STUDIES OF SYNTHETIC METHODOLOGIES FOR INTRODUCTION OF HYDROXY GROUPS ONTO STEROL RING C AND RING D

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ABSTRACT

In the last few decades, research became more and more interested in this area. However the chemistry aspect has somehow held up the research of oxysterols. Despite continuing advances in studies in the chemistry of oxysterols, most researchers rely on commercial materials that are unfortunately very limited with regard to structure types, available quantities, and unreasonable costs. Many naturally occurring potent oxysterols have not been well studied simply because they are not chemically synthesized and therefore not commercially available.

In this project, we developed a synthetic route to prepare C-, D-ring oxygenated cholesterol in an acceptable yield. Two useful intermediates, Cholest-7, 14-dien-6-one **22** and cholest-7-en-6-one **20** were also synthesized by a new method, from which introduction of hydroxy groups into ring C and D could be achieved in one or two steps.

It was suggested in our experiments that hydroxy groups on sterol ring A and B could more or less decrease the yield of an important intermediate, cholest-7-en-6-one, which however is of great importance to introduce hydroxy groups onto ring C and D. To prepare this cholest-7-en-6-one in good yield, several synthetic routes were tried in this project. Synthesis of cholest-7-en-6-one with 3-OH and 5-OH on sterol ring was not successful. Despite all the effort, only 20% yield of 5α-cholestan-3β, 5α-diol-7-en-6-one-3-acetate **5** was given in the experiment. In the preparation of cholest-7-en-6-one with 3-OH and 4-OH on sterol rings, an important reaction, 7-bromination, however ended up in multiple products and hence the following step could not be carried out. A compound **15** with no hydroxy groups on sterol rings was synthesized by treating 3-chloro-cholesterol **14** with Na/ethanol at –50 °C. From this compound, the cholest-7-en-6-one **20** was finally achieved in 76% yield and a 14-OH sterol **21** was synthesized by allylic oxidation in over 90% yield.

Key words: Oxysterol, Cholesterol, Sterol, Bromination, Hydroxy group, allylic oxidation.

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ABRBREVIATIONS

Ac	actetyl
COSY	correlated spectroscopy
CT	cholesterol-3 β ,5 α ,6 β -triol
DCM	dichloromethane
DMF	dimethylformamide
DMP	3,5-dimethylparazole
DNA	deoxyribonucleic acid
Et	ethyl
FCS	fetal calf serum
HMG-CoA	hydroxymethylglutaryl-coenzyme A
In vacuo	latin in the vacuum
In situ	latin in the normal, natural, original
In vivo	latin occurring or make to occur within a living oranism
IR	infrared
LDL	low density lipoprotein
***m*CPBA**	m-chloroperbenzoic acid
Me	methyl group
mRNA	messenger ribonucleic acid

NBS	N-bromosuccinimide
NK cell	natural killer cell
NMR	nuclear magnetic resonance
Py	pyridine
SRE	sterol regulatory element
SREBP	sterol regulatory element binding protein
TBHP	t-butyl hydroperoxide
THF	tetrahydrofuran
TLC	thin layer chromatrograpy
TNF	tumour necrosis factor
LXR	liver X receptor
FXR	Farnesoid-X-activated receptor
UV	ultraviolet
MS	mass spectrum

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CHAPTER ONE

INTRODUCTION

1.1 Steroids and Their Structures

Steroids represent a large group of naturally occurring family of organic molecules with biochemical and medical interest, which are extensively distributed in the animal and plant kingdoms. The basic structure to which all the steroids are related is that of fully reduced phenanthrene to which is fused a five-numbered ring structure. The complete structure is shown in **Figure 1.1**.

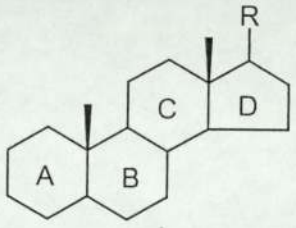
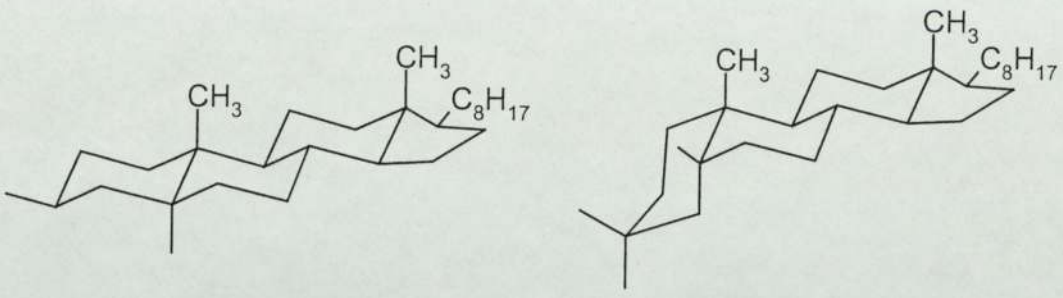


Figure 1.1 Structure of Steroids

However, this structure doesn't fully represent the structure of steroids, as the true structure is a three dimensional molecule, which have a length about 2nm, width 0.75 nm and thickness 0.45 nm. The three dimensional structure of Steroids is shown in **Figure 1.2** with the cyclohexane rings A, B, C in the chair form. The alternative boat form for the steroid rings is less stable and does not normally occur.



