial stocks. The radioactive analog of AdoMet, [*methyl* - $^3H_3$] S-adenosyl-L-methionine, was purchased from PerkinElmer (>97% pure). The specific activity of the radio-label varies from 5-15 Ci/mmol to 55-85 Ci/mmol with at total concentration of 0.55 mCi/mL. This will be equivalent to 36.6 μM (considering 15 Ci/mmol) or 6.5 μM (considering 85 Ci/mmol). Since saturating AdoMet concentration of 100 μM was a requirement for most of our kinetic analysis, a series of working stocks were prepared by combining the radiated AdoMet with cold AdoMet stock solution to compensate the required molarity and to maintain enough radioactivity to detect enzyme activity. The first cocktail was made by combining 181.8 μL of [*methyl* - $^3H_3$] AdoMet from the original bottle, 81.67 μL of 6.0 mM cold AdoMet, and 536.53 μL of 0.01 M H_2SO_4. The purpose of using H_2SO_4 is to ensure the longevity of the AdoMet solution. The final specific activity was 200 μCi/μmol or 125 μCi/mL. Taking from this first cocktail, a second cocktail was made that was added directly to assays. It was formulated by adding 144 μL of the first cocktail, 285 μL of 6.0 mM AdoMet, and 2.571 mL of the same buffer as the protein buffer. The final specific activity of the second cocktail is 10 μCi/μmol or 6 μCi/mL or 0.6 mM final stock solution in buffer of choice. The addition

of the buffer to the second cocktail was to ensure pH and buffer environment competence to the protein buffer.

*2.1.9 Purification of Sterol Substrates and Inhibitors***.** Purification of substrates and inhibitors (i.e. zymosterol and 26, 27 DHZ) was performed at room temperature on a HPLC system having an ISCO 2350 Model pump and an ISCO V4 Absorbance Detector (ISCO Inc., Lincoln, NE), which the detection wavelength was set at 205 nm. The columns were C18-TSK-gel analytical column (ODS 120 A, 5 μm, 4.6 mm ID x 25 cm), C18-TSK-Gel semi-prep column (ODS 120 A, 10 μm, 10 mm ID x 250 mm; Phenomenex, Torance, CA), and a C18 Selectosil column. Optimal separation of sterol substrates was achieved using an HPLC-grade methanol with a flow rate of 2.5 mL/min on the C18-TSK-gel semi-prep column and 4.0 mL/min on the C18 Selectosil column. The optimal flow rate on the C18-TSK analytical column was 1.0 mL/min. Mass of any sterol substrate was determined through gas chromatography in a Hewlett Packard 5890 Series II GC system. This instrument was equipped with a 2 mm IDx50 cm glass column packed with Chromosorb W-HP coated with 3% SE-30 (Alltech Associates, Inc. Deerfield, IL), a FID detector, and a HP 3395 integrator. It uses He as the carrier gas with a pressure of 6.5 psi, and the pressure of the hydrogen and air were set at 15 and 30 psi, respectively. The oven temperature was set at 245°C and the detector at 300°C. Purity of sterol substrates was verified with mass spectra given by Gas Chromatography/Mass Spectroscopy (GC/MS) analysis. The instrument used for the analysis was a Hewlett Packard 6890 GC interfaced to a 5973 mass selective detector at 70 eV. The GC was equipped with DB-5 capillary column (J&W, Folson, CA) with

dimensions of 250 mm IDx30 mm and film thickness of 0.25 mm. Chromatography was performed at a constant flow rate of 1.2 mL/min. Injection volume of the sterol sample was 1 μL, and the injection model was pulsed splitless at a pressure of 25 psi. The oven temperature was programmed during the chromatography as followed: initial temperature was 170°C to 280°C and maintained at 280°C fro the next 15 minutes. The temperature for the GC to MS interface, MS ion source, and the quadrupole were set at 280°C, 250°C, and 230°C, respectively.

2.2 DEVELOPMENT AND GROWTH OF STEROL METHYLTRANSFERASE ENZYMES

*2.2.1 Transformation of the ERG6 gene into Escherichia coli BL21 (DE3) host cells***.** The plasmid pRG458, which contained the wild type ERG6 gene that encodes for the (S)-adenosyl-L-methionine:$\Delta^{24(25)}$ to $\Delta^{24(25)}$-sterol methyltransferase enzyme from *S. cerevisiae*, was acquired from Dr. Martin Bard (Department of Biology, Indiana University, Indianapolis, IN)[25]. Resulting in the construction of a 1281 base pair (bp) fragment insert analogous to the ERG6 DNA, the open reading frame of the SMT gene was subcloned into the plasmid pBR322[19]. Involving a progression of steps, an NdeI-HinIII fragment containing the ERG6 gene from pBR322 was subcloned into plasmid pET23a(+)[12]. The resulting plasmid was then transformed into *Escherichia coli* (*E. coli*) BL21(DE3) host cells (from Novagen) according to the fast freezing/thawing method[26]. The plasmid pET23a(+) also contains a gene for the resistance against ampicillin. The Y81W mutant SMT was produced via the QuikChange™ site-directed mutagenesis kit

(Strategene) according to the manufacturer's instructions. The recombinant pET23a-ERG6 was used as the mutagenesis template[27]. The point mutation was introduced into the bp 318-320 sequence of the wild type ERG6 gene. The resulting mutant plasmid was transformed into host cells as described in the previous paragraph. Mutated and wild type DNA sequences were verified through automated DNA sequencing. For general investigative purposes, both *E. coli* BL21(DE3) host cell cultures containing the ERG6 gene for *S. cerevisiae* yeast wild type and Y81W mutant SMT enzymes were develop in the same manner, as described below.

*2.2.2 Development of E. coli BL21 (DE3) Host Cell Pellet***.** Luria Bertani (LB) medium used in growing cell cultures was composed of 10 mg/mL Difco™ bacto-tryptone, 5 mg/mL Difco™ bacto-yeast extract, and 10 mg/mL NaCl. M9 minimal medium consisted of 11.28 g/L of Difco™ M9 minimal salts (5x). Both media were autoclaved before use. A LB agar plate had a volume of 30 mL consisting of the same composition as LB media with 15 mg/mL agar and 30 μL of 50 mg/mL ampicillin stock. A single colony, taken from an LB agar plate was placed into a 500 mL Erlenmeyer flask containing 200 mL of LB media, along with 200 μL of ampicillin at a concentration of 50 mg/mL, and allowed to incubated overnight at 37°C and 185 rpm in a shaker. A 25 mL portion of this "starter" culture was then added to a 2.7 L Fernbach flask holding 975 mL of fresh LB media with 1 mL of ampicillin at the same concentration as stated above. The cells were allowed to grow, under the same conditions, until the OD at 600 nm reached between 0.6 and 0.8 absorbance units on a spectrophotometer. This period would usually take between two and half and three hours. The cell culture was blanked

against 1 mL of fresh LB medium. At this point, 1 mL of a 400 mM IPTG solution was added to the cell culture for induction of the SMT enzyme. The cell culture grew 4 more hours under the same conditions. Approximately 350 mL of cell culture was placed into 500 mL centrifuge bottles and balanced with respect to their weight. The centrifuge bottles, with the cell culture, were then inserted into a J-10 centrifuge rotor. The cells were collected using a centrifuge set at 7500 rpm and 4°C for 20 minutes. The supernatant was discarded and the cell pellet was distributed into 5 g portions and put into 50 mL Falcon® tubes. The Falcon® tubes, with the cell pellet, were stored at -80ºC until further use. One liter of cell culture produced between 4 and 5 g of cell pellet. To obtain the *E. coli* BL21 (DE3) host cell pellet, a Beckman Coulter Avanti J-26 XPI Centrifuge was utilized under 4°C and 7500 rpm conditions for 20 minutes. After the cells are lysed at the first of the purification process, the cells are ultracentrifuged using a Beckman Coulter Optima™ L-80 XP ultracentrifuge at 4°C and 33,000 rpm (100,000xG) for 1 hour. The samples were placed into 26.3 mL ultracentrifuge tubes (polycarbonate, 25x89 mm, produced by Bechman) that fit into the Ti-70 rotor. The *E. coli* BL21 (DE3) host cell pellet suspended in Qa buffer was lysed using a SLM Instruments French® Pressure Cell Press under 12,000 psi pressure. All work done using a spectrophotometer was done on a Beckman Coulter DU® 530 UV/Vis Spectrophotometer.

2.2.3 Development of Escherichia coli BL21 (DE3) Host Cell Pellet Containing the Y81W Mutant Strain for X-ray Diffraction Analysis. Glycerol stocks of each respective cell culture were also made using the "starter" culture. 200 µL of autoclaved glycerol and 800 µL of "starter" culture were mixed together in a 1.5 mL Eppendorf tube, quickly

brought down to below -80°C by liquid nitrogen, and stored at -80°C. Approximately 10 glycerol stocks were made of each cell culture at one time. One glycerol stock can inoculate one "starter" culture by placing 5 μL, completely thawed, into 200 mL of fresh LB media. If kept frozen and taking only a loop full from an inoculum loop to inoculate 200 mL of fresh LB media, one glycerol stock can be reused. The growth of the E. coli BL21(DE3) host cell cultures containing the ERG6 gene for S. cerevisiae yeast Y81W mutant SMT enzyme was performed under a different method for X-ray crystallography. Ragu Kaagasabai developed this method, and it differs from the normal growing protocol because of the addition of the seleno-methionine component for X-ray diffraction purposes. Six 900 mL portions of M9 media were made and placed in six 2.7 L Fernbach flasks, and a 300 mL portion of M9 media was also made and placed into a 1 L flask. All M9 media was autoclaved and saved for later use. 1500 mL of LB media was made, and 250 mL portions were distributed into six 1 L flasks and autoclaved. After allowing them to cool, 5 μL of thawed out Y81W mutant cell glycerol stock was added to each flask, along with 250 μL of ampicillin at a concentration of 50 mg/mL. All six flasks were incubated at 185 rpm and 37°C, overnight. In 6 sterile 500 mL centrifuge bottles, centrifuge the "starter" cell cultures in a J-10 rotor at 7500 rpm and 4°C for 20 minutes. The supernatant was discarded, and the cell pellet was resuspended in 300 mL of M9 media. Total mass of the cell pellet should be around 7.4 g. After resuspension, put 50 mL of this M9 cell culture into each of the Fernbach flasks containing 900 mL of M9 media. Also place 50 mL of the glucose solution and 1 mL of 50 mg/mL ampicillin stock into each of the flasks. The total volume in each flask should be 1 L at this point. The cell cultures were incubated at 200 rpm and 37°C until the OD at 600 nm reached 0.5

absorbance units. At this stage, 50 mL of the amino acid solution were added to each flask. After another 30 minutes of incubation under the same conditions, 1 mL of 400 mM IPTG was added to each flask to induce the production of the desired Y81W mutant SMT enzyme. The cell culture was then incubated, with the same parameters, for 5 more hours. The cells were collected as described earlier in the methods section.

*2.2.4 Purification of Wild Type and Y81W Mutant SMT Enzymes***.** Both *S. cerevisiae* wild type and Y81W mutant yeast SMT enzymes were purified in the same manner, as described below. A 5.0 g portion of cell pellet was taken out of refrigeration and allowed to thaw in ice. 25 mL of Qa buffer were added to each pellet still in the Falcon® tube and vortexed. After the pellet dissolved off the wall of the tube, the solution was transferred to a 100 mL beaker and stirred thoroughly at 4ºC for 15 min until complete suspension was achieved. At this time, the volume reached 28 mL. The cell pellet solution was French pressed twice at 12,000 psi. The cylinder was cooled before use in a -20ºC refrigerator for 15 minutes. A 0.5 mL portion of the lysed cells (lysate) was taken and stored at -20ºC. The lysate was split into two equal volumes and placed in two separate 33 mL ultracentrifuge tubes. These tubes were ultracentrifuged at 4ºC and 33,000 rpm (100,000 x G) for 1 hour. A 0.5 mL supernatant portion was saved from each sample and stored at -20ºC. All Chromatography work done from this point was performed at 4ºC. Q-Sepharose® Fast Flow (anion-exchange, wet bead size – 45 to 165 μm, and made by Sigma-Aldrich) was used as the matrix in the first two columns when purifying SMT enzymes. The glass columns had dimensions of 27 mm in diameter and 135 mm in length. With each fraction collected, a 0.5 mL portion was taken for SDS-

PAGE and quantification purposes. These quantities were stored at -20ºC. The supernatant from the ultracentrifugation step was obtained and placed on a column loaded with 25 mL of Q-Sepharose Fast Flow equilibrated with Qa buffer without detergent. The first 10 mL of flow through was not collected since this was the void volume of the column containing only Qa buffer. After the first 10 mL had eluted, the flow through was collected in a 50 mL Falcon® tube. 10 mL of Qb buffer without detergent was then added when the supernatant had passed through. This step is necessary to elute the entire supernatant out of the column. The elution from the first column should be slightly less then 30 mL. A 1.2 mL portion of 10% (by volume) Polyoxyethylene-10-tridecyl ether detergent suspended in Qa buffer was vortexed into the supernatant pass through of the first column. (Final concentration of detergent was 0.4%.) An additional 30 mL of Qb without detergent was added to the first column in order to clean the Q-Sepharose matrix but was collected in a separate 50 mL Falcon® tube. This fraction was kept, as well. The supernatant flow through, with 0.4% detergent, was placed onto another column of 25 mL Q-Sepharose matrix now equilibrated with Qa buffer with 0.4% Polyoxyethylene-10-tridecyl ether detergent. Adhering to the process utilized on the first column, the flow through was collected. Instead of using Qb buffer to elute the sample, a NaCl gradient was employed, starting at 10% (by volume) Qa +D (100 mM NaCl in Qa buffer with detergent). A total of 30 mL of 0.1 M NaCl Qa+D fraction was collected, and the process was repeated with 0.15 M, 0.165 M, 0.18 M, 0.2 M, 0.225 M, and 1.0 M (Qb+D) NaCl Qa+D buffer used on the same column, in succession. At each gradient step, the total volume collected was approximately 30 mL, and each fraction was stored in a Falcon® tube at -20ºC until the next day. Using the saved 0.5 mL portions and 12% SDS-PAGE

gel, SDS-PAGE analysis was ran on the fractions of the lysate, supernatant, first column pass through, second column pass through, 0.1 M NaCl Qa+D, 0.15 M NaCl Qa+D, 0.165 M NaCl Qa+D, 0.18 M NaCl Qa+D, 0.2 M NaCl Qa+D, and 0.225 M NaCl Qa+D. A Bradford protein assay at 595 nm on a UV-Vis Spectrophotometer was also ran on the saved samples. Using a standard graph of known concentrations of bovine serum albumin on the same spectrophotometer, one can get a high estimate of the protein concentration. From the data obtained from the SDS-PAGE analysis, the 0.165 M and 0.18 M fractions were combined and concentrated down to 10 mL and then washed three times with 10 mL of Qa+D buffer each time using a 50 mL Amicon® ultrafiltration system. The final volume was concentrated under 5 mL. The concentrated sample was loaded onto a Gel Permeation Chromatography (GPC) column. GPC was performed on a HiPrep™ 26/60 Sephacryl™ S-300 GPC column connected to a Fast Protein Liquid Chromatography (FPLC) system made by Pharmacia LKB®. The sample was eluted at a flow rate of 1 mL/min, and sensitivity at 0.1. Fractions were collected in 2 mL aliquots. The fractions underwent analysis with SDS-PAGE and Bradford methods. The purest fractions were pooled and concentrated and washed, as described in the above text. The concentrated sample of no more than 2 mL was loaded onto a anion exchange column, and the desired fractions collected. The column used in purifying the SMT enzyme was the Q-Sepharose (Mono-Q) column produced by Pharmacia Biotech®, Code No. 17-0556-01, with a volume of 8.0 mL. All Mono-Q runs had the same parameters of a range of 0.2, chart speed of 0.5 mm/min, a flow rate of 1 mL/min, and an introduced NaCl gradient that started at 0.05 M NaCl Qa+D and increased step-wise 0.05 M every 20 minutes to 25% NaCl Qa+D. It then increased to 100% NaCl Qa+D to wash the column

for 30 minutes. 2 mL fractions were collected, starting at 50 minutes and ending at the 135 minute mark. Collection was performed using a Pharmacia LKB® fraction collector, FRAC-100 (Code No. 18-1000-77). The fractions were stored in 13x100 mm glass culture tubes. The purity of the SMT enzyme was confirmed by SDS-PAGE analysis with a 12% SDS gel. Only fractions with one band corresponding to 43 kDa were considered pure and kept for further investigative purposes. These fractions were washed and concentrated, as directed before in this section, to 10 mL. The mass of the SMT enzyme was found by detecting how much absorbance the enzyme had at 280 nm. Fractions that contained both the 43 kDa pure SMT band and the degradative 40 kDa band, but were otherwise pure, were concentrated and washed once more, as directed previously in this section. The final 2 mL volume produced from the concentration process was placed again on the Mono-Q column controlled by the same FPLC column, under the same parameters. Verification of the purity of the SMT enzyme was done with the same SDS-PAGE and A_{280} analysis, and collection was also done corresponding to the same methods outlined above. After concentrating and washing of the second round pure SMT enzyme, the sample was combined with the first round of pure SMT.

2.3 CHEMICAL AFFINITY LABELING OF WILD TYPE AND Y81W MUTANT SMT WITH [3-^{3}H] 26,27 DHZ

*2.3.1 Preliminary Work and Purification of SMT Samples***.** Before any chemical affinity labeling could take place, the radioactivity of the [3-^{3}H] 26, 27 DHZ was tested. Using a GC, the exact amount of [3-^{3}H] 26, 27 DHZ was calculated in the stock solution, by comparing the integral value to a stock 1 mg/mL Cholesterol sample. This set of data could then be used to determine the dpm/μg and, therefore, how much radioactivity is present in a complexed SMT-[3-^{3}H] 26, 27 DHZ adduct. The purity of the radiated 26, 27 DHZ solution was analyzed by GC/MS. Cell pellets of 4.9 g and 4.5 g (WT) and 5.1 g and 4.9 g (Y81W) of *E. coli* BL21(DE3) host cell cultures were used in this study. The purification of both SMT strains was done in Tris buffer (Method described in the HEPES buffer purification section). A SDS-PAGE analysis was performed on each purified NaCl gradient fraction using a 12% polyacrylamide gel to verify the purity and presence of the SMT enzyme in each strain. Using the Amicon® Ultrafiltration (50 mL) device, the 15% and 16.5% fractions from both the WT and Y81W mutant purification samples purified from the second Q-sepharose column were washed three times with Tris+D buffer and concentrated to 8 mL and 10.5 mL, respectively. The concentrated samples of WT and Y81W mutant SMT enzymes underwent a Bradford quantification method, and a weight of 25.8 mg/8 mL and 25.8 mg/10.5 mL, respectively, was found. This is a 40% overestimation as described in the comparison of Bradford and A_{280} methods in the results and discussion chapter. A correction factor of 0.85 was used to determine the amount of SMT because the samples were of 85% purity as determined through visual inspection of SDS-PAGE. This amount was calculated to be 21.9 mg/8

mL and 21.9 mg/10.5 mL for WT and Y81W samples, respectively. 2.5 mL of Tris+D buffer was added to the WT sample to even the volumes between the two samples. An activity assay using (^{3}H) AdoMet was performed on both samples to ensure the presence of active enzymes.

