PURIFICATION AND INHIBITION OF HYDROXYMETHYLGLUTARYL COENZYME A SYNTHASE

By

KAREN LESLEY BELL, B.Sc.

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AUTHOR: Karen Lesley Bell, B.Sc. (Brock University)

SUPERVISOR: Professor Paul Harrison

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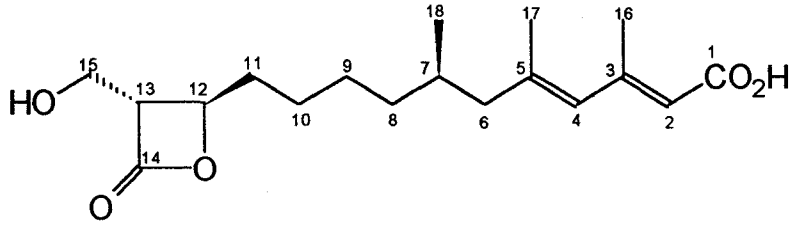
ABSTRACT

Hydroxymethylglutaryl-CoA synthase (HMG-CoA synthase) catalyzes the formation of HMG-CoA from acetyl-CoA and acetoacetyl-CoA. F-244 **(1)**, a naturally occurring β-lactone isolated from *Fusarium sp.* ATCC 20788 and other species, is known to be a potent and specific inhibitor of HMG-CoA synthase isolated from rat liver.



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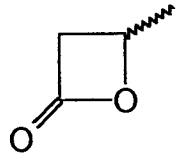

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This thesis describes the 48 fold purification of HMG-CoA synthase from bakers yeast in a three step procedure involving ethanol fractionation followed by ammonium sulfate precipitation and then hydroxylapatite chromatography. This procedure was found to be reproducible and yields a preparation of specific activity 0.14 units (μmol/min)/mg in an overall yield of 8%.

In our study, F-244 was found to be a potent irreversible inhibitor of HMG-CoA synthase isolated from bakers yeast, with an IC_{50} value of 0.009 μM. This value is almost identical to the inhibitory activity of F-244 on rat liver HMG-CoA synthase that has been reported in the literature.

Tritium labeled F-244 was prepared, for the first time, by feeding *methyl*-[^{3}H]-methionine to cultures of *Fusarium sp.* The [15,16,17,18-^{3}H] F-244 isolated had a specific activity of 1.3 x 10^6 DPM/mg. This tritiated F-244 was then used as an affinity

label for HMG-CoA synthase. Attempts to isolate the enzyme-inhibitor complex were unsuccessful due to the low level of radioactivity associated with the tritiated F-244.

HMG-CoA synthase was also shown to be inhibited in a time-dependent irreversible manner by (±)-β-butyrolactone **(2)**. The rate of inactivation (k_2) was found to be 0.4697 s^{-1} and the inhibition constant (K_I) was found to be 9 mM. The inactivation was found to be irreversible over several hours.

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

A	absorbance
AcAcCoA	acetoacetyl-CoA
AcCoA	acetyl-CoA
AcOH	acetic acid
AU	absorbance units
c	concentration
CE	counting efficiency
CM	carboxymethyl
CoA	coenzyme A
CPM	counts per minute
d	path length of light through the cuvette
DEAE	diethylaminoethyl
DMSO	dimethylsulfoxide
DPM	decompositions per minute
ε	extinction coefficient
Enz	enzyme
EtOH	ethanol
HIC	hydrophobic interaction chromatography
HPLC	high pressure liquid chromatography
HMG	β-hydroxy-β-methylglutaric acid
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
K_I	inhibition constant
K_M	Michaelis-Menten constant
N	total number of radioactive nuclei

N_O	initial number of radioactive nuclei
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
pI	isolelectric point
ppt	precipitate
R	average radioactivity
SAM	S-adenosylmethionine
SDS	sodium dodecyl sulfate
t	time
$t_{½}$	half life
TEMED	tetramethylethylenediamine
TMS	tetramethylsilane
UV	ultraviolet
V	rate of catalysis
V_{max}	maximum rate of enzyme activity

DEFINITIONS

For the purposes of this thesis, the following definitions are made.

assay - an analytical procedure for determining the disappearance of an enzymatic substrate or the appearance of products.

autolysis - the disruption of cells, allowing enzymes to be released into solution.

fold purification - the degree to which an enzyme is purified from the initial autolysis step.

homeostasis - a tendency toward keeping a relatively stable internal environment in the bodies of higher animals by means of complex physiological interactions.

IC_{50} - the concentration of inhibitor required to inhibit 50% of the enzyme activity.

isoelectric point - the pH at which the net charge of a protein is zero.

ping pong - an enzymatic reaction in which one or more products are released before all the substrates are bound.

specific activity (enzyme) - the total enzyme activity present per unit weight of total protein.

step fold purification - the degree to which an enzyme is purified from one purification step to the next.

total activity - the amount of substrate that can be converted into product per unit time.

unit of enzyme activity - one unit of enzyme activity is defined as the amount of enzyme required to transform 1 μmole of substrate into product per minute.

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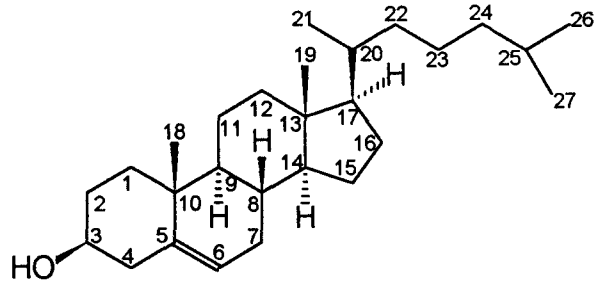
Chapter 1

INTRODUCTION

1.1.0 ♦ General

Steroids are complex fat-soluble molecules with four fused rings. The most abundant steroids are the steroid alcohols known as sterols, which are present largely as ester derivatives. The major sterol found in mammals is cholesterol **(1)**. Cholesterol is essential for the growth and viability of cells in higher organisms. Cholesterol is present in the plasma membranes of eukaryotic cells in order to modulate the fluidity of the cell membranes and it is the precursor of steroid hormones such as progesterone, testosterone, estradiol and cortisol, as well as of the bile salts that help to solubilize fats for digestion. Cholesterol can be obtained from the diet or it can be synthesized *de novo* primarily in the liver, although appreciable amounts are also formed by the intestine.[1]



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1.2.0 ♦ Biosynthesis of Cholesterol

The extraordinarily complex biosynthetic pathway of cholesterol was elucidated from the work of Konrad Bloch, Feodor Lynen, and John Cornforth, who received Nobel prizes in 1961 for their success.[1]

Early evidence concerning the synthesis of cholesterol was obtained from isotopic labeling experiments in which two kinds of isotopically labeled acetates were fed to rats. The two acetates were labeled with ^{14}C; one was labeled at the methyl carbon, the other was labeled at the carboxyl carbon. The cholesterol was then isolated from the tissues of rats fed the two different types of labeled acetate. The labeled cholesterol obtained in each case was then degraded, step by step, by known chemical reactions to yield characteristic products. Determination of the radioactivity of these products revealed the location in the cholesterol molecule of the carbon atoms derived from the methyl carbon of acetate and those derived from the carboxyl carbon (**Figure 1**). This information became the blueprint for working out the enzymatic steps in the biosynthesis of cholesterol.[2]

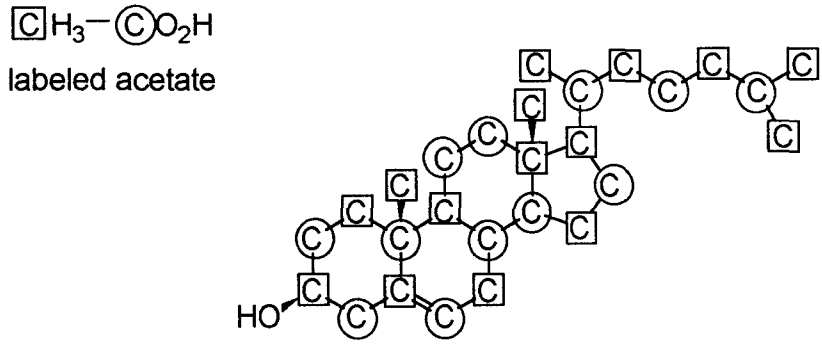
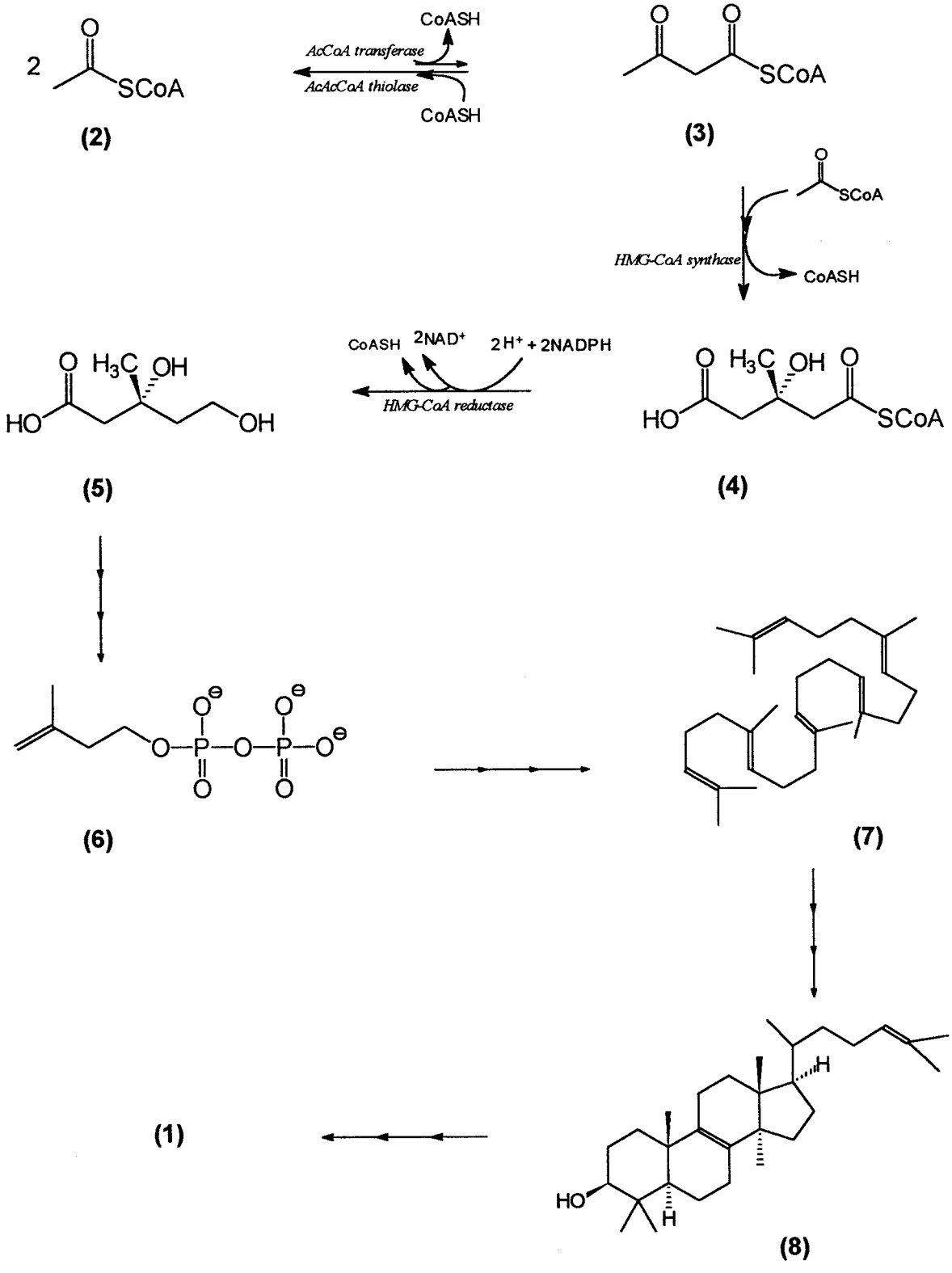


Figure 1. The origin of the carbon atoms of cholesterol

Acetate is present as it's coenzyme A (CoA) ester, acetyl-CoA, *in vivo*. The principle source of acetyl-CoA is pyruvic acid, which is in turn formed from glucose. If glucose levels are low, β-oxidation of fatty acids contributes to the acetyl-CoA pool.

The biosynthesis of cholesterol **(Scheme 1)** begins with the condensation of two acetyl coenzyme A **(2)** units, catalyzed by acetyl-CoA transferase, to produce acetoacetyl-CoA **(3)**. Acetoacetyl-CoA then condenses with another acetyl-CoA unit to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) **(4)**; this reaction is catalyzed by HMG-CoA synthase. The next step, often referred to as the committed step in cholesterol biosynthesis, is the irreversible formation of mevalonic acid **(5)**. This reaction is catalyzed by HMG-CoA reductase. Three phosphate groups are then attached to mevalonic acid and then decarboxylation of the resulting phosphorylated mevalonate leads to the formation of Δ^3-isopentenyl pyrophosphate **(6)**, an activated form of an isoprene unit. Six isopentenyl groups are then assembled, with the loss of their pyrophosphate groups, to yield the hydrocarbon squalene **(7)**. Squalene then undergoes a series of complex enzymatic reactions in which its linear structure is folded and cyclized to form lanosterol **(8)**. The bioconversion of lanosterol into cholesterol involves the loss of three methyl groups, the saturation of the side-chain double bond and migration of the $\Delta^{8,9}$ double bond to the $\Delta^{5,6}$ position.[3]


2
SCoA
(2)
CoASH
AcCoA transferase
AcAcCoA thiolase
CoASH
SCoA
(3)
SCoA
HMG-CoA synthase
CoASH
CoASH
2NAD+
2H+ + 2NADPH
HMG-CoA reductase
HO
OH
OH
(5)
HO
OH
SCoA
(4)
(6)
(7)
(1)
HO
H
H
(8)

Scheme 1

1.2.1 ◊ Regulation of Cholesterol Biosynthesis

Although the presence of cholesterol is essential for life, elevated cholesterol levels can cause disease and death by contributing to the formation of atherosclerotic plaques in arteries throughout the body, potentially leading to heart attacks, strokes and peripheral vascular disease.[4] Thus, it becomes evident that cholesterol metabolism must be precisely regulated.

The rate of cholesterol biosynthesis is highly responsive to the amount of cholesterol absorbed from dietary sources. Dietary cholesterol suppresses the synthesis of HMG-CoA reductase in the liver and inactivates existing enzyme molecules. Cholesterol biosynthesis is also controlled by the concentration of a specific protein, sterol carrier protein, which binds the water-insoluble intermediates of the sequence and thus makes them more readily available for the subsequent enzymatic steps.[1,5]

In attempts to reduce elevated cholesterol levels by intervention, control of the dietary intake of saturated fat and cholesterol is the first line of treatment; if this fails, then drug therapy is initiated.[4]

The most commonly used drugs in the treatment of elevated cholesterol levels are the bile salt sequestrants. Bile salts are polar derivatives of cholesterol which promote the absorption of dietary cholesterol. Bile salt sequestrants are positively charged polymers that bind negatively charged bile salts and decrease the extent to which they are reabsorbed in the intestine.[4]

Recently another line of drug therapy has evolved, directed towards inhibiting the biosynthesis of cholesterol. The target enzyme is HMG-CoA reductase, which catalyzes the rate determining step in cholesterol biosynthesis. The HMG-CoA reductase inhibiting drugs lovastatin and compactin have proven to be effective in lowering serum cholesterol levels in cultured animal cells,[6] in intact animals *in vivo*,[7,8] and in humans.[9,10]

However, there are toxic side effects associated with these drugs such as: angina, gastrointestinal problems, jaundice, skin rashes, visual disturbances, hypertension, and development or worsening of congestive heart failure to name but a few.[11]

1.2.2 ◊ HMG-CoA synthase as a potential target for lowering cholesterol levels

The inhibition of a specific enzyme in the biosynthetic pathway of cholesterol, HMG-CoA reductase, has proven to be an effective means of lowering overall cholesterol levels. This discovery has sparked considerable interest in finding other potential sites of regulation.

A number of studies have shown that HMG-CoA reductase and HMG-CoA synthase are transcriptionally regulated by similar mechanisms.[12-15] Both enzymes contain an oligonucleotide sequence in the promoter region of their genes which has been shown to be essential for sterol mediated regulation. The sterol mediated regulation is believed to be involved in cholesterol homeostasis, controlling the levels of these two enzymes. Lovastatin, a cholesterol lowering drug, interferes with this homeostasis by reducing cholesterol biosynthesis,[16] which results in an up-regulation of HMG-CoA reductase levels. Since the regulation is similar, inhibitors of HMG-CoA synthase are also expected to reduce serum cholesterol levels. The activity of this enzyme is affected by agents that alter cholesterol biosynthesis in the livers of chickens,[17] and rats.[18-20] Studies in Chinese hamster ovary cells,[21,22] HeLa,[23] C-6 glia cells,[24,25] and L-M cells[26] have also demonstrated that cholesterol biosynthesis is sensitive to changes in the HMG-CoA synthase activity. Sections **1.3.0** through **1.3.3** will take a detailed look at HMG-CoA synthase.

1.3.0 ♦ HMG-CoA Synthase

While studying the biosynthesis of β-hydroxy-β-methylglutaric acid (HMG) in 1956, Rudney obtained a soluble rat liver microsome preparation which synthesized HMG from acetyl-CoA and acetoacetyl-CoA. He designated the enzyme catalyzing this reaction as the "HMG-condensing enzyme";[27] the name of this enzyme has since been changed to HMG-CoA synthase. Rudney subsequently demonstrated the presence of HMG-CoA synthase in partially purified beef liver homogenates and in cell free extracts of baker's yeast.[28]

Since its initial discovery, HMG-CoA synthase has been successfully purified, in most cases to homogeneity, from chicken liver,[17,29-32] rat liver,[33,34] hamster liver,[35] ox liver[36-38] and yeast.[39-43]

The HMG-CoA synthases isolated from these various sources possess the same catalytic properties, but exhibit some different physical properties. Some of these physical properties are detailed below:

1. Molecular Weight

In most cases it has been determined that the enzyme is dimeric, composed of two subunits of equal molecular weight. However, the molecular weights of these subunits differ depending upon the source of the enzyme.