(5α structure) (5β structure)

Figure 1.2 Three-Dimensional Structures of Steroids

We can see from the structure above that the free valencies of carbon atom involved in the rings are not all in the plane of the molecule. One bond of each atom is perpendicular to the plane, which is called "a" bond (axial). While the other one who makes an angle of 30 degree to the plane is called "e" bond (equatorial). It has been agreed by convention that the angular methyl groups and the side-chain all define the upper or β side of the molecule and the lower side is referred to as the α side. So, when depicting a steroid in the two-dimensional way, it is conventional to show β groups in full line and α group in broken line.

1.2 Bile acid, Steroid hormones and Vitamin D...

Steroids differ considerably one from another in the degree of saturation of each of the four hydrocarbon rings and in the side-chain substituents as R attached to these rings. With wide variations in structure, they encompass compounds of vital importance to life, such as cholesterol, the bile acids, vitamin D, sex hormones,

corticoid hormones, cardiac aglycons, antibiotics and insect moulting hormones etc. Some examples of these steroids are shown in **Figure 1.3**.

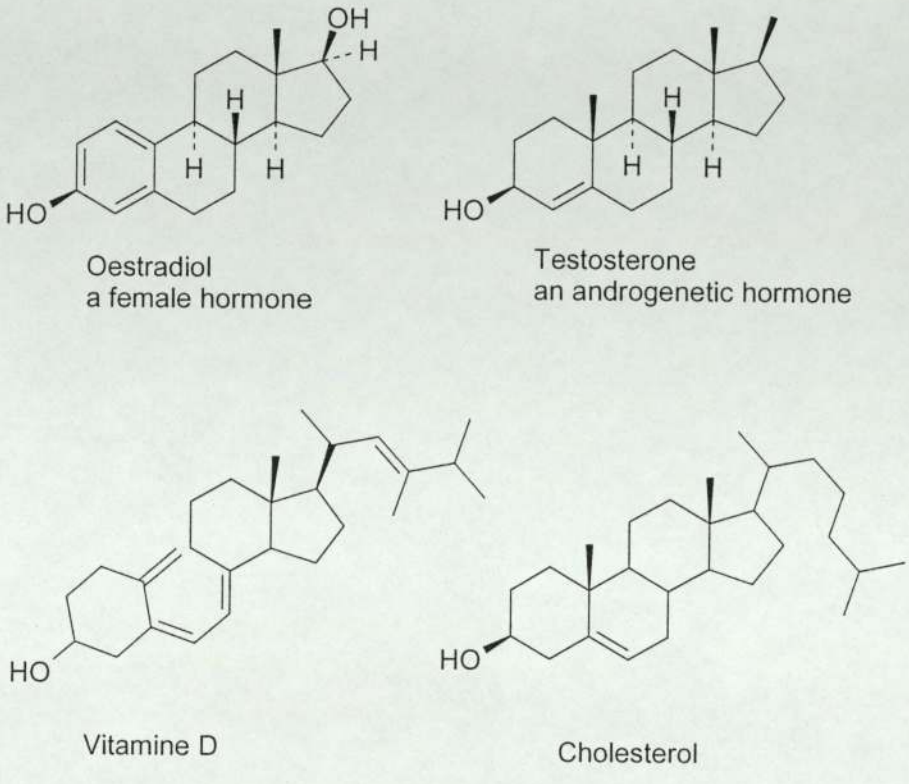


Figure 1.3 Examples of Steroids.

1.3 Cholesterol

Among these steroids, the most abounded compound is cholesterol, a kind of unsaturated crystallized alcohol of formula $C_{27}H_{45}O$, existed in fat and cholelith. It was first isolated around 1770 by Poulletier de la Salle from gallstones. In 1815, cholesterol was found as a component of human gallstones and isolated from the unsaponifiable fraction of animal fats by M. E. Chevrevl, a young French chemist,

who named it cholesterine. Cholesterol has the characterized structure—the four-ring system with an attached eight-carbon side chain and two angular methyl groups at C10 and C13 respectively. The structure and numbering sequence of cholesterol is shown in **Figure 1.4** and the 3D structure[1] is shown in **Figure 1.5**.