2.3.2 Complex of the Wild Type and Y81W Mutant SMT with [3-^{3}H] 26, 27 DHZ. Using a ratio of 1 (SMT sample): 4 ([3-^{3}H]26, 27 DHZ): 5 (AdoMet), both samples were complexed with the [3-^{3}H]26, 27 DHZ, separately. In a 13x100 mm glass tube, 650 μL (110.5 mg) [3-^{3}H] 26, 27 DHZ, taken from a stock solution, was added to 120 μL of 5% Tween 80 in ethanol to help dissolve the inhibitor in the SMT solution. After the combination of these two components was blown down via N_2 gas, a small amount of Tris+D buffer was used to wash the inside of the glass tube, and this liquid was placed in a 50 mL Falcon® tube. To this tube, 5 mL (10.5 mg) of SMT sample from the second Q-sepharose column and 55 μL of 6 mM AdoMet were added. The final concentrations of the components in 5.5 mL were 11 μM of the SMT enzyme, 52 μM of 26, 27 DHZ, and 66 μM AdoMet. The tube was capped and sealed with parafilm and incubated in a water bath set at 35°C and shook at 100 rpm for 6 hours. A nonradiated complex was also performed using nonradiated 26, 27 DHZ in the same manner and incubated with the radiated samples to ensure the same conditions were maintained. There were four total samples complexed: 2 radiated samples (WT and Y81W) and 2 nonradiated samples (WT and Y81W). A separate fraction of poorer quality from both strains was also concentrated in the same manner to 5 mL each. These poorer quality fractions were also complexed according to the same method stated above. However, these assays used 2.3

mL (3 mg) of the respective SMT sample and an adjusted amount of components. There were also four different assays using these poorer quality samples - 2 radiated samples (WT and Y81W) and 2 nonradiated samples (WT and Y81W). These samples were stored at -20°C and not used further in the tryptic digest study. This step was to ensure that usable material would remain if any mistakes were made.

*2.3.3 Tryptic Digest of Complex Samples***.** Using the four samples of good quality and a Slide-A-Lyzer cassette with a 10 kDa cutoff, both the non-radiated and radiated samples were dialyzed to exchange the buffers. The buffers were exchanged from a Tris+D to a 50 mM AMBIC buffer (ideal for tryptic digest). This step was performed by first washing each membrane (4 – radiated and non-radiated for each sample) with 5 mL of 50 mM AMBIC buffer in order to check for leaks and prepare the membrane for dialysis. The sample from each complex assay was inserted via a syringe inside the membrane. The entire membrane apparatus, including a foam piece that helps it float, was placed into a liter beaker containing 500 mL of 50 mM AMBIC buffer and a magnetic stirring rod. The beaker with everything inside was placed on a magnetic stirrer, and the membrane allowed to spin. Every three hours, the 50 mM AMBIC buffer was changed with fresh buffer twice. The third fresh change of buffer was kept for another three hours. The trypsin used for these digestions were of proteomics grade and bought from Sigma. For tryptic digestion to be in optimal conditions, the ratio of substrate (SMT) to enzyme (trypsin) must equal 20. With 10.5 mg of SMT enzyme used in each complex assay, this ratio was used to calculate that 0.55 mg of trypsin is needed for each tryptic digestion (5 total – 4 complex assays and a negative control containing only trypsin). For 5 tryptic

digestion assays, a total of 2.75 mg trypsin was taken and dissolved in 50 μL of 50 mM acetic acid. To each complex solution (radiated WT and Y81W and non-radiated WT and Y81W, approximately 5 mL in each solution) and negative control (5 mL of 50 mM AMBIC buffer), 10 μL of the dissolved trypsin solution was added. The assays were incubated in 50 mL Falcon® tubes placed in a water bath with a temperature of 37°C and shaken at 100 rpm for 8 hours.

2.3.4 H_2O_2 Digestion. After the trypsin digestion, a hydrogen peroxide (H_2O_2) digestion assay was performed, using an 18% polyacrylamide gel, on various stages of the process. Stages analyzed with this assay were the steps: concentrated sample before complexing, before digestion, after digestion, and the negative control after complexing. Both radiated and non-radiated samples were placed on their separate 18% polyacrylamide gel along with a low range and polypeptide molecular weight standard. The electrophoresis conditions were set at 100V for 2 hours. Non-radiated samples underwent SDS-PAGE analysis to compare with the radiated gel to confirm that both samples were digested the same. After the radiated gels were stained for 30 minutes and destained for 2 hours, 2 mm bands were cut from the entire length of the digested sample well. The undigested, complexed band at 43 kDa was also cut out in its separate well. Each band was cut into smaller pieces and place into their respective scintillation vial. To each scintillation vial, 1 mL of 50% H_2O_2/ddH_2O solution was added. Each vial was capped and placed in a water bath set at 70°C and shaking at 80 rpm. After 20 hours, 5 mL of PerkinElmer Optiphase 'HiSafe' 3 scintillation cocktail was added to each vial, vortexed for 30 minutes with the cap on, and the radioactivity counted. This entire H_2O_2 digestion assay

was repeated again; however, in the second attempt, the electrophoresis conditions were at 80V for 2 hours and the digested, complexed sample well was cut into three broad bands. The top band contained the 43 kDa band, the second band was the middle of the gel, and the bottom band consisted of the rest of the gel, including the portion below the coomassie blue stain line.

*2.3.5 RP-HPLC Analysis of the [3-^{3}H] 26, 27 DHZ Bound Peptide Fragments***.** The digested, complexed fraction from all four samples was filtered using a Centricon filter device with a 10 kDa filter size. The pass through (peptides < 10 kDa) and top layer (peptides > 10 kDa) samples were blown down via N_2 gas. After this step, the radiated WT and Y81W pass throughs had a volume of 290 μL and 300 μL, respectively. From this volume, 50 μL of each strain were loaded onto a Vydac C18 column attached to a HPLC system with a flow rate of 0.5 mL/min. The solvents used in this chromatography work were A.) 0.1% TFA in dd$H_2$0 and B.) 0.1% TFA in ACN. The sample was eluted by a linear gradient of 1-99% solvent B. The RP-HPLC chromatograms of the two different strains of SMT-26, 27 DHZ complexes were achieved by an Agilent 1100 series HPLC system with a diode array detector set at 15°C, and signal detected at 210 nm. Fractions were collected in 0.5 mL volumes every minute. Detailed protocols on the resolution of tryptic-digests using RP-HPLC on C_{18} Vydac^{TM} column has been reviewed by Matsudaira *et al*.[28] The radioactivity of each fraction was then counted in the normal method. This data pinpointed the minutes where radioactivity was the highest and, therefore, when the possible [3-^{3}H] 26, 27 DHZ bound peptide eluted. The non-radiated pass through of the WT (310 μL) and Y81W (310 μL) samples were then added to their

respective radiated pass through to increase the signal output on the HPLC. For each strain, 95 μL aliquots were loaded onto the same HPLC column until all of the volume for each respective strain was has expired. Minutes 30-45 were collected, pooled, and volume reduced by blowing down the liquid with N_2 gas.

2.3.6 Saponification of [3-^{3}H] 26, 27 DHZ Bound Peptide Fragments and RP-HPLC Analysis of the Sterol Products. To the volume blown down for each strain (1.8 mL – WT and 2 mL – Y81W), an equal amount of 10% KOH MeOH was added. Each solution was heated in a water bath at 85°C for 15 minutes. Next, hexane and ethylacetate extractions were performed to obtain the hydrophobic sterol that was bound to a peptide fragment. A 4 mL portion of hexane was added to both solutions, vortexed for 30 seconds, and centrifuged for 30 seconds at 10, 000 rpm. The top (organic, hydrophobic) layer was extracted using a 1000 μL micropipette. This step was repeated twice more with hexane and another 2 times with ethylacetate. All of the extractions for each respective strain was collected, pooled, and dried under N_2 gas. After drying, the sample of each strain was resuspended in 250 μL of MeOH. Of this volume 15 μL went to radioactivity counting and the remainder (235 μL) was blown dried with N_2 gas to 75 μL. 70 μL of each strain and of pure 26, 27 DHZ (1.5 mg/mL) and cholesterol (1 mg/mL) were then loaded, in separate runs, onto a TSK-gel S-C18 column connected to a HPLC system with a flow rate of 1.0 mL/min. DHZ and Cholesterol were put onto the column as standards for comparison. For each strain, the fractions were collected every minute (1 mL fractions). Each fraction was blown dried by N_2 gas, and their

radioactivity counted by normal means. Quantificational analysis on key steps along the chemical affinity labeling study was taken via the A_{280} method to tract the flow of protein concentration.

2.3.7 Analysis of the Top Layer (peptides > 10 kDa). For each strain, the top layer (peptides > 10 kDa) from the Centricon filtration step also underwent this HPLC analysis. First, H_2O_2 digestion assays were performed, as directed above, on this top layer of the radiated complexes (WT and Y81W) using a 20% polyacrylamide gel. A 50 μL portion of the top layer was placed on the same Vydac C18 column, under the same conditions, to determine the elution profile of the top layer for each strain. The 0.5 mL fractions were collected, and their radioactivity counted in the normal method. The two radiated strains were saponified, as previously outlined, separately. The extractions were collected, pooled, and blown dried with N_2 gas. For each strain, a portion was set aside to be counted for radioactivity, and the remaining sample was loaded onto the TSK-gel S-C18 column connected to a HPLC system with a flow rate of 1.0 mL/min. The 1 mL fractions that were collected every minute were counted for radioactivity adhering to the normal method. Quantificational analysis on key steps along the chemical affinity labeling study was taken via the A_{280} method to tract the flow of protein concentration.

2.4 IMMUNOBLOTTING ANALYSIS OF THE Y81W MUTANT SMT ENZYME

*2.4.1 Antibody Purification***.** The antibody purified is polyclonal and specific to the *S. cerevisiae* yeast SMT enzyme. Invitrogen Life Technologies grew the antibody for our lab. They produced the antibody by injecting rabbits with the yeast SMT enzymes over a span of two to three months. In that span, the rabbits created antibodies in response to the SMT protein circulating through their blood. A blood sample was taken and the antibody was isolated, placed in vials, and shipped to our lab on October 13, 2004. The vials, containing 19 to 48 mL of antibody samples, were stored at -20ºC. The purification of the antibody serum from Invitrogen was performed using the Bio-Rad® Econo-Pac® Serum IgG Purification Kit. Starting with the Econo-Pac 10 DG desalting column, the buffer was discarded from above the top frit. 20 mL of Rabbit Application buffer was added to the top of the desalting column and allowed to flow through the column. A 2 mL fraction of the antibody serum created by Invitrogen, in addition to 1 mL of rabbit application buffer, was first put onto the desalting column. The first 3 mL of the flow through were discarded. Four mL of rabbit application buffer were added to elute the serum from the column. The next 4 mL of flow through was collected for IgG purification. The buffer above the top frit of the Econo-Pac serum IgG purification column was discarded. The IgG column was washed with 40 mL of regeneration buffer and then equilibrated with 40 mL of Rabbit Application buffer. The flow through from the desalting column was then placed on the IgG column. The first four mL of flow through was discarded. The sample was then eluted with 22 mL of Rabbit Application buffer. The flow through was collected in 2 mL fractions. This process was repeated three more times. Eleven fractions were collected on the first and second purification

runs. Ten and eight fractions were collected on the third and fourth runs, respectively. The fractions, held in 13x100 glass tubes and covered in Para film, were stored at 4ºC. A Bradford Protein Quantification Assay was performed on the fractions from all four runs through the purification columns. Assuming that fractions 1-3 of the runs 2-4 would coincide with that of fractions 1-3 of the first (0.00 mg/mL), those fractions were not analyzed. From the information presented by the Bradford Protein Quantification Assay, fractions 9,10, and 11 from Run 1, fractions 5, 6, 9, 10, and 11 from Run 2, fractions 7, 8, and 9 from Run 3, and fractions 5, 6, 7, and 8 from Run 4 were checked for purity with a 12% SDS-PAGE gel. SDS-PAGE electrophoresis was carried out according to Laemmli[29]. Fractions surrounding the purest fractions (fractions 5, 6, 7, 8 of Run 1, fractions 3, 4, 7, 8 of Run 2, fractions 5, 6, 10, 11 of Run 3, and fractions 3, 4 of Run 4) were also analyzed through SDS-PAGE analysis, but no significant bands corresponding to the antibody could be visualized (Data not shown). A SDS-PAGE analysis of the approximate weight of the antibody to be used in western blot studies was performed against a low range SDS-PAGE standard.

*2.4.2 Western Blot Analysis on the Y81W SMT Enzyme***.** A 12% polyacrylamide gel was used to perform a SDS-PAGE of various stages of the purification process. Ten μg of total protein of the lysate, pellet from ultracentrifugation stage, supernatant from ultracentrifugation stage, first Q-sepharose column flow through, sample that was placed on the Mono-Q, and Mono-Q purified sample was placed in their respective wells. The SDS-PAGE was run at 120 V until the coomassie blue stain line reached the bottom of the gel. The edges of the gel was then trimmed, a nitrocellulose membrane was cut to

match the dimensions of the gel. A nick was put on the left, top corner of both the gel and the membrane to give a place of reference. The gel, membrane, 4 pieces of Waltman filter paper, and 2 thin sponges were soaked in PBS buffer for 30 minutes. The layers of materials in the clamp were put together inside the clamp in this specific order: one sponge, 2 pieces of Waltman filter paper, the gel, the membrane, the final 2 pieces of Waltman filter paper, and then the other sponge. All of the components were placed on black side of the clamp, and the clear side clamped down on the membrane side, not the gel side, of the clamp. The nicks in the membrane and gel were made sure to align properly in the clamp. A protein transfer from the gel to the membrane was completed in a 1xPBS buffer using a Bio-Rad Mini Trans-Blot® Cell set at 60 V for 90 minutes. After the Y81W mutant SMT was transferred to the membrane, it was soaked in PBST buffer twice for 5 minutes each. The membrane was then incubated on a slow rocker in 3% blocker solution for 1 hour. The membrane was taken out of the blocking solution and wash twice with PBST solution for 5 minutes each time. It was incubated again with the primary SMT antibody, appropriately diluted in the antibody dilution buffer for 1 hour. Again, the membrane was washed with PBST in the same manner described above. Next, it was incubated with 1:3000 dilution of GAx-HRP secondary antibody for another hour. PBST was used to wash the membrane. The membrane was incubated in the Bio-Rad Amplification Reagent for 10 minutes. At this step, the membrane was then washed three times, five minutes each time, in 20% DMSO/PBST. It was then washed with PBST in normal fashion. The 1:1000 dilution of streptavidin-HRP was used to incubate the membrane for 30 minutes. It was washed again in PBST buffer. The final step of the western blot was to incubate the membrane in the Opti-4CN substrate solution until the

desired color intensity was viewed (approximately 3-5 minutes). The membrane was washed in ddH_2O for 15 minutes and then air-dried. It was put into an empty agar plate for storage.

2.5 X-RAY DIFFRACTION ANALYSIS ON THE YEAST Y81W MUTANT SMT ENZYME

*2.5.1 Buffer Comparative Analysis***.** The Y81W mutant SMT enzyme was tested in three different buffers (Tris, potassium phosphate, and HEPES) to determine which buffer contributed to the best activity of the enzyme. This comparison was performed by doing an activity assay on the supernatant obtained from the ultracentrifugation step of the purification in each of the three buffers. A Bradford method quantificational analysis was done on each of the buffer's supernatant in order to add the same amount of protein to each activity assay. For each buffer system, a 25 and 100 μM (final concentration) of zymosterol was tested. In a 13x100 mm glass tube, the appropriate amount of zymosterol was added to 12 μL of 5% Tween 80, vortexed, and blown dry with N_2 gas. To each tube (9 total – 0, 25, and 100 μM zymosterol for each buffer), 0.5 μM of Y81W mutant SMT supernatant from each buffer system was added to their respective 25 and 100 μM zymosterol tubes. 100 μL of 50 μM at 0.6 μCi [^{3}H] AdoMet (three different cocktails diluted with the three different buffers) was added to their respective buffers' tubes, as well. The volume of each assay was adjusted to 600 μL with the corresponding buffers. The blank tubes for each buffer contained no zymosterol (0 μM). The assay was performed in a water bath at a temperature of 35°C and gently rocked for 45 minutes.

The assay was immediately quenched with 10% KOH MeOH by adding 600 μL of the solution to each tube. The tubes were then place in another water bath with a temperature of 85°C for 15 minutes. A hexane extraction was performed by adding 2.5 mL of hexane to each tube, vortexing for 30 seconds, and centrifuging at 10, 000 rpm for 30 seconds. The top (organic, hydrophobic) layer was collected by a 1.0 mL plastic pipette and placed in a scintillation vial (one vial per tube). To these vials, 5 mL of scintillation fluid was added. The vials were capped, vortexed for 30 seconds, and the radioactivity counted for each vial. A Beckman LS 6500 Liquid Scintillation Counter took radioactivity measurements. 5 mL of ScintiVerse® BD cocktail, FischerChemical, was used in each assay, unless stated otherwise, and were automatically quenched correctly. This procedure was repeated, but the next vials contained two hexane extractions from the same, corresponding assay tubes. The volume was allowed to air-dry overnight. Again, 5 mL of scintillation fluid was added to each vial. The vials were capped, vortexed for 30 seconds, and placed in the scintillation counter for the radioactivity to be counted. The two sets of data obtained were extrapolated to give approximate K_{cat} values for the Y81W mutant SMT enzyme in each buffer system. This process was repeated in HEPES buffer with Y81W samples of the lysate, supernatant, 0.15 M, 0.165 M, and 0.18 M purification steps before complexing the enzyme with 26, 27 DHZ. The DPM counts were converted to pmol/min by dividing the DPM counts by 22.2. This comes from the relationship that 1 μCi equals 2.22 x 10^6 DPM, which makes 1 pmol of 10 μCi/μmol (concentration of the second cocktail) [*methyl* $^3H_3$] AdoMet to be equivalent to 22.2 DPM. This number can further by divided by protein concentration of the sample to give the V_{max} value of pmol/min/mg.

*2.5.2 Purification of the Yeast Y81W Mutant SMT Enzyme for X-Ray Diffraction***.** The protocol for purifying the SMT enzyme from taking the Y81W mutant 5 g cell pellet and lysing the cells to the stage where the two Q-sepharose columns were employed was the same as in the previous section. However, to purify the necessary amount for X-ray diffraction, three 5 g cell pellets of the Y81W SMT host cells, grown in M9 media, was purified. From the data obtained from the SDS-PAGE analysis, the 16.5% and 18% fractions of all three purification runs were combined and concentrated to 20 mL using a 50 mL Amicon® ultrafiltration system. The sample was then wash once with 20 mL of Qa+D buffer. Final volume of the sample after washing once and concentrating was 20 mL. 40 mL of Qa+D buffer was added to this volume, and three 20 mL portions were placed in their respective 125 mL Erlenmeyer flask. Next, the concentrated sample was complexed with the inhibitor 26, 27 Dehydrozymosterol (DHZ) in a one to five ratio, respectively. S-Adenosyl-L-Methionine (AdoMet) at a concentration of 6mM was also used in a one to five ratio to the amount of Y81W mutant SMT enzyme, i.e. 12.5 g of SMT enzyme was concentrated with 0.143 mg of 26, 27 DHZ and 160 µL of AdoMet (Calculation was done from one 5 g cell pellet). In a 13x100 mm glass tube, the desired amount of the 26, 27 DHZ, taken from the stock solution, was first added to 160 µL of 5% Tween 80 in ethanol to help better dissolve the inhibitor in the SMT solution. After the combination of these two components were blown down via N_2 gas, 2 mL of Qa+D buffer was used to wash the inside of the glass tube and this liquid was distributed in one of the 125 mL Erlenmeyer flask containing a 20 mL portion of the purified Y81W mutant SMT enzyme. To this flask, the AdoMet was also added. The flask was covered in parafilm and incubated at 37°C and 185 rpm for 4.5 hours. This process was repeated

twice more with the other flasks containing purified Y81W mutant SMT enzyme. After incubation, all three flasks were combined, washed two more times with Qa+D buffer, and concentrated to 10 mL using the 50 mL Amicon® ultrafiltration system. The amount of enzyme was tested again with a Bradford and A_{280} assay. At 2 mL portions, the sample was put on the Mono-Q column using the same FPLC system and under the same parameters depicted in the previous section (total of 5 purification runs). Verification of the purity of the enzyme was also done in the same manner as described in the previous section. A second stage of Mono-Q purification was also ran on the impure fractions (from SDS-PAGE analysis, 43 kDa band with the break-down product 40 kDa band, but no more impurities). All pure fractions were combined, washed three times with Qa+D, and concentrated to 2 mL using the 50 mL Amicon® ultrafiltration system. A buffer exchange was performed on this sample with 25 mM HEPES buffer with 0.2% B-D-G using a Centrifugal filter device. The resulting buffer with the Y81W mutant SMT enzyme had one final A_{280} assay performed on it before it was sent for X-ray Diffraction analysis.