2. Isoelectric Point

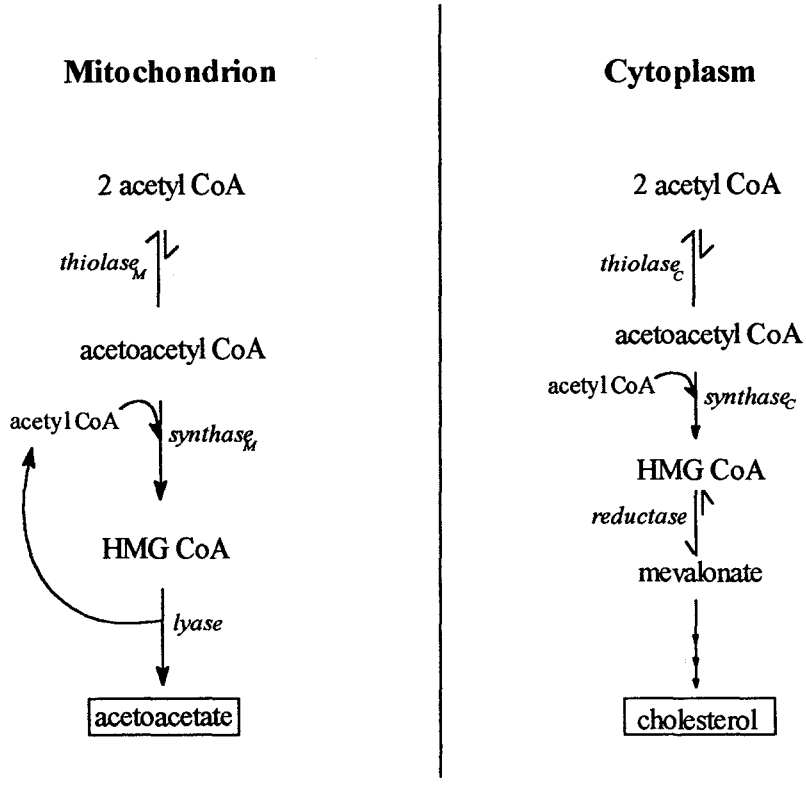
Another characteristic property of enzymes is their isoelectric point (pI). Enzymes are proteins composed of various amino acids: some of these amino acids are acidic and some basic, giving the protein an overall charge. The isoelectric point of a protein is the pH at which its net charge is zero. Molecules of HMG-CoA synthase

isolated from different sources exhibit different isoelectric points, ranging from 5.2 for cytosolic chicken liver enzyme, to 7.2 for mitochondrial chicken liver enzyme.[17]

3. Amino Acid Sequence

In addition, the amino acid sequences differ slightly for the various forms of the enzyme; however all of the sequenced enzymes indicate that a cysteine residue is present in the enzyme's active site.[33,44-48]

The HMG-CoA synthase in liver is further complicated by the existence of two distinct forms of the enzyme: a mitochondrial form and a cytoplasmic form. The mitochondrial and cytosolic forms of the enzyme have been proven to be physically, catalytically and immunologically distinct. The two forms of hepatic synthase have been shown to participate in two different biosynthetic pathways in animal liver, the synthesis of cholesterol and of acetoacetate; cytoplasmic HMG-CoA is involved in cholesterol biosynthesis, and the mitochondrial enzyme is involved in the biosynthesis of ketone bodies as illustrated in **Scheme 2**.



Scheme 2

In addition to the mitochondrial and cytosolic forms of liver HMG-CoA synthase, the chicken and rat liver cytosolic enzyme are themselves both composed of multiple forms of the enzyme.[31,34] Chicken liver cytosolic synthase consists of four molecular species, of these four forms three are interconvertible and the fourth form bears close resemblance to the mitochondrial synthase.[17] It has been speculated that this fourth form is a cytoplasmic precursor of the mitochondrial enzyme. Similarly, rat liver is composed of at least three molecular species, one of which closely resembles the mitochondrial enzyme.[34] However, it is presently unknown why multiple forms of cytosolic synthase exist.

Some of the properties of the various HMG-CoA synthases that have been isolated as well as the degree of purification and homogeneity are summarized in **Table 1**.

Table 1. Properties of HMG-CoA synthase isolated from various sources

Source	molecular weight	dimeric (mw subunit)	pI	fold purification	homogeneous	sequenced
chicken liver:						
-mitochondria	105,000	yes (53,000)	7.2	4,[30] 23[32]	yes	yes
-cytosol: type 1	90,000	yes (52,000)	6.6	3,[17]	yes	no
type 2	94-100,000	yes (55,000)	5.4	2,[17]	yes	no
type 3	100,000	yes (58,000)	5.2	300,[17]	yes	no
type 4	unknown	yes (58,000)	unknown	175,[17]	yes	no
yeast	130,000	unknown	5.6[r]	13,[40] 30,[39] 40,[43] 250,[41] 10[42]	no	no
ox liver:						
-mitochondria	95-119,000	yes (47,900)	6.3	212[38]	yes	yes
rat liver:						
-mitochondria	105,000	yes (53,000)	7.5	400[34]	yes	yes
-cytosol: type 1	120,000	yes (55,000)	6.5	450[34]	yes	no
type 2	269,000	unknown	7.2	650[34]	yes	no
type 3	186,000	unknown	7.2	650[34]	yes	no

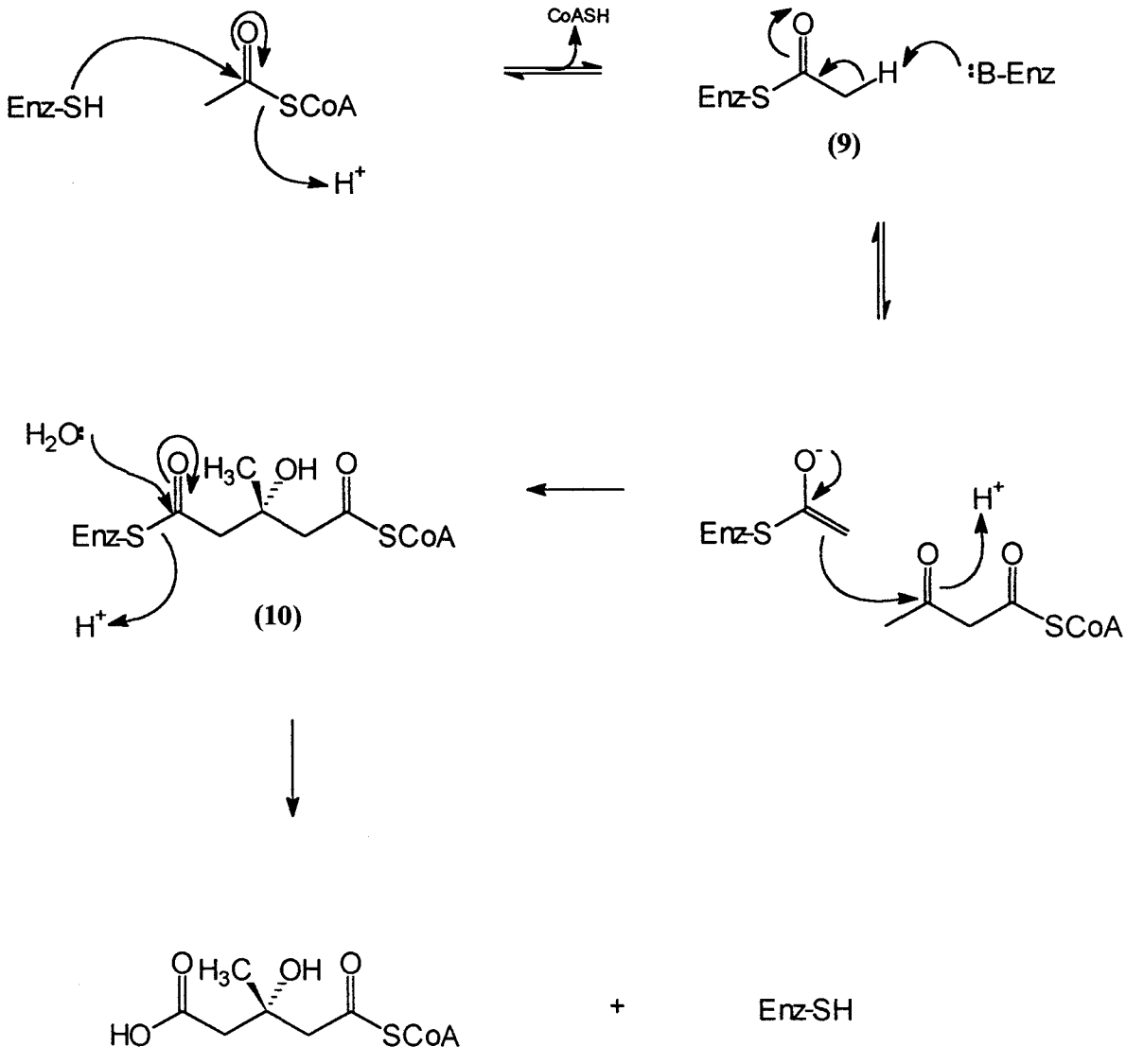
Despite the physical differences between HMG-CoA synthase isolated from various different sources, the catalytic requirements of the enzyme, specifically the substrate specificity and mode of action, remain the same.

1.3.1 ◊ Mode of Action of HMG-CoA synthase

Kinetic and chemical experiments on HMG-CoA synthase isolated from yeast, ox and chicken livers have established that the enzymatic formation of HMG-CoA proceeds through a covalently modified form of the enzyme and an ordered series of binary complexes without the formation of kinetically significant amounts of ternary complex.[49,50] Such a mechanism has been termed "ping pong" by Cleland.[51]

The mode of action of the enzyme has been shown to involve the nucleophilic attack of a cysteine sulfur atom in the enzyme's active site, on the carbonyl carbon of acetyl CoA to form an S-acetyl enzyme intermediate **(9)**. There is substantial evidence supporting the formation of such an intermediate. Middleton and Tubbs[52,53] have demonstrated that stoichiometric release of CoA from acetyl-CoA occurred when equimolar quantities of enzyme were mixed with acetyl-CoA. They also successfully isolated [^{14}C]acetyl-enzyme and observed its conversion into free enzyme and [^{14}C]acetyl-CoA in the presence of CoA. Miziorko et al[54] have reported similar results and have identified a cysteinyl sulfhydryl as the site of acetylation. Deprotonation of this S-acetyl enzyme species produces an enolate ion, which subsequently undergoes an aldol-type condensation with acetoacetyl CoA to form a 1,5-dithioester of 3-hydroxy-3-methylglutarate **(10)**. This intermediate has been trapped and partially characterized by Miziorko.[55] Using either acetoacetyl-S[^{3}H]CoA or [^{14}C]acetyl-S-enzyme, they determined that the label from either of these sources could be trapped in the accumulated intermediate with carbon-14 activity appearing in the HMG moiety. They also determined

that the breakdown of **(10)** to HMG-CoA and free enzyme is slow relative to the rate of formation of acetoacetyl-CoA and acetyl-S-enzyme. Hydrolysis of the thioester linkage between the enzyme and the condensation product results in the release of HMG-CoA and accounts for the irreversible nature of the overall reaction. The proposed mechanism of action of HMG-CoA synthase is outlined in **Scheme 3**.



Scheme 3

Detailed studies of acyl-CoA binding to HMG-CoA synthase have been conducted by electron spin resonance (ESR) using a spin-labeled acyl-CoA probe (3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl-CoA) as a substrate.[56-59] It was found that acyl-CoA binds to identical, independent enzyme subunits, that there is one CoA binding pocket per active site, and that bound acyl-CoA could be displaced by each of the enzyme substrates or products.

1.3.2 ◊ Substrate Specificity

The HMG-CoA synthases isolated from different sources have the same substrate specificity:

1. The enzyme is absolutely specific for an acetylated substrate in order for a productive condensation with acetoacetyl-CoA to occur. Evidence to support this substrate specificity comes from studies done on the yeast, chicken and ox liver enzymes. Middleton has demonstrated that for the yeast enzyme propionyl- or butyryl-CoA cannot replace acetyl CoA and 3-ketohexanoyl-CoA cannot replace acetoacetyl CoA;[53] similar results have been found for the ox liver enzyme.[36] In contrast, the mitochondrial chicken liver enzyme will bind to both acetyl-CoA and propionyl-CoA to form a covalent acyl-S-enzyme species; however, only the acetyl-S-enzyme species reacts further to form products. The chicken liver enzyme will bind to bulkier acyl-CoAs at the acetyl-CoA site, however these compounds fail to acylate the active site cysteinyl -SH. Nevertheless, the acyl group of an acyl-CoA bound at the acetyl-CoA site is highly immobilized in the noncovalent complex.[17,30]

2. The enzyme is not absolutely specific for the CoA moiety of its substrates. The yeast enzyme has been shown to accept acetyl-3'-dephospho-CoA,[29] acetylpantetheine, and acetylglutathione as acetyl-CoA analogs in the reaction. However, only acetoacetyl-acyl carrier protein (AcAcACP)[60] has been found to replace acetoacetyl-CoA as a substrate. The mitochondrial chicken liver enzyme will also accept acetyl-3'-dephospho-CoA as an acetyl-CoA analogs and N-acetyl-S-acetoacetyl-cysteamine will serve as an acetoacetyl-CoA analog.[17,30] In addition, the thioester functionality is not an absolute requirement in the substrate which condenses with the acetyl-enzyme intermediate. 3-Oxobutyl-CoA, a thioether, reacts slowly with acetyl-S-enzyme to produce the thioether analog of HMG-CoA.[17]

1.3.3 ◊ Kinetic Properties

For most enzymes, the rate of catalysis (V) varies with the substrate concentration. At very low substrate concentrations the rate of the reaction is very low, but it will increase with increasing substrate concentrations until a plateau is reached, called the maximum rate (V_{max}), in which the enzyme is "saturated' with its substrate and can function no faster, as seen in **Figure 2**. Since the curve expressing this relationship has the same general shape for most enzymes, Michaelis and Menten defined a constant, K_M, that is useful in establishing the precise relationship between the substrate concentration and the velocity of the enzyme-catalyzed reaction. K_M, the Michaelis-Menten constant, is defined as the concentration of the specific substrate at which a given enzyme yields one-half its maximum velocity.[61]

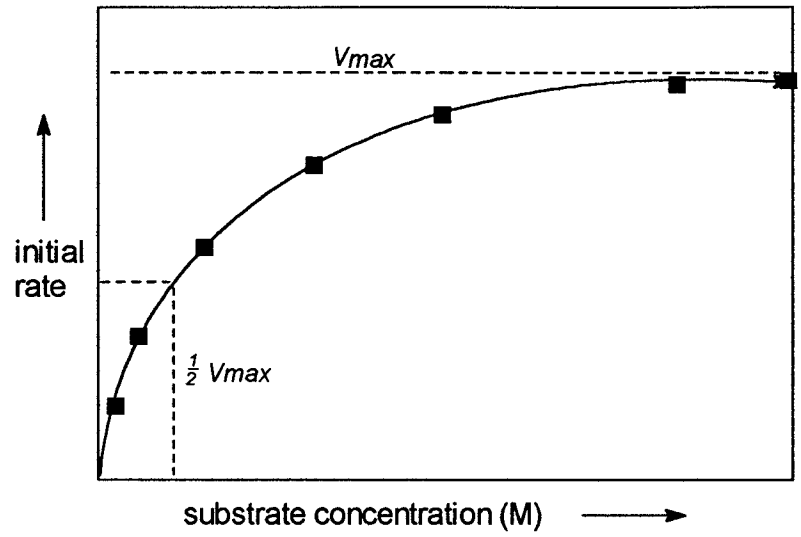


Figure 2. Plot of enzyme rate as a function of substrate concentration

Each enzyme has its own characteristic K_M values and consequently, the HMG-CoA synthase isolated from different sources was found to have different K_M values. In all cases it was found that the apparent K_M for acetoacetyl-CoA was extremely low and K_M for acetyl-CoA was much greater, but the exact values varied. The following table summarizes the K_M values found for HMG-CoA synthase isolated from different sources.

Table 2. Apparent K_M values for HMG-CoA synthase isolated from different sources

Source of enzyme	K_M (acetoacetyl-CoA)* (μM)	K_M (acetyl-CoA)$ (μM)
chicken liver:!		
-mitochondria	<5	1000
-cytosol: type 1	<2	290
type 2	<2	310
type 3	<2	310
type 4	unknown	unknown
Baker's yeast!	<<0.4	14
ox liver#	<1	>100
rat liver:!		
-mitochondria	<0.1	551
-cytosol: type 1	18	127
type 2	18	130
type 3	18	130

* 50 μM acetyl-CoA used
$ 200 μM acetoacetyl-CoA used
! measured at pH 8.0
measured at pH 7.8

All forms of HMG-CoA synthase are strongly inhibited by acetoacetyl-CoA. This substrate inhibition appears to be competitive with respect to acetyl-CoA binding and is affected by the pH; it is less pronounced at pH values above 8.0.

1.4.0 ♦ β-Lactone Antibiotic F-244

The naturally occurring antibiotic β-lactone F-244 was first isolated in 1971 by Aldridge, during the screening for physiologically active compounds from *Cephalosporium sp.*[62,63] Subsequently, it has been isolated independently from *Scopulariopsis sp.*[64] and *Fusarium sp.*[65] The structure and stereochemistry of F-244 have been identified as (*E,E*)-11-[3'-(hydroxymethyl)-4'-oxo-2'-oxetanyl]-3,5,7-trimethyl-2,4-undecadienoic acid with an absolute configuration of 2'*R*, 3'*R* and 7*R*.[66] Its total synthesis has also been reported[66-69] as has its biosynthesis.[70,71] The structure of the β-lactone F-244 is shown in **Figure 3**.

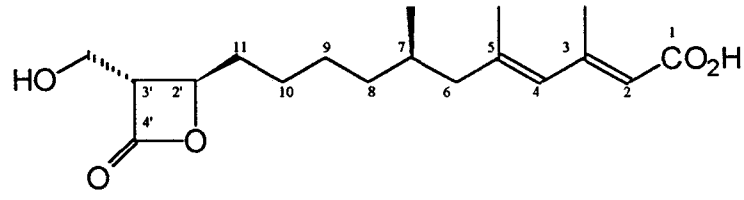


Figure 3 - Structure of the β-Lactone F-244

Also known as 1233A and L-659,699, this antibiotic and its derivatives are the only known specific inhibitors of HMG-CoA synthase both in cell culture and in cell-free enzyme extract.[4,72] As a result, this compound is a potential candidate as an antihypercholesterolemic drug.

1.4.1 ◊ Mechanism of Inhibition of HMG-CoA synthase by F-244

The mechanism for the inhibition of HMG-CoA synthase by F-244 is unknown and is the subject of active research. A mechanism has been proposed by Mayer el al[73] **(Figure 4a)**. Since the initial step in the catalytic cycle involves nucleophilic attack by an

active site cysteine residue on the carbonyl group of acetyl CoA, Mayer proposed that the inactivation of the enzyme is due to the β-lactone functionality. This hypothesized mechanism involves the attack of the sulfhydryl on the carbonyl carbon of the β-lactone ring. The subsequent ring-opening reaction would proceed to give a covalently bound enzyme-inhibitor complex, effectively inactivating the enzyme.

A second possible mechanism **(Figure 4b)** involves the attack of the enzyme's active site sulfhydryl on the β-carbon atom, instead of the acyl carbon. This attack would result in cleavage of the C-O bond to open the four-membered ring and relieve the angle strain. Such a mechanism has been termed $B_{AL}2$ (base catalyzed alkyl cleavage via an S_N2 mechanism),[74] and it is a known and precedented mechanism for β-lactone cleavage.[75,76] Inhibition by this mechanism is especially attractive since the thioether formed would be more stable than the thioester formed in Mayer's mechanism.