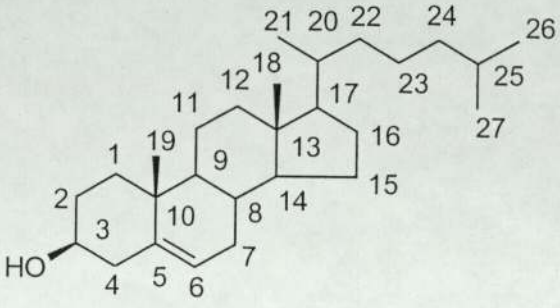
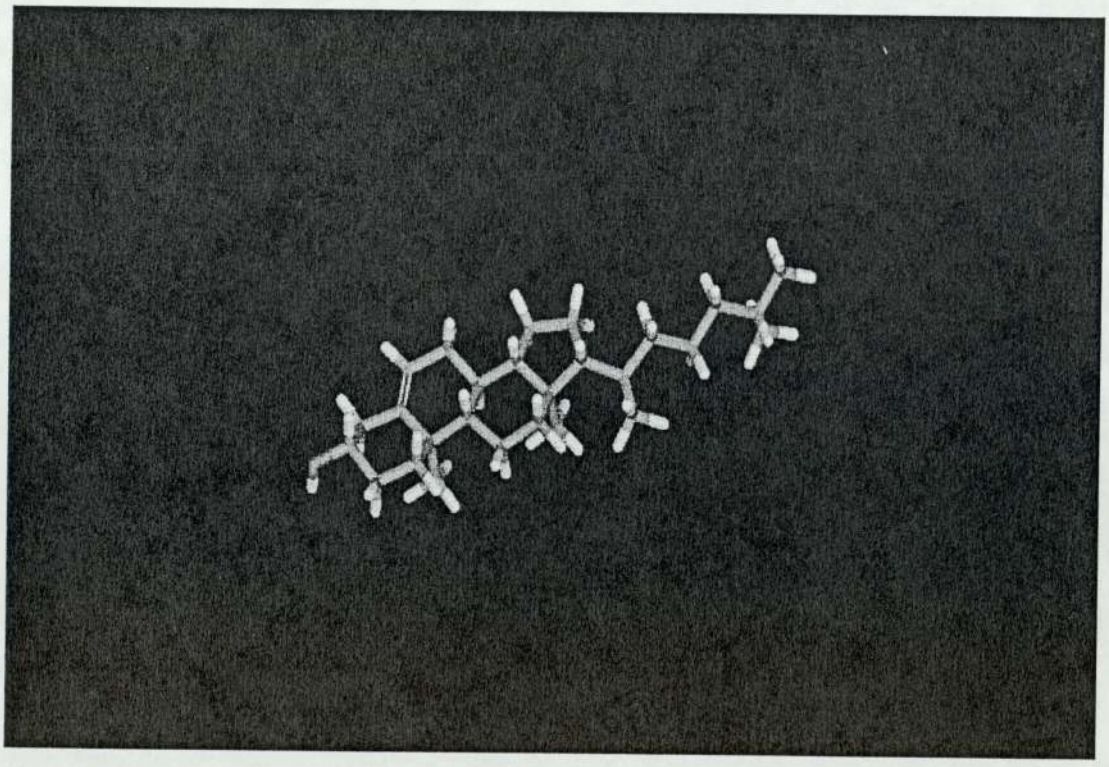


Figure 1.4 Structure and Numbering Sequence of Cholesterol.



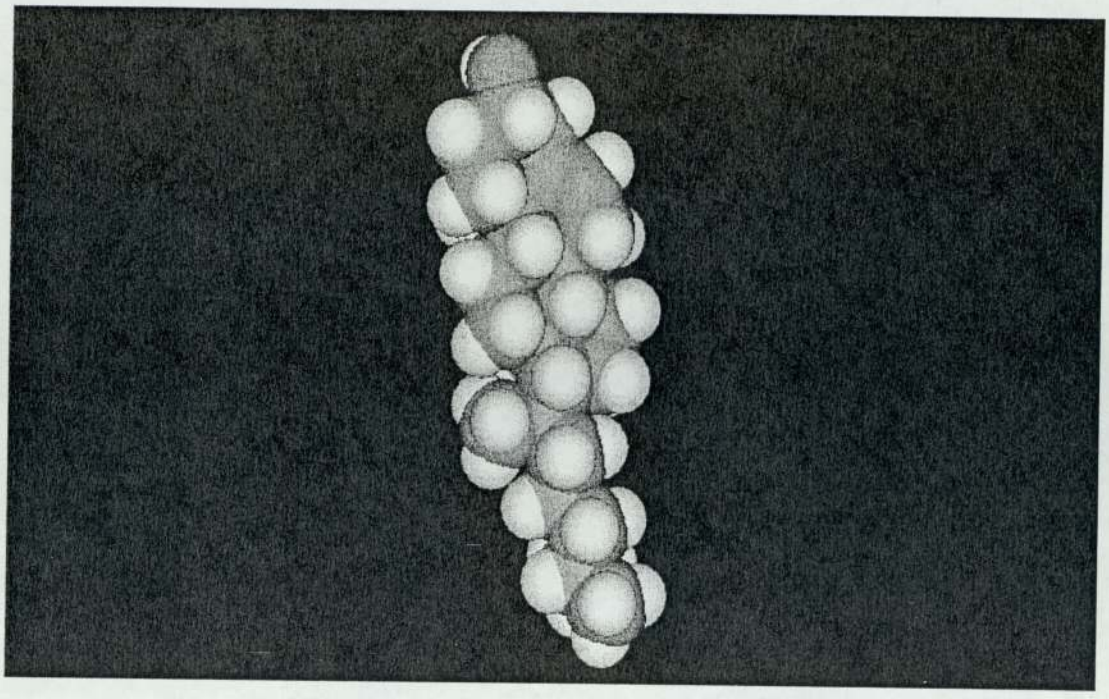


Figure 1.5 3D Structure of Cholesterol.