*2.5.3 Crystal Formation and X-ray Diffraction Analysis***.** The purified sample was sent to the laboratory of Dr. D.W. Christianson at University of Pennsylvania for crystal formation and X-ray diffraction analysis conducted by Dr. Luigi Di Costanzo. The complex between SMT-Xxx and zymosterol was crystallized by the hanging drop vapor diffusion method at 277 K. An initial Index Screen (Hampton Research) identified at least twelve different conditions under which microcrystals appeared in about one week. In particular, because of the formation of fewer nuclei and bigger crystals, one condition

was most suitable for growing larger crystals. Drops containing 3.0 μL of enzyme solution [5.0 mg/mL SMT, 0.2% BDGP, 50.0 mM HEPES (pH 7.5)] and 3.0 μL of precipitant buffer [0.1 M Sodium Formate, 10-20% (w/v) PEG-3350] were equilibrated against a 1 mL reservoir of precipitant buffer. This crystal screen was designed as a 96 reagent crystallization screen than combined the strategies of the Grid Screen, Sparse Matrix, and Incomplete Factorial with classical, contemporary, and new crystallization reagent systems into a highly effective and efficient format. Prior to X-ray diffraction data collection, crystals were cryoprotected in mother liquor containing 28% glycerol prior to flash-cooling. Analyzing the crystals was performed at the Brookhaven National Laboratory (Upton, NY) on beamline X29A (l=1.000 Å, 100 K) using an ADSC Quantum 315 detector. Data reduction was achieved with HKL2000 and data were scaled with Scalepack. Crystals belong to space group C2 with unit cell parameters of a= 122.3, b=68.6, c=127.2 Å (Beta angle=111.28 degree).

CHAPTER III
RESULTS AND DISCUSSION

3.1 STEROL METHYLTRANSFERASE PURIFICATION

In the purification of WT and Y81W sterol methyltransferase enzymes, two modifications were implemented to help increase the yield at the end of the process. The first addition to the purification protocol that had been established by Dr. W.D. Nes and coworkers was the use of Gel Permeation Chromatography (GPC) after the first anion-exchange Q-sepharose column stage. It was thought that this step would help discard any extremely massive or small proteins that might elute with the same polarity as the SMT enzyme in the Q-sepharose columns. It was also thought that decreasing the protein load on the Q-sepharose (Mono-Q) columns connected to the FPLC system would help sustain the productivity of the column. However, this modification did not prove fruitful because the purity of the SMT sample did not improve to the degree as first predicted. As proven through SDS-PAGE analysis, the purity of the SMT enzyme after the GPC stage was not significantly increased as compared to the sample that was placed on the GPC column (Figure 3.11). It also increased the time necessary to obtain a pure sample by three days due to concentrating and washing the sample before and after the GPC step. In purifying the Y81W mutant SMT enzyme for X-ray Diffraction, time is critical with the native conformation and activity decreasing with every day. The GPC step was intended to be placed before complexing SMT to 26, 27 DHZ. Due to the placement of the step and that no real advancement in yield or purity of the enzyme was achieved, the GPC step was discarded. One positive result from the GPC studies of the Y81W mutant SMT enzyme was that the molecular weight, as predicted by a GPC standard graph, coincided closely

to the weight of the WT SMT enzyme found through the same studies[24] (Y81W – 170 kDa, WT – 172 kDa; 1% Difference) (Figure 3.12). The next modification that was implemented was the expansion of the NaCl gradient used in the first anion-exchange Q-sepharose column stage. The previous protocol instructed that the gradient have 0.1 M, 0.2 M, 0.3 M, and 1.0 M NaCl steps in the second anion exchange Q-sepharose column (equilibrated with 0.4% detergent). In this version, the SMT enzyme eluted in the 0.2 M step. The revised gradient was expanded to include the following steps in the same column: 0.1 M, 0.15 M, 0.165 M, 0.18 M, 0.2 M, 0.225 M, and 1.0 M NaCl. This expansion helped us pinpoint the elution better while cleaning the protein further without losing any significant time. With the introduction of this gradient system, the protein eluted with the 0.165 M and 0.18 M steps, mostly in the former step in the Y81W SMT sample (Figure 3.13). Coinciding with the information stated above, the elution profile matches that of earlier work in the Nes lab. Before there was no NaCl elution steps in between that of 0.1 M and 0.2 M, allowing all of the protein to elute in the 0.2 M NaCl step. However, with the advent of the three elution steps in between the previous steps, the protein can elute in a more specific region of polarity. This result led to a slightly purer sample of SMT enzyme. Utilizing SDS-PAGE analysis, the protein was at best 90% pure after this stage of purification. With the same SDS-PAGE analysis, it was also determined that the molecular weight of the monomeric unit of the Y81W SMT enzyme corresponded with the WT SMT enzyme (43 kDa).[12] During the purification process of both the WT and Y81W mutant SMT enzymes, the chromatographic properties of both strains were investigated using the Q-sepharose (Mono-Q) column connected to the FPLC system. On this column with the same parameters, the retention times of the WT

and Y81W SMTs both came off in the 0.2 M NaCl elution step within 8 minutes of each other (WT – 79 min, Y81W – 87 min) (Figures 3.14, 3.15). Even though the two SMTs eluted in the same gradient step, they eluted in slightly different retentions times. The WT and Y81W samples eluted slightly differently in the second anion-exchange Q-sepharose column with the WT coming off mostly in the 0.18 M NaCl step, and the Y81W SMT coming off mostly in the 0.165 M NaCl step (Data not shown). However, in both samples, the elution came in both gradient steps. Nevertheless, with this observation and the data obtained from the FPLC column, it was suggested that the point mutation might force the mutant SMT to possess different chromatographic properties and, therefore, perhaps different conformational shapes. This data led to an experiment of combining previously purified WT and Y81W SMTs (1mg of each sample), and load the combination onto the same column with the same parameters. The resulting chromatogram showed a co-migration where the two enzymes eluted out of the column at the same time of 79 minutes in the 0.2 M NaCl gradient step (Figure 3.16). This co-migration disproves the idea that the two different enzyme strains have different chromatographic properties. This suggests that the conformations of the two different SMTs are either identical or so similar that no difference can be discerned through chromatographic means of investigation. This conclusion is backed by previous Circular Dichroism (CD) work on both strains of SMTs. This work showed that the secondary structure of the Y81W and WT SMT enzymes was in good agreement with each other.[24] The data showed little difference in the far ultra-violet circular dichroism spectrum. This region can calculate the percentage of the amino acids in each specific secondary structure (i.e. α-helix, β-sheets, and random coils). CD Data for the Y81W mutant SMT

enzyme has not been published. Also, CD data from the soybean WT and Y83F mutant SMT enzymes show no significant difference in the same region of the spectrum[30]. Due to a difference of nomenclature, the amino acid position 83 in the soybean SMT is equivalent to that of amino acid position 81 in the yeast SMT. This research also was also intended to show that no difference was discovered by changing the native aromatic amino acid with a different aromatic amino acid, as seen in the Y81W mutant SMT enzyme. Using data obtained from other previous studies, a spatial arrangement of the secondary structure was developed with respect to interaction with a sterol substrate and AdoMet (Figure 3.17).[31] Other chromatography work done with the FPLC was to obtain a correlation between peak height and amount of protein in pure samples of Y18W mutant SMT. Peak height was determined by measuring the vertical distance from the horizontal line where the chromatogram started to the exact location where the chromatogram comes to a point in the SMT peak. This study showed a direct link between the peak height and how much protein was in the fraction pertaining to the peak. The peak fraction contained 34% of the Y81W SMT purified in each respective Mono-Q purification run. This connection was continued to how much protein was actually loaded onto the column with respect to the peak height (Figure 3.18). If 3 mg of SMT enzyme sample from the second Q-sepharose column purification stage is loaded onto the Mono-Q column, it will produce a SMT peak approximately 60 mm high. Also, the total amount of SMT (fractions containing either only the 43 kDa band or the 43 and 40 kDa bands) obtained from one purification process through the Mono-Q stage was 15.91 mg by A_{280} method. This amount is 85% of the amount loaded onto the Mono-Q column of 18.73 by A_{280} method. This finding agrees with the visual inspection of SDS-PAGE

analysis that also showed that the SMT sample to be loaded onto the Mono-Q column to be of 85% purity. Another means of tracking the progression of active Y81W SMT was performing a Western Blot on important stages of the purification. Before a Western Blot could be performed on the Y81W mutant SMT, the SMT-antibody needed to be purified and quantified (Figure 3.19). The antibody was also ran against a marker to approximately determine its mass of ~50 kDa (see Appendix Figure 7.1). One question that was addressed in this research was to know if there was any active Y81W mutant SMT being left in the pellet after the 100,000xG stage. With this immunoblotting technique, a polyclonal antibody specific to the yeast SMT was used to find any trace of SMT in each specific purification step (Figure 3.110). It was found that there was a small amount of SMT in the pellet, but again, the time necessary to attain this insignificant amount would be damaging to the main objective of obtaining the largest amount of active SMT enzyme possible in the shortest period of time. Also with my efforts of purifying the Y81W mutant SMT, discovered that the Bradford Method of quantification was drastically over-calculated as compared to the more reliable method of A_{280} quantification. Our enzyme is rich in aromatic amino acids, and, therefore by using this region of the spectrum that is most susceptible in detecting aromatic amino acids, the amount of enzyme can easily be calculated using the Beer-Lambert Law. The result of comparing the two methods came to be that the Bradford method was a 40% overestimation to that of the A_{280} method. This finding is in agreement with that of some previous work by Stoscheck and coworkers. They found that the Bradford method is extremely sensitive to Bovine Albumin Serum (the standard first used in this research). It was discovered that the increased sensitivity was as large as a factor of 2 compared to

"average" proteins.[32, 33] In response to this data, I formulated a purified Y81W mutant SMT Bradford Method dose response curve for future work on this enzyme (see Appendix Figure 7.2). This standard should give a better approximation of the amount of enzyme using the Bradford method. An independent verification of the Nes lab spectrophotometer was conducted with the Nanodrop ND-1000 spectrophotometer (Experimental Sciences Building, Texas Tech Univ., Lubbock, TX). A Y81W mutant SMT sample was previously purified and found to have a concentration of 1.03 mg/mL with the Nes Lab spectrophotometer. Testing the same sample with the Nanodrop instrument, the concentration increased 14% to 1.2 mg/mL (see Appendix Figure 7.3).

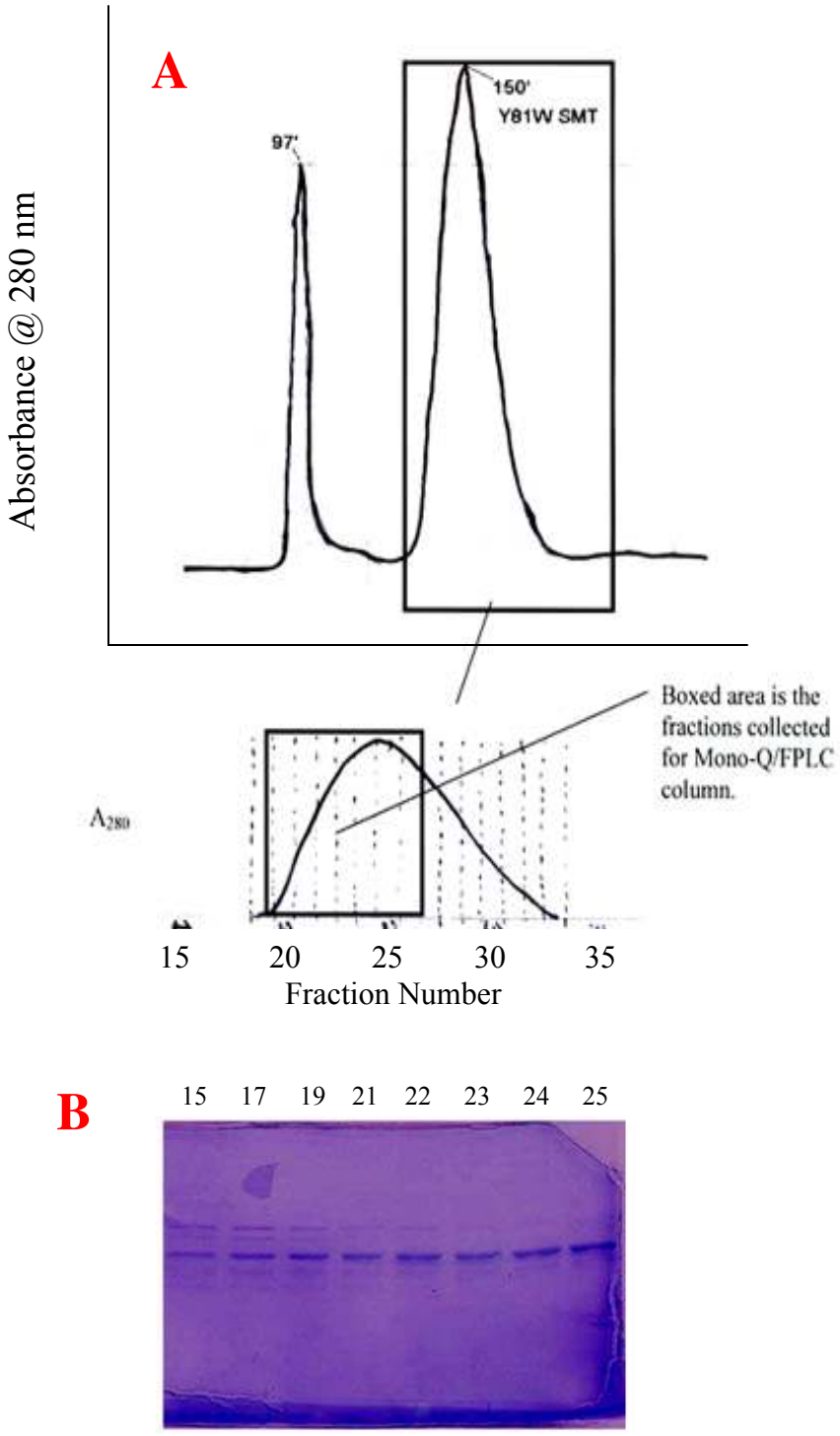


Figure 3.1.1. A. Gel Permeation chromatogram of Y81W mutant SMT enzyme. GPC was performed on a HiPrep™ 26/60 Sephacryl™ S-300 GPC column connected to a FPLC system at a flow rate of 1 mL/min, and sensitivity at 0.1. By Bradford Method, a total of 3 mg of Y81W mutant SMT in a volume of 3 mL were loaded onto the column. **B.** 12% polyacrylamide SDS-PAGE gel of eluted fractions from the GPC purification. Fraction numbers are indicated above the gel.

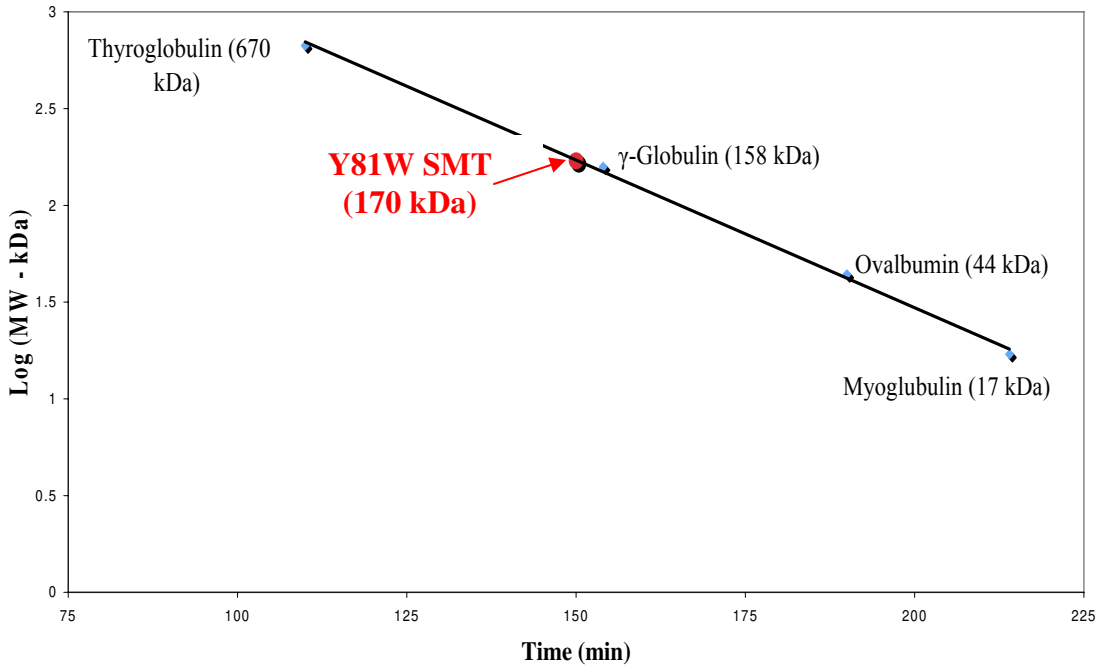


Figure 3.1.2. Calibration curve of the molecular weight standards for the gel permeation chromatography obtained by plotting logarithmic molecular weight against the elution time. Molecular weights used are as labeled on the curve. Note by using the curve, the molecular weight of the Y81W mutant SMT is 170 kDa.

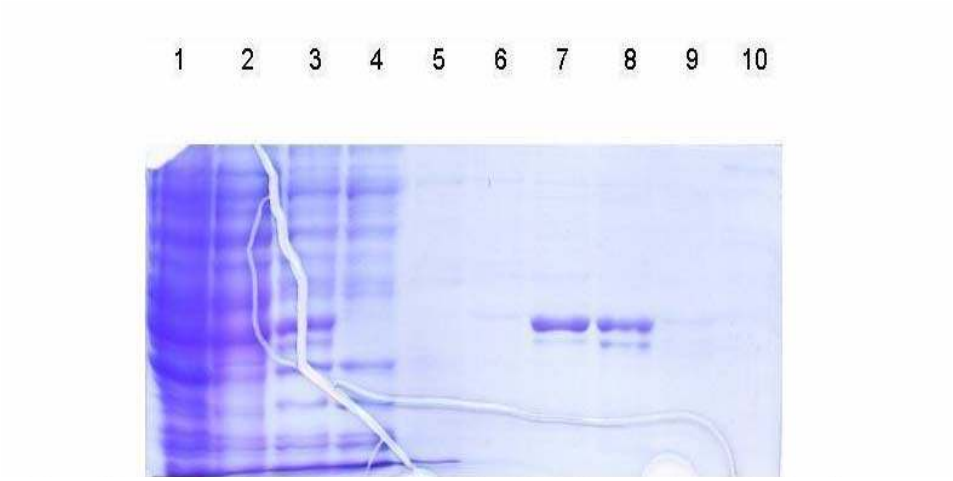


Figure 3.1.3. 12% polyacrylamide SDS-PAGE gel of the first two Q-sepharose column fractions from the Y81W mutant SMT. The gel is Coomassie-blue stained. The columns were equilibrated with Qa buffer (as described in Material and Methods Chapter). Lane 1 – Lysate, Lane 2 – Supernatant, Lane 3 – 1st Q-sepharose column pass through, Lane 4 – 2nd Q-sepharose column pass through, Lane 5 – 0.1 M NaCl Qa+D, Lane 6 – 0.15 M NaCl Qa+D, Lane 7 – 0.165 M NaCl Qa+D, Lane 8 – 0.18 M NaCl Qa+D, Lane 9 – 0.2 M NaCl Qa+D, Lane 10 – 0.225 M NaCl Qa+D. According to Bradford Method, 4.0 µg of 0.165 M NaCl Qa+D fraction and 4.71 µg of 0.18 M NaCl Qa+D fraction were loaded onto their respective well. The migration of the 0.165 and 0.18 M NaCl Qa+D fraction coincides with a 43 kDa size protein and the monomer of the Y81W mutant SMT with a slight 40 kDa band in co-migration.