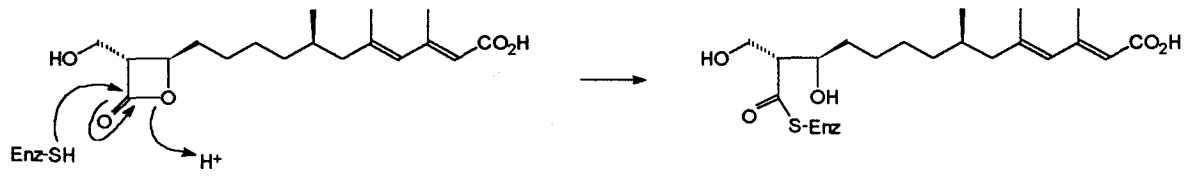


Figure 4a. Mechanism 1 - nucleophilic attack at the acyl carbon

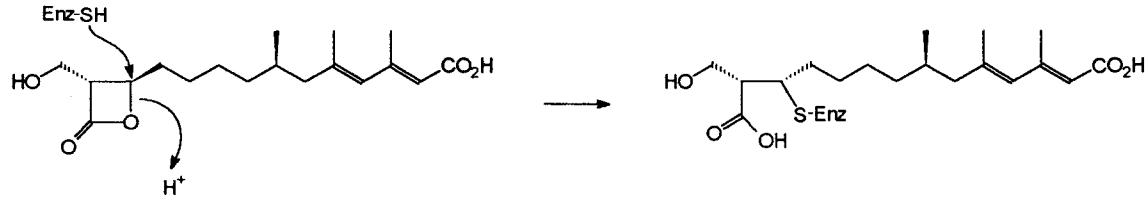


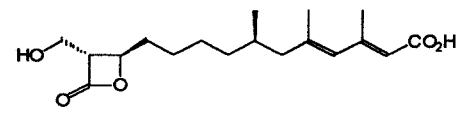
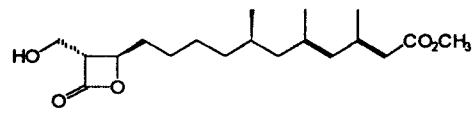
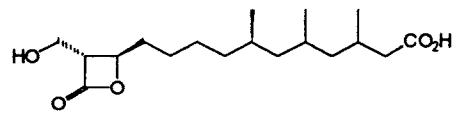
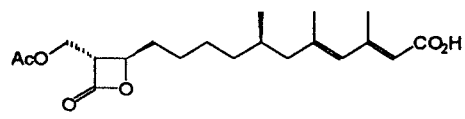
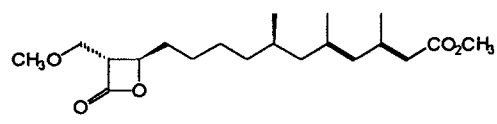
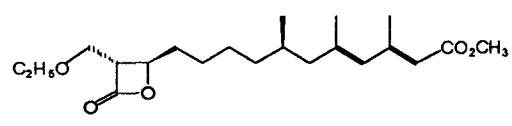
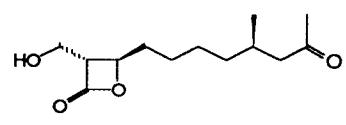
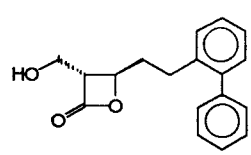
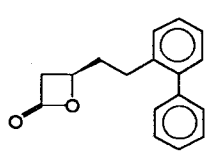
Figure 4b. Mechanism 2 - nucleophilic attack at the alkyl carbon

However, it is important to note that the β-lactone ring is not the only factor required for inhibition of HMG-CoA synthase. There have been numerous independent studies directed towards determining the components of F-244 necessary for inhibition of HMG-CoA synthase.[73,77-79]

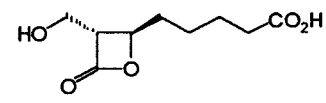
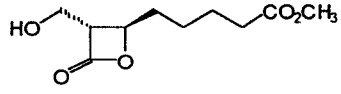
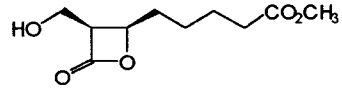
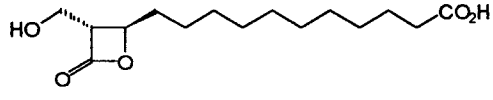
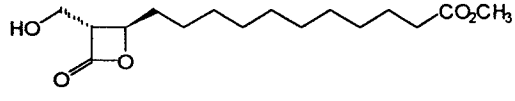
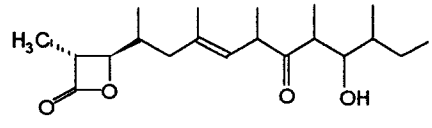
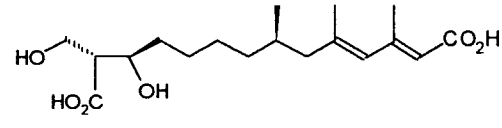
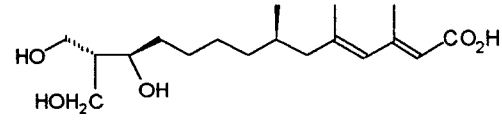
It has been found that the saturated derivative of F-244 was about 50% as active as the native polyunsaturated compound, indicating the importance of the double bonds at the 2 and 4 positions.[78] Not only is the presence of these double bonds essential, but their geometry is also important. It has been found that only the *trans* β-lactone was active against HMG-CoA synthase, whereas the *cis* β-lactone showed no inhibitory activity.[77] The methyl ester derivative exhibited activity comparable to F-244, demonstrating that the carboxyl group can be tampered with without loss of activity.[78,79] A derivative in which the hydroxyl residue was acetylated, reduced the inhibition considerably. Thus, the hydroxymethyl moiety of F-244 is necessary for potent inhibition, perhaps in spacial interaction with the enzyme.[78,79] Opening the β-lactone ring resulted in complete loss of inhibitory activity, showing that the lactone ring is essential for potent inhibitory activity against HMG-CoA synthase. Reducing or lengthening the carbon side chain dramatically reduced the inhibitor potency, indicating that the length of the carbon side chain is another factor affecting the inhibitory activity.[73,77,79]

A brief summary of some of these investigations is shown in **Table 3**.

Table 3. Inhibitory Abilities of various F-244 analogs

F-244 Analog	IC_{50} (μM)*				
	Omura[77]	Tomoda[78]	Mayer[73]	Mayer#[73]	Green-span#[79]
	0.34	0.20	0.27	0.01	0.10
	-	0.27	-	-	0.10
	-	0.43	-	-	0.40
	-	4.51 (32%)	-	-	2.0
	-	-	-	-	0.23
	-	-	-	-	0.74
	-	-	115	1.5	1.6
	-	-	1.0	0.06	-
	-	-	9.0	12.0	-

(Table 3 continued)

F-244 Analog	IC_{50} (μM)*				
	Omural[77]	Tomodal[78]	Mayer[73]	Mayer#[73]	Green-span#[79]
	0.99	-	-	-	-
	2.08	-	-	-	-
	> 2.31	-	-	-	-
	0.42	-	-	-	-
	0.47	-	-	-	-
	-	no activity	-	-	-
	-	no activity	-	-	no activity
	-	no activity	-	-	-

* IC_{50} is the concentration of inhibitor required to inhibit 50% of the enzyme activity
! enzyme activity measured without prior incubation with the inhibitor
enzyme activity measured after 5 minute incubation with the inhibitor

1.4.2 ◊ Reversibility of F-244 Inhibition of HMG-CoA synthase

There is some discrepancy concerning the reversibility of the inhibition of HMG-CoA synthase by F-244. Greenspan and co-workers have reported that the inhibition is reversible upon dilution of the enzyme-inhibitor complex.[79] However, this claim has been disputed by Mayer et al[73] and Omura et al,[77] who claim the inhibition to be irreversible over several hours even upon dilution of the enzyme-inhibitor complex. This matter remains to be clarified.

Chapter 2

RESULTS AND DISCUSSION

2.1.0 ♦ OBJECTIVES:

The primary objective of this study was to purify the enzyme HMG-CoA synthase from bakers yeast in order to study the interaction of this enzyme with its only known specific inhibitor, F-244. It was also hoped that the question of the reversibility of F-244 inhibition of HMG-CoA synthase would be resolved by this study.

A second objective was to study the effect of other β-lactone compounds on the activity of HMG-CoA synthase to determine whether or not these compounds are inhibitors of the enzyme.

A third objective was to prepare tritiated F-244 to use as an affinity label for HMG-CoA synthase. The resulting enzyme-inhibitor complex would then be isolated in order to obtained "labeled" enzyme for future studies.

2.2.0 ♦ General

The first step in any enzyme purification is to find a source of the enzyme. Once the raw material has been identified, it is then necessary to find a method for disrupting the cells in order to release the enzymes into solution. But before any purification steps can be done, it is essential to develop an analytical technique for detecting the presence of the desired enzyme in solution; this is known as an assay.

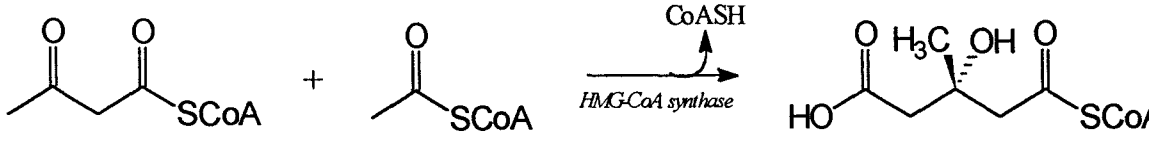
Section **2.1.1** will describe the assay used to detect and measure the activity of HMG-CoA synthase in this study.

2.2.1 ◊ Assay of HMG-CoA Synthase Activity

The amount of enzyme in a given solution or extract may be assayed quantitatively in terms of the catalytic effect it produces. In order to do this it is necessary to know:

(1) The overall equation of the reaction catalyzed by the enzyme

As explained in sections **1.2.0** and **1.3.1**, HMG-CoA synthase catalyzes the condensation of acetyl-CoA with acetoacetyl-CoA to form HMG-CoA (**Scheme 5**).

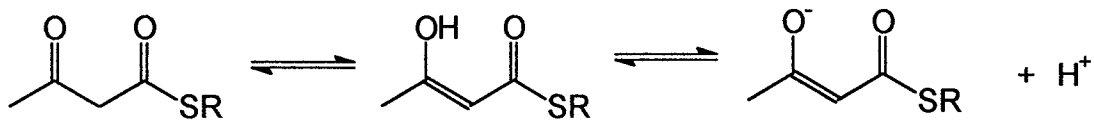


Scheme 5

(2) An analytical procedure for determining the disappearance of the substrate or the appearance of the reaction products

Lynen and co-workers[80,81] were the first to make use of the optical properties of acetoacetic thio CoA esters in developing sensitive enzyme assays. They discovered that these acetoacetic thio esters possessed a strong absorption band between 280 and 320 nm, with a maximum around 303 nm. Since the intensity of this band was pH dependent,

increasing with increasing pH, they attributed it to the formation of the enolate ion **(11)** **(Scheme 6)**.



(11)

Scheme 6

Thus, the activity of HMG-CoA synthase was measured spectrophotometrically by measurement of the disappearance of one of the substrates, acetoacetyl-CoA, in the presence of the other substrate, acetyl-CoA, with respect to time at 303 nm.

(3) Whether the enzyme requires cofactors such as metal ions or coenzymes

HMG-CoA synthase requires no cofactors; however, it has been shown by Stern[82-84] and by Beinert[85] that the presence of magnesium ion, although it has no direct effect on the enzyme being studied, increases the apparent extinction coefficient of acetoacetyl-CoA. It is believed that a 1:1 chelate is formed between acetoacetic thio esters of CoA and the divalent magnesium ion. Stern[82] has suggested the formation of an "internal" chelate (**Figure 5**) in which the ligand groups are the enol and the carbonyl oxygen atoms of the acyl moiety and the carbonyl oxygen atoms (or possibly nitrogen atoms) of the two peptide bonds.

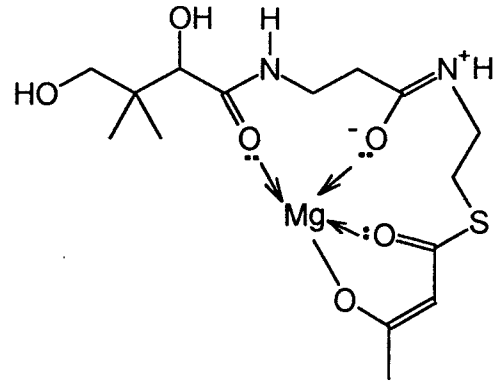


Figure 5. Stern's representation of the "internal" chelate

Thus, the assay was performed in the presence of magnesium ion in order to achieve a reasonably high sensitivity in the assay of the enzyme.

(4) The dependence of the enzyme activity on substrate concentration (K_M of the substrate)

The dependence of HMG-CoA synthase activity on the concentrations of acetoacetyl-CoA and acetyl-CoA was studied by Miller and co-workers.[86] They optimized the assay by using 0.048 mM acetoacetyl-CoA and 0.190 mM acetyl-CoA in the assay mixture. Thus, these same concentrations of substrate were used in this work.

(5) The pH at which the enzyme activity is maximal

Middleton and Tubbs[41] determined that the enzyme activity was maximal at pH 8.2; thus, the assays performed in this work were also at pH 8.2.

(6) A temperature zone in which the enzyme is stable and has high activity

The spectrophotometric assay of HMG-CoA synthase isolated from bakers yeast has been performed at 30°C[41] and at room temperature[39] and no significant differences in activity were observed. In this study all of the assays were performed at room temperature for convenience purposes.

2.2.2 ◊ Sample Assay of HMG-CoA Synthase Activity

HMG-CoA synthase activity was assayed in this work by first measuring the rate of decrease of acetoacetyl-CoA absorbance at 303 nm in the presence of the enzyme. In the absence of acetyl-CoA, acetoacetyl-CoA is consumed by acetoacetyl-CoA thiolase, an enzyme which catalyzes the hydrolysis of acetoacetyl-CoA. Acetyl-CoA was then added, and the increase in acetoacetyl-CoA disappearance was taken to represent the HMG-CoA synthase activity. A representative assay is shown in **Figure 6**.

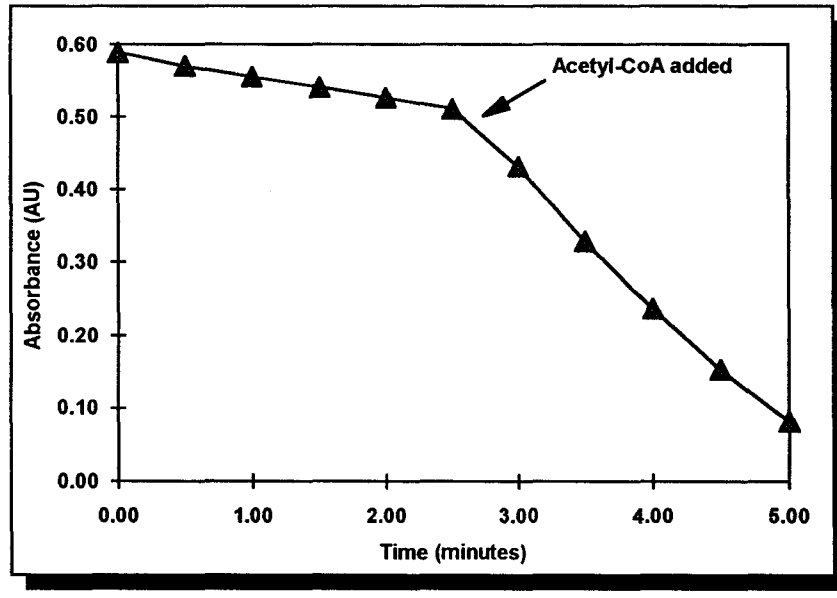


Figure 6. Typical Assay of HMG-CoA synthase activity (at 303 nm)

The data represented in **Figure 6** is actual experimental data obtained from assaying 15μL of enzyme solution after the disruption of the cells (total volume of solution was 80 mL). The raw data used to construct this graph is shown in **Table 4**.

Table 4. Data obtained from the assay of HMG-CoA synthase activity

Time (minutes)	Absorbance (AU)
0	0.589
0.5	0.570
1	0.555
1.5	0.541
2	0.526
2.5	0.511
3	0.432
3.5	0.328
4	0.237
4.5	0.153
5	0.081

Since the plot in **Figure 6** is linear between 0 to 2.5 min and between 3 to 5 min, a linear regression analysis was performed in order to determine the slopes of these two lines. The regression data is shown in **Table 5**.

Table 5. Regression output for the assay of HMG-CoA synthase activity

Regression Output:	From 0 to 2.5 minutes:	From 3 to 5 minutes:
Constant	0.58695	0.94780
Std Err of Y Est	0.00151	0.01098
R squared	0.99779	0.99532
No. of Observations	6	5
Degrees of Freedom	4	3
X Coefficient	-0.03063	-0.1754
Std Err of Coeff	7.21E-4	6.7E-3

In order to ensure that the assay was indeed measuring HMG-CoA synthase activity, several control experiments were performed. In the first control the rate of spontaneous acetoacetyl-CoA hydrolysis was measured. In this experiment the reference and sample cells both contained 0.2 M tris-HCl buffer, pH 8.2, and the experiment was initiated by the addition of acetoacetyl-CoA to the sample cell. It was found that the rate of acetoacetyl-CoA hydrolysis was only 0.003 absorbance units per minute.

A second control measured the absorbance of the protein solution. In this experiment both the sample and reference cells contained tris-HCl buffer, protein solution was then added to the sample cell and the absorbance was measured at 303 nm. It was found that the protein solution had a weak absorbance of 0.3 AU that remained constant for over 5 minutes.

The third control experiment measured the rate of acetoacetyl-CoA absorbance after the addition of acetyl-CoA. This experiment was performed in much the same way as the first control. The rate of spontaneous acetoacetyl-CoA hydrolysis was first

measured and then acetyl-CoA was added and the hydrolysis rate of acetoacetyl-CoA was measured again. It was found that after the addition of acetyl-CoA the rate of acetoacetyl-CoA hydrolysis was unchanged (0.003 absorbance units/min).

The final control experiment involved assaying a denatured protein solution. A sample from a protein solution was first assayed as previously described and it was shown to have activity, according to the assay. The remainder of the protein solution was boiled for 5 minutes in order to denature the protein; if the assay were working properly then assaying the boiled protein solution should show no increase in acetoacetyl-CoA absorbance after the addition of acetyl-CoA. The results of this experiment were exactly as predicted, indicating that the assay was working properly.

2.2.3 ◊ Calculation of Total and Specific Activities, Fold Purification and Yield of HMG-CoA Synthase

1. Calculation of Total Activity

The total activity of an enzyme is defined as the amount of substrate that can be converted into product per unit time. In the case of HMG-CoA synthase, 1 unit is defined as the amount of enzyme required to transform 1 μmol of substrate into product per minute. The total activity of HMG-CoA synthase can be calculated from the data in **Tables 4** and **5.**

The decrease in acetoacetyl-CoA absorbance due to HMG-CoA synthase activity can be calculated by subtracting the difference in slope after the addition of acetyl-CoA from the slope prior to the addition, which represents thiolase activity. Using the data shown in **Figure 6** as an example, the rate of decrease of acetoacetyl-CoA absorbance due to HMG-CoA synthase activity would be: (-0.1754 AU/min) - (-0.03063 AU/min) = -0.1448 AU/min.

From this value the rate of change in acetoacetyl-CoA concentration can be calculated using Beer's law:

$$A = \varepsilon dc$$

(where A= absorbance; ε=extinction coefficient of acetoacetyl-CoA; d= path length of light through the cuvette and c=concentration)

Since the absorbance (0.1448 Au/min), path length (1 cm) and extinction coefficient of acetoacetyl-CoA (16,100 Au/cm mol/L) are known, the equation can be solved for rate of change of the concentration of acetoacetyl-CoA:

$$c = A/d\varepsilon$$

$$c = \frac{0.1448 \text{ Au/min}}{(1\text{cm})(16{,}000 \text{ au/cm mol/L})}$$

$$c = 9.0 \times 10^{-6} \text{ mol/L/min}$$

Thus, the acetoacetyl-CoA concentration is changing at a rate of 9.0×10^{-6} mol/L per min.

Since the total volume of the assay is known (1.5 mL), the activity of the enzyme can be expressed as moles of acetoacetyl-CoA disappearing per minute:

$$(9.0 \times 10^{-6} \text{ mol/L/min})(0.0015 \text{ L}) = 1.35 \times 10^{-8} \text{ mol/min}$$

$$= 1.35 \times 10^{-2} \text{ μmol/min}$$

Acetoacetyl-CoA is disappearing at a rate of 1.35×10^{-2} μmol per minute under the conditions of the assay. Since the volume of enzyme added to the assay is known (15 μL), the activity can be expressed in terms of the volume of enzyme present:

$$\frac{1.35 \times 10^{-2} \text{ μmol/min}}{15 \text{ μL enzyme added}} = 9.0 \times 10^{-4} \text{ μmol/min/μL}$$

$$= 9.0 \times 10^{-4} \text{ units/μL}$$

The total volume of the enzyme solution is also known (80 mL). Thus, the total enzyme activity is:

$$(9.0 \times 10^{-4} \text{ units/μL})(80 \times 10^{3} \text{ μL}) = 72 \text{ units}$$

Thus, the total activity of the enzyme is 72 units.