1.3.1 The Role of Cholesterol in Membrane System

Lipid studies 70 years ago introduced the conception of a phospholipid bilayer as the basic structure of a biological membrane. As research in this area advanced, the idea over the next 10 years was modified specifically to allow for the presence of protein and overcome the low permeability of unmodified phospholipid bilayer to polar materials.[2] Today, in the model of membrane, phospholipid and protein are the two essential components. Cholesterol and other components are often but by no means always in minor amount, modify the basic protein/phospholipid pattern in specific ways, with particular functional consequences.

The cholesterol molecule inserts itself in the membrane with the same orientation as the phospholipid molecules. **Figure 1.6** shows the phospholipid molecules with a cholesterol molecule in between. Note that the polar head of the cholesterol is aligned with the polar head of the phospholipids.[3]

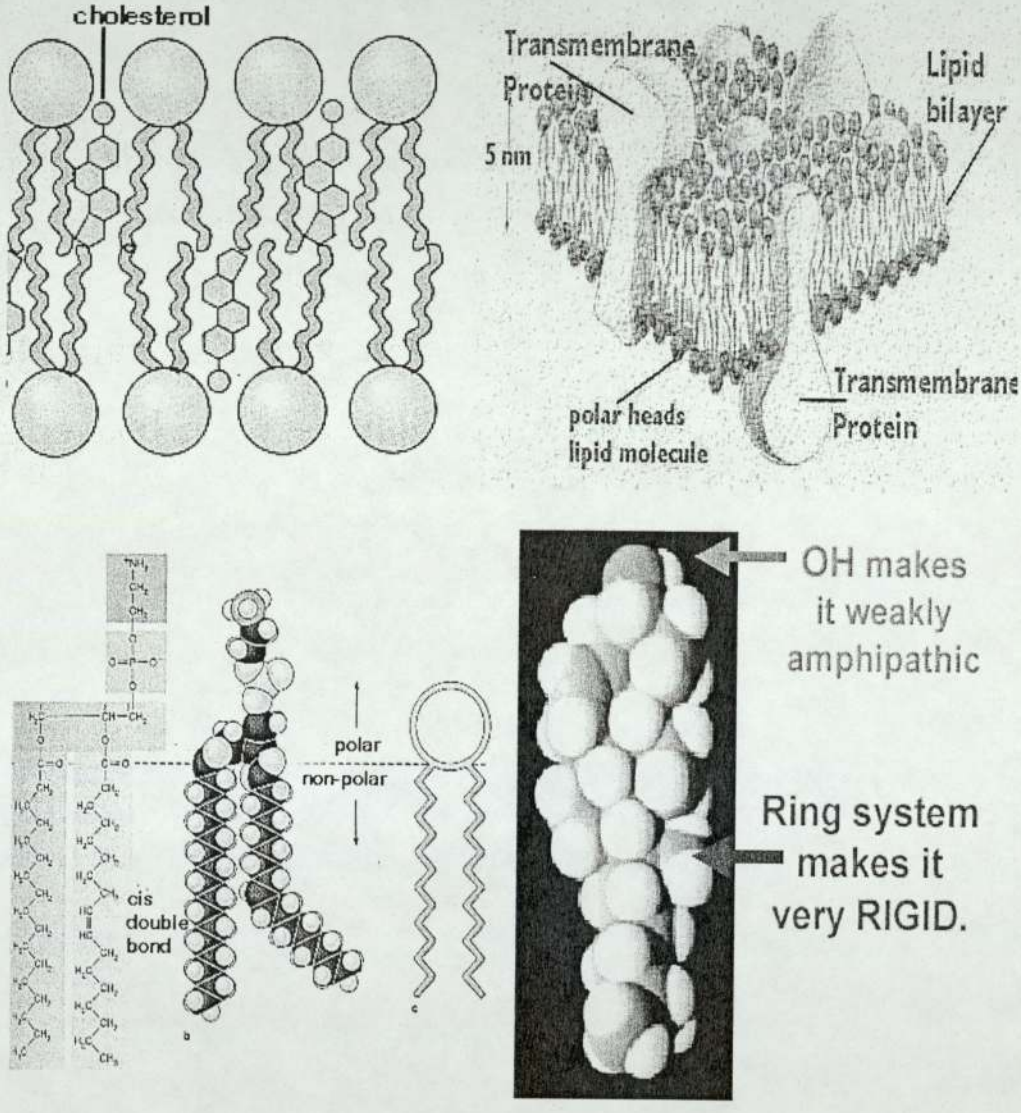


Figure 1.6 Cholesterol in the Phospholipid Molecule.

Cholesterol exerts a significant effect on the mobility or fluidity of phospholipids, an effect critically upon the temperarure.[4] Below the transition temperature the hydrocarbon chains of phospholids are relatively rigid or gel-like and in this situation cholesterol has a disruptive influence, allowing increased movement of the chain. With phospholipids above the transition temperature, however, the steroid nucleus prevents flexing of the acyl chains and thus reduces fluidity of the membrane. In addition to its ability to modify the fluidity of phospholipid, Cholesterol could also modify the permeability of membrane. All the components of a biological membrane participate in its role of maintaining a distinction between the compartments on either side. Protein are obviously important, particularly where an active transport mechanism exists, However, the more mechanical barrier properties of membranes depend to a large extent on their lipid content, in particular on the nature of phospholipid hydrocarbon chains, the interaction with sterols, and the chemistry of the phospholipid headgroup.[5] Thus the presence of more or less cholesterol not only alters the structure qualities of membrane but also influence their permeability, an effect shown in both natural and artificial membranes. Studies carried out on liposome suggested that cholesterol had great effects on the permeability of glucose,[6] glycerol[7] and chloride.[8]