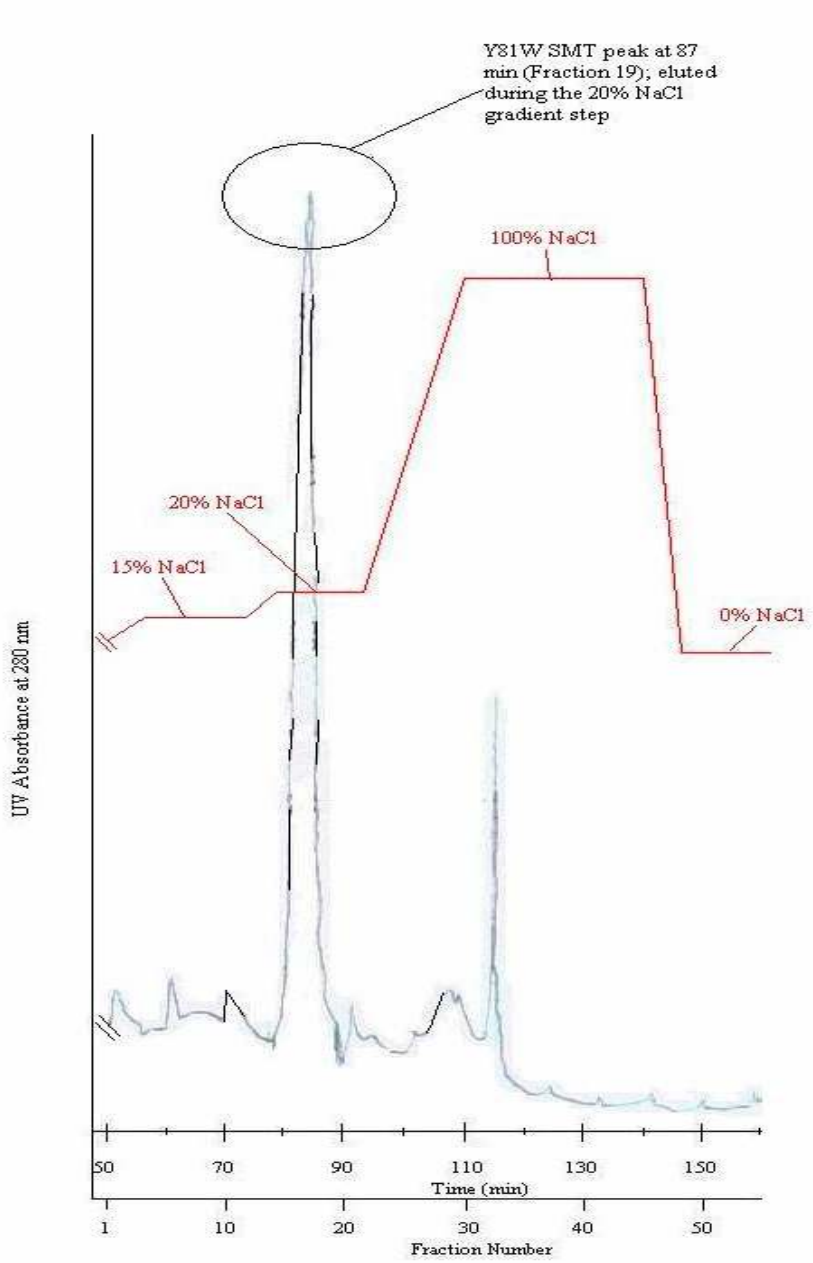


Figure 3.1.4. FPLC chromatogram from the uncomplexed Y81W mutant SMT sample. The column used was a Q-sepharose (Mono-Q) column made by Pharmacia Biotech®, vol. of 8.0 mL. The column was equilibrated with Qa+D buffer (as described in the Materials and Methods Chapter) and controlled by a Fast Protein Liquid Chromatography (FPLC) system made by Pharmacia LKB®. The column had the parameters of a Range of 0.2, flow rate of 1.0 mL/min, and a recorder speed of 0.5 mm/min. According to Bradford quantitation analysis, 6 mg of the uncomplexed Y81W sample in 1.7 mL Qa+D buffer (<0.02 M NaCl) was loaded onto the Mono-Q column. The uncomplexed Y81W SMT had a peak that eluted at 87 minutes and in the 0.2 M NaCl gradient step. Gradient is depicted in red.

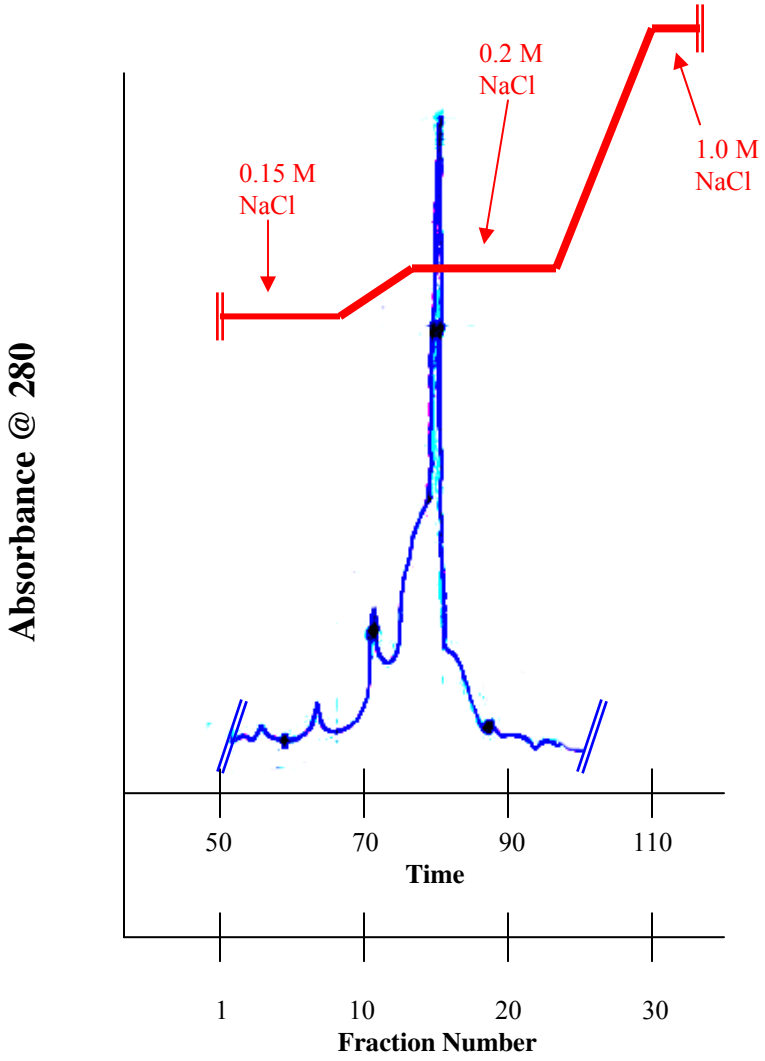


Figure 3.1.5. FPLC chromatogram from the uncomplexed WT SMT sample. The column used was a Q-sepharose (Mono-Q) column made by Pharmacia Biotech®, vol. of 8.0 mL. The column was equilibrated with Qa+D buffer (as described in the Materials and Methods Chapter) and controlled by a Fast Protein Liquid Chromatography (FPLC) system made by Pharmacia LKB®. The column had the parameters of a Range of 0.2, flow rate of 1.0 mL/min, and a recorder speed of 0.5 mm/min. According to Bradford quantitation analysis, 3 mg of the uncomplexed WT sample in 2.0 mL Qa+D buffer (<0.02 M NaCl) was loaded onto the Mono-Q column. The uncomplexed WT SMT had a peak that eluted at 79 minutes and in the 0.2 M NaCl gradient step. Gradient is depicted in red.

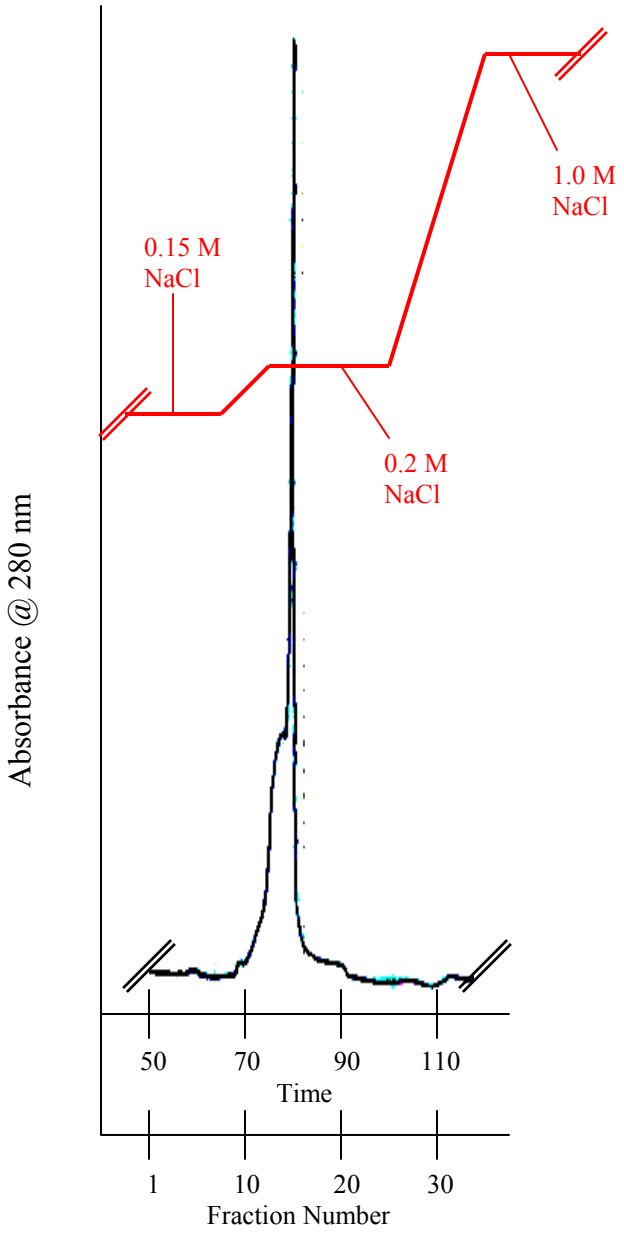


Figure 3.1.6. FPLC chromatogram from the uncomplexed WT and Y81W SMT sample. The column used was a Q-sepharose (Mono-Q) column made by Pharmacia Biotech®, vol. of 8.0 mL. The column was equilibrated with Qa+D buffer (as described in the Materials and Methods Chapter) and controlled by a Fast Protein Liquid Chromatography (FPLC) system made by Pharmacia LKB®. The column had the parameters of a Range of 0.2, flow rate of 1.0 mL/min, and a recorder speed of 0.5 mm/min. According to A_{280} quantitation, 1 mg each of the uncomplexed WT and Y81W combined sample in 2.0 mL Qa+D buffer (<0.02 M NaCl) was loaded onto the Mono-Q column. The uncomplexed WT+Y81W SMT co-migrated with peak that eluted at 78 minutes and in the 0.2 M NaCl gradient step. Gradient is depicted in red.

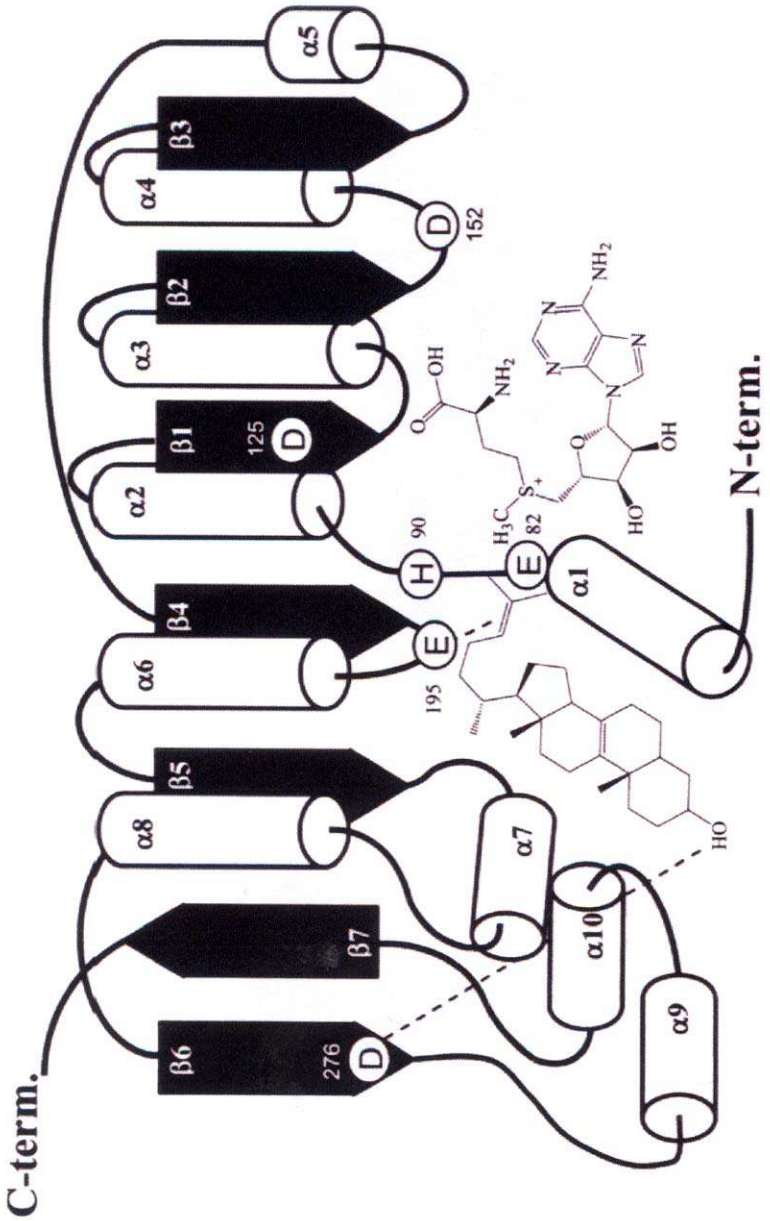


Figure 3.1.7. Schematic representation of the methyltransferase fold of *Erg6*p. Spatial arrangement of the secondary structure elements in relation to sterol and AdoMet substrates is shown along with possible contact amino acids that interact with the substrates. Adapted from Reference 31.

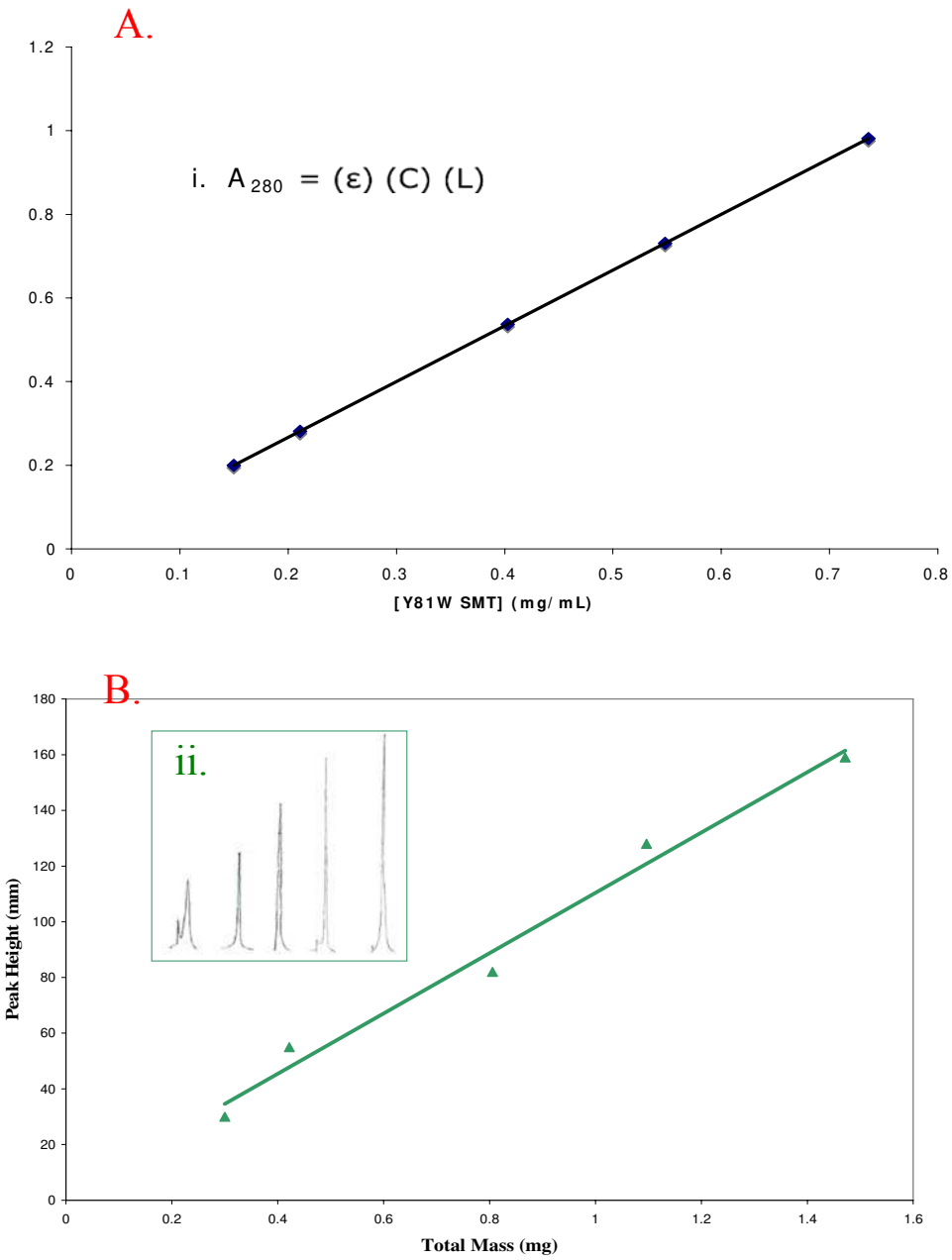
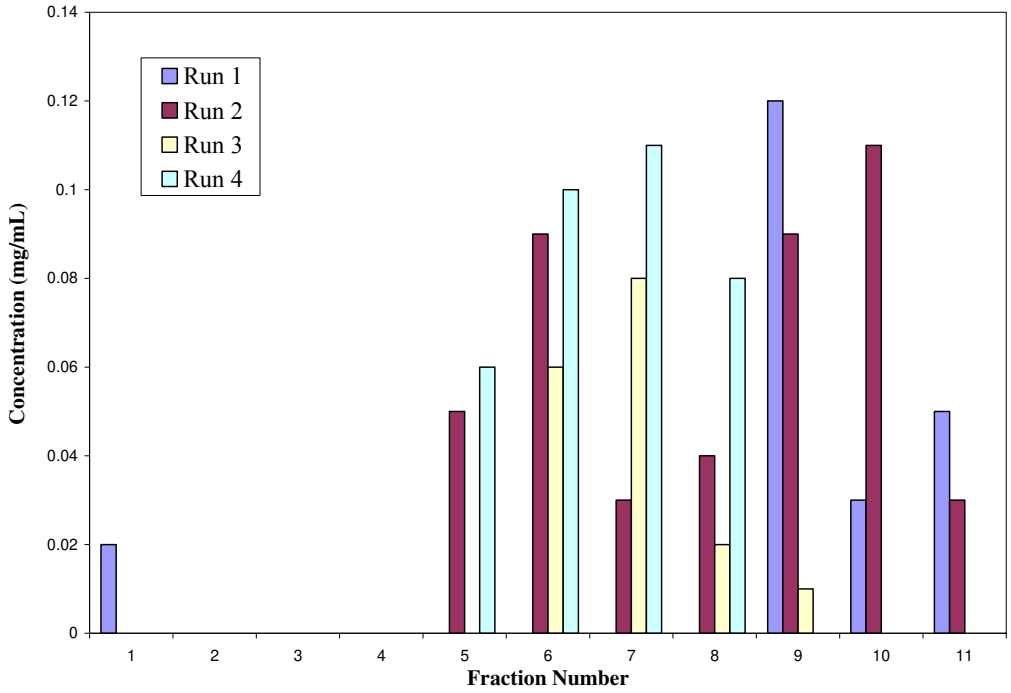


FIGURE 3.1.8. A. Chart depicting the relationship between absorbance at 280 nm and the concentration of Y81W SMT in the peak fraction. **Insert i.** Beer-Lambert Equation (A_{280} is the absorbance at 280 nm, ε is the extinction coefficient for WT and mutant yeast SMT enzymes (58,370 M^{-1} cm^{-1} or 1.36 $(mg/mL)^{-1}$ cm^{-1}), and L is the path length of the cell in the spectrophotometer (1 cm) **B.** Chart depicting the correlation of the total mass of Y81W mutant SMT in the 2 mL peak fraction to the peak height. The peak fraction represents 34% of the total Y81W SMT purified from the Mono-Q (Q-sepharose) column connected to a FPLC system. Fractions were collected in the 0.2 M NaCl gradient step at an average of 87 min. **Insert ii.** The actual chromatograms used with this study were detected at 280 nm.