2. Calculation of Specific Activity

The specific enzyme activity of an enzyme is the total enzyme activity present per unit weight of total protein. For the purpose of the present work, the specific activity was measured in units per milligram. If the specific activity of an enzyme remains constant after sequential purification steps, then the enzyme is considered to be pure.

The specific activity of HMG-CoA synthase was calculated from the total activity, the total volume of enzyme solution and the total amount of protein present in the enzyme solution.

The total protein was determined using a total protein determination kit obtained from Sigma. This method employed a modified Lowry procedure which combines the biuret reaction and the reaction of the folin-Ciocalteau reagent with proteins. The biuret method is based on the binding of proteins to copper species whereas the folin-Ciocalteau reagent reacts with phenols such as tyrosine in proteins. A strong dark blue

color is produced in the presence of protein, the intensity of this color increases with greater protein concentrations and can be measured at 725 nm.

The specific activity was calculated as follows:

$$\text{Specific Activity} = \frac{\text{total enzyme activity}}{\text{total volume of solution}} \times \frac{1}{\text{total protein}}$$

$$= \frac{72 \text{ units}}{80 \text{ mL}} \times \frac{1}{86 \text{ mg/mL}}$$

$$= 0.01 \text{ units/mg}$$

Thus, the specific activity is 0.01 units/mg.

3. Calculation of Fold Purification

The degree to which an enzyme is purified from one purification step to the next is the fold purification. Fold purification is calculated by dividing the specific activity after the step to the specific activity before the step:

$$\text{Fold Purification} = \frac{\text{specific activity after}}{\text{specific activity before}}$$

4. Calculation of Yield

The overall yield of an enzyme purification process is determined by dividing the total activity by the initial total activity and multiplying by 100:

$$\text{Yield} = \frac{\text{total activity}}{\text{initial total activity}} \times 100\%$$

This calculation assumes a 100% yield after the initial autolysis step.

2.3.0 ♦ Purification of HMG-CoA synthase

At the outset of this project it was believed that the purification of HMG-CoA synthase from bakers yeast would be reasonably simple. Bakers yeast was chosen as the source of the enzyme since it is a cheap and convenient material to handle and also the HMG-CoA synthase isolated from bakers yeast exists in only one form (see section **1.3.0**). Furthermore, the enzyme from this source has not yet been sequenced. There are several published procedures found in the literature for the isolation of HMG-CoA synthase from bakers yeast;[39-43] the method of Middleton and Tubbs[41] was chosen since it gave the greatest fold purification (250 fold).

The first challenge was to find a good source of bakers yeast. Initially, fresh yeast was obtained from a bakery and it was autolyzed in toluene as described by Middleton and Tubbs;[41] however the HMG-CoA synthase from this source of yeast was found to have very low activity. Subsequently, freeze-dried yeast was obtained from Sigma. According to Sigma, this yeast was supposed to autolyze in aqueous buffer at 37° C. In order to determine the optimum time required for autolysis, an experiment was performed in which 0.2M Tris-HCl buffer pH 8.2, was added to the yeast and the mixture was incubated at 37°C. Samples from this mixture were withdrawn after various time periods and assayed for HMG-CoA synthase activity (**Figure 7, Table 6**). It was found that an incubation period of four hours yielded the greatest HMG-CoA synthase activity. Thus, freeze-dried yeast was used as the source of HMG-CoA synthase in this work and it was autolyzed in tris-HCl buffer for four hours.

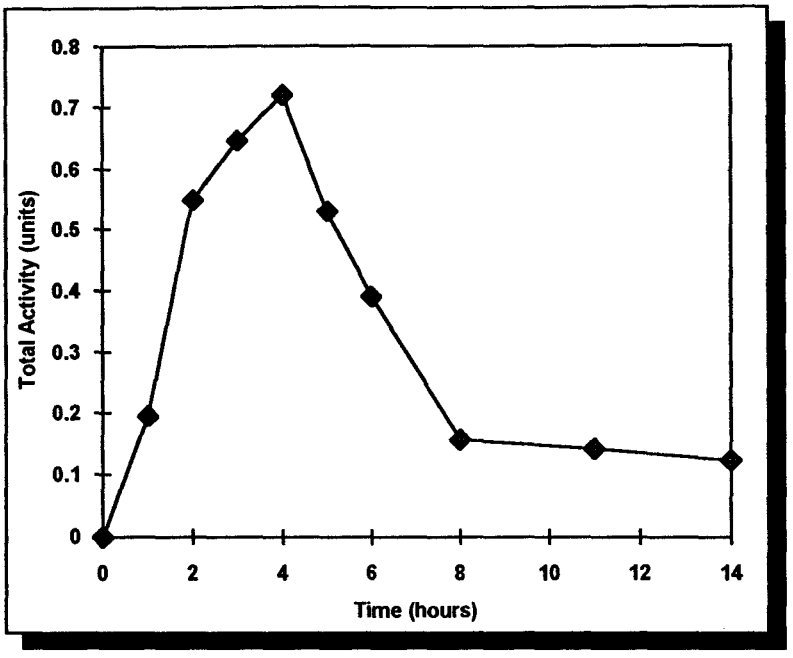


Figure 7. Time dependence of autolysis

Table 6. Total activity of HMG-CoA synthase after various autolysis times

Time (hours)	Total Activity (units)
1	0.195
2	0.549
3	0.647
4	0.721
5	0.530
6	0.392
8	0.156
11	0.142
14	0.123

After the autolysis of the yeast, the first purification step described by Middleton and Tubbs[41] was an ethanol fractionation step. Ethanol addition to the aqueous protein-containing extract decreases the solvating power of water for a charged, hydrophilic enzyme molecule by reducing the dielectric constant of the solvent. Ordered

water structure around hydrophobic areas on the protein's surface can be displaced by molecules of ethanol, leading to a relatively higher "solubility" of these areas. Consequently the water-soluble proteins experience a decrease in solubility to the point of aggregation and precipitation whereas hydrophobic proteins experience an increase and remain in solution. By controlling the amount of solvent added proteins can be selectively precipitated: generally, the larger the protein molecule, the lower percentage of organic solvent required to precipitate it. The large molecules aggregate faster because they have a greater chance of possessing a charged surface area that matches up with another protein molecule.[87]

In the ethanol fractionation procedure of Middleton and Tubbs,[41] ethanol was added to the autolysate to a final concentration of 25% v/v and the mixture was stirred at room temperature for two hours. The protein that had precipitated was unwanted denatured protein and it was removed by centrifugation. The supernatant was then cooled to -3°C in order to precipitate the HMG-CoA synthase. This procedure was repeated in the present work and it was found that the enzyme could be purified about 5 fold, which was comparable to that reported by these workers; however, the yield was only about 14% compared to the 85% yield reported by Middleton and Tubbs.[41] It was reasoned that this low yield could be due to a failure to precipitate much of the enzyme from solution. It was thought that the addition of more ethanol to the supernatant obtained after the removal of the unwanted protein, may cause more of the enzyme to precipitate out of solution. Thus the method of Middleton and Tubbs was modified and ethanol was added to the autolysate to 25%, 30% or 40% v/v final concentration, in an attempt to precipitate the enzyme from solution. In practice it was found that a final ethanol concentration of 30% v/v was optimum. This modification resulted in a 6 fold purification of the enzyme with a yield of 47%. Although the yield was still much lower than that reported by Middleton and Tubbs, the procedural modification was regarded as a success. In his book

about enzyme purification Scopes states that "fractionations are always a compromise between recovery of activity and degree of purification. If the fraction is the first step from a readily available starting material, recovery can be sacrificed for purity".[87] It was thus decided that by using this modification, both the yield and fold purification were acceptable and hence this procedure was used in this work. A comparison of HMG-CoA synthase activity before and after this procedural modification can be seen in **Table 7**.

Table 7. Purification of HMG-CoA synthase from yeast: comparison of unmodified and modified ethanol procedures

Procedure	Volume (mL)	Total Activity (units)*	Total Protein (mg/mL)	Specific Activity (units/mg)	Fold Purification	Yield (%)
Autolysis:	114 (128)	34 (30)	50 (48)	0.006 (0.005)	- -	- -
Ethanol:	4 (6)	5 (14)	42 (92)	0.030 (0.028)	5 (6)	15 (47)

* 1 unit of enzyme activity is defined as the amount of enzyme necessary to transform 1 μmol of substrate into product per minute under the conditions of the assay.
(values in parenthesis are the values obtained for the modified ethanol procedure)

The next purification step in the procedure of Middleton and Tubbs[41] was an isoelectric precipitation with acetic acid. In an isoelectric precipitation the pH of the protein solution is lowered to the isoelectric point of the desired protein. As a result, the desired protein will have an overall charge near zero resulting in an electrostatic attraction of proteins with similar properties to form aggregates which precipitate out of the solution.[87] In this step the enzyme was reported to precipitate upon lowering the pH to the isoelectric point of the enzyme, 5.6 (see **Table 1**), with acetic acid. This step was reported to give a 23 fold purification and a 70% yield.[41] This step was found to be unreproducible in our hands; the resulting precipitated enzyme had a lower specific activity (0.009 units/mg) than the ethanol precipitate (0.032 units/mg). Attempting the acetic acid precipitation step immediately after the autolysis was also unsuccessful as the enzyme did not precipitate; the HMG-CoA synthase activity was found in the supernatant.

The failure of the isoelectric precipitation is not easy to explain. This procedure was repeated several times each time trying to dissolve the ethanol precipitate in the minimum amount of buffer to try to make the protein solution as concentrated as possible; however no purification was obtained in any of these attempts. It may be possible that the isoelectric point of the HMG-CoA synthase isolated from this particular source of yeast is different from that reported in the literature, and as a result the enzyme does not precipitate at pH 5.6.

Due to the difficulties experienced in the isoelectric precipitation step, it was decided to skip this step and continue on with the published procedure. Middleton and Tubbs' next purification step was a diethylaminoethyl (DEAE) cellulose chromatography step.[41] DEAE cellulose is an anion exchanger. Proteins bind to ion exchangers by electrostatic forces between the protein's surface charges and the dense clusters of charged groups on the exchangers. In order to become attached, the protein must displace the

counterions on the ion exchanger; generally the net charge on the protein will be the same sign as the counterions displaced, hence the term "ion exchange". Proteins of the opposite charge do not bind to the ion exchanger and can be removed. Bound protein can be eluted from the exchanger by the addition of salt. The salt can directly displace the protein by occupying the charged sites on the ion exchanger, thereby blocking reattachment by the protein. Alternatively, the system can be considered as an equilibrium in which even strongly bound proteins spend some time not adsorbed; the presence of the salt ions between the unattached protein and the adsorbent greatly weakens the attraction between the two. In either case, the desorbed protein is replaced by counterions.[87]

The isoelectric point of HMG-CoA synthase is reported to be 5.6,[60] thus the protein is negatively charged and requires an anion exchanger such as DEAE cellulose. In the DEAE cellulose chromatography step, the ethanol precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.8, and the pH of the solution was adjusted to 7.8 with 0.1 M potassium hydroxide. The protein solution was then applied to a preequilibrated column of DEAE cellulose, pH 7.8, and the column was washed with one column volume of potassium phosphate buffer before a salt gradient (0 to 0.5 M KCl) was initiated. Middleton and Tubbs reported that the enzyme was eluted by the addition of 0.075 M KCl to the buffer,[41] however it was found that the enzyme did not bind to the column at all, but rather came off in the first few fractions. Furthermore, no purification was obtained; the enzyme that was recovered had a lower specific activity than the ethanol precipitate. This experiment was repeated several times and different grades of DEAE cellulose were tried (DE 11, DE 32 and DE 52), however the result was the same in all cases.

The failure of the enzyme to bind to the DEAE cellulose column could possibly be due to the enzyme having a different isoelectric point from that reported in the literature. Thus, it may bind to a cation exchanger.

Since purification using anion exchange chromatography was unsuccessful, a cation exchanger was attempted. Carboxymethyl (CM) cellulose was used as the cation exchanger and it was equilibrated with 0.01 M potassium phosphate buffer, pH 7.8. The column was eluted with the addition of potassium chloride to the buffer in a linear gradient (0 to 2 M KCl). Once again the HMG-CoA synthase activity appeared in the first few fractions, indicating that it did not bind to the column. The specific activity of the recovered enzyme was lower than the starting activity; hence no purification was achieved.

There are a few possible explanations for the lack of purification obtained from the ion exchange chromatography steps. The failure of the enzyme to bind to either exchanger could be due to an uneven distribution of charges over the protein surface or to nonelectrostatic interactions, such as hydrophobic and van der Waals forces, which prevented the protein from binding. The loss of activity in the enzyme recovered from the column could be due to the enzyme denaturing on the gel.

The ion exchange chromatography step was abandoned and another one of Middleton and Tubbs purification steps was attempted: a calcium phosphate gel adsorption step. Calcium phosphate gel is the gelatinous form of calcium hydroxyphosphate. This inorganic adsorbent does not have a readily explainable mode of action. The surface of this material is made up of charged ions, with associated water of hydration, so undoubtedly an electrostatic interaction is an important component of the adsorption of proteins to it. However, every positive area on the surface has negative areas very close by, and vice versa: consequently the interaction is more likely to be a polar dipole-dipole bonding than an ion exchange effect. Proteins have the greatest probability of having adjacent positive and negative groups present at neutral pH; thus, adsorption is most likely between pH 6 and 9, regardless of the isoelectric point of the

protein.[87] Adsorption is encouraged by low salt concentrations and elution of proteins occurs at higher salt concentrations.

In the calcium phosphate gel adsorption step, the ethanol precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.8 and the pH was adjusted to 6.8 with the addition of an appropriate amount of acetic acid. Calcium phosphate gel was then added in the proportion of 1.5 mg/mg of protein present and the suspension was stirred for 5 min at 4°C. The gel was collected by centrifugation; the supernatant was assayed and no enzyme activity was detected, thereby ensuring that the enzyme had been adsorbed onto the gel. The enzyme was eluted from the gel with 0.2 M potassium phosphate buffer, pH 8.3. The enzyme recovered from the gel had a lower specific activity (0.017 units/mg) than the enzyme put on the gel (0.041 units/mg); thus this step was unsuccessful. Middleton and Tubbs reported that the enzyme activity recoverable from the gel decreased rapidly with time and that the presence of glycerol stabilized the enzyme on the gel.[41] This experiment was repeated several times with the addition of glycerol (20-30% v/v) but with no success; in each case the enzyme recovered from the gel had a lower specific activity than before it was put on the gel.

The failure of the calcium phosphate gel adsorption step is not readily explainable. It was initially thought that perhaps a more concentrated salt solution was required to completely elute the enzyme from the gel. However, it was found that eluting the gel with 0.4 M potassium phosphate buffer, pH 8.3 did not increase the specific activity of the enzyme recovered from the gel, suggesting that perhaps the enzyme was being denatured on the gel.

With the failure of yet another one of Middleton and Tubbs purification steps, alternative purification steps were sought. At this point it was decided to perform gel electrophoresis on the enzyme preparations after the autolysis and ethanol precipitation steps, in order to see the degree of purity.

Electrophoresis is a powerful means of separating proteins and it is based on the principle that a molecule with a net charge will move in an electric field. Proteins are separated largely on the basis of mass by electrophoresis in a polyacrylamide gel under denaturing conditions. The mixture of proteins was first dissolved in a solution of sodium dodecyl sulfate (SDS), an anionic detergent that disrupts nearly all noncovalent interactions in native proteins. β-Mercaptoethanol was also present in order to reduce disulfide bonds. Anions of SDS bind to main chains at a ratio of about one SDS for every two amino acid residues, which results in the formation of an SDS-denatured protein complex with a large net negative charge roughly proportional to the mass of the protein. The SDS complexes with the denatured proteins are then electrophoresed on a polyacrylamide gel and the proteins are visualized by staining the gel with a dye such as Coomassie blue, which reveals a series of bands. The smaller the molecular weight of the protein the farther the protein will migrate down the gel; this is due to the fact that the continuous polymer framework of the gel impedes the movement of large molecules.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on the autolysate and ethanol precipitate and the molecular weights of the protein bands were compared with the known molecular weights of a protein standard present on the same gel. The autolysate and ethanol precipitate appeared identical on the gel: both contained numerous bands, with the majority of the bands occurring near the bottom of the gel. Unfortunately, too many bands were present to allow the identification of the band corresponding to HMG-CoA synthase. The presence of so many protein bands indicates that the enzyme is very impure.

The observation that the majority of protein bands corresponded to small protein molecules and HMG-CoA synthase is a large protein, suggested that significant purification of the enzyme could be obtained if these small protein molecules were removed. One method of separating proteins by their molecular weights is gel filtration.

Sephadex is a sizing gel and sephadex gel filtration separates proteins according to their size. Smaller molecules can enter the beads of the gel but larger ones cannot. The result is that small molecules are distributed both in the aqueous solution inside the beads and between them, whereas large molecules are located only in the solution between the beads. Large molecules flow more rapidly through this column and emerge first because a smaller volume is accessible to them. Gel filtration is an excellent technique for separating proteins according to their molecular weights.

Sizing gels are usually performed at a late stage in the purification process, when the enzyme is more stable. However, a gel filtration step was attempted as the second step in this purification because it was believed that the column could be run on such a small scale that the time of completion and loss of enzyme activity would be minimal. The column had a total volume of 10 mL and it was equilibrated with 0.02 M tris-HCl buffer, pH 7.5, and the ethanol precipitate was applied. Fractions were collected every 0.5 mL and were assayed in order to locate the enzyme. Unfortunately, there was no HMG-CoA synthase activity in any of the fractions collected. The failure of this experiment may have been caused by the instability of the enzyme solution due to contamination with proteases.

The literature on HMG-CoA synthase purification was again consulted. In an earlier purification procedure, Ferguson and Rudney[39] used an ammonium sulfate precipitation to purifiy the enzyme. Salting out is largely dependent on the hydrophobicity of the protein. A typical protein molecule in solution has hydrophobic patches (due to side chains of phenylalanine, tyrosine, tryptophan, leucine, methionine and valine). Forcing these hydrophobic groups into contact with the aqueous solvent causes an ordering of water molecules and effectively freezes them around the side chains. This ordering is thermodynamically unstable since it represents a large decrease in entropy compared with the unsolvated protein plus free water molecules. In the presence of high

salt concentrations, as the salt ions become solvated they start to pull off the ordered "frozen" water molecules from the hydrophobic side chains, exposing the bare fatty areas. Proteins with a larger number of such residues on their surface will aggregate and precipitate sooner, whereas proteins with few nonpolar surface residues may remain in solution even at the highest salt concentration. Many enzymes are precipitated from solution over a sufficiently narrow range of salt concentration to make this procedure a highly effective method of fractionation.[87]

In the salting out procedure, the ethanol precipitate was dissolved in 0.01 M potassium phosphate buffer, pH 7.8, and finely crushed ammonium sulfate was added with stirring to achieve 35% saturation. The protein that precipitated at this level of saturation was discarded and more ammonium sulfate was added to 55% saturation. HMG-CoA synthase was found to precipitate out in the 35 to 55% saturation range to give an overall thirteen fold purification at a 92% recovery of enzyme activity (**Table 8**). Although the yield for this step was very high, the degree of purification was only about two fold. However, one great advantage of ammonium sulfate fractionation over virtually all other techniques is the stabilization of proteins that occurs. An ammonium sulfate suspension of protein precipitate is often quite stable and it is the normal packaging method for commercial enzymes. This stabilization arises from the fact that the high salt concentration reduces the effective water activity, making the environment more comparable to the natural environment occurring in the cell.[87] It was found in this work that an ammonium sulfate pellet containing HMG-CoA synthase was stable for about three days without much loss of enzyme activity.