1.4 Oxysterols

Oxysterol comprises the compounds formed from the oxidation of naturally

occurring sterols. In the human body, oxysterols are produced by endogenous oxidation and may also be derived from food. Absorption of oxysterols has been demonstrated,[9] and some oxysterols have been isolated from some fresh foods and many processed foods, such as dairy, eggs, meat and fish products.[10] Early researches on oxysterols showed that oxysterols, from endogenous origin, were formed solely by enzymatic processes, mainly in the biosynthesis of steroid hormones and bile acids. However, it is now known that non-enzymatic processes can also occur in the body.[11] **Table 1.1** presents the major naturally occurring oxysterols from both enzymatic and non-enzymatic origins.

Enzymatic origin	Non-enzymatic origin
Cholest-5-en-3β, 7α-diol	5,6α-Epoxy-5α-cholestan-3β-ol
Cholest-5-en-3β, 25-diol	5,6β-Epoxy-5β-cholestan-3β-ol
(25R)-Cholesterol-5-3β, 26-diol	Cholest-5-en-3β, 7β-diol
(25S)-Cholesterol-5-en-3β, 26-diol	5α-Cholestane-3β, 5α, 6β-triol
(20S)-Cholest-5-en-3β, 20-diol	3β-Hydroxycholest-5en-7-one
(22R)-Cholest-5-en-3β, 22-diol	Cholest-5-en-3β, 7α-diol
(20R, 22R)-Cholest-5-en-3β, 20,22-triol	Cholest-5-en-3β, 25-diol

Table 1.1 Major Naturally Occurring Oxysterols.

Though oxysterols can be generated from cholesterol by autoxidation,[12] they have

different biological effects from cholesterol. Studies suggested that these oxygenated derivatives of cholesterol, but not highly purified cholesterol itself, caused an inhibition of sterol biosynthesis and lowed of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase activity in cultured mammalian cells. These discoveries stimulated a tremendous amount of research on oxystrerols, including their actions on a wide variety of other processes and the chemical preparation of a large number of natural and synthetic oxygenated sterols. Up to date, an increasing amount of evidence indicates that oxysterols represent major natural regulators of sterol synthesis and HMG-CoA reductase. The discovery of the metabolic defect in familial hypercholesterolemia (FH) and the identification of the low-density lipoprotein (LDL) receptor[13] resulted in an explosion of research on LDL, including its roles in the control of sterol biosynthesis and a large variety of other important cellular processes, and studies on the levels of oxysterols in LDL. A considerable amount of research has been focused on the possible involvement of oxysterols, as a component of oxidatively modified LDL, in the pathogenesis of atherosclerosis. Resent research has shown that oxysterol can also induce programmed cell death in a variety of cell types and can affect the development of calcification in vascular cells. In addition, Gene regulation effect of oxysterols has also been reported.[14] It can affect the critical enzymes involved in the biosynthesis of cholesterol and other important lipids.

Due to the significance of oxysterols in biological processes, the studies involving

oxysterols grow rapidly. Several biological effects of oxysterol, such as cytotoxicity, gene regulation, atherogenesis, mutagenesis, and carcinogenesis, have been well studied. Among them, cytotoxicity is of great interest due to its possible utility in the development of antitumor agent.

1.5 Oxysterol and Gene Regulation

Studies in the 1960s and 1970s demonstrated that addition of cholesterol to either cells in culture or in animal diets inhibited the expression of HMG-CoA reductase, HMG-CoA synthease and the LDL receptor. Detailed studies by Kandutsch and his colleagues demonstrated that various oxysterols were far more potent repressors than cholesterol.[15] Indeed, the slight repression by cholesterol could easily be explained by the production of oxysterols during prior storage of the sterol or following addition of the sterol to the culture medium. A major breakthrough for understanding the mechanism involved in feedback repression came when the gene encoding the LDL receptor was isolated; the promoter of this gene was shown to contain a 10 bp *cis* element that was named sterol regulatory element 1 (SRE-1). The activity of an LDL receptor promoter-receptor gene increased when the transfected cells were incubated in cholesterol-free medium and decreased when LDL or oxysterols were added to this lipid-poor medium.[16] Such studies finally led to the purification and cloning of a family of transcription factors termed sterol regulatory element binding proteins (SREBPS) that bind to the SRE-1, or to one of the many

variants of this 10 bp sequence (**Table 1.2**), and activate transcription.[17] Thus, SREs function as conditional positive elements that are necessary for activated transcription in sterol-derived cells. The SREs are not required for basal transcription that occurs in cholesterol-loaded cells.