A.



B.

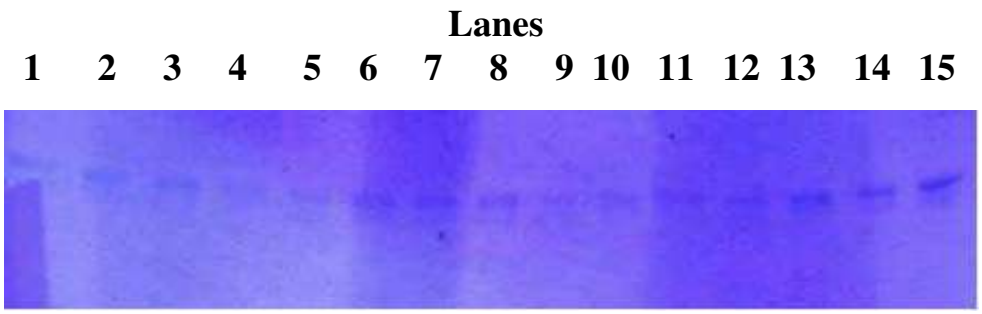


Figure 3.1.9. A. Bar graph representation of the Bradford Protein Quantitation data of the SMT-Antibody Purification. Each purification run is highlighted with its own color. **B.** 12% polyacrylamide SDS-PAGE gel of different fractions collected from the purification of the yeast SMT-antibody; Lanes 1 – 3: fractions 9-11 of Run1, Lanes 4-8: fractions 5, 6, 9, 10, 11 of Run 2 (in order), Lanes 9-11: fractions 7-9 of Run 3, Lanes 12-15: Fractions 5-8 of Run 4.

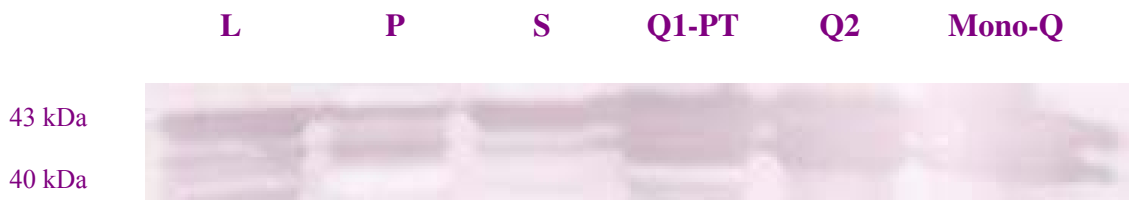


Figure 3.1.10. Western Blot analysis of Y81W mutant SMT enzyme at various key stages of the purification process. Analysis was performed with yeast SMT-antibody purified by Bio-Rad® Econo-Pac® Serum IgG Purification Kit. Actual Western Blot was achieved through the use of the Bio-Rad® Opti-4CN Kit. 10 µg of total protein was loaded per well. **L** – Lysate after French Press, **P** – 100,000xG Pellet, **S** – 100,000xG Supernatant, **Q1-PT** – first Q-sepharose column pass through, **Q2** – 0.165 M and 0.18 M NaCl fractions from the second Q-sepharose column that was loaded onto the FPLC controlled Mono-Q column, **Mono-Q** – purified sample from the FPLC controlled Mono-Q column.

3.2 CHEMICAL AFFINITY LABELING STUDIES ON WILD TYPE AND Y81W MUTANT STEROL METHYLTRANSFERASE

This study was performed in attempt to map the reaction channeling of the Y81W mutant SMT as compared to WT SMT with the mechanism-based inactivator 26, 27 Dehydrozymosterol (DHZ). 85% pure samples of both strains of SMT enzymes were used in this project. The samples were taken from the 0.165 M and 0.18 M NaCl gradient steps, and the purity was verified through SDS-PAGE analysis. In order to tract the presence of 26, 27 DHZ in the enzyme adduct and discover any new sterol product, the radioactive [3-^{3}H] 26,27 DHZ analog was employed. The radioactivity can easily be measured with a scintillation counter while not changing the chromatographic properties of the tryptic digested peptide-sterol adduct in the Vydac C_{18} column or new sterol product in the TSK-gel S-C_{18} column after saponification. The [3-^{3}H] 26,27 DHZ was also chosen to be radioactive component because any *E. coli* proteins produced from the host cells that might have co-migrated with the SMT enzymes does not use zymosterol and thusly its mechanism-based inactivator analog as substrates. As mentioned in a previous chapter, bacteria such as *E. coli* does not produce sterols and theoretically would not have any enzymes with the capability to bind to the 26, 27 DHZ. However, AdoMet is a universal methyl donor across many species including bacteria. If AdoMet was used as the radioactive component, one might believe that there would be a large possibility of background radioactivity due to interaction with bacterial proteins. To ensure how much radioactivity was used from the start of the project, a GC chromatogram and radioactivity count was taken to determine that the [3-^{3}H]26,27 DHZ had a concentration of 4.12×10^4 DPM/μg or 0.0186 μCi/μg. Therefore, using the ratio of 1:4 (SMT Enzyme:DHZ),

complexing 10.5 mg of both each respective SMT strain required 110.5 μg of [3-^{3}H] 26,27 DHZ with a total activity of 4.6 x 10^6 DPM counts for each assay. Quantitation of the enzyme was done by the Bradford method with taking in account of the 85% purity of each sample. The assay also required 1:5 ratio of SMT enzyme to non-radiated AdoMet, which resulted is using 55 μL of 6.0 mM AdoMet to each assay. An increase in SMT molar concentration compared to previous attempts was thought to increase the amount of peptides that could be used for the analysis of the exact residue covalently bound to the [3-^{3}H]26,27 DHZ (Table 3.21). This investigation into the labeled residue was not pursued due to the lack of digestion by trypsin, and the production of sterols changed as described below. After the complex assay, a partial (8 hr) tryptic digest was performed. Trypsin is an endopeptidase that catalyzes the hydrolysis of peptide bonds on the carboxyl side of the positively charged lysine and arginine amino acids. In a full tryptic digestion of the yeast SMT, there are 44 catalyzed cleavages. To examine to what extent the tryptic digest acted on each SMT enzyme; a H_2O_2 digestion study was performed on the SMT enzymes. This study did not produce any repeatable or beneficial data to show exactly where the [3-^{3}H] 26,27 DHZ bound peptide fragment was and determine the size of the fragment. An effort in altering the voltage and/or changing the time during the electrophoresis was attempted, but no clear conclusion could be made except that, comparatively, the radioactivity was present in the middle of the gel (Data not shown). This indecisive information was also fueled by the lack of radioactivity found in the gel. An approximately 0.5% sample was taken from each tryptic digested sample which should equal to 2.3 x 10^4 DPM (compared to the value complexed with the SMT). However, the largest count obtained was a full 10 fold less than that expected value. It

was decided to continue with the project with filtering the samples with a 10 kDa membrane. From previous work in the Nes lab, it was believed after an 8-hour tryptic digest, the 26, 27 DHZ bound peptide fragment was less than 5 kDa.[35] For this reason, the filtrate (peptides<10 kDa) was first analyzed with the RP-HPLC-RC. The total radioactivity count of this filtrate was only 3.7% and 2.6% of the WT and Y81W, respectively, to that of the starting count (Table 3.22). The analysis of the peptide fragments from the filtrate found that the radioactivity eluted in a single peak around the 38-minute mark in both samples (Figure 3.21). After saponification of the peptide fragments, the sterol products were extracted with hexane and analyzed. One single peak eluted at 4 minutes for both samples (Figure 3.22). Compared to the elution profile of the 1 μg/μL standard cholesterol that came off at 50 minutes, and the 1.5 μg/μL 26, 27 DHZ standard that eluted at 25 minutes, the investigated samples eluted at an extremely fast retention time. This result suggests that, in Reverse Phase chromatography where more polar substances elute in a faster time, that the sterol in both samples is the same compound due to the exact retention time, and it is more polar than either cholesterol or 26, 27 DHZ. A more polar nature possibly implies more hydroxyl groups. These hydroxyl groups are formed after the covalent bonds, more specifically ester bonds, are hydrolyzed during the saponification process. These ester bonds are believed to form between the carbocation formed during the ES* complex and negatively charged amino acids such as the acidic amino acids glutamic and aspartic acid. One specific amino acid, E68, was found to directly bind to the 26, 27 DHZ in this fashion[27] (Figure 3.23). Different formation of sterol products can be directly linked to the retention time in the RP-HPLC and different interactions within the active site of the SMT enzyme that shifts

the carbocation along the side chain on 26, 27 DHZ. Previous work done by Nes and coworkers on the Y81W mutant has already isolated two possible diol (term used to signify the existence of two hydroxyl groups located on the 3β and side chain positions) products along with the monol (term used to signify the existence of one hydroxyl group at the 3β position) turnover product formed after binding with 26, 27 DHZ (Figures 1.52, 3.24). These diols are formed through reaction channeling after the positive charge of the carbocation shifts in the ES* complex. It is believed that due to the mutation at position 81 substituting tryptophan for tyrosine and thusly increasing the π-electric density, the carbocation is longer-lived and allowed to shift more easily. The research done on the filtrate did not coincide with these findings because the α_c (0.08) of the unknown sterol did not match with either of the two diols isolated in the previous work (α_c = 0.17, 0.22, respectively). In an attempt to find the diol products, all of the work that was performed with the filtrate was repeated on the samples that did not pass through the 10 kDa membrane. The H_2O_2 digestion performed with an 18% polyacrylamide gel on both samples showed that most of the radioactivity was still present nearest the region of the undigested sample (Figure 3.25). With SDS-PAGE analysis, the height of the band indicates the mass of the protein. The placement of the peptide was also indicative of the 43 kDa mass of the monomer of the SMT. This digestion, along with the H_2O_2 digestion performed on the total digestion, reveals that perhaps the SMT of both samples was not properly digested. A reason for this lack of digestion could be that the tryptic digestion was partially completed (8 hrs). After saponifying both samples from the peptides greater than 10 kDa and extracting the sterols with a hydrophobic layer extraction method, the samples were separately loaded onto the same TSK-gel-column. The

radioactivity elution of the WT sterol production mirrored that of the Y81W sterol production (Figure 3.26). In each sample, there was the peak with the α_c of 0.10 and another major peak with a α_c of 0.58. The peak with a α_c of 0.10 is the same peak that had a α_c of 0.08 in the sample that came from peptides less than 10 kDa (only a difference of 1 minute). The identity of this sterol was not determined. The peak with a α_c of 0.68 is most likely the monol turnover product due to its relationship to the retention time of pure 26, 27 DHZ. In a previous study performed by Jayasimha,[24] the α_c's of the monol and 26, 27 DHZ are in the same relationship but frame shifted 0.1 units more than that of what my research found. With this frame shift taken in account, the peak with a α_c of 0.10 would coincide with the α_c's of the two diols found in the Jayasimha study (0.17, 0.22).

Table 3.21. The different protocols for complexing Y81W SMT with 26, 27 DHZ used in the chemical affinity labeling studies.

PROTOCOL	SMT (μM)	DHZ Conc. (μM)	AdoMet Conc. (μM)
Jayasimha[24] *	0.15	100	100
Marshall[34]	0.8	100	100
Veeramachaneni[35] **	0.83	10	2
Soape/Nguyen ***	11	52	66

* Protocol did not include a tryptic digest of the SMT samples after complexing. Retention times of sterol products from Soape/Nguyen protocol were compared to this study.
** Protocol used a 16 hour complex assay
*** Protocol used a 6 hour complex assay

Table 3.22. Account of the radioactivity at key stages of the Chemical Affinity Labeling Studies with [3-3H] 26,27 DHZ to WT and Y81W mutant SMT samples.

	WT		Y81W	
SAMPLE	**TOTAL RADIOACTIVITY (DPM)**	**%**	**TOTAL RADIOACTIVITY (DPM)**	**%**
[3-^{3}H] 26,27 DHZ (Beginning)	**4.6 x 10^6**	**100**	**4.6 x 10^6**	**100**
Filtration (<10 kDa)	**1.7 x 10^5**	**3.7**	**1.2 x 10^5**	**2.6**
Hot+Cold of Filtrate	1.1 x 10^5	2.4	9.4 x 10^4	2.1
Sample to Collect 30-45 min	7.5 x 10^4	1.6	6.2 x 10^4	1.3
Saponified Sample	1.0 x 10^4	0.2	9.0 X 10^3	0.2
Saponification from TSK column	2.4 x 10^4 **	0.5	1.6 x 10^4 **	0.3
Filtration (>10 kDa)	**3.8 x 10^6**	**83**	**3.3 x 10^6**	**72**
HPLC Peptide Analysis	5.0 x 10^5	10.9	4.1 x 10^5	9
Saponification from TSK column	1.9 x 10^6	41.3	1.8 x 10^6	39
H_2O_2 Digestion (2 x's)	3.8 x 10^5	8.3	3.2 x 10^5	7

** Increase in radioactivity count could be due residual radioactivity left in the column that can have affect on small amounts of radioactivity.

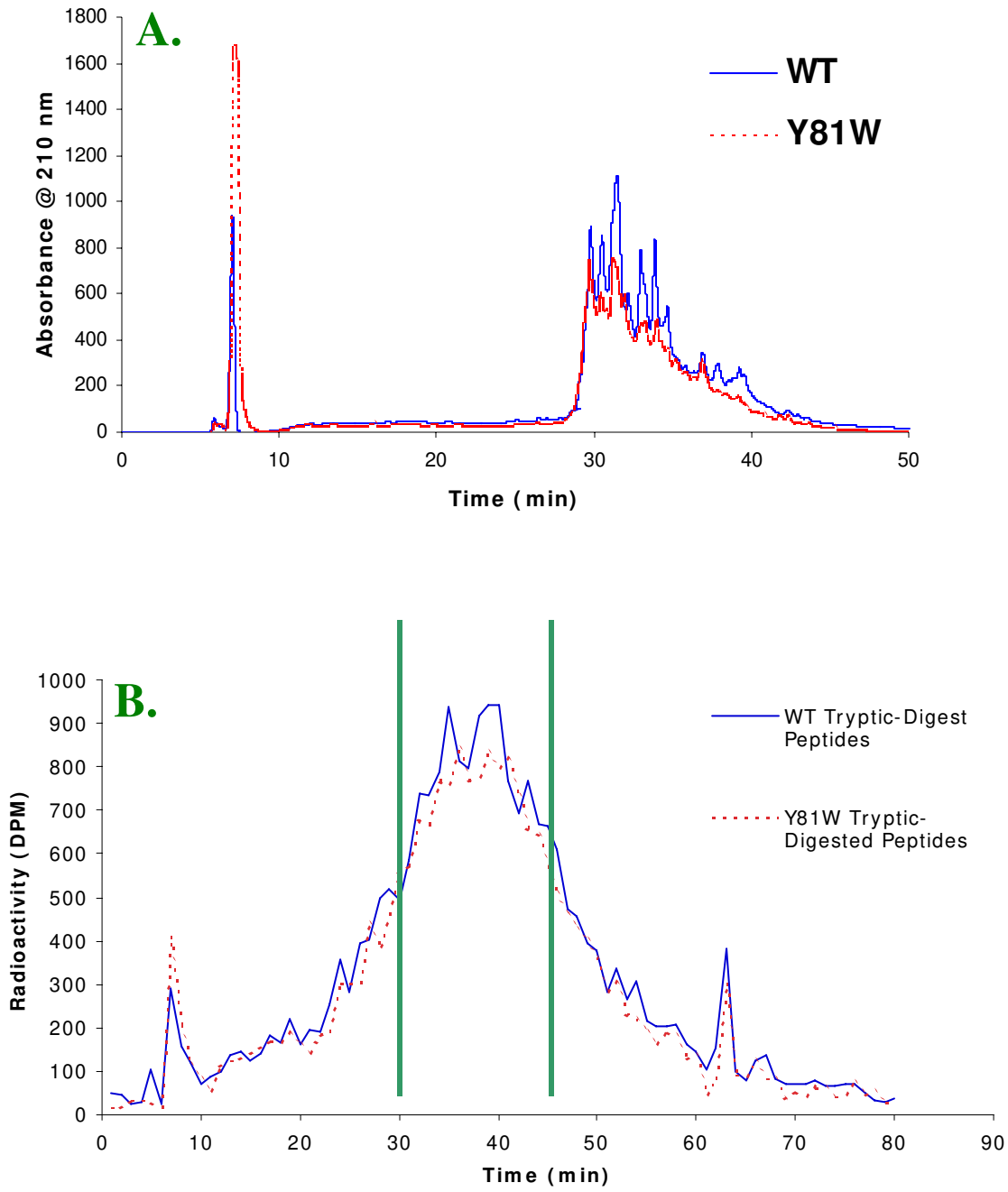


Figure 3.2.1. A. RP-HPLC chromatogram of tryptic-digested peptides of both WT and Y81W mutant SMTs. Minutes 30-45 were collected and a portion was reinjected on the column. HPLC was performed using a Vydac^{TM} C_{18} column, and detection was set at 210 nm. Fractions were collected every minute in 0.5 mL aliquots, and the radioactivity of each fraction counted with a scintillation counter. **B.** Radioactivity of each fraction is represented with the radioactivity in DPMs graphed against time in minutes. For each strain, fractions 30-45 were collected from the entire sample and pooled for saponification. The region collected is highlighted between the green bars.

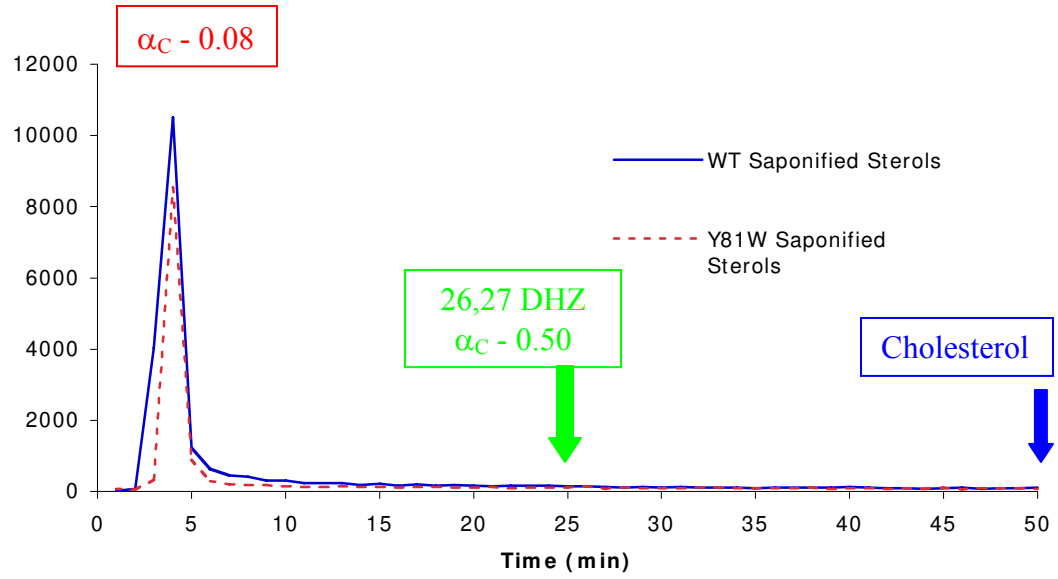


Figure 3.2.2. Representation of the radioactivity in DPMs per minute (0.5 mL fractions) of the saponified sterols that were bound to the tryptic-digested peptide fragments from the <10 kDa samples. The sterol was isolated using a RP-HPLC C_{18} TSK-gel-column. Radioactivity was performed by a scintillation counter. A single radioactive peak was found at 4 minutes (α_C – 0.08) for both samples.