Table 8. Purification of HMG-CoA synthase from yeast: typical results from ammonium sulfate precipitation

Procedure	Volume (mL)	Total Activity (units)	Total Protein (mg/mL)	Specific Activity (units/mg)	Fold Purification	Yield (%)
Autolysis:	119	31	38	0.006	-	100
Ethanol	6	13	54	0.040	6	42
$(NH_4)_2SO_4$ pptn (35-55%)	3	12	52	0.076	13	39

The redissolved ammonium sulfate precipitate had a high salt concentration which had to be removed before proceeding to any further purification steps. Desalting was accomplished by passage through a sephadex G-25 column equilibrated with 0.01 M potassium phosphate buffer pH 7.8.

The next purification step attempted was a hydroxylapatite column. Hydroxylapatite is the crystalline form of calcium hydroxyphosphate and it works by the same principles as calcium phosphate gel adsorption. Although the calcium phosphate gel step was unsuccessful, it was thought that hydroxylapatite may work since it can be packed in a column and eluted with a salt gradient which would theoretically provide a better separation. A small column of hydroxylapatite was first attempted which had a total volume of 5 mL. The column was equilibrated with 10 mM potassium phosphate buffer,

pH 6.8, and the sample was applied. The column was then eluted with a step gradient of 10 mM to 400 mM potassium phosphate, pH 6.8. It was found that the enzyme did not come off the column in the first few fractions, but it eluted when the more concentrated phosphate buffer was run through the column. These results indicate that the enzyme did initially bind to the column and was eluted at high salt concentrations.

With the success of this preliminary experiment, several other experiments were performed, using different concentrations of potassium phosphate buffer, in order to determine the salt concentration at which the enzyme eluted from the column. It was found that the enzyme was eluted from the column at 150 mM potassium phosphate, pH 6.8.

A larger scale hydroxylapatite column was then attempted. This column was eluted with a linear gradient of 10 mM to 200 mM potassium phosphate buffer, pH 6.8, and fractions were collected every few millilitres. The fractions containing HMG-CoA synthase activity were slightly colored and as such were easy to identify. The recovery of HMG-CoA synthase from this procedure was only 24 %; however an overall 48 fold purification was obtained, which compensates for the low yield. The results of this experiment are shown in **Table 9**.

Table 9. Purification of HMG-CoA synthase from yeast: typical results after hydroxylapatite column

Procedure	Volume (mL)	Total Activity (units)	Total Protein (mg/mL)	Specific Activity (units/mg)	Fold Purification	Yield (%)
Autolysis:	55	5	28	0.003	-	100
Ethanol	1.5	2	81	0.016	5	41
$(NH_4)_2SO_4$ pptn (35-55%)	1	1.8	64	0.028	9	36
Hydroxyl-apatite column	1.5	0.4	2	0.143	48	8

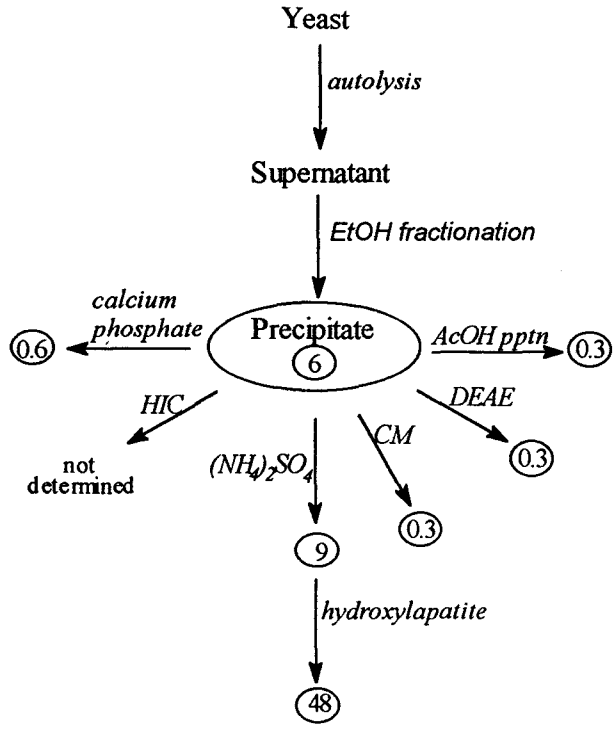
Since the ammonium sulfate precipitation step resulted in such a low degree of purification, it was decided to replace this step with a superior step. Hydrophobic interaction chromatography was attempted. Hydrophobic interaction chromatography is based on the same principles as ammonium sulfate precipitation since the hydrophobic interactions increase in strength with increasing salt concentration. Consequently at high salt concentrations most proteins can be absorbed to hydrophobic groups attached to inert matrices. Decreasing the salt concentration decreases the hydrophobic interactions

between the protein and the column and results in the elution of the protein from the column.

Hydrophobic interaction chromatography with a methyl support was attempted on a small scale. The 5 mL column was equilibrated with 100 mM sodium phosphate buffer, pH 6.8 containing 2.4 mM ammonium sulfate, and the ethanol precipitate was applied. The column was eluted with a step gradient of 2.4 to 0 M ammonium sulfate present in the phosphate buffer. It was found that the HMG-CoA synthase activity was eluted from the column after the 0 M ammonium sulfate buffer was added. This preliminary experiment indicates that the enzyme is binding to the column in the presence of high salt concentrations, and is eluted from the column when the salt concentration is reduced. Although this step seems promising, due to time constraints it was not possible to explore this technique any further. Additional work must now be done on this experiment in order to determine the exact concentration of buffer needed to elute HMG-CoA synthase from the column.

2.2.1 ◊ Summary of HMG-CoA Synthase Purification Steps

The following **Scheme** 7 summarizes the fold purification obtained from the various purification steps that were attempted in the purification of HMG-CoA synthase.



(NOTE: the numbers in the circles represent the fold purification achieved by each step)

Scheme 7

2.4.0 ♦ Inhibition of HMG-CoA Synthase By F-244

As was previously mentioned, the only known specific inhibitor of HMG-CoA synthase is the β-lactone F-244, which has proven to be quite potent.[73,77-79]

It was decided to test the inhibitory ability of F-244 on a crude enzyme preparation, using the method of Mayer and co-workers.[73] The F-244 was dissolved in dimethyl sulfoxide (DMSO) and added to the enzyme at a final concentration which was 20 times greater than the IC_{50} value of F-244[77] in order to ensure complete inhibition of enzyme activity. It was reported by Mayer and co-workers that DMSO did not affect

HMG-CoA synthase activity at a concentration of 10% v/v,[73] nevertheless a control was also prepared which contained only enzyme and DMSO. Both samples were preincubated at 30°C for 5 minutes and were subsequently assayed. As expected, it was found that in the presence of F-244, there was no HMG-CoA synthase activity (**Figure 8** and **Table 10**). The slope of the graph before acetyl-CoA addition (-0.02569 ± 6.24E-4; r^2 = 0.99884) was almost identical to the slope after (-0.02600 ± 3.83E-4; r^2 = 0.99959). In addition, the control did exhibit HMG-CoA synthase activity since the slope of the graph before acetyl-CoA addition (-0.03909 ± 1.11E-4; r^2 = 0.99838) was much smaller than after the addition (-0.1014 ± 1.67E-4; r^2 = 0.99948).

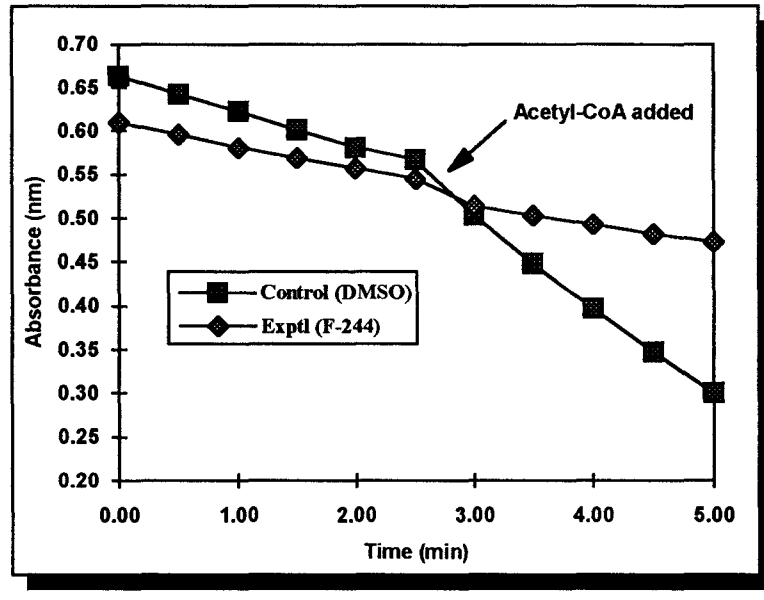


Figure 8. Assay of HMG-CoA synthase activity in the presence of F-244

Table 10. Data obtained from assay of HMG-CoA synthase activity in the presence of F-244

Time (minutes)	Absorbance (AU)	
	Exptl (F-244)	Control (DMSO)
0	0.610	0.663
0.5	0.596	0.642
1	0.581	0.622
1.5	0.569	0.601
2	0.557	0.581
2.5	0.545	0.567
3	0.514	0.503
3.5	0.503	0.448
4	0.491	0.397
4.5	0.480	0.347
5	0.469	0.300

Since the ability of F-244 to inhibit the crude enzyme preparation had been demonstrated, the IC_{50} value was determined. It was seen from **Table 3** that the IC_{50} values are dependent on the time of F-244 exposure to HMG-CoA synthase: when the enzyme was preincubated with the inhibitor, the IC_{50} value was lower than that obtained without preincubation. Mayer and co-workers reported an IC_{50} value of 0.01 μM when the enzyme and inhibitor were preincubated for 5 minutes,[73] however, Greenspan and co-workers reported a value of 0.1 μM obtained under the same conditions.[79]

Using the method of Mayer[73] and Greenspan,[79] the IC_{50} value was determined. Enzyme solution was incubated with various concentrations of F-244 and then assayed. The activity of the enzyme was then compared with the activity of a

control, containing no inhibitor, in order to determine the percent inhibition. The percentage inhibition of HMG-CoA synthase activity as a function of F-244 concentration is shown in **Figure 9** and **Table 11**

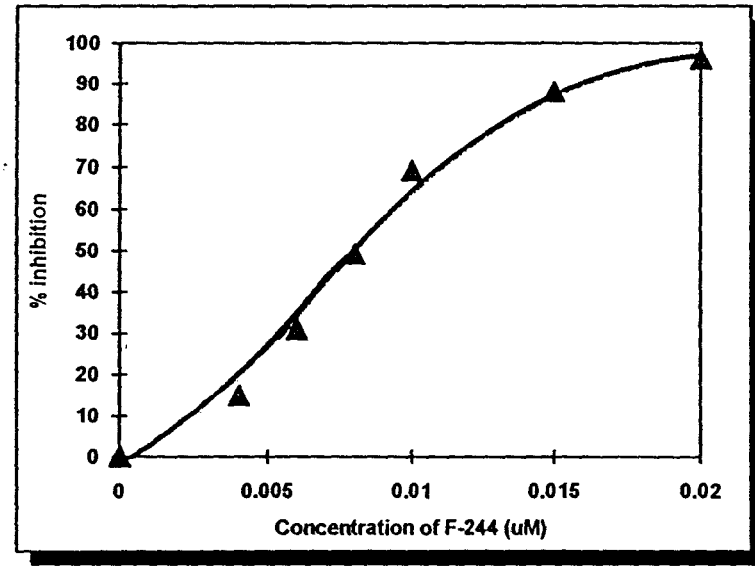


Figure 9. Effect of F-244 concentration on HMG-CoA synthase activity

Table 12. Effect of F-244 concentration on HMG-CoA synthase activity

F-244 concentration (μM)	% inhibition
0	0
0.004	15
0.006	31
0.008	49
0.01	69
0.015	88
0.02	96

From the data in **Figure 9**, it can be seen that the IC_{50} value is close to 0.009 μM, which is comparable with the value obtained by Mayer and co-workers.[73]

2.4.1 ◊ Reversibility of F-244 Inhibition of HMG-CoA Synthase

As mentioned in section **1.4.2** there is some discrepancy surrounding the reversibility of F-244 inhibition of HMG-CoA synthase. Greenspan and co-workers have reported that this inhibition is reversible upon dilution of the enzyme-inhibitor complex;[79] however this claim has been disputed.[73,77] It was hoped that this discrepancy could be resolved in the present work.

Greenspan's experimental procedure of diluting the enzyme-inhibitor complex[79] was repeated in the present work. In this experiment, HMG-CoA synthase was preincubated with enough F-244 (0.04 μM) to cause complete inhibition of the enzyme. After 5 minutes an assay of the enzyme solution revealed 100% inhibition of the enzyme by F-244. The enzyme-inhibitor complex was then diluted into assay mixtures which contained sufficient F-244 to bring the concentration of F-244 to 0.004, 0.008, 0.012 and 0.04 μM. After an additional 5 minute incubation at 30°C, the solutions were assayed. In contrast to Greenspan's results, it was found that the enzyme remained 100% inactivated in all cases (**Table 12**). These results imply that the inhibition of HMG-CoA synthase by F-244 is not reversible upon dilution of the enzyme-inhibitor complex, contrasting Greenspan's earlier reports.[79] In addition, the inhibited enzyme and a control containing no F-244 were allowed to stand for up to two hours at room temperature and were then assayed. HMG-CoA synthase activity was found to be present in the control but not in the previously inhibited enzyme sample. The results of these experiments suggest that the inhibition of HMG-CoA synthase by F-244 is *irreversible.*

Table 12. Effect of dilution on F-244 inhibited HMG-CoA synthase

Concentration of F-244 (μM)	% INHIBITION	
	Enzyme preincubated with F-244	Enzyme not preincubated with F-244
0.04	100	100
0.02	100	95
0.008	100	48
0.004	100	12

2.5.0 ♦ Affinity Labeling of HMG-CoA Synthase

As mentioned in section **2.2.0**, the isolation of HMG-CoA synthase from bakers yeast was not as easy as originally anticipated. However, a three step sequence was developed which allowed purification of the enzyme by about 48 fold. However, this enzyme is still too crude to be used in experiments designed to probe the inhibitory mechanism of F-244. Since the purification of HMG-CoA synthase had proven to be difficult, it was decided to use radioactively labeled F-244 as an affinity label for the enzyme in the hope that the labeled enzyme-inhibitor complex would be easier to purify.

An affinity label, or active-site-directed irreversible inhibitor, is a chemically reactive compound that is designed to resemble the substrate of an enzyme, so that it binds specifically to the active site and forms covalent bonds with the protein residues.[88-90] Affinity labels are very useful for identifying catalytically important residues at the active site of the target enzyme.

Since F-244 is the only known specific inhibitor of HMG-CoA synthase, and since a procedure for the isolation of F-244 from *Fusarium sp.* had been developed in our laboratory,[91] it was decided to prepare [15, 16, 17, 18 - ^{3}H] F-244 as an affinity label for HMG-CoA synthase.

2.5.1 ◊ Preparation of [15, 16, 17, 18 - ^{3}H] F-244

Studies on the biosynthesis of F-244 done in our laboratory have revealed that F-244 is derived from 4 methionine and 7 acetate units (**Figure 10**). The 7 acetate units condense together to form a heptaketide via the polyketide pathway. The 4 methyl carbons originate from methionine and are introduced into the main skeleton by the activated methyl donor *S*-adenosylmethionine (SAM). This result was demonstrated, in our laboratory, by incorporation of *methyl*-[^{13}C]-methionine into growing cultures of *Fusarium sp.* followed by isolation and NMR analysis (15% incorporation of label).

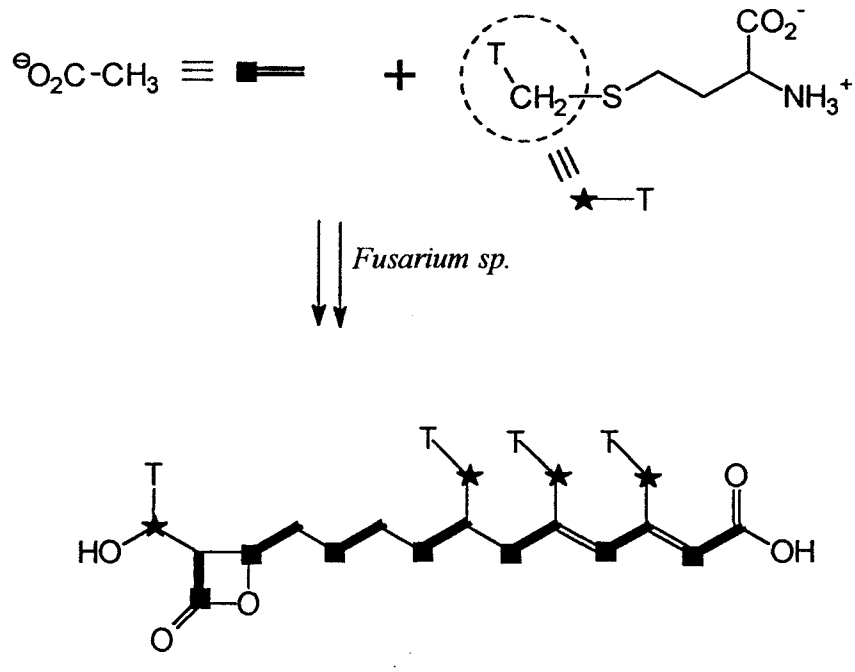


Figure 10. Biosynthetic labeling pattern of F-244

By feeding *methyl*-[^{3}H]-methionine to cultures of *Fusarium sp.*, before and during production of F-244, it was possible to incorporate the [^{3}H] methyl residue of methionine into the side chain of F-244. The [15, 16, 17, 18 - ^{3}H] F-244 was then isolated according to the procedure established in our laboratory. The isolated [15, 16, 17, 18 - ^{3}H] F-244 was determined to be pure by comparison of its ^{1}H-NMR spectrum with that of an authentic sample of pure F-244. This was the first reported preparation of [15, 16, 17, 18 - ^{3}H] F-244. The amount of radioactivity present in the tritiated F-244 was then determined as follows:

1. Determination of Specific Activity:

Radioactivity is the spontaneous disintegration of unstable atomic nuclei. The initial number of radioactive nuclei (N_0) present decreases over time (t). The half life of the reaction ($t_{½}$) is the time required for half of the nuclei to decay. At any given time, the total number of radioactive nuclei (N) present can be calculated from the following equation: $N = N_0 e^{(-0.693t/t_{1/2})}$

To determine the activity of a [^{3}H]-toluene standard compound, in terms of disintegrations per minute (DPM), several factors must be known or determined. The initial radioactivity (N_0) is known to be 2.06 x 10^6 DPM/mL as calibrated on January 2, 1990. The time elapsed since the calibration date and the date on which a portion was counted was 3.230 years. The half life ($t_{½}$) of tritium is known to be 12.35 (± 0.01%) years. Substitution of these values into the above equation gives the activity of the standard:

$$N = (2.06 \times 10^6 \text{ DPM/mL}) e^{(-0.693 \times 3.230)/12.35 (\pm 0.01\%)}$$

$$N = 1.72 \times 10^6 \text{ DPM/mL } (\pm 0.01\%)$$

Since a series of 4 µL samples from the [^{3}H]-toluene standard were prepared and counted by liquid scintillation, the average radioactivity (R) will be:

$$R = [1.72 \times 10^6 \text{ DPM/mL } (\pm 0.01\%)] \times [4 \times 10^{-3} \text{ mL } (\pm 1\%)]$$

$$R = 6.88 \times 10^3 \text{ DPM } (\pm 1\%)$$

This value can be used to determine the counting efficiency (CE) of the sample, or to compare the activities of samples which are counted with different efficiencies.