Gene	SRE sequence
LDL receptors	ATCACCCCAC
HMG-CoA synthase	CTCACCCCAC
HMG-CoA reductase	GCCACCCTAC
FPP synthase	CTCACACGAC
Squalene synthase	ATCACGCCAG
SREBP-2	ATCACCCCAC
Acetyl-CoA carboxylase	GGAGGACCAT
Fatty acid synthase	ATCACCCCAC
Stearoyl-CoA desaturase1	AGCAGATTGCG
Stearoyl-CoA desaturase2	AGCAGATTGTG
Glycerol-3-phosphate acyltransferase	CTCAGCCTAG
ATP-citrate layase	TCAGGCTAG

Table 1.2 Nucleotide Sequences of Sterol Regulatory Elements of Defined Genes

1.5.1 Sterol Regulatory Element Binding Proteins

Sterol regulatory element binding proteins, designated as SREBP-1a, SREBP-1c, and SREBP-2, are transcription factors that are synthesized as 125 kDa membrane-bound precursors, localized on the nuclear envelope and the endoplasmic reticulum.[18] SREBP-1a and SREBP-1c differ only in the length of the amino terminal transactivation domain. As compared to SREBP-1c, SREBP-1a has 29 additional acidic-rich amino acids at the amino terminal end of the protein and consequently is a more potent transcriptional activator.[19]

SREBPs belong to the family of basic helix-loop-helix leucine zipper (bHLH-zip) transcription factors in which three domains can be distinguished. The NH_2-terminal domains harbor a typical consensus bHLH-zip sequence of which basic region binds DNA and the helix-loop-helix motif determines dimerization.[20] In addition, this domain contains at its extreme NH_2-terminal ends an acidic trancription activation region. The NH_2 segment is connected to the membranes by a hairpin anchor formed by two hydrophobic regions, corresponding to the membrane spanning segments, and a short hydrophilic loop of approx 30 amino acids that resides in the lumen. Finally, the COOH-terminal fragment, which projects into the cytoplasm, serves as a regulatory domain for sterol-mediated cleavage.

As the concentration of intracellular sterols declines, the inactive membrane-bound

precursor protein is proteolytically cleaved in a two-step process, resulting in the release of an active and soluble 68 kDa NH_2-terminal segment. This active form is subsequently targeted to the nucleus where it transactivates sterol response genes.[21]

1.5.2 The Nuclear Receptors LXR, SF-1, LRH-1 and FXR

Nuclear hormone receptors are ligand-activated transcription factors that mediate the transcriptional activity of small lipophilic signaling molecules such as steroids, retinoids and thyroid hormones. A growing number of proteins have been identified that process the structural features of nuclear hormone receptors, but that lack known ligands. Known as orphan receptors, these proteins represent targets for novel signaling molecules. Identification ligands for this class of receptors has accelerated to a major extent the physiological characterization of the pathways they control. Recently, several independent studies have demonstrated that three distinct orphan receptors are activated by oxysterols, which have a feedforward regulation to the cholesterol metabolism.[22]

Liver X receptor (LXR) is a nuclear receptor originally isolated from liver. Two different LXR genes exist, LXRα and LXRβ. At present, LXRα is the best-characterized form. LXRα is expressed to a high extent in liver with lower levels being present in kidney, intestine, spleen and adipose tissue. In contrast to LXRα, LXRβ is more ubiquitously expressed.[23] LXR binds as a heterodimer with

9-cis retinoic acid receptor (RXR) to an LXR response element (LXRE). The RXR/LXR heterodimers can be activated on the one hand by oxysterol intermediates of the cholesterol/bile acid/steroid hormone synthesis pathways and on the other hand by RXR ligand,[24] which mimic the presence of an LXR ligand. The finding that oxysterols are LXR activators is based on the fact that most ligands for nuclear receptors are lipophilic derivatives of acetyl-CoA and several of them are cholesterol metabolites (i.e. the steroid hormones). Interestingly, only a very confined group of metabolites of the steroid/bile acid synthesis pathways are powerful activators and ligands. Structure activity study have in-fact demonstrated that position-specific monooxidation of the sterol side chain is required for LXR binding and activation. Several naturally occurred oxysterols have been described as potent ligands and activators based on this rule. For example, 24,25(s)-epoxy-cholesterol was found to be the most effective activator and 24-hydroxy-cholesterol which was equally potent but less effective.[25]

Studies showed that oxysterols are not the only compounds capable of modulating LXR activity. It was reported that transactivation by ligand-activated LXRs may be further modulated through other signal transduction pathsways involving phosphorylation by protein kinases, such as PKA and PKC.[26] Consistent with this report, Tamura and his colleagues demonstrated that LXR plays an important role in inducing both rennin and c-Myc trancription after activation of the camp signal transcription cascade.[27] In addition, these report suggested that LXRs binds both the

classical DR-4 and a newly identified LXR response element corresponding to the binding sequence of the camp-inducible transcription activator (CNRE), thereby mediating camp responsiveness of a subset of target genes.

Resent identification of a series of new target genes for LXR, however, has underscored an unequivocal role of this receptor in almost every aspect of cholsterol homostasis. First, LXRα controls ATP cassette binding protein (ABC)-mediated reverse cholesterol transport from the peripheral tissues to the liver.[28] By regulating the expression of the cholesterol ester transfer protein (CETP), LXRα furthermore favors transfer of cholesterol to the liver. In the liver, LXR controls cholesterol catabolism through its regulatory effects on CYP 7A1.[29] Finally, in the gut activation of LXRα has been shown to decrease intestinal cholesterol absorption through its effects on intestinal ABC expression.[30] All of these actions of LXRα activation seem to favor cholesterol catabolism and excretion and hence contribute to lowering total body cholesterol level.