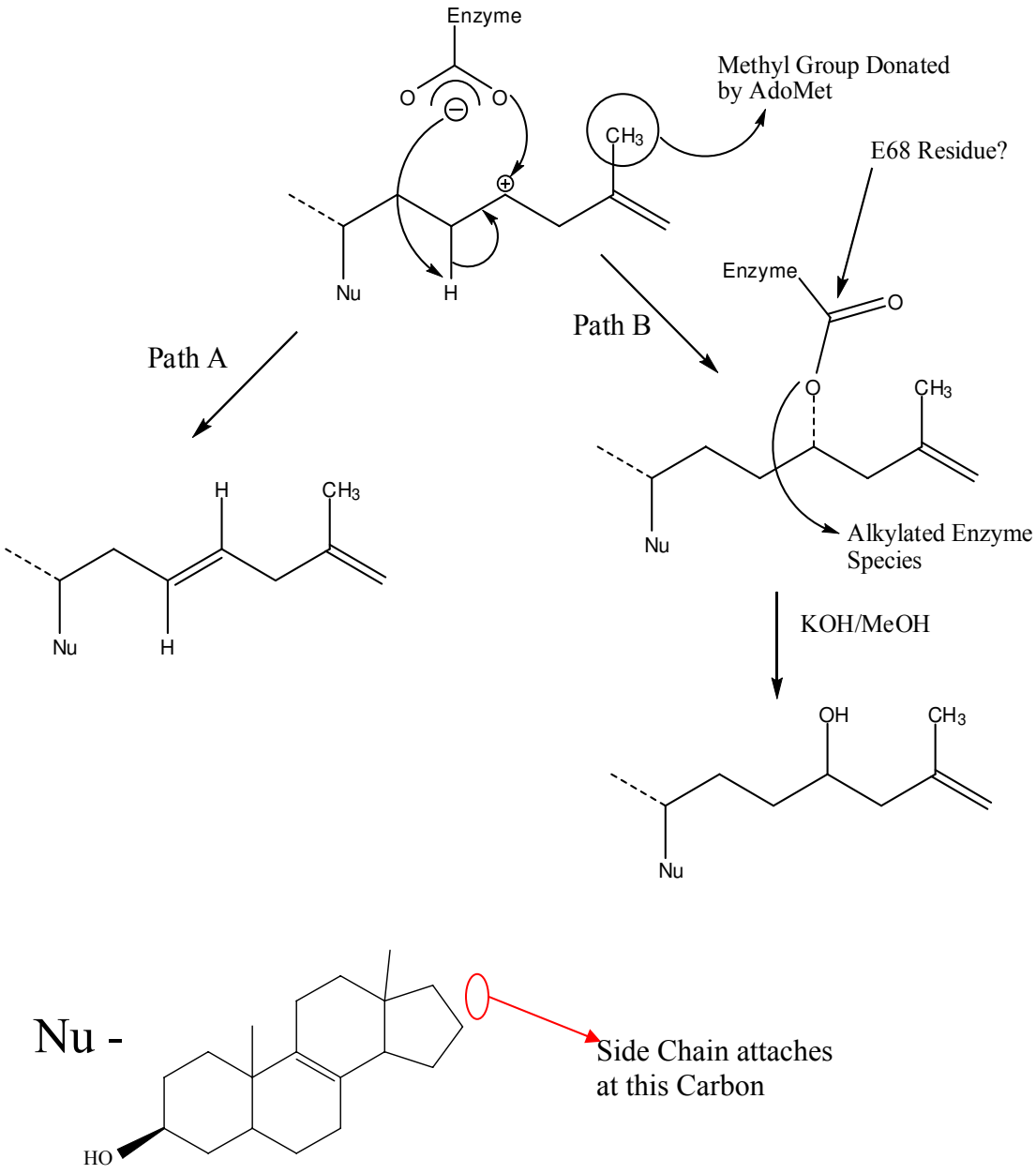
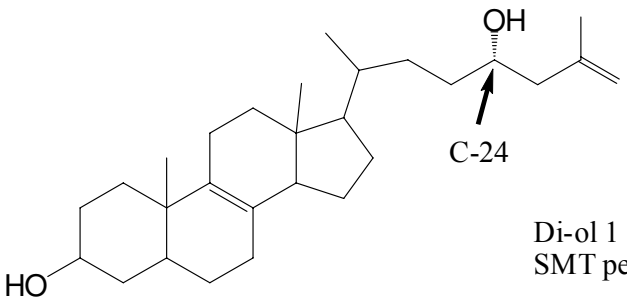
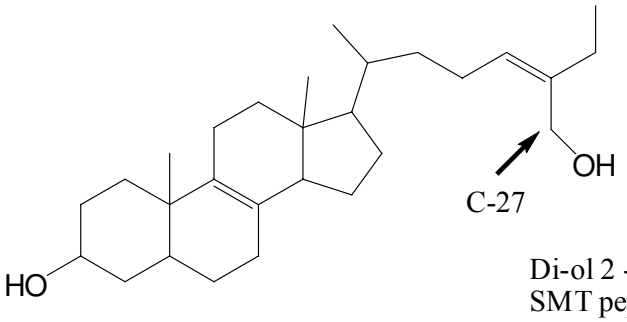


Figure 3.2.3. Yeast wild type SMT interaction with 26, 27 DHZ. Path A depicts the formation of the monol turnover product. Path B depicts the formation of the enzyme bound diol product. The carbocation on the mechanism-based inactivator forms an ester bond with the negative charged glutamic acid at position 68. This position is suspected to be linked to Region I. After saponification of the peptide-bound sterol, a second hydroxyl group is created by the hydrolysis of the ester.



Di-ol 1 - Saponified from Y81W mutant SMT peptide fragment



Di-ol 2 - Saponified from Y81W mutant SMT peptide fragment

Figure 3.2.4. Established Structures of the diols isolated after saponifying the Y81W peptide fragments bound to 26, 27 DHZ. Diol 1 is formed with the interaction with Region I. Diol 2 is believed to be formed through reaction channeling due to a longer-lived carbocation, which interacts with an undetermined amino acid in Region III.

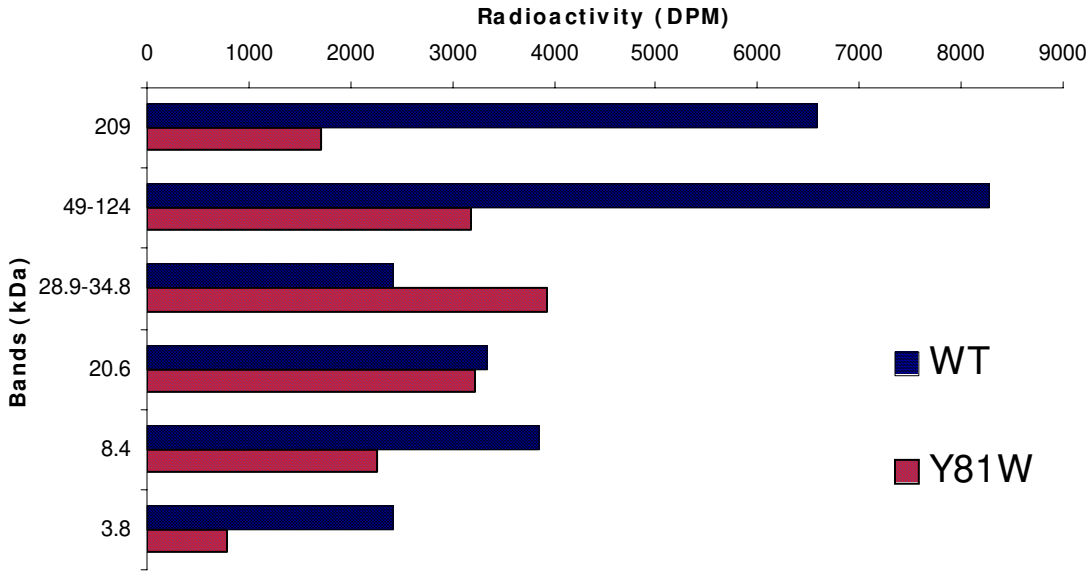


Figure 3.2.5. Radioactivity counts of a H_2O_2 digestion of an 18% polyacrylamide SDS-PAGE gel of the "top layer" (peptides > 10 kDa). Six bands were taken from the length of the well. The y-axis represents the region or mass that each band represents, as compared to the Bio-Rad Broad Range Prestained Molecular Weight Marker that ran beside the digested samples. Radioactivity is expressed in DPMs. Majority of the radioactivity fell in the region of the undigested SMT (43 kDa).

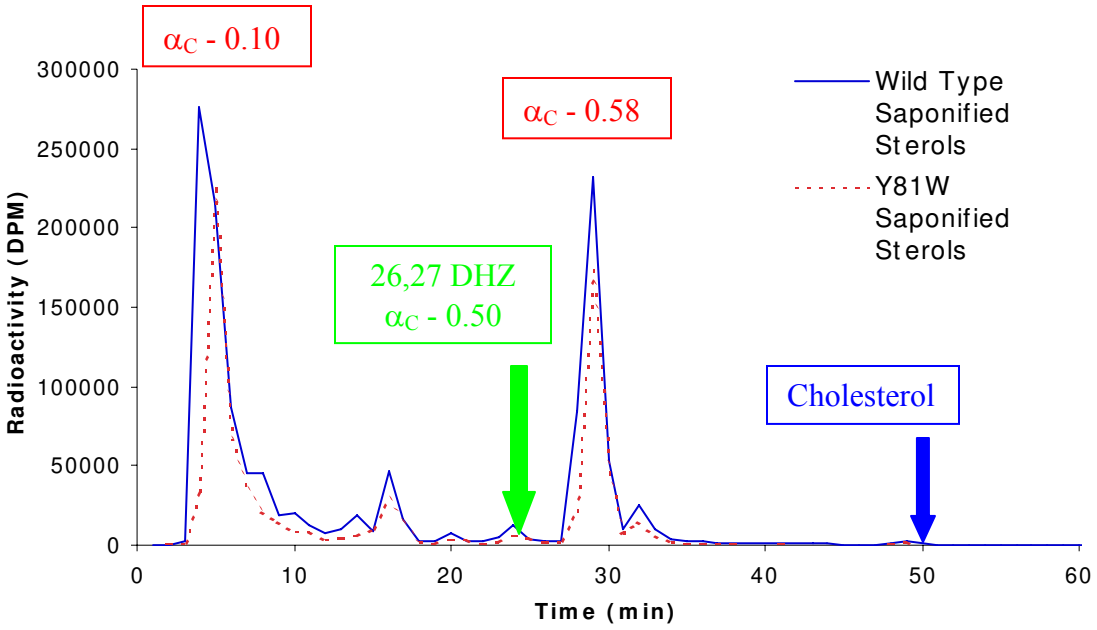


Figure 3.2.6. Representation of the radioactivity in DPMs per minute (0.5 mL fractions) of the saponified sterols that were bound to the tryptic-digested peptide fragments from the >10 kDa samples. The sterols were isolated using a RP-HPLC C_{18} TSK-gel-column. Radioactivity was performed by a scintillation counter. Two significant radioactive peaks were found at 5 (α_C - 0.10) and 29 (α_C - 0.58) minutes for both samples. The retention time of 26, 27 DHZ and cholesterol are as indicated on the graph (25 and 50 min., respectively).

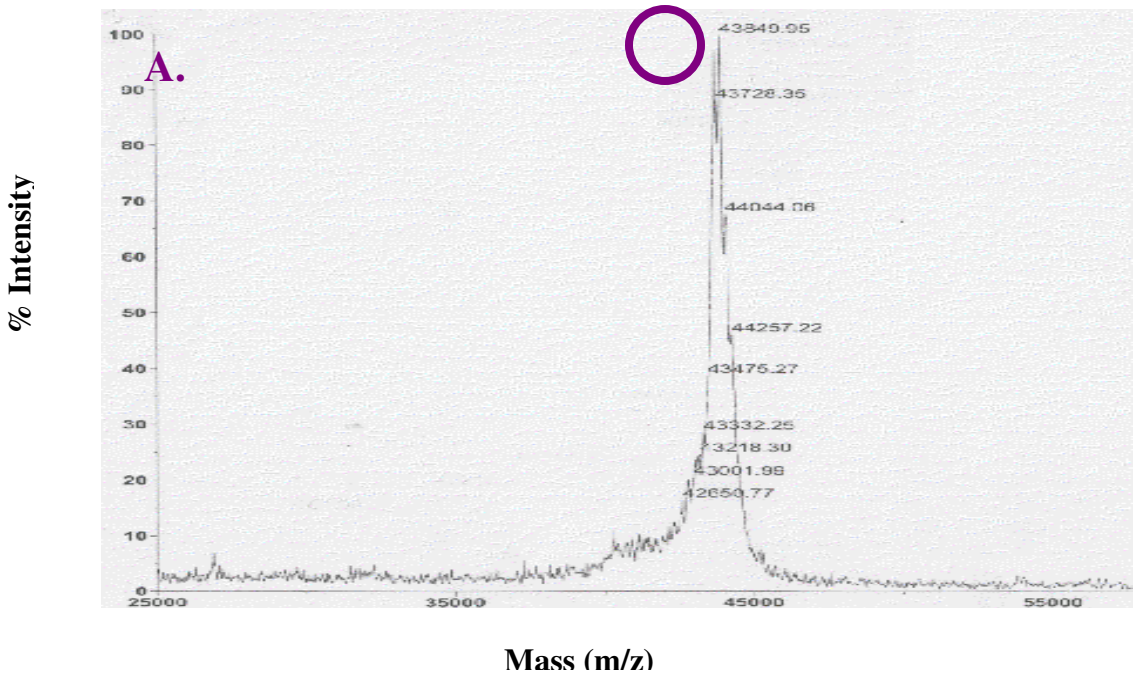
3.3 X-RAY DIFFRACTION ANALYSIS

With this discrepancy of data from my research and previous works, the actual three-dimensional interaction of the Y81W mutant SMT with 26, 27 DHZ is greatly needed to clarify any questions. The alternative of X-ray diffraction analysis was utilized in attempt to explore the purpose of these investigated domains. X-ray diffraction works off the principle that x-rays are scattered when they strike the electrons of atoms, much like in the manner that light waves are scattered by the engraved lines of a diffraction grating. Using the crystal of the protein as a three-dimensional diffraction grating, a monochromatic beam of x-rays is scattered. The resulting diffraction gives a pattern in which the diffracted rays reinforce and do not destructively interfere. More accurately, the distribution of the electron density, and thus the protein 3-D structure, may be calculated from the diffraction pattern by Fourier transformation.[36] Perhaps, the biggest problem in these calculations is the determination of the phase of each diffracted ray. This setback is resolved by a method called isomorphous replacement.[37] With this method, a heavy metal atom is bound at specific sites in the crystal without disturbing its structure or packing. These heavy atoms give a frame of reference because the metal scatters x-rays more than do the atoms of the protein, and its scattering is added to every diffracted ray. Data about the phases of the diffracted rays (information necessary for Fourier transformation) can then be obtained from the changes in strength, depending on whether they are reinforced or diminished by the scattering from the heavy atoms. This project used selenium in the role of the heavy metal. It was introduced into the SMT by adding seleno-methionine that was incorporated into the primary structure of the Y81W mutant SMT enzyme during the growth of host cells. To ensure that the addition of the

selenium did not alter the mass of the SMT enzyme, a Matrix Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) analysis was carried out to determine the difference of mass between the Y81W SMT with selenium and without selenium (Figure 3.31). A difference of only 550 Da was found between the two samples. To ensure that the bulk seleno-methionine was incorporated, high concentrations of isoleucine, lysine, and threonine were introduced during the growth of the host cells. These amino acids block the methionine biosynthesis in *E.* coli by inhibiting aspartokinases. Next, the HEPES buffer was tested to determine if the solution environment was at optimal conditions. A phosphate, Tris, and HEPES buffer was tested in separate activity runs with the 100,000xG supernatant to verify that the HEPES buffer used in purification was the best choice for the project. The phosphate buffer is ideal for circular dichroism studies, and the Tris buffer had been used previously in this X-ray diffraction study. In the comparison study, HEPES buffer did display better activity as compared to the other two buffers (data not shown). Another reason to use HEPES buffer was to continue the same principle buffer throughout the entire process. The SMT was also crystallized in HEPES buffer. Using this buffer, purification was performed on the Y81W mutant SMT. SDS-PAGE analysis with a 12% polyacrylamide gel (Figure 3.13) and Bradford Method and an activity assay (Figure 3.32) were performed on key purifications steps before the complex to 26, 27 DHZ. In the complex assay, corrections in the purity (85%) and the Bradford method (40% increase) (see Appendix Figure 7.4) were taken into account before calculations on how much 26, 27 DHZ, AdoMet, and 5% Tween 80 would be added. Before 26, 27 DHZ was added, the purity and amount were verified through GC/MS and GC, respectively. A standard cholesterol dose response curve was created

on the GC to determine the concentration of 26, 27 DHZ (see Appendix Figure 7.5). If using 10 mg of Y81W SMT, 1.5 mg/mL 26, 27 DHZ stock solution, and 6 mM AdoMet stock solution in a ratio of 1:5:5, respectively, 110 µg of 26, 27 DHZ (73 µL of stock), 100 µL of AdoMet stock, and 60 µL of 5% Tween 80 was combined as described in the Materials and Methods Chapter. After the assay, the complex was further purified with the Mono-Q column connected to a FPLC system. Chromatography work done at this stage discovered that the retention time for the uncomplexed sample done previously (Figure 3.14) matched exactly with that of the complexed sample of Y81W mutant SMT (Figure 3.33). A_{280} quantitational and SDS-PAGE analysis were performed on the fractions collected (Figure 3.34). Only the fractions with the pure 43 kDa band were collected for X-ray diffraction analysis. In earlier studies, the lower band (40 kDa) was excised from a 12% polyacrylamide SDS-PAGE gel, and the protein sequenced. It was discovered that this protein was actually a truncated form of the SMT. For this reason, it was believed that it was a product of degradation and not considered for this study. Another round of Mono-Q column purification was completed with the impure fractions. The entire process was repeated numerous times to produce as much 26, 27 DHZ-Y81W SMT complexes as possible. In my attempt, a total of 4.72 mg of pure Y81W SMT complexed with 26, 27 DHZ was purified. The sample was flash frozen with liguid N_2. All purification attempts were sent to the University of Pennsylvania for X-ray diffraction. Each attempt gave varied results (Table 3.31). In the first attempt, crystallization conditions had a precipitant reservoir solution composed of 16% Peg3350 (w/v) with 0.2 M Na-formitate. A 1 mL portion of reservoir solution was set to equilibrate against a drop composed of 4uL of protein (4-5 mg/mL buffer: 25 mM

HEPES, 0.2% detergent) with 4uL of reservoir solution. Prismatic-like crystals grew to typical dimensions of 0.1 mm x 0.1 mm x 0.2 mm in two-three weeks. These crystals gave the best results with diffraction at 4.15 Å (Figure 3.35). The space group of the crystal is F222, and the asymmetric unit contains 1 molecule. Even though diffraction at 4.15 Å cannot give sufficient resolution to determine the location of amino acid side chains or interactions with the 26, 27 DHZ, it was determined with this data that the enzyme was indeed a homotetramer with a size of 172 kDa. Along with supporting information given by my GPC studies, this data gives a small glimpse into the 4° structural design (Figure 3.36). This number also agrees with the multiplication of 4 monomers (43 kDa) to give the whole homotetremeric enzyme mass of 172 kDa. According to SDS-PAGE analysis, the monomer size has been proven on several occasions to be 43 kDa. As stated before, no information on the interactions of any domains in the SMT have been obtained. However, the University of Pennsylvania does have good quality crystals of the sample sent from my research. The crystallization conditions were the same as in previous attempts, but the scaled data set from the 4.15 Å attempt was going to be used to aid in increasing the resolution. No specific data has been received from this latest X-ray diffraction analysis; however, the morphology of the crystals were of better quality than past attempts, but the resolution was not as great as the first attempt. This unwanted result could be due to the presence of only the 43 kDa monomer sample sent for analysis. It was thought that the degradative 40 kDa monomer was keeping this study from achieving a better result from the diffraction analysis, but perhaps the additional presence of the degradative 40 kDa monomer actually created larger crystals which are better for diffraction.