2. Determination of Counting Efficiency

The counting efficiency (CE) is a ratio of the number of observed counts per minute (CPM) to the number of disintegrations per minute (DPM) occurring in the

sample. It is necessary to calculate the counting efficiency in order to determine the specific activity of the sample. One of the basic methods used to determine CE is internal standardization. This method involves counting a known amount of non-quenching radioactive standard, such as [^{3}H]-toluene. An identical amount of this standard is then added to a known amount of sample and the resulting mixture is counted. The difference between the counts of the sample plus standard and of the sample alone is then divided by the DPM of the standard:

$$CE = \frac{[(CPM_{st+s}) - (CPM_s)]}{[DPM_{st}]} \times 100$$

If the DPM of the standard are known, then this equation becomes:

$$CE = \frac{[(CPM_s)]}{[DPM_{st}]} \times 100$$

$$CE = \frac{[4089\ CPM\ (\pm 3\ \%)]}{[6.88 \times 10^3\ DPM\ (\pm 1\%)]} \times 100$$

$$CE = 59.4\ (\pm 3.3\ \%)$$

$$CE = (59.4 \pm 2.0)\%$$

The specific activity of [^{3}H] F-244 can now be determined from the CE value.

3. Determination of the Specific Activity of [15, 16, 17, 18,- ^{3}H] F-244

The specific activity of [15, 16, 17, 18 - ^{3}H] F-244 was determined by counting 250 µL (±1%) samples of a 60 µL (± 2%) solution in 95% ethanol. The DPM

values for these aliquots are shown in **Table 13**. The DPM values were calculated from the measured, corrected CPM values of the samples and the CE was determined as described above. Since the radioactivity associated with the 250 μL samples and the concentration of the sample were known, the specific activity of [15, 16, 17, 18 - ^{3}H] F-244 was calculated to be 1.3 x 10^6 DPM/mg (± 6.6%).

Table 13. CPM and DPM values of [15,16,17,18-^{3}H] F-244 and the[^{3}H]-toluene standard

[^{3}H] F-244!, A	CPM	2SIG%	DPM*
250 μL	3720	1.99	6262
250 μL	3580	1.99	6026
250 μL	4266	1.98	7182
[^{3}H]-toluene$, B	**CPM**	**2SIG%**	**DPM**
4 μL	3955	1.99	6658
4 μL	3701	2.00	6231
4 μL	4607	1.98	7756
A & B	**CPM**	**2SIG%**	**DPM**
254 μL	7565	1.98	12735
254 μL	8564	1.97	14418
254 μL	7523	1.98	12666

! Corrected CPM values obtained by subtracting the background CPM from the measured CPM value of the sample. The background CPM correction value was calculated from a series of 5 mL scintillation cocktail samples, the average CPM value was 14.05.
* These values were calculated by using the CE value determined from the [^{3}H]-toluene standard.
$ Each sample was removed from the 10 mL standard, du Pont Lot # S004008, and delivered into a scintillation vial containing 5 mL cocktail.

The tritiated F-244 that was prepared had a lower level of radioactivity than expected. As a result, the specific incorporation of *methyl*-[^{3}H]-methionine into F-244 was calculated. The specific activity of [15, 16, 17, 18 - ^{3}H] F-244 was 1.3 x 10^6

DPM/mg. By multiplying this value by the molecular weight of F-244, the DPM present per mmole of F-244 was determined:

$$(1.3 \times 10^6 \text{ DPM/mg}) \times 324.4 \text{ mg/mmole} = 4.2 \times 10^8 \text{ DPM/mmole F-244}$$

Since there are 11 possible sites in F-244 which could have the tritium label, dividing this value by 11 gives the DPM per mmole per site.

$$\frac{4.2 \times 10^8 \text{ DPM/mmole F-244}}{11 \text{ sites}} = 3.8 \times 10^7 \text{ DPM/mmol per site}$$

The methyl-[^{3}H]-methionine used in this experiment had an activity of 185.00 mCi/mmole, multiplying this value by the conversion factor of 2.22×10^9 DPM/mCi gives the activity in DPM/mmole:

$$(185.00 \text{ mCi/mmol})(2.22 \times 10^9 \text{ DPM/mCi}) = 4.11 \times 10^{11} \text{ DPM/mmol}$$

Since there are 3 tritiums present in the *methyl*-[^{3}H]-methionine, dividing this value by 3 gives the DPM per mmole per site.

$$\frac{4.11 \times 10^{11} \text{ DPM/mmol}}{3 \text{ tritiums}} = 1.4 \times 10^{11} \text{ DPM/mmol/site.}$$

The specific incorportation of *methyl*-[^{3}H]-methionine into [15, 16, 17, 18 - ^{3}H] F-244 can now be calculated:

$$\text{specific incorporation} = \frac{3.8 \times 10^7 \text{ DPM/mmol/site}}{1.4 \times 10^{11} \text{ DPM/mmol/site}} \times 100\%$$

$$= 0.03\%$$

Since the specific incorporation of *methyl*-[^{3}H]-methionine into the tritiated F-244 was found to be only 0.03%, it was decided to modify the procedure and attempt the preparation of [15, 16, 17, 18 - ^{3}H] F-244 again.

It was discovered in our laboratory, that the addition of 40 mg of *methyl*-[^{13}C]-methionine to a culture of *Fusarium sp.* resulted in a 15% incorporation of the label into F-244. In the above experiment, only 0.42 mg of *methyl*-[^{3}H]-methionine was added to the culture to give a 0.03% incorporation; it is therefore likely that the concentration of methionine was too low to allow for a good incorporation. Thus, a second attempt was made at preparing [^{3}H] F-244, with the hope of obtaining a sample with a greater level of radioactivity. This time, 3.2 mg of *methyl*-[^{3}H]-methionine was combined with 36.8 mg of unlabeled methionine in order to give a final weight of 40 mg. This 40 mg of methionine was then fed to the culture of *Fusarium sp.* as described earlier. However, during this second attempt problems with the growth of *Fusarium sp.* were encountered and the tritiated F-244 that was isolated was even less radioactive than the first sample (specific activity = 8.9 x 10^{5} DPM/mg). As a result, the first sample of tritiated F-244 prepared was used in incubation experiments with HMG-CoA synthase.

2.5.2 ◊ Preparation of [15, 16, 17, 18 - ^{3}H] F-244 labeled HMG-CoA Synthase

Partially purified HMG-CoA synthase, taken after the ethanol precipitation step, was concentrated by ultra filtration through a membrane with a molecular weight cut-off of 30,000. The concentrated enzyme was estimated to contain 534 μg (4.1 x 10^{-9} moles, assuming a molecular weight of 130,000) of HMG-CoA synthase. This enzyme was then incubated for 5 minutes at 30°C with twice as many moles of tritiated F-244, in order to ensure complete inhibition of the enzyme. The enzyme was then assayed and

found to contain no HMG-CoA synthase activity compared to a control lacking inhibitor. The enzyme-inhibitor complex was then separated from unbound [15, 16, 17, 18 - ^{3}H] F-244 by ultra filtration through a membrane with a molecular weight cut-off of 30,000. Liquid scintillation counting of the filtrate yielded an activity of 2017 DPM, corresponding to unbound tritiated F-244. The residue was also counted and found to have an activity of 1448 DPM, corresponding to [15, 16, 17, 18 - ^{3}H] F-244 bound to HMG-CoA synthase.

The labeled enzyme protein mixture was then separated by gel electrophoresis. Two gels were run simultaneously: the first one contained labeled enzyme mixture and unlabeled F-244 inhibited enzyme was run on the second. The nonradioactive gel was stained with Coomassie blue and the radioactive gel was sliced into twenty 3 mm segments. These segments were then added directly to 5 mL of scintillation cocktail and were counted. The procedure used in this experiment is diagrammed in **Figure 11**.

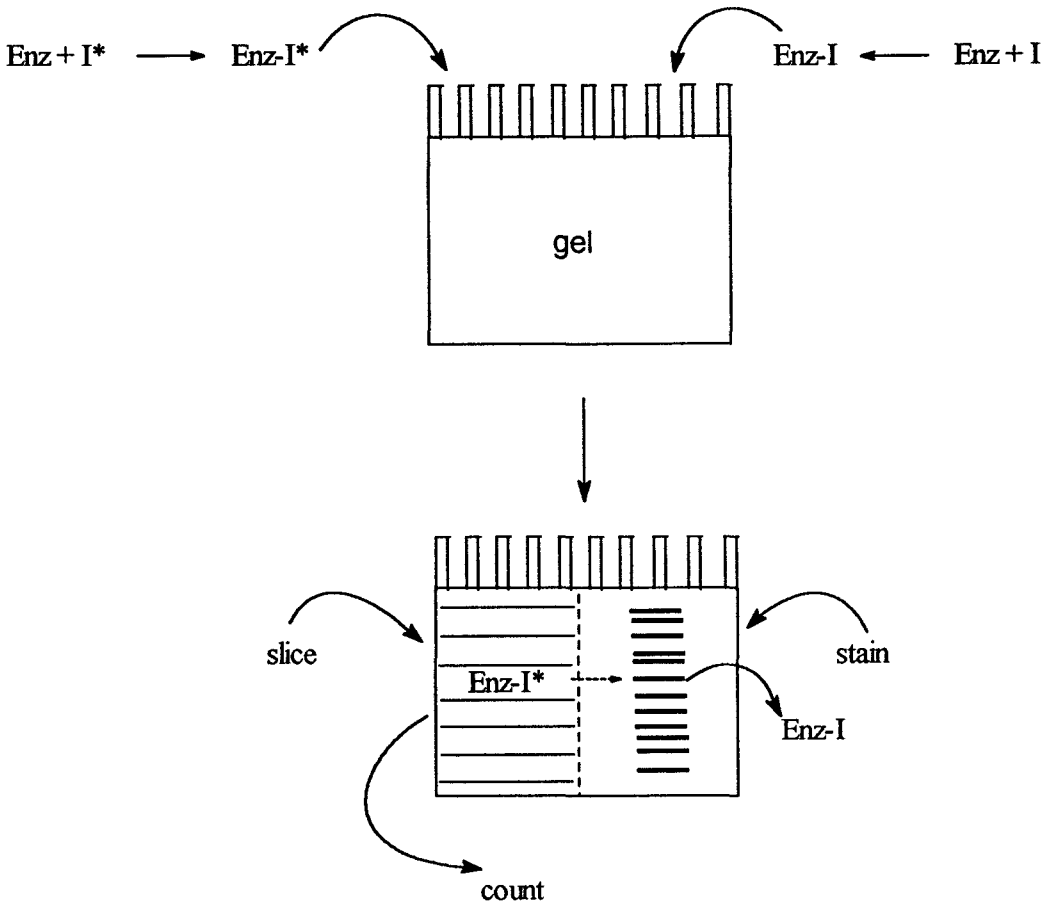


Figure 11. Schematic representation of affinity labeling of HMG-CoA synthase

It was expected that the segment containing [15, 16, 17, 18 - ^{3}H] F-244 inhibited HMG-CoA synthase would show a higher degree of radioactivity than the other segments, however this was not observed. Most of the segments were found to contain a low level of radioactivity, but none of these segments exhibited the higher degree of radioactivity anticipated. Consequently, it was not possible to identify the segment corresponding to [15, 16, 17, 18 - ^{3}H] F-244 inhibited HMG-CoA synthase.

The failure of this experiment was most likely due to the low level of radioactivity in the tritiated F-244 that was prepared. A tritiated F-244 sample containing a higher level of radioactivity should allow for easier detection of the enzyme-inhibitor

complex. Due to time restrictions, it was not possible to prepare such a sample; however this should be done in future experiments.

2.6.0 ♦ Effect of Other ß-Lactone Compounds on HMG-CoA Synthase Activity

As discussed in section **1.4.1**, the β-lactone ring of F-244 is not the only factor required for potent inhibition of HMG-CoA synthase. A number of studies have shown the importance of the hydroxymethyl moiety, the length of the carbon side chain, and the presence and geometry of the double bonds.[73,77-79]

In this work, the HMG-CoA synthase inhibitory potential of (±)-β-butyrolactone and a β-lactone compound of unknown structure were tested and compared to F-244. (±)-β-Butyrolactone was chosen because it was readily available and it would be interesting to see if a β-lactone of such a simple structure would inhibit HMG-CoA synthase. The β-lactone of unknown structure was a sample obtained from an industrial collaborator. Due to a secrecy agreement, the structure of this β-lactone is not reported.

In order to test the inhibitory activity of these two samples, various concentrations of the samples were prepared and then incubated with HMG-CoA synthase for 5 min at 30°C. These samples were then assayed and their HMG-CoA synthase activity was compared to that of a standard.

From these experiments it was found that the unknown β-lactone did not inhibit HMG-CoA synthase activity at all, at concentrations as high as 1.5 mM. On the other hand, (±)-β-butyrolactone did inhibit HMG-CoA synthase activity, with an IC_{50} value of approximately 2 mM (**Table 14**). However, (±)-β-butyrolactone was a very weak inhibitor compared to F-244 (**Table 15**).

Table 14. Effect of (±)-β-butyrolactone on HMG-CoA synthase activity

Concentration of (±)-β-butyrolactone (mM)	% Inhibition
0.5	5
1	28
1.5	41
2	52
4	72

Table 15. IC_{50} values of (±)-β-butyrolactone, unknown β-lactone and F-244

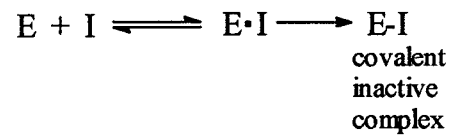
Inhibitor	IC_{50} (μM)
F-244	~0.009
(±)-β-butyrolactone	~2000
unknown β-lactone	>>1500

Since it was discovered that (±)-β-butyrolactone was an inhibitor of HMG-CoA synthase, although not a very potent one, the next question to answer is whether this inhibition is reversible or irreversible.

In the case of reversible inhibition, the enzyme binds to the inhibitor to form an enzyme-inhibitor complex. This complex rapidly dissociates to give free enzyme and free inhibitor. However, an irreversible inhibitor dissociates very slowly from its target enzyme because it becomes very tightly bound to the enzyme, either covalently or noncovalently **(Scheme 8)**.

1. Reversible Inhibition

2. Irreversible Inhibition



Scheme 8

The extent of irreversible inhibition is dependent upon time, whereas reversible inhibition is not. In the case of irreversible inhibition the longer the enzyme is exposed to the inhibitor, the more covalent inactive complex is irreversibly formed; thus the enzyme becomes more inactivated with time. In contrast, in reversible inhibition the enzyme-inhibitor complex rapidly forms and then dissociates; thus the enzyme would not become more inactivated with respect to time.

Several experiments were performed in order to determine the reversibility of HMG-CoA synthase inhibition by (±)-β-butyrolactone. In the first experiment HMG-CoA synthase was preincubated with 2mM inhibitor for 5 minutes and then assayed. The enzyme was found to be 51% inactivated compared to a control lacking inhibitor. An assay was then performed in which (±)-β-butyrolactone was added to the enzyme solution and assayed immediately, with no preincubation. It was found that the HMG-CoA synthase activity was only 10% inactivated compared to the control containing no inhibitor. These results suggest that the inhibition is irreversible.

In a second experiment, HMG-CoA synthase was preincubated with 4mM (±)-β-butyrolactone for 5 minutes. An assay revealed that HMG-CoA synthase activity was inhibited by ~80% compared to a control lacking inhibitor. The enzyme-inhibitor complex was then diluted into buffer to achieve a final (±)-β-butyrolactone concentration of 0.5 mM. An assay was then performed, however the enzyme remained ~80% inactivated

inactivated compared with the control. In addition, allowing the inhibited enzyme to stand for up to two hours before assaying it for HMG-CoA synthase activity did not result in any recovery of activity, nor was there appreciable loss of activity in the control with no inhibitor. From the results of the above experiments it can be concluded that (±)-β-butyrolactone inhibition of HMG-CoA synthase is irreversible.

Since the inhibition of HMG-CoA synthase by (±)-β-butyrolactone was found to be irreversible, a time dependent study of the inactivation was performed. In this study 1, 2, 4 and 8 mM (±)-β-butyrolactone were used and the activity of HMG-CoA synthase was measured after preincubation times of 0, 2, 4, 8 and 10 minutes. The results of this experiment are shown in **Table 16**. A plot of the log of activity remaining versus time for each concentration of inhibitor yields linear plots as shown in **Figure 12**.

Table 16. Time dependent inhibition HMG-CoA synthase with various concentrations of (±)-β-butyrolactone

Concentration of inhibitor (mM)	% Activity remaining				
	0 min	2 min	4 min	8 min	10 min
1	100	81	65	43	34
2	99	69	48	22	16
4	100	63	27	6	4
8	98	34	12	1	0

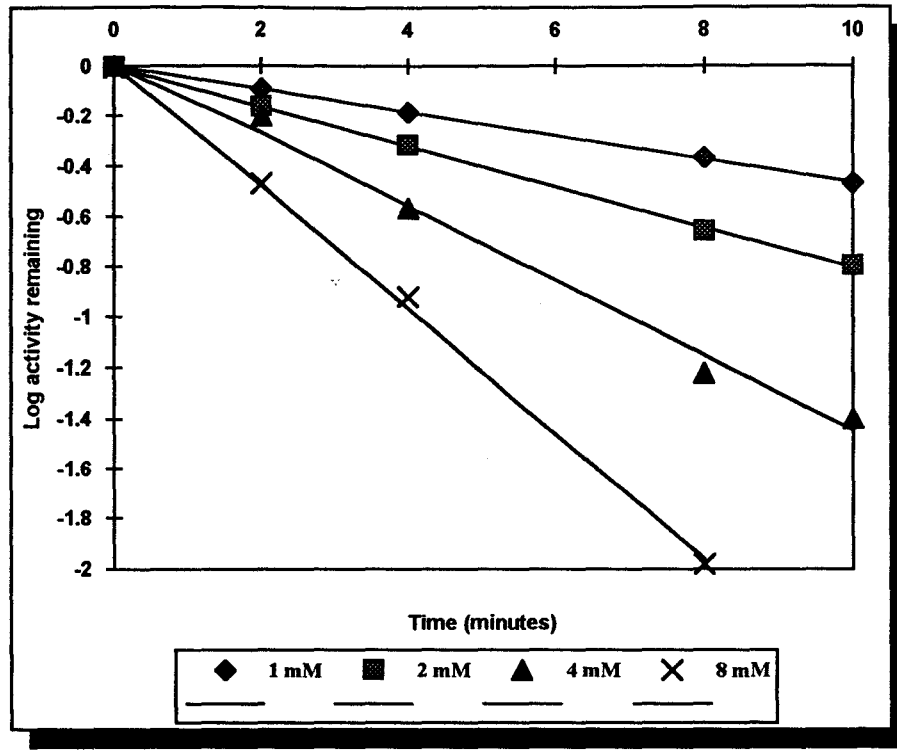


Figure 12. Time dependent inhibition of HMG-CoA synthase by (±)-β-butyrolactone

From the plots in **Figure 12** it is seen that the time-dependent loss of HMG-CoA synthase activity shows first order kinetics. A secondary reciprocal plot can now be constructed of 1/slope versus 1/concentration of inhibitor (**Figure 13**). This plot yields two important pieces of information related to the following equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I \xrightarrow{k_2} E{\cdot}I \qquad K_I = \frac{k_{-1}}{k_1}$$

The limiting rate constant for inactivation (k_2) can be determined from the vertical intercept of the reciprocal plot, which is $1/k_2$. The horizontal intercept is $-1/K_I$, this allows for the determination of K_I, the dissociation constant of inhibitor from the E·I complex, which provides a measure of affinity of the inhibitor for the enzyme (**Table 18**).