Farnesoid-X-activated receptor (FXR) was original identified and cloned in rat as an orphan nuclear hormone receptor based on hybridization with a degenerate oligonucleotide designed from the highly conserved nuclear hormone receptor DNA binding domain.[31] FxR functions as a heterodimer with RXR and binds to sequence elements in the promoters of target genes. The FXR/RXR heterodimer binds with highest affinity to inverted repeats separated by 1bp (IR-1) and with low affinity to

direct repeats separated by 4 and 5 bp (DR-4 and DR-5).[32] As is the case for other nuclear hormone receptors, FXR regulates target gene activity in response to ligand. While initial studies suggested that farnesol and retinoid metabolites were likely ligands for FXR, it now appears that bile acids may be more physiologically relevant activators for FXR.

Based on recent studies, binding of bile acid to FXR could initiate the downregulation of CYP7A, the rate limiting enzyme in the conversion of bile acid to cholesterol.[33] The mechanism for this regulation is that the FXR/RXR heterodimer induces the expression of small heterodimer partner 1 (SHP-1) which in turn inhibits a positive regulation of CYP7A promoter, LRH-1.[34] Current data demonstrated that FXR is a bile acid sensor that plays a integral role in the bile acid synthesis and transport.

Steroidogenic factor-1 (SF-1 or NR5A1) is another receptor activated by oxysterols. This receptor is a member of the growing Fushi Tarazu factor-1 (Ftz-F1)-related subgroup of nuclear receptors.[35] Several genes involved in steroidogenesis such as cytochrome P-450 steroid hydroxylases (P450c17) 3β-hydroxysteroid dehydrogenase (3β-HSD) and the steroidogenic acute regulatory protein (StAR) are directly regulated by SF-1. Recently, studies showed that the SF-1 activity could be enhanced by several oxysterols in CV-1 cells, but it is not known whether these compounds bind directly to the receptor or not. Structure-activity relationship

analysis has identified that oxysterols with 25, 26, 27 hydroxy groups were the most effective SF-1 activators in CV-1 cells.[36]

1.5.3 Gene Control of Cholesterol Level

Intracellular and extracellular cholesterol levels are tightly maintained within a narrow concentration range by an intricate transcriptional mechanism. Excess cholesterol can be converted into oxysterols, signaling molecules, which modulate the activity of a number of transcription factors, limiting accumulation of excess cholesterol. Oxysterols are regulators of cholesterol synthesis and metabolism. The regulation of gene expression by oxysterols is involved in cholesterol and lipid metabolism.[37] Resent studies showed that oxysterols are both positive and negative regulators of gene expression. As positive effectors, they bind to and activate the nuclear receptor liver x receptors LXR,[38] which increase transcription of the cholesterol 7α-hydroxylase gene, the rate-limiting enzyme in the formation of bile acids.[39] This activation stimulates the convention of cholesterol into bile acid. Excessive dietary cholesterol leads to increased oxysterol formation, on the other hand, Oxysterol binds to liver X receptors and thereby induces transcription of cholesterol 7α-hydoxylase, thus increase the removal of cholesterol as bile acid.[40] As negative regulators, oxysterols suppress the cleavage of two transcription factors known as sterol regulatory element binding protein-1 (SREBP-1) and sterol regulatory element binding protein-2 (SREBP-2).[41] When intracellular cholesterol

levels decline, SREBPs can be cleaved to release an active form as described in 1.5.1. This amino-terminal fragments then migrate to the nucleus where they bind with a high affinity to the sterol regulatory element (SRE), thus activating the transcription of a network genes involved in cholesterol synthesis and supply[42] to restore the intracellular cholesterol levels. Another membrane-embedded protein, SREBPs cleavage activating protein (SCAP), is required for the cleavage to occur. Recent studies showed that Oxysterols for example 25-hydroxycholesterol could inhibit the cycling of SREBP cleavage activating protein between endoplasmic reticulum and Golgi.[43] **Figure 1.7**[44] gives the whole idea of gene regulation of cholesterol synthesis and metabolism.

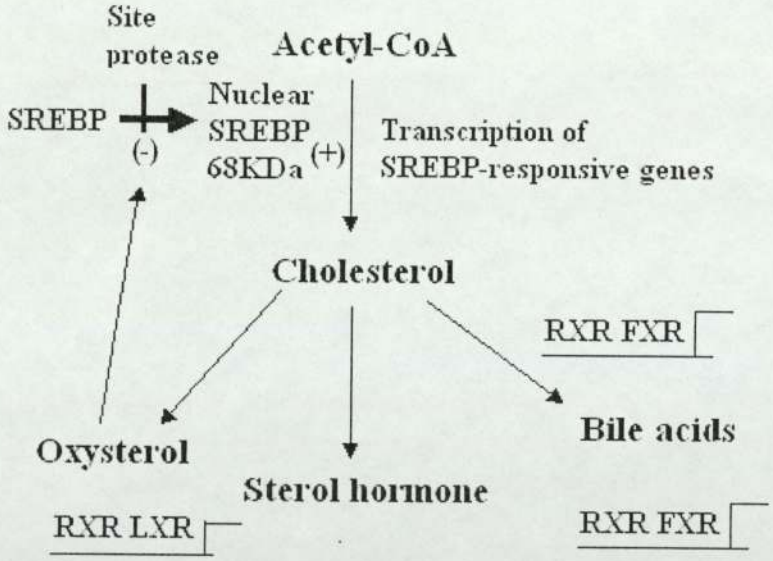


Figure 1.7 Gene Control of Cholesterol Level.