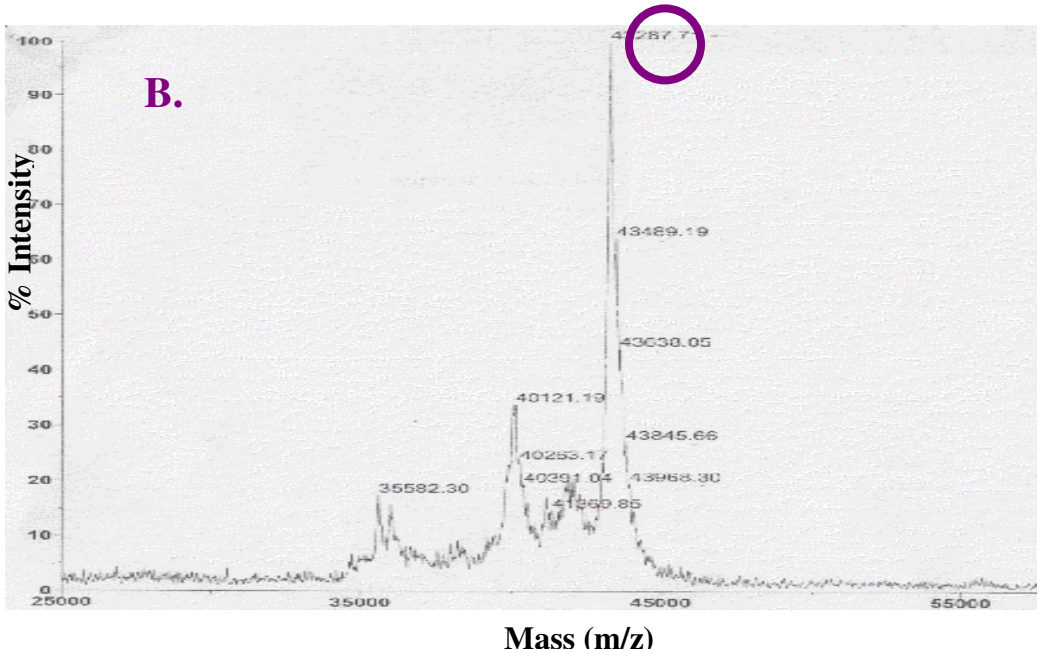


Figure 3.3.1. MALDI-TOF spectra of the Y81W mutant SMT **A.** with seleno-methionine and **B.** without seleno-methionine. Both spectra give a mass of approximately 43 kDA for the monomer of the Y81W mutant SMT (with – 43.85 kDa, without – 43.29 kDa). Work was done by Di Costanzo at the University of Pennsylvania.

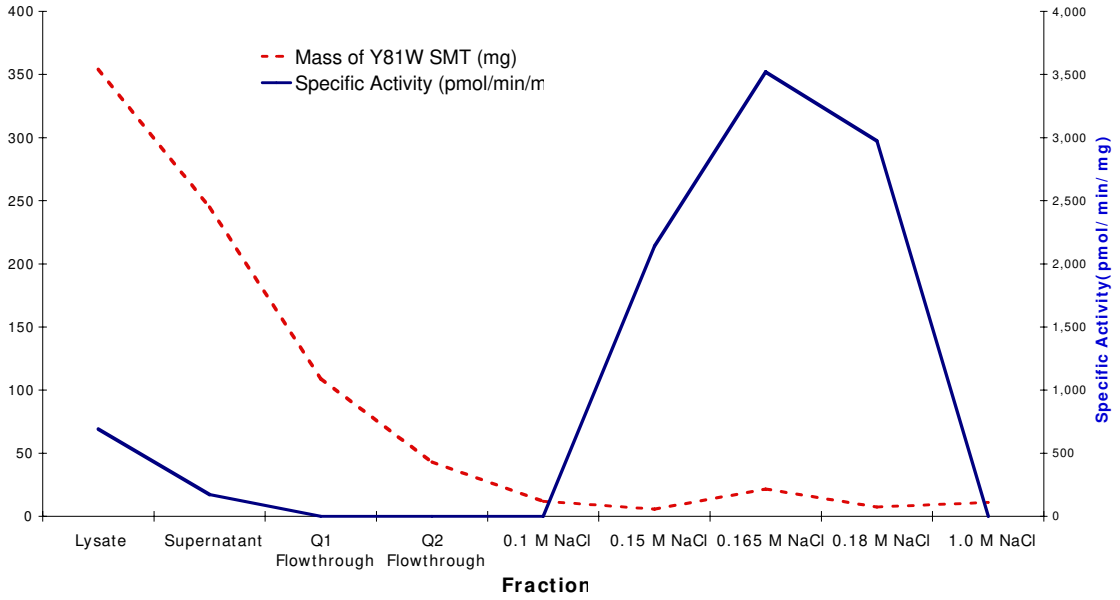


Figure 3.3.2. Representation of the relationship of the mass of the Y81W mutant SMT enzyme in each fraction compared to the specific activity of fractions at the beginning and ones collected to complex with 26, 27 DHZ. Mass was determined through the Bradford method, with a BSA standard curve. Activity assays were executed as described in Materials and Methods Chapter. Along with information obtained from SDS-PAGE analysis on the same fractions, 0.165 M and 0.18 M NaCl fractions were collected to complex with 26, 27 DHZ. For the activity assay, 100 μM zymosterol and 0.6 μM AdoMet were held constant. Pure Y81W SMT concentration varied from sample to sample: Lysate – 1.8 μM, Supernatant – 1.2 μM, 0.15 M NaCl – 0.09 μM, 0.165 M NaCl - 0.35 μM, and 0.18 M – 0.12 μM.

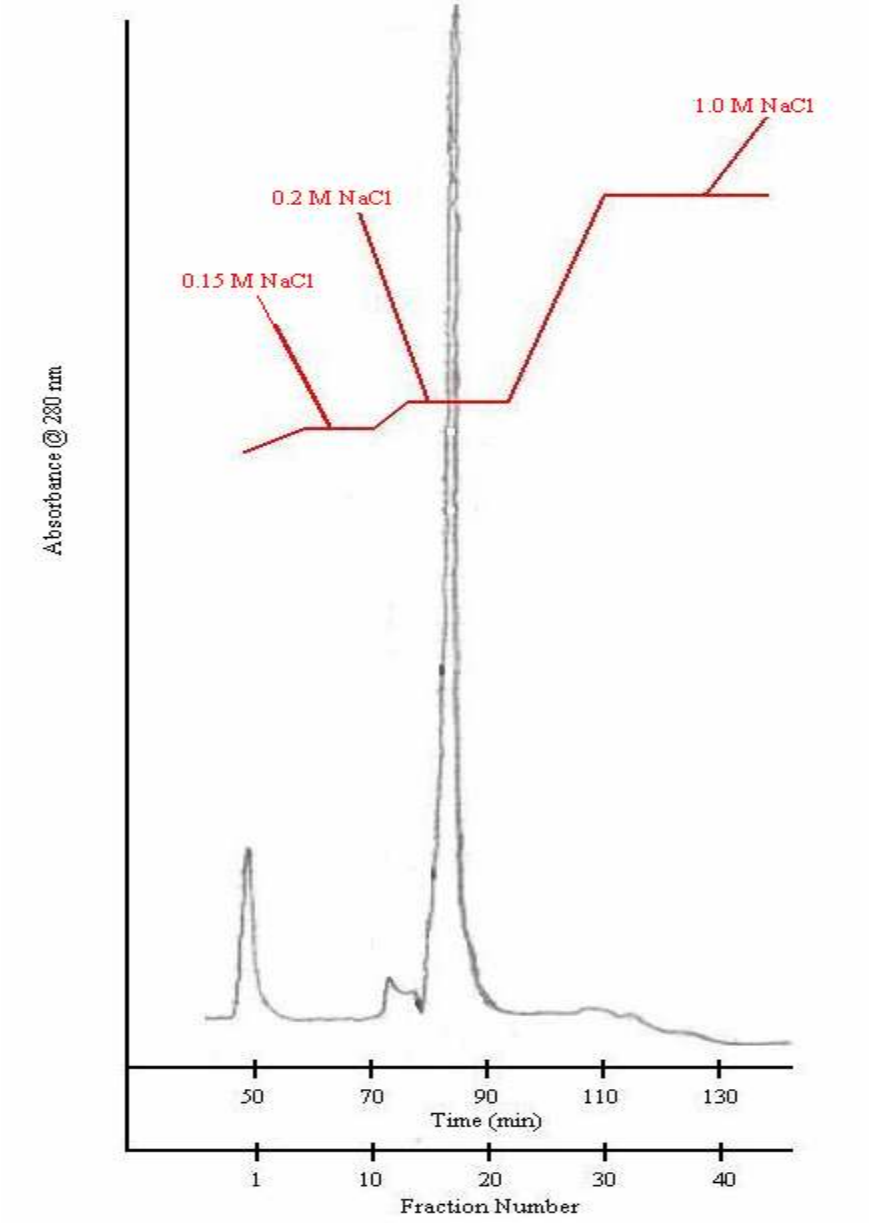


Figure 3.3.3. FPLC chromatogram from the Y81W SMT sample complexed with 26, 27 DHZ. The column used was a Q-sepharose (Mono-Q) column made by Pharmacia Biotech®, vol. of 8.0 mL. The column was equilibrated with Qa+D buffer (as described in the Materials and Methods Chapter) and controlled by a Fast Protein Liquid Chromatography (FPLC) system made by Pharmacia LKB®. The column had the parameters of a Range of 0.2, flow rate of 1.0 mL/min, and a recorder speed of 0.5 mm/min. According to Bradford quantitation analysis, 3.5 mg of the uncomplexed Y81W sample in 1.4 mL Qa+D buffer (<0.02 M NaCl) was loaded onto the Mono-Q column. The complexed Y81W SMT had a peak that eluted at 87 minutes and in the 0.2 M NaCl gradient step. Gradient is depicted in red.

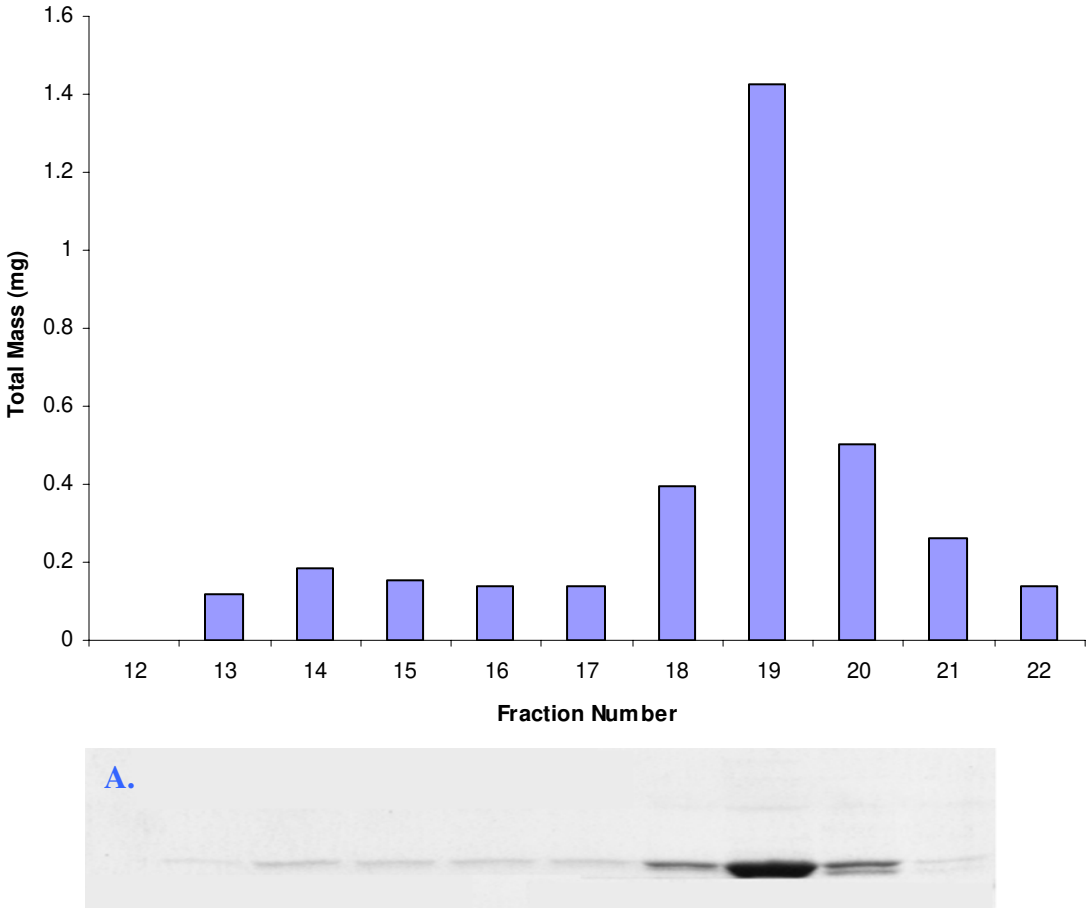


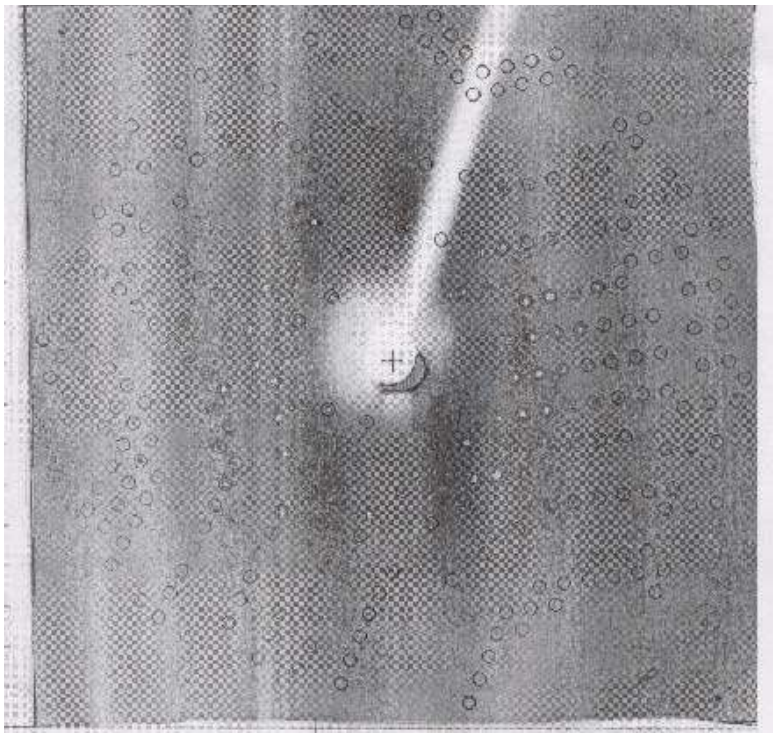
Figure 3.3.4. Bar graph representing the total mass of each fraction pertaining to the peak of the complexed Y81W SMT in the chromatogram of Figure 3.33. Quantitation was done by A_{280} method. **Insert A**. The SDS-PAGE analysis on a 12% polyacrylamide gel of each fraction in the graph. Note how the intensity of the SDS-PAGE bands increase or decrease in respect to the amount of SMT. For X-ray diffraction, only fractions with pure bands at 43 kDa (Upper band) were used. In this case, Fractions 13-18 were considered pure and collected. As vividly illustrated in Fractions 20 and 21, the peak at Fraction 19 also included a lower band (40 kDa) that cannot be seen in the figure. Fraction 22 did not reveal any bands.

Table 3.31. Attempts at obtaining X-ray diffraction data on the 26, 27 DHZ-Y81W SMT complex.

ATTEMPT	SMT PURIFIED (mg)	RESULT	REMARKS	CRYSTAL SOLUTION COMPOSITION
R. Kanagasabai (Jan., 2005)	5	Diffraction at 4.15 Å (See Figure 3.35)	Crystallized the 43 and 40 kDa bands/ Purified with Tris buffer	16% (w/v) PEG 3350 & 0.2 M Na-formate
Z. Song (Nov., 2005)	3	Crystal formation / No Diffraction	Crystallized the 43 and 40 kDa bands/ Purified with Tris buffer	16% (w/v) PEG 3350 & 0.2 M Na-formate
M. Soape (Nov., 2006)	0.5	No Crystal formation	Crystallized the 43 kDa band/ Purified with HEPES buffer	N/A
M. Soape (Jan., 2007)	4.72	Crystal formation / Diffraction Resolution not adequote for data analysis	Crystallized the 43 kDa band/ Purified with HEPES buffer	16% (w/v) PEG 3350 & 0.2 M Na-formate

* Analysis of the crystals was performed at the Brookhaven National Laboratory (Upton, NY) on beamline X29A (l=1.000 Å, 100 K) using an ADSC Quantum 315 detector.Ź Data reduction was achieved with HKL2000 and data were scaled with Scalepack.

A.



B.

Unit Cell Lengths:	80.3478	126.5557	201.7222
Unit Cell Angles:	90.0000	90.0000	90.0000
Unit Cell Volume:	2.0512×10^6		
Orientation Angles:	-78.7029	9.2761	-178.0298
Mosaicity:	0.410		

Figure 3.3.5. A. Diffraction picture of the 26, 27 DHZ-Y81W SMT complex crystal being bombarded by a single beam of x-rays. The circles are the detection of the x-rays being diffracted off the crystal. Picture produced by the work done by Kanagasabai at a resolution at 4.15 Å. **B.** X-ray diffraction analysis data.