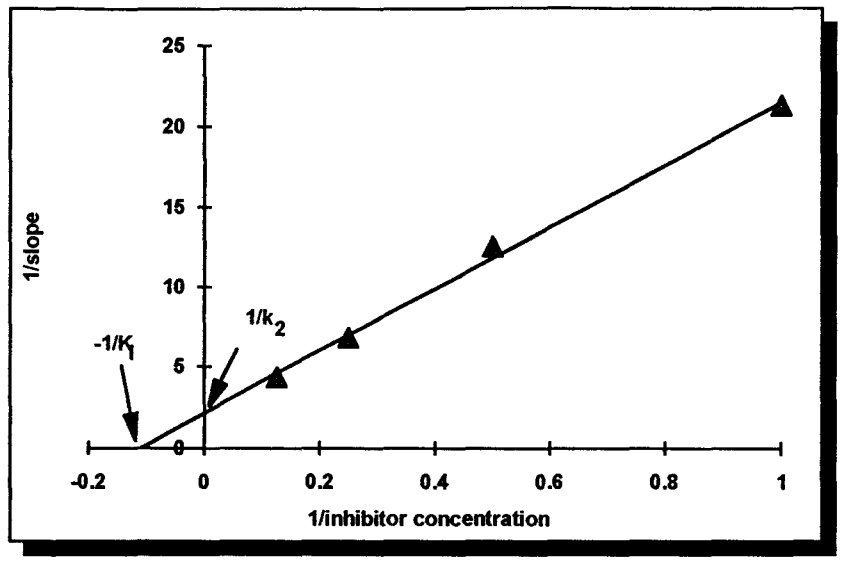


Figure 13. Double reciprocal plot of slope vs. (±)-β-butyrolactone concentration

Figure 17. Data for Figure 13

$[Inhibitor]^{-1}$	$Slope^{-1}$
0.13	4.3
0.25	6.8
0.50	12.5
1.0	21.3

Regression Output:	
Constant: *(1/k₂)*	2.1291
Std Err of Y Est	0.4412
R squared	0.9978
No. of Observations	5
Degrees of Freedom	3
X Coefficient	19.4246
Std Err of Coeff	0.5210

From the above data, $1/k_2$ was found to be 2.1291; thus k_2 would be 0.4697 s^{-1}. The value of $-1/K_I$ was found to be approximately -0.11, and hence K_I is 9 mM. Mayer and co-workers have tried to construct a similar set of plots for F-244.[73] However, they found that the rate of inactivation, k_2, was too rapid to be accurately measured.

2.7.0 ♦ Conclusions and Recommendations for Future Work

The isolation of HMG-CoA synthase from bakers yeast was attempted using the published procedure of Middleton and Tubbs,[41] however, this procedure proved to be irreproducible in our hands. Consequently, the procedure was modified. Thus, it was found that ethanol fractionation followed by ammonium sulfate precipitation and then hydroxylapatite chromatography, gave material which was purified 48 fold with an overall yield of 8%. This procedure was found to be reproducible. Preliminary experiments using hydrophobic interaction chromatography to further purify the enzyme have been promising and should be explored further. Work is currently ongoing in our laboratory in order to purify this enzyme to homogeneity.

The β-lactone F-244 was found to be a potent irreversible inhibitor of HMG-CoA synthase isolated from bakers yeast, with an IC_{50} value of 0.009 μM. This result is consistent with the inhibitory activity of F-244 on rat HMG-CoA synthase that has been reported in the literature. Further work is still needed in order to determine the mechanism of inhibition.

Tritium labeled F-244 was prepared by feeding *methyl*-[^{3}H]-methionine to cultures of *Fusarium sp.* The [15, 16, 17, 18 - ^{3}H] F-244 isolated had a specific activity of 1.3 x 10^6 DPM/mg. This compound was then used as an affinity label for HMG-CoA synthase. Incubation of the enzyme with [15, 16, 17, 18 - ^{3}H] F-244 resulted in complete loss of enzyme activity. Attempts to locate the enzyme-inhibitor complex on a gel were unsuccessful, probably owing to the low level of radioactivity associated with the tritiated F-244.

The use of [15, 16, 17, 18 - ^{3}H] F-244 as an affinity label has several possible applications. First, it offers an alternative approach to purifying the enzyme-inhibitor complex. Once isolated, it may be possible to crystallize this complex which would allow

an X-ray crystal structure of the inhibitor attached to the enzyme's active site to be obtained for the first time. Second, the radiolabeled enzyme-inhibitor complex could allow the enzyme active site to be sequenced. The labeled enzyme could be subjected to reagents that cleave polypeptides at a specific site, such as cyanogen bromide which cleaves only those peptide bonds adjacent to methionine residues. The radioactive polypeptide fragment, containing a short sequence of amino acids could then be analyzed by an amino acid sequencing machine.

(±)-β-Butyrolactone was also found to be an irreversible inhibitor of HMG-CoA synthase, although not a very potent one, with an IC_{50} value of 2 mM. From time dependent inhibition studies, it was found that $k_2 = 0.4697\ s^{-1}$ and $K_I = 9$ mM, where k_2 and K_I are defined by the following equation:

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{-}I \xrightarrow{k_2} E\text{-}I \qquad K_I = \frac{k_{-1}}{k_1}$$

In the future, radiolabeled (±)-β-butyrolactone could be synthesized which contains a high level of radioactivity. Although the IC_{50} of (±)-β-butyrolactone is quite high, it may be possible to synthesize sufficient quantities of the radiolabeled compound in order to use it as an affinity label for HMG-CoA synthase.

Chapter 3
EXPERIMENTAL

3.1.0 ♦ General

Ultraviolet (UV) absorption data were obtained with a Perkin-Elmer Lambda 9 double beam spectrophotometer. HMG-CoA synthase assays were taken at 303 nm and total protein determinations at 725 nm. The cuvettes used were methylacrylate disposable obtained from CanLab (# S7360-2).

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AM-200 spectrometer equipped with a 5mm dual frequency ^{1}H-^{13}C probe. Chloroform-d was used as the solvent and tetramethylsilane (TMS) was used as an internal standard. The chemical shifts are reported in δ values (ppm), followed in brackets by the multiplicity symbol (s= singlet, d= doublet, dd= doublet of doublets, t= triplet, q= quartet, m= multiplet), the coupling constants where appropriate, the number of protons, and the type of group.

All masses were measured on a mechanical Gram-Atic balance from Fisher Scientific Co. which determined weights to the fourth decimal place.

Plastic tipped automatic pipettes from Nichiryo, Model 5000, were used to deliver microliter volumes. The accuracy and precision are 1-2% and 0.5% respectively.

All the water used in this work was obtained from a millipure purification system.

Samples were centrifuged in a Beckman Model J2-32 centrifuge containing a JA-14 rotor.

High pressure liquid chromatography (HPLC) was performed on a Beckman model 110B using a RP 18 reverse phase silica gel column. The solvent used was acetonitrile (60%), phosphoric acid (0.1%) in water and the flow rate was 1.0 mL/min. A UV detector was used and it was set at 275 nm.

Liquid scintillation counting was performed on a Beckman LS 5801 scintillation counter using Fisherbrand 20 mL plastic screw-cap vials and Beckman Ready Safe liquid scintillation cocktail for aqueous and non-aqueous samples.

Preparation of Buffers

Tris buffers were prepared from tris base and then neutralized with HCl. Potassium dihydrogen phosphate buffer was adjusted to the desired pH with KOH. Sodium phosphate buffer was adjusted to the desired pH with HCl. The pH values were recorded at room temperature using an Orion Research model SA250 pH meter. Buffers were degassed prior to use by evacuation to 30 mm Hg on a water aspirator with stirring.

General Procedure for the Assay of HMG-CoA Synthase Activity

Solutions of magnesium choride (1 M), acetoacetyl-CoA (4 mM) and acetyl-CoA (8 mM) were prepared fresh daily.

In the standard assay, the reference and sample cells both contained tris-HCl buffer, pH 8.2 (1.47 mL, 0.2 M), magnesium chloride solution (15 μL) and enzyme (15 μ L), in a 1 cm light path cuvette. Acetoacetyl-CoA (18 μL) was added to the sample side and the acetoacetyl-CoA absorbance was measured every 0.5 minutes for a total of 2.5 minutes at 303 nm. To measure the activity of HMG-CoA synthase, acetyl-CoA (36 μL) was added to both the reference and the sample cells and the decrease in absorbance was measured every 0.5 minutes for another 2.5 minutes. The increase in acetoacetyl-CoA disappearance was taken to represent HMG-CoA synthase activity.

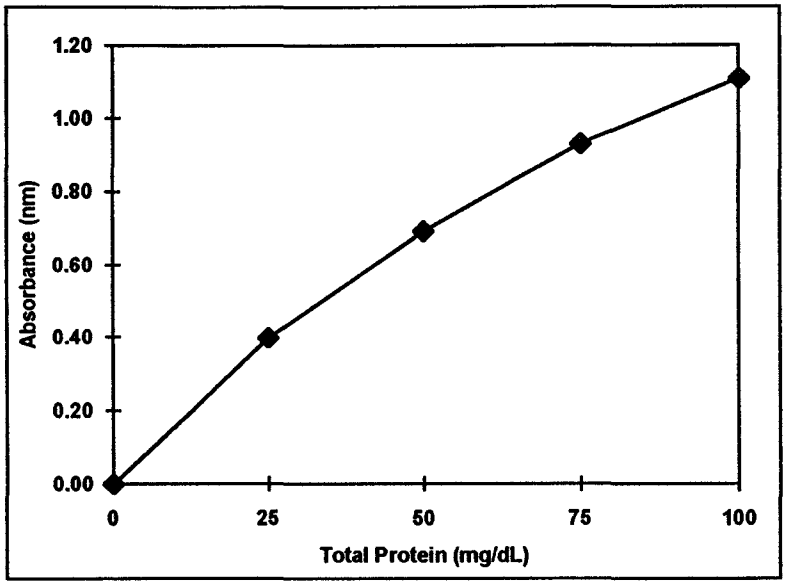
General Procedure for the Determination of Total Protein

The total protein present in the enzyme preparations was determined using a micro protein determination kit purchased from Sigma (# 690-A) which utilized a modified Lowry procedure.

Calibration Curve:

A calibration curve was generated by diluting a protein standard of albumin (0.5 mL) to 50 mL with 0.1 M sodium chloride solution. Five samples of varying protein concentration were prepared. The first sample was a reference and contained only sodium chloride solution (0.20 mL), the other four samples contained varying amounts of protein standard (0.05 mL, 0.10 mL, 0.15 mL and 0.20 mL) and sodium chloride solution (0.15 mL, 0.10 mL, 0.05 mL and 0 mL). Biuret reagent (2.2 mL) was added to each of these samples, the samples were mixed thoroughly and they were left to stand for 10 minutes at room temperature. Folin and Ciocalteu's phenol reagent (0.1 mL) was then added and after thorough mixing, the samples were left standing for 30 minutes at room temperature. The absorbance of each of these samples was then measured at 725 nm, using the reference sample in the reference cell of the spectrophotometer.

From the absorbance data a calibration curve was constructed relating protein concentration to absorbance:



Total protein (mg/dL)	Absorbance (AU)
0	0
25	0.40
50	0.69
75	0.93
100	1.11

Determination of Total Protein in a Sample:

The enzyme solution (175 μL) was diluted to 20 mL with 0.1 M sodium chloride solution, a reference sample was also prepared which contained only sodium chloride solution (0.2 mL). Biuret reagent (2.2 mL) was then added to both solutions (0.2 mL). After 10 minutes, folin and Ciocalteu's phenol reagent (0.1 mL) was added and the solutions were left to stand at room temperature for 30 minutes. The absorbance of the solutions was then measured at 725 nm using the reference sample in the reference cell and the enzyme solution in the sample cell. The total protein of the enzyme solution was

then determined from the calibration curve, by finding the total protein corresponding to the measured absorbance.

General Procedure for Gel Electrophoresis (SDS-PAGE)

Gel electrophoresis was performed on a Bio Rad Mini-PROTEAN II apparatus. The apparatus was assembled according to the manufacturer's instructions.

Preparation of Stock Solutions

30% Acrylamide/bis was prepared by combining acrylamide (87.6 g), N,N'-bis-methylene-acrylamide (2.4 g) and water (300 mL). The mixture was then filtered and stored at 4°C in the dark for a maximum of 30 days.

10% SDS was prepared by dissolving SDS (10 g) in water (100 mL) with gentle stirring.

Sample buffer was prepared by combining water (4.0 mL), 0.5 M tris-HCl, pH 6.8 (1.0 mL), glycerol (0.80 mL), 10% (w/v) SDS (1.6 mL), 2-β-mercaptoethanol (0.4 mL) and 0.05% (w/v) bromophenol blue (0.2 mL).

Electrode buffer was prepared by combining tris base (9 g), glycine (43.2 g), SDS (3 g) and water (600 mL). It was stored at 4°C.

General Procedure for the Preparation of Gels

The separating gel was prepared by combining water (3.35 mL), tris-HCl buffer, pH 8.8 (1.5 M, 2.5 mL), 10% (w/v) SDS (100 μL) and 30% acrylamide/bis (4.0 mL), which had been degassed for 15 minutes. The solution was then degassed for 15 minutes and then freshly prepared ammonium persulfate (APS) (50 μL of a 10% solution) and tetramethylethylenediamine (TEMED) (5 μL) were added. The gel was poured and

immediately overlayed with water. The gel was allowed to polymerize for 1.5 hours, was rinsed with water and the surface was dried with filter paper.

The stacking gel was prepared by combining water (6.1 mL), tris-HCl buffer, pH 6.8 (0.5M, 2.5 mL), 10% (w/v) SDS (100 μL), and 30% acrylamide/bis (1.3 mL) which had been degassed for 15 minutes. The solution was degassed for 15 minutes and then freshly prepared ammonium persulfate (APS) (50 μL) and TEMED (10 μL) were added. A comb was placed between the two glass plates and the stacking gel was poured. The gel was allowed to polymerize for 1.5 hours. The comb was removed and the wells were rinsed with water.

Electrode buffer (60 mL) was diluted with water (240 mL) and some of this solution (115 mL) was added to the upper buffer chamber of the electrophoresis apparatus. The remainder of the diluted buffer was added to the lower buffer chamber.

The protein samples were prepared by diluting the sample (10 μL) with sample buffer (40 μL) and heating at 95°C for 4 minutes. The sample (5 μL) was then applied to the gel along with protein standards (5 μL).

The gels were run at 200 volts, constant voltage setting until the bromophenol blue tracking dye reached the bottom of the gel (approximately 45 minutes).

The gels were stained for 0.5 hours with 0.1% Coomassie blue in fixative (methanol:acetic acid:water, 4:1:5). Destaining was performed with methanol (40%) and acetic acid (10%) in water.

3.2.0 ♦ Purification of HMG-CoA Synthase

3.2.1 ◊ Autolysis

Freeze dried *Saccharomyces cerevisiae* was obtained from Sigma. Tris-HCl buffer pH 8.2 was added to the dried yeast (0.2M, 40mL/10g yeast). The mixture was

then placed in a water bath at 38°C and stirred for 4 hours. The resulting suspension was then centrifuged (18,000 g for 20 minutes) at room temperature to yield a yellowish brown supernatant.

	Autolysate
Volume (mL)	61
Total Activity (units)	16
Total Protein (mg/mL)	34
Specific Act. (units/mg)	0.007

3.2.2 ◊ Ethanol Fractionation - *unmodified procedure*

To the extract at room temperature (18-20°C), cold (-10°C) 96% ethanol was slowly added, with stirring, to a final concentration of 25% (v/v). The preparation was then stirred at room temperature for 2 hours. The thick white precipitate of denatured protein was then removed by centrifugation (12,000 g for 20 minutes) at room temperature. The supernatant was cooled to -3°C in an ice-salt bath and left at this temperature for 40 minutes with occasional stirring. The reddish-brown sticky precipitate was collected by centrifugation (2000 g for 20 minutes) at -3°C and redissolved in potassium phosphate buffer, pH 7.8 (1 mL/10g yeast, 0.01 M).

	Autolysate	EtOH ppt.
Volume (mL)	114	4
Total Activity (units)	34	5
Total Protein (mg/mL)	50	42
Specific Act.(units/mg)	0.006	0.030
Fold Purification	-	5
Yield (%)	100	15

3.2.3 ◊ Ethanol Fractionation - *modified procedure*

To the extract at room temperature (18-20°C), cold (-10°C) 96% ethanol was slowly added, with stirring, to a final concentration of 25% (v/v). The preparation was then stirred at room temperature for 2 hours. The thick white precipitate of denatured protein was then removed by centrifugation (12,000 g for 20 minutes) at room temperature. More 96% ethanol was added to the orangy brown supernatant to a final concentration of 30% (v/v) and the solution was cooled to -10°C and left at this temperature for 40 minutes with occasional stirring. The reddish-brown sticky precipitate was collected by centrifugation (2500 g for 20 minutes) at -5°C. It was found that this precipitate could be kept for 3 days at -10°C without much loss of activity. The precipitate was redissolved in potassium phosphate buffer, pH 7.8 (1 mL/10g yeast, 0.01 M).

	Autolysate	EtOH ppt.
Volume (mL)	128	6
Total Activity (units)	30	14
Total Protein (mg/mL)	48	92
Specific Act.(units/mg)	0.005	0.028
Fold Purification	-	6
Yield (%)	100	47

3.2.4 ◊ Acetic Acid Precipitation

Sodium acetate buffer pH 4.3 (2M), was added slowly, with stirring to the redissolved ethanol pellet (section **3.2.3**), in order to adjust the pH to 5.6 at 4°C. The white precipitate was collected by centrifugation (10,000 g for 20 minutes) at 4°C and dissolved in potassium phosphate buffer, pH 7.8 (1 mL/10 g yeast, 0.05 M).

	EtOH ppt.	AcOH ppt.
Volume (mL)	7	1
Total Activity (units)	18	0.2
Total Protein (mg/mL)	81	20
Specific Act.(units/mg)	0.032	0.009
Fold Purification	6	0.3
Yield (%)	34	1

3.2.5 ◊ DEAE Cellulose Chromatography

Potassium hydroxide (0.1 M) was added to the redissolved ethanol pellet (section **3.2.3**) in order to adjust the pH to 7.8. The protein solution was then applied to a column of DEAE cellulose (1.5cm x 15cm) that had been previously equilibrated with potassium phosphate buffer, pH 7.8 (0.01 M), containing dithiothreitol (0.5 mM). The column was washed once with one column volume (25 mL) of the potassium phosphate buffer and then a linear gradient of phosphate buffer containing 0 to 0.5 M KCl (50 mL) was applied. The flow rate was set at 1 mL/min and fractions were collected every 2 mL. The fractions were assayed for HMG-CoA synthase activity. The enzyme activity was found in the first four fractions, which were pooled.

	EtOH ppt.	DEAE
Volume (mL)	7	12
Total Activity (units)	19	0.1
Total Protein (mg/mL)	86	1
Specific Act.(units/mg)	0.031	0.008
Fold Purification	6	0.3
Yield (%)	41	0.5

3.2.6 ◊ CM Cellulose Chromatography

The pH of the enzyme solution from section **3.2.3** was adjusted to 7.8 with potassium hydroxide (0.1 M) and the solution was applied to a column of CM cellulose (1.5cm x 15cm) which had previously been equilibrated with potassium phosphate buffer, pH 7.8 (0.01 M). The column was washed with one column volume (25 mL) of phosphate buffer and then a linear gradient of phosphate buffer, containing 0 to 2 M potassium chloride, was applied. The flow rate was set at 1 mL/min and fractions were collected every 2 mL. The fractions were then assayed and HMG-CoA synthase activity was detected in the first four fractions, which were pooled.