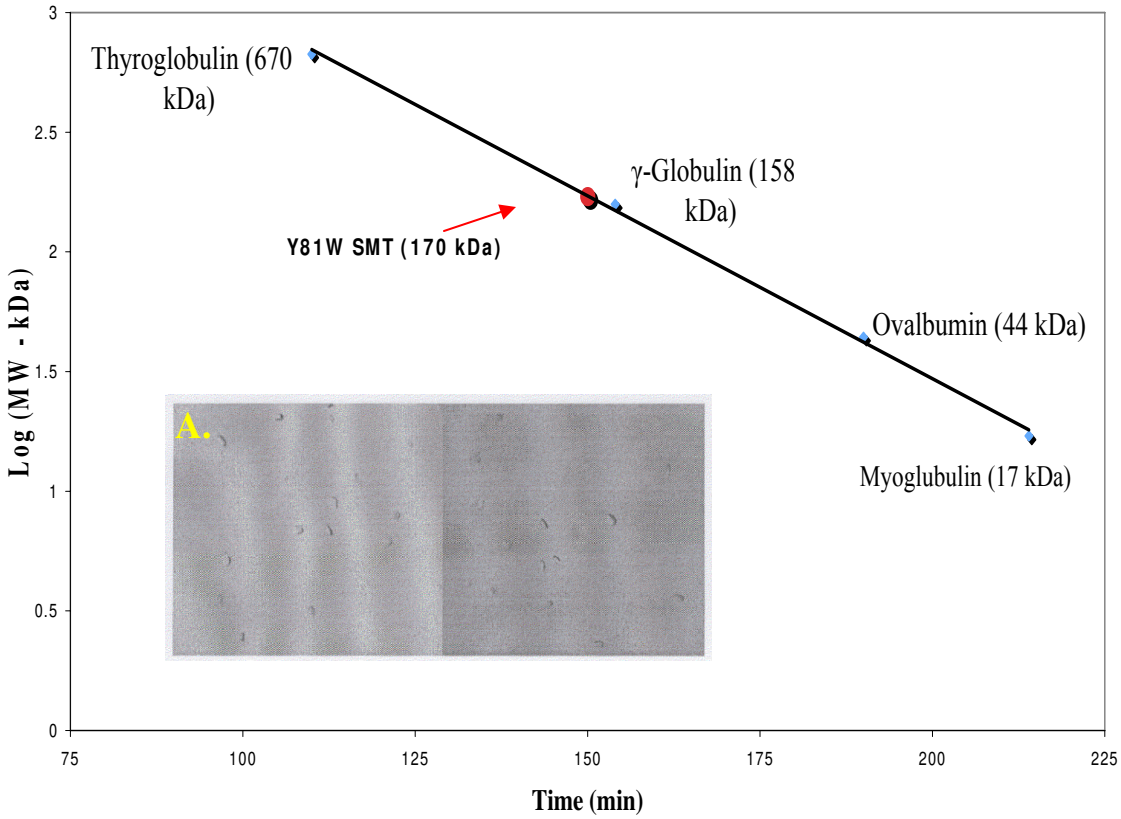


Figure 3.3.6. GPC standard graph displaying the reported mass of the Y81W SMT to be 170 kDa. **Insert A.** Y81W mutant SMT bound to 26, 27 DHZ crystals formed before X-ray diffraction analysis. The crystals diffracted at 4.15 Å, and this data confirmed the size of the Y81W mutant SMT to be more accurately measured at 172 kDa (1% Difference from the GPC studies).

CHAPTER IV
CONCLUSION

SMT is an extremely beneficial enzyme to study and gain knowledge of how the sterol has developed in various species of plants, fungi, and animals. The differences in the sterols and what enzymes cause the divergence of pathways have become the forefront of medical applications and understanding how the enzyme evolved from its development from bacteria. This research project has hopefully aided in this effort, or at least, gave a protocol of what not to do. In the first portion of this thesis, employing a GPC stage in the middle of the purification of the Y81W mutant SMT was found to prolong the purification without producing a significant difference in the generation of pure protein. The GPC application did give another verification of the total mass of the Y81W mutant SMT enzyme (170 kDa). However, in a separate endeavor to refine the purification, an extended NaCl gradient did prove to aid in the isolation of the Y81W mutant with better efficiency and without adding time to the overall protocol. Also, the chromatographic properties of the WT and Y81W mutant SMT in an anion-exchange column (Mono-Q column connected to a FPLC system) were the same in all cases studied. In the chemical affinity labeling and tryptic-digest studies with Y81W mutant SMT and 26, 27 DHZ, a different production of sterols was discovered. Comparing to previous investigations, these results could be the result of an increase in protein concentration during the complex assay, and/or a decrease in the amount of trypsin used in the digestion. With whatever circumstances, the sterol production between both WT and Y81W samples was basically identical in retention time and intensity. The last segment of my research included purifying an abundance of Y81W mutant SMT

complexed with 26, 27 DHZ for X-ray diffraction analysis. If successive, this method could finally answer many question involving the important domains located in the SMT enzyme. It will give a detailed 3-dimensional picture of the enzyme and the interaction with the mechanism-based inhibitor. In this effort, a small but significant accomplishment has been made with a diffraction of the crystal at 4.15 Å. At this resolution, it was calculated that the total mass of the SMT was 172 kDa. This result meant that the enzyme was made of four monomers (43 kDa x 4 = 172 kDa). More crystals have been generated and will be analyzed in the near future. This work has brought a better understanding of the nature of the Y81W mutant SMT, an understanding that can be used to compare and contrast with the WT counterpart.

CHAPTER V
FUTURE WORK

Present work on the SMT enzyme includes an ongoing look into the Region I and III, and their substrate specificity. Such work as additional site-directed mutagenesis on conserved residues within these regions and selectively swapping residues between SMT1 and SMT2 isoforms will address if these signature motifs are sufficient enough in establishing substrate specificity. As stated in the passage above, the challenge of obtaining the 3-dimensional picture will be a constant effort in the Nes lab. The post-translational modifications of lack there of will also be investigated in determining what other factors can affect the activity of the enzyme (i.e. phosphorylation).

Other SMTs in different species are also being examined in the Nes lab such as *T. brucei*. This pathogen is the cause of the African sleeping sickness, and work is in progress in developing potent inhibitors of its SMT1 to enhance medical applications in the fight against this disease. In another devastating pathogen, *C. albicans*, morphological changes from single cell to hyphal structures have been implicated in method of infection. A possible study into this phenomenon is to look at the levels of SMT species and the factors such as ATP and/or NADP that might affect the formation of the pathogenic strains.

With the differences of the isoforms within and across species boundaries, along with the different factors that can affect SMTs, research into this enzyme will be a long and arduous journey. As new information is gathered, beneficial applications will be developed that will impact millions of lives.

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APPENDIX

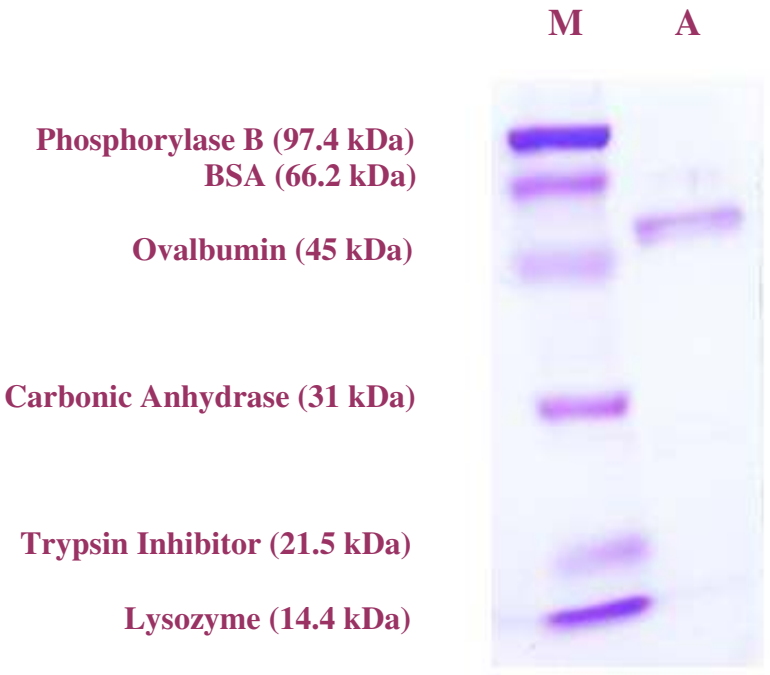

Figure A.1. 12% poloyacrylamide SDS-PAGE gel of the SMT-specific antibody produced by Invitrogen® and purified by a Bio-Rad® IgG Purification Kit. The approximate size of the antibody is 50 kDa. **M –** Bio- Rad® Low Range Molecular Weight Marker **A. –** Purified SMT-specific antibody

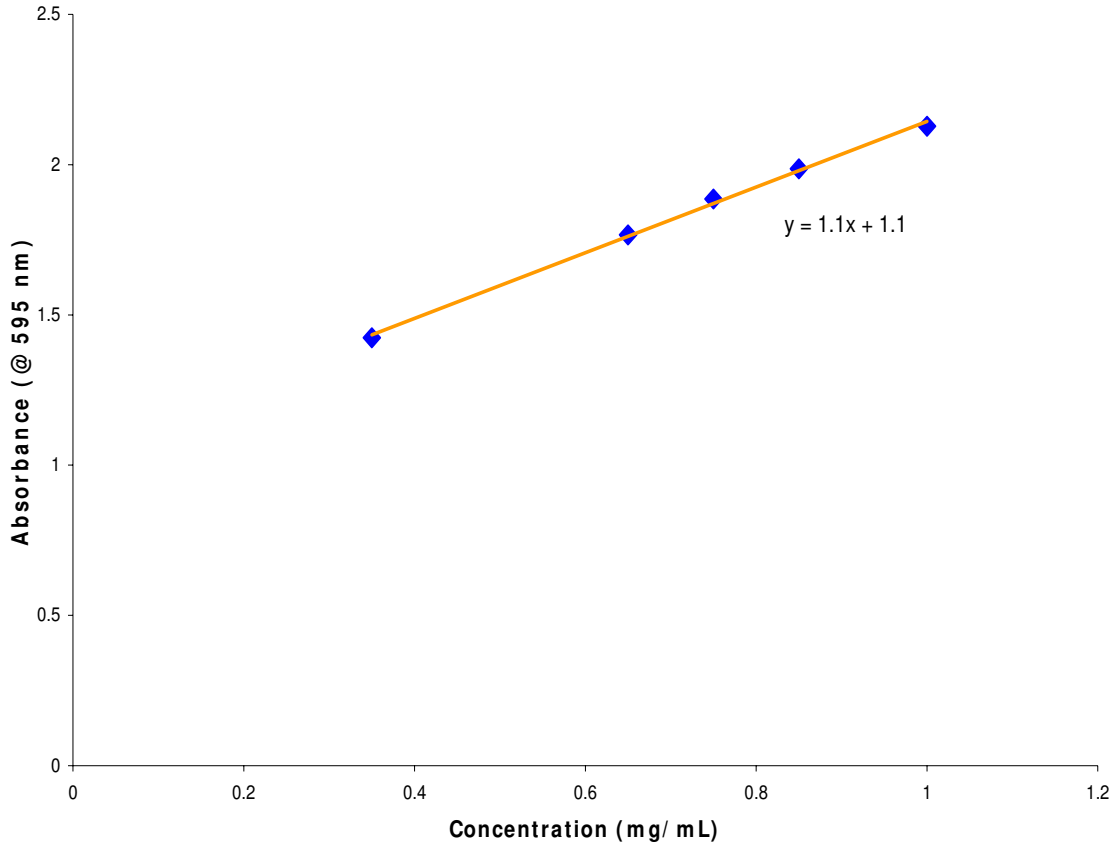

Figure A.2. Bradford dose response curve using pure Y81W mutant SMT enzyme as the standard. The Bradford Method was performed as directed in Materials and Methods Chapter with a varied concentration of purified Y81W mutant SMT enzyme.

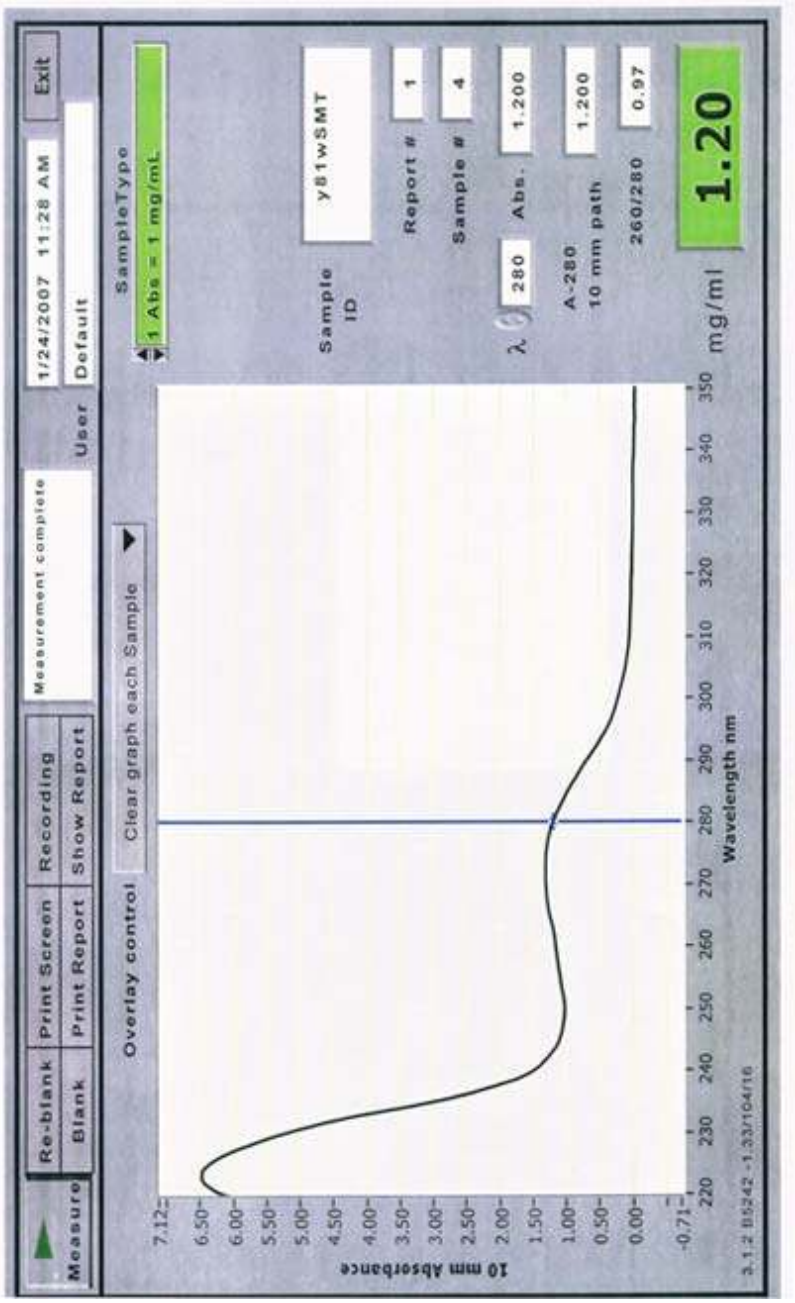

Figure A.3. Printscreen of the display given for the Nanodrop Nd-1000 Spectrophotometer. This instrument gave a purified Y81W mutant SMT enzyme concentration of 1.2 mg/mL for a previously determined concentration of 1.03 mg/mL from the Nes lab spectrophotometer.

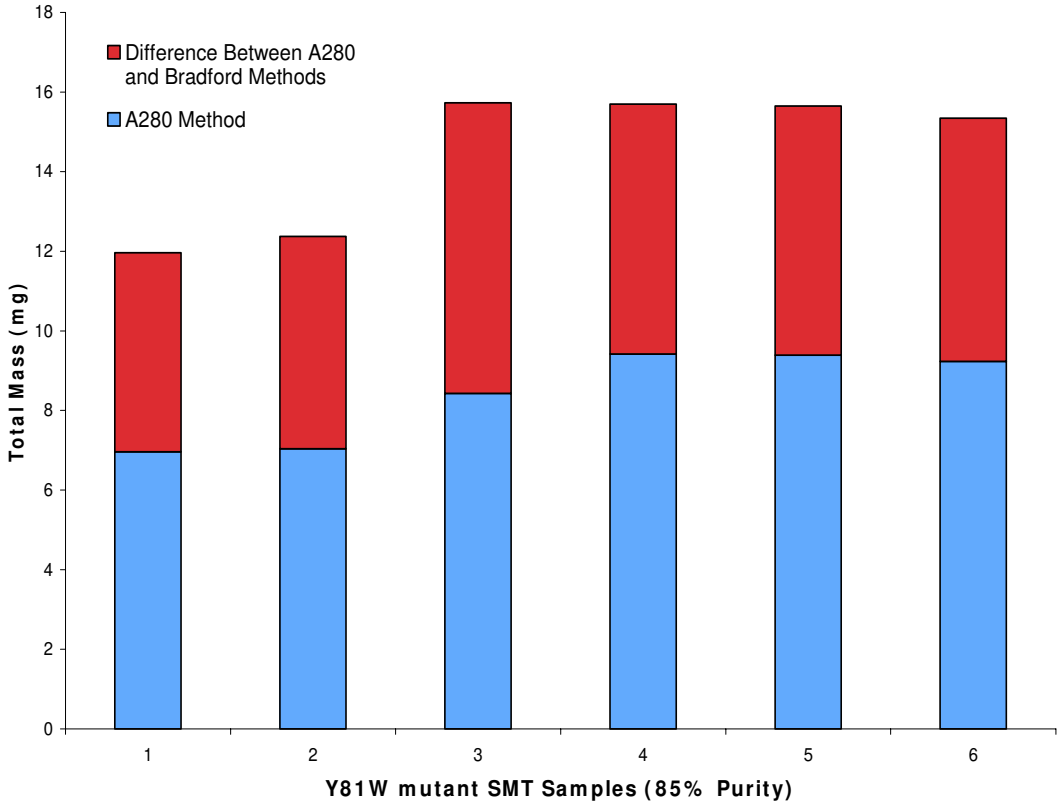

Figure A.4. Representation of the calculated mass difference between the A_{280} (Blue portion) and (Total length of the column) Bradford methods. An average of 42% is the difference (Red Portion) between each respective method. Each column is a separate 85% pure Y81W mutant SMT sample that was to be placed onto the Mono-Q purification column.

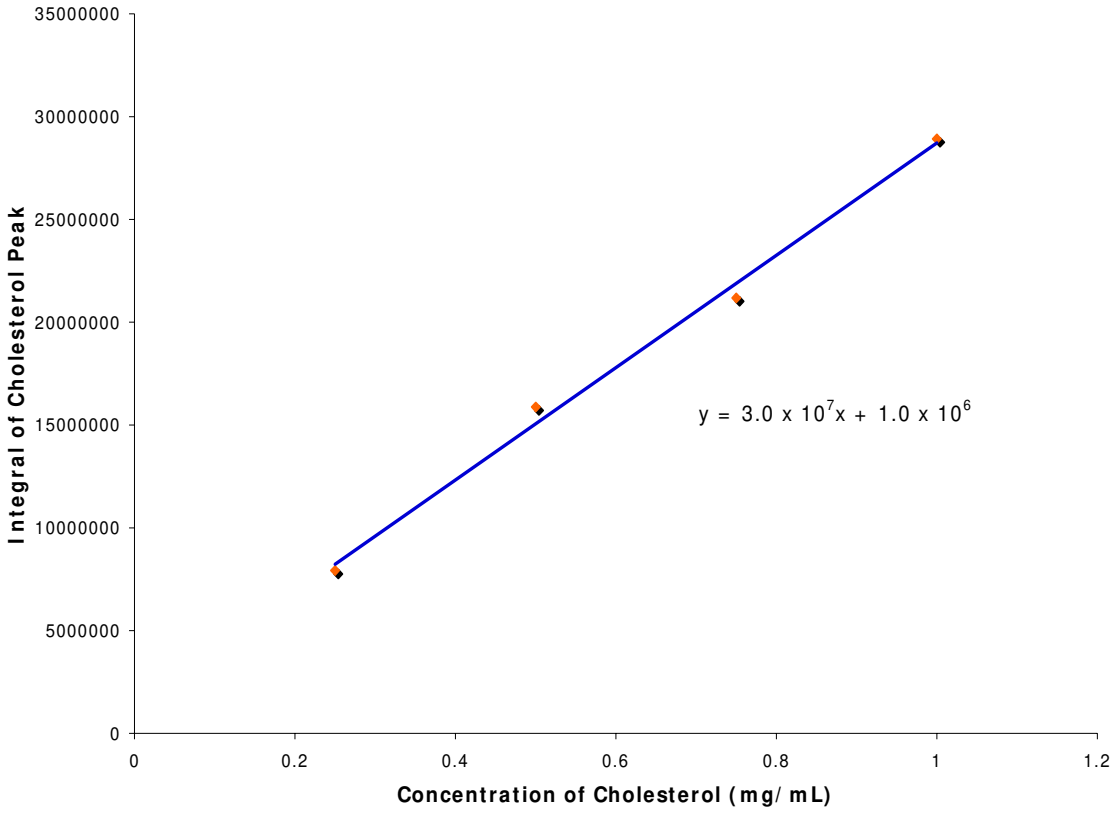

Figure A.5. Cholesterol dose response curve formulated from known concentrations of cholesterol as compared to their integral value on their respective GC chromatogram. Cholesterol has a retention time of 6.5 minutes, a
and 26, 27 DHZ has a retention time of 9 minutes.

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