	EtOH ppt.	CM
Volume (mL)	5	14
Total Activity (units)	12	0.1
Total Protein (mg/mL)	85	1
Specific Act.(units/mg)	0.028	0.007
Fold Purification	5	0.3
Yield (%)	43	1

3.2.7 ◊ Calcium Phosphate Gel Adsorption[g]

Acetic acid (1M) was added to the redissolved ethanol precipitate (section **3.2.3**) to adjust the pH to 6.8. Calcium phosphate gel was added in the proportion 1.5 mg/mg of protein present. After stirring for 5 minutes at 4°C, the gel was collected by centrifugation (2500g for 20 minutes) and the light yellow supernatant was discarded. Potassium phosphate buffer (0.2M), pH 8.3, was immediately added to the gel. After stirring for 20 minutes the gel was collected by centrifugation (2500g for 20 minutes) and the light yellow supernatant was kept. The gel was then suspended in more potassium

phosphate buffer and a second elution was performed. The supernatants were combined and assayed for activity and the gel was discarded.

	EtOH ppt.	Gel
Volume (mL)	6	4
Total Activity (units)	14	0.6
Total Protein (mg/mL)	77	9
Specific Act.(units/mg)	0.030	0.017
Fold Purification	5	0.6
Yield (%)	44	4

3.2.8 ◊ Gel Filtration on Sephadex G-150

The precipitate from the ethanol step (section **3.2.3**) was dissolved in tris-HCl buffer, pH 7.5, (0.02M, 1 mL/10 g yeast). The enzyme solution (1 mL) was then loaded on a Sephadex G-150 column (1.5cm x 10 cm) equilibrated with tris-HCl buffer, pH 7.5 (0.02 M), containing dithiothreitol (0.5 mM) and 10 % (v/v) glycerol. Fractions were collected every 1 mL and then assayed. No HMG-CoA synthase activity was detected.

3.2.9 ◊ Ammonium Sulfate Precipitation

Finely crushed ammonium sulfate was added slowly with stirring to the redissolved ethanol precipitate (section **3.2.3**) to achieve 35% saturation at room temperature. Stirring was maintained for 20 minutes at room temperature after all of the ammonium sulfate had dissolved. The off-white precipitate was collected by centrifugation (10,000 g for 10 minutes) at room temperature and discarded. Ammonium sulfate was slowly added to the yellowish brown supernatant to reach 55% saturation. After all of the ammonium sulfate had dissolved, stirring was continued for 20 minutes at room temperature. The precipitate was collected by centrifugation (10,000 g for 10

minutes) at room temperature and the clear yellow supernatant was discarded. The off-white precipitate was dissolved in tris-HCl buffer, pH 8.2 (0.2M, 1mL/10g yeast).

The precipitate was desalted on a sephadex G-25 column (1 cm x 10 cm) equilibrated with potassium phosphate buffer, pH 7.8 (0.01 M).

	EtOH ppt.	$(NH_4)_2SO_4$ ppt.
Volume (mL)	6	3
Total Activity (units)	13	12
Total Protein (mg/mL)	54	52
Specific Act.(units/mg)	0.040	0.076
Fold Purification	6	2
Yield (%)	42	92

3.2.10 ◊ Hydroxylapatite Chromatography

The precipitate from the ammonium sulfate precipitation step (section **3.2.9**) was dissolved in potassium phosphate buffer, pH 6.8 (1 mL/10 g yeast, 0.01 M). The enzyme solution was adjusted to pH 6.8 with acetic acid (1 M) and was then loaded onto a column of hydroxylapatite (1.5 cm x 15 cm) equilibrated with potassium phosphate buffer, pH 6.8 (0.01 M). The column was washed with one column volume (25 mL) potassium phosphate buffer (0.01 M) and then a linear gradient of 0.01 M to 0.2 M potassium phosphate buffer, pH 6.8, was applied. The flow rate was about 1 mL/min and fractions were collected every 2 mL. The fractions were then assayed for HMG-CoA synthase activity and those fractions containing the enzyme were pooled.

	$(NH_4)_2SO_4$ ppt.	Hydroxlyapatite
Volume (mL)	1	2
Total Activity (units)	1.8	0.4
Total Protein (mg/mL)	85	2
Specific Act.(units/mg)	0.028	0.143
Fold Purification	9	5
Yield (%)	36	22

3.2.11 ◊ Hydrophobic Interaction Chromatography

The precipitate from the ethanol precipitation step (section **3.2.3**) (250 μL) was dissolved in sodium phosphate buffer, pH 6.8 (0.1 M), containing ammonium sulfate (1 mL/10 g yeast, 2.4 M). The enzyme solution was adjusted to pH 6.8 with acetic acid (1 M) and was then loaded onto the hydrophobic interaction column (1cm x 5cm) (Bio-Rad methyl-HIC # 156-0080), which was previously equilibrated with sodium phosphate buffer, pH 6.8 (0.1 M), containing ammonium sulfate (2.4 M). The column was washed with one column volume (5 mL) of sodium phosphate buffer, pH 6.8 (0.1 M), containing ammonium sulfate (2.4 M). The column was then washed with one column volume (5 mL) of sodium phosphate buffer, pH 6.8 (0.1 M). Fractions were collected every 1 mL and were then dialyzed for 2 hours against 500 mL of tris-HCl buffer, pH 8.2 (0.05 M), with the buffer changed after 1 hour. The fractions were then assayed for HMG-CoA synthase activity and the enzyme activity was found to be present in the last fraction.

3.3.0 ♦ Inhibition of HMG-CoA Synthase By F-244

DMSO (1.0 mL) was added to F-244 (1.0 mg) to produce a 3 mM solution. A serial dilution was performed by taking some of the F-244 solution (50 μL) and diluting it

with more DMSO (1 mL) to produce a 150 μM solution. Some of this diluted F-244 solution (150 μL) was added to a partially purified enzyme sample (50 μL). A control was also prepared which contained enzyme (50 μL) and DMSO (150 μL). The resulting solutions were then incubated at 30°C for 5 minutes and were then assayed for HMG-CoA synthase activity.

3.3.1 ◊ Determination of the IC_{50} value of F-244

Enzyme solution was incubated with 0, 0.004, 0.006, 0.008, 0.01, 0.015 and 0.02 μM F-244 solution. A control was also prepared which contained only enzyme solution (15 μL). The samples were then incubated at 30°C for 5 minutes and were then assayed for HMG-CoA synthase activity. The % inhibition was measured by comparison of the activity of the F-244 samples with the activity of the control.

3.3.2 ◊ Dilution of F-244-HMG-CoA Synthase Complex

The procedure used was the same as that reported by Greenspan.[av] Enzyme solution (120 μL), tris-HCl buffer, pH 8.2 (0.5 M, 1080 μL), and F-244 (7.9 μM, 64 μL) were combined and incubated at 30°C for 5 minutes. A control was also prepared which contained DMSO (64 μL) in place of F-244. These solutions (50 μL) were assayed and no HMG-CoA synthase activity was detected in the F-244 containing sample; however enzyme activity was detected in the control. The remainder of these solutions was divided into four equal portions (291 μL). The first sample had magnesium chloride (30 μL), F-244 (7.9 μM, 150 μL), and tris-HCl buffer, pH 8.2 (0.5 M, 2.529 mL) added to give a final F-244 concentration of 0.4 μM. The second sample had magnesium chloride (30 μL), F-244 (7.9 μM, 32 μL), and tris-HCl buffer, pH 8.2 (0.5 M, 2.647 mL) added to give a final F-244 concentration of 0.12 μM. The third sample had magnesium chloride (30 μL), F-244 (7.9 μM, 16 μL), and tris-HCl buffer, pH 8.2 (0.5 M, 2.663 mL) added to give

a final F-244 concentration of 0.08 μM. Finally, the fourth sample had magnesium chloride (30 μL), and tris-HCl buffer, pH 8.2 (0.5 M, 2.679 mL) added to give a final F-244 concentration of 0.04 μM. The controls had DMSO added instead of F-244. These samples were incubated at 30°C for 5 minutes and assayed. No HMG-CoA synthase activity was present in the F-244 samples, however, enzyme activity was detected in the controls.

3.4.0 ♦ Procedure for the Affinity Labeling of HMG-CoA Synthase

3.4.1 ◊ General Procedure for the Growth of *Fusarium sp.* ATCC 20788

The *Fusarium sp.* was initially cultured on a potato agar slant and stored at 0 °C. The fermentation began with the preparation of the growth medium containing: corn meal (0.5 g , Primo brand), tomato paste (4.0 g , Primo brand), oat flour (1.0 g), dextrose (1.0 g), and water (100 mL) in a 500 mL Erlenmeyer flask, which was subsequently autoclaved at 121°C for 20 minutes. The *Fusarium* was then transferred from the slant to the flask as a spore suspension in water. The flask was incubated in an incubator-shaker at 28°C and 212 rpm for 3 days.

A corn meal medium was prepared which contained: corn meal (4.0 g), trace element solution (25 mL) containing $MgSO_4.7H_2O$ (0.1 g/L), sodium tartrate (0.1 g/L), $FeSO_4.7H_2O$ (0.01 g/L) and $ZnSO_4.7H_2O$ (0.01 g/L), and water (175 mL). Three 500 mL flasks containing corn meal medium (200 mL), which had been autoclaved at 121°C for 20 minutes, were each innoculated with the growth medium (3 mL). The flasks were then incubated in an incubator-shaker for 10 days at 25°C and 220 rpm.

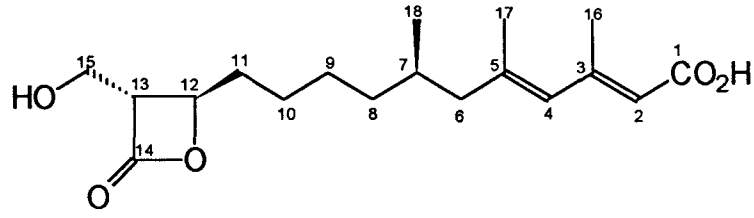
3.4.2 ◊ Assay for Production of F-244

After 3 days in the corn meal medium, some of the suspension (5 mL) was removed from one of the flasks and was combined with an equal volume of acetonitrile. The suspension was stirred for 15 minutes at room temperature and filtered under vacuum. The filtrate was evaporated to dryness *in vacuo* and a solution of 60 % acetonitrile / 0.1% phosphoric acid was added (5 mL) in a volumetric flask. Some of this solution (20 µL) was injected onto the HPLC and the UV detector was set at 275 nm in order to monitor the presence of F-244.

3.4.3 ◊ Incorporation of *methyl*-[^{3}H]-methionine into F-244

After 3 days in the corn meal medium, *methyl*-[^{3}H]-methionine (0.07 mL, 0.06 mg, 0.07 mCi, 185 mCi/mmol) was fed to each of two flasks every day for a total of 7 days.

3.4.4 ◊ Isolation of [15, 16, 17, 18 - ^{3}H] F-244



The corn meal broth was collected and centrifuged at 18600 g for 10 minutes. The supernatant was adjusted to pH 4 with acetic acid, was extracted with dichloromethane (4 x 150 mL) and the organic phases were collected. The mycelial cake was stirred with 5% methanol in ethyl acetate (200 mL) until the liquid became bright orange. The liquid phase was separated and collected, and the solid phase was again

stirred with the same solvent mixture until the liquid phase no longer turned orange. All the organic extracts were then combined and concentrated *in vacuo*. The residue (160 mg) was then dissolved in 30% ethyl acetate in hexane with 1% acetic acid (1 mL) and put on a column of silica gel 60 (230 - 400 mesh ASTM, E. Merck) in a ratio of 25:1 to the weight of the crude sample. The column was eluted with a step gradient of ethyl acetate in hexane (30%, 40%, 50%, 60% and 70%) containing 1% acetic acid. Fractions were collected every 3 mL and TLC was used to detect which fractions contained the F-244. The fractions containing F-244 were combined and concentrated *in vacuo*. The crude product (80 mg) was dissolved in chloroform, methanol and acetic acid in a ratio of 97:2:1 and reapplied to a smaller column of silica gel 60 (0.5 x 7 cm) eluted with the methanol, chloroform, and acetic acid solvent (97:2:1). The polarity of the solvent was gradually increased to 4%, 6%, 8% and 10% of methanol while maintaining the 1% acetic acid. The F-244 containing fractions were then combined, concentrated, and dried *in vacuo* for 4 hours. The impure F-244 (30 mg) was then dissolved in dichloromethane, hexane and methanol in a 10:10:1 ratio and passed through a column of Sephadex LH-20 eluted with methanol. The F-244 containing fractions were then combined and concentrated. The F-244 still appeared impure by TLC and was subjected to another silica gel 60 column. The impure residue (12 mg) was dissolved in ethyl acetate and hexane in a 4:6 ratio containing 1% acetic acid. The column was eluted with the same solvent and the F-244 containing fractions were combined and concentrated *in vacuo*. The purified F-244 (2 mg) appeared as an orangy-red solid; ^{1}H NMR (200.13 MHz, $CDCl_3$) δ (ppm) 0.84 (d, $J_{18\text{-}7}$=6.6 Hz, 3H, C*H*$_3$-18), 1.14 (m, 1H, C*H*-8'), 1.24-1.44 (m, 5H, C*H*-8,9,9',10,10'), 1.66 (m, 1H, C*H*-7), 1.76 (m, $J_{11\text{-}12}$=4.1 Hz, 1H, C*H*-11), 1.79 (s, 3*H*, CH_3-17), 1.86 (dd, $J_{6\text{-}6'}$=13.2 Hz, 1H, C*H*-6), 1.90 (m, $J_{11'\text{-}12}$=4.1 Hz, 1H, C*H*-11'), 2.08 (dd, $J_{6'\text{-}7}$=8.2 Hz, 1H, C*H*-6'), 2.23 (s, 3H, C*H*$_3$-16), 3.39 (m, $J_{13\text{-}15}$=5.0 Hz, 1H, C*H*-13), 3.88 (dd, $J_{15\text{-}15'}$=11.6 Hz, 1H, C*H*-15), 4.04 (dd, $J_{15'\text{-}13}$=4.2 Hz, 1H, C*H*-15'), 4.58 (m, $J_{12\text{-}13}$=4.1 Hz, 1H, C*H*-12), 5.67 (s,

1H, C*H*-2), 5.72 (s, 1H, C*H*-4). NMR assaignments based on a detailed one and two-D NMR analysis by E. Saepudin. The specific activity of [15, 16, 17, 18 - ^{3}H] F-244 was determined by liquid scintillation counting to be 1.3 x 10^6 DPM/mg.

3.5.0 ♦ Preparation of [15, 16, 17, 18 - ^{3}H] F-244 Labeled HMG-CoA Synthase

Partially purified HMG-CoA synthase (1 mL, 24 mg/mL, 0.04 units/mg), taken after the ethanol precipitation step, was concentrated by ultra filtration through a membrane with a molecular weight cut-off of 30,000, at 4°C. The enzyme (0.5 mL) was then incubated with [15, 16, 17, 18 - ^{3}H] F-244 (2.2 mM, 1.5 μL, 1.3 x 10^6 DPM/mg) at 30°C for 5 minutes. An assay was performed on a portion of this solution (20 μL) and no HMG-CoA synthase activity was detected. The solution was then subjected to ultra filtration at 4°C. Liquid scintillation counting yielded an activity of 2017 DPM for the filtrate and 1448 DPM for the residue. The residue (~ 0.3 mL) was then subjected to gel electrophoresis. Some of the residue (10 μL) was mixed with sample buffer (40 μL) and was then heated at 95°C for 4 minutes. The entire sample was then applied to the gel. Another gel was also prepared which contained HMG-CoA synthase inhibited with unlabeled F-244 (see section **3.6.0**). Both of these gels were run simultaneously as described in the general procedure described above.

The nonradioactive gel was stained with Coomassie blue, as described above. The radioactive gel was carefully sliced into twenty 3 mm segments. These segments were then added to 5 mL of liquid scintillation cocktail and then counted.

3.6.0 ♦ Effect of Other β-Lactone Compounds on HMG-CoA Synthase Activity

A solution of (±)-β-butyrolactone (100 mM) was prepared by the addition of (±)-β-butyrolactone (6.1 μL) to DMSO (1 mL). A solution of the β-lactone of

undisclosed structure (~ 10 mM) was prepared by dissolving the compound (2 mg) in DMSO (0.5 mL).

The ability of (±)-β-butyrolactone to inhibit HMG-CoA synthase was tested. HMG-CoA synthase (30 μL, 19 mg/mL, 0.04 units/mg) was incubated with various amounts of (±)-β-butyrolactone (15, 30, 45, 60 and 120 μL of 100 mM solution), magnesium chloride (30 μL) and tris-HCl buffer, pH 8.2 (2.925, 2.910, 2.895, 2.880 and 2.820 mL) to give (±)-β-butyrolactone concentrations of 0.5, 1, 1.5, 2 and 4 mM. These solutions were incubated at 30°C for 5 minutes and then assayed. Their activities were compared to that of a control which lacked inhibitor (0.04 units/mg).

The β-lactone of unknown structure was tested as an inhibitor of HMG-CoA synthase. HMG-CoA synthase (30 μL, 19 mg/mL, 0.04 units/mg) was incubated with various amounts of this β-lactone (0.3, 300 and 450 μL), magnesium chloride (30 μL) and tris-HCl buffer, pH 8.2 (2.939, 2.640 and 2.490 mL) to give inhibitor concentrations of 0.001, 1 and 1.5 mM. These solutions were incubated at 30°C for 5 minutes and were then assayed. Their activities were compared to that of a control which lacked inhibitor (0.04 units/mg).

3.6.1 ◊ Reversibility of (±)-β-Butyrolactone Inhibition of HMG-CoA Synthase

The reversibility of (±)-β-butyrolactone inhibition of HMG-CoA synthase was tested by performing two experiments.

In the first experiment, HMG-CoA synthase (20 mg/mL, 0.021 units/mg) was incubated with (±)-β-butyrolactone (2 mM) at 30°C for 5 minutes. The solution was then assayed for HMG-CoA synthase activity and it was found to be 51% inactivated (0.010 units/mg) compared to a control lacking inhibitor (0.021 units/mg).

In the second part of this experiment, HMG-CoA synthase (20 mg/mL, 0.021 units/mg) was added to (±)-β-butyrolactone (2 mM) and assayed immediately, with no

preincubation period. It was found that the enzyme was ~10% inactivated (0.019 units/mg) compared to the control lacking inhibitor (0.021 units/mg).

In the second experiment HMG-CoA synthase (60 μL, 20 mg/mL, 0.021 units/mg) was incubated with (±)-β-butyrolactone (20 μL, 100 mM) and 420 μL of tris-HCl buffer, pH 8.2, at 30°C for 5 minutes to give a final inhibitor concentration of 4 mM. The mixture was then divided in two (2 x 250 μL). To the first half, (±)-β-butyrolactone (110 μL, 100 mM), magnesium chloride (30 μL) and tris-HCl buffer (2.610 mL) were added in order to keep the inhibitor concentration at 4 mM. To the second half, (±)-β-butyrolactone (5 μL, 100 mM), magnesium chloride (30 μL) and tris-HCl buffer (2.715 mL) were added in order to dilute the inhibitor concentration to 0.5 mM. Both of these solutions were incubated at 30°C for 5 minutes and then assayed for HMG-CoA synthase activity. It was found that the enzyme was ~80% inactivated (0.004 units/mg) in both solutions compared to a control which lacked inhibitor (0.021 units/mg).

3.6.2 ◊ Time Dependent Inhibition of HMG-CoA Synthase by (±)-β-Butyrolactone

Various amounts of (±)-β-butyrolactone (30, 60, 120 and 240 μL) were incubated with HMG-CoA synthase (30 μL, 66 mg/mL, 0.02 units/mg), magnesium chloride (30 μL, 1M) and tris-HCl buffer, pH 8.2 (2.910, 2.880, 2.820, 2.700 mL of 0.2M) to give final (±)-β-butyrolactone concentrations of 1, 2, 4 and 8 mM. These solutions were then incubated at 30°C for time periods of 0, 2, 4, 8 and 10 minutes and then immediately assayed for HMG-CoA synthase activity. The amount of HMG-CoA synthase activity remaining after each of these time periods was determined in comparison to a control lacking inhibitor (0.02 units/mg) which was subjected to the same conditions.

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