1.5.4 Gene Regulation of HMG-CoA and LDL Receptor

The rate-limiting step of cholesterol biosynthesis in cells is catalyzed by an enzyme, HMG-CoA reductase. The supply of exogenous cholesterol to cells is regulated via the uptake of LDL by the LDL receptor. Therefore the genes encoding both HMG-CoA reductase and LDL receptor are transcriped at a relatively high rate when the cells require cholesterol. This regulation is mediated by SRE-1 located in the promoter regions of both HMG-CoA reductase and LDL receptor Genes.[45] Beside the SRE, the promoters of these genes also contain binding sites for sp1 (LDL receptor), and NF1 (HMG-CoA reductase) transcription factors.[46] These elements might also be implicated in the modulation of cell response to oxysterols. The SREBPs binding to SREs are thought to ensure the "concert regulation" of HMG-CoA reductase and LDL receptor as described in 1.5.

Some reports shows that cells independently regulate cholesterol uptake from their biosynthesis, and different oxysterols can repress HMG-CoA reductase gene expression via different mechanisms.[47] Different oxysterols have different effects on HMG-CoA reductase receptors and LDL receptors. 25-hydroxycholseterol was found to be a powerful regulator of SREBP-mediated transcription. This compound co-coordinately represses the transcription of HMG-CoA reductase and LDL receptor genes by blocking the proteolysis of SREBPs, by interacting with SCAP. However, the proteins that trigger this effect have not yet been identified. Several

studies suggest that a well known cytoplasmic oxysterol carrier, OSBP, is implicated in this role.[48] Indeed, it has been shown that in CHO-K1 cells overexpressing OSBP, the mRNA levels of HMG-CoA reductase, synthase and LDL receptor are elevated.[49]

Some other oxysterols, for example 15-ketocholesterol (K15), 5α-cholset-8-en -3β-ol-15-one, and 3β-(2-hydroxyethoxy)-5α-cholset-8-en-15-one (CK15) efficiently inhibit the biosynthesis of cholesterol in hepatocytes and Hep G2 cells and bind with a high affinity to OSBP.[50] K15 inhibits HMG-CoA reductase and LDL receptor activities in Hep G2 cells. CK15 was also reported to repress HMG-CoA reductase activity in Hep cells. It is possible that these 15-ketosterols may influence the transcription of sterol sensitive genes in the same way as 25-hydroxycholseterols. However, when investigating the effects of 15-ketosterols on mRNA levels of HMG-CoA reductase, LDL receptor and OSBP in Hep G2 cells. These oxysterols were found to decrease the level of HMG-CoA reductase mRNA, but unlike 25-hydroxycholesterol, they did not affect the level of LDL receptor mRNA. In addition, the level of OSBP mRNA was not affect by oxysterols including 25-hydroxycholesterol.

1.6 Oxysterols cytotoxicity

A number of studies have looked into the cytotoxicity of various oxygenated sterols.

At present, there is frequently no clear differentiation of toxic actions due to the primary or secondary effects of the compounds and actions due to the induction of apoptotic changes that might be natural, physiological actions of some of the concerned compounds. A variety of changes have frequently been taken as indication of cytotoxicity, including changes in cell growth, cell viability, cell detachment, plating efficiency of various aspects of morphology, transport of small molecules, protein synthesis and DNA synthesis.

Cytotoxicity of the 5α, 6α- and 5β, 6β-epoxides of cholesterol and 5α, 6β-diOH-cholesterol in cultured rabbit aortic endothelial cells have been reported by Sevanian et al.[51] The results indicated that the triol was more potent than the epoxides in this aspect. The 5β, 6β-isomer was more potent than the 5α, 6α epoxide. Chisolm and his colleages[52] reported on the cytotoxicity of 7β-hydroperoxycholest-5-en-3β-ol in human fibroblasts. In further studies from the same laboratory, 7β-hydroperoxy-cholesterol, under similar experimental conditions, was found to show higher cytotoxicity than 7β-OH-cholesterol, 7-keto-cholesterol and 5α, 6α-epoxy-cholsterol with human skin fibroblasts. The 7β-hydroperoxide was also reported to show higher cytotoxicity than 7β-OH-cholesterol with rabbit and human aortic smooth muscle cells.

1.6.1 Mechanisms of Cytotoxicity

The cytotoxicity attributable to oxysterols is mainly derived from two factors. One is their ability to inhibit HMG-CoA reductase activity, leading to reduced endogenous cholesterol synthesis, the other is replacement of cholesterol by oxysterol molecules in membrane, perturbing permeability, stability and other membrane properties.

Cholesterol plays an important role in the membrane system as described in 1.3.1. Reduction of the cholesterol level will disturb the membrane stability. The cholesterol level is maintained by the balance between synthesis and metabolism, which could be regulated by sterol sensitive gene as introduced before. In the biosynthesis of cholesterol, HMG-CoA reductase is a key enzyme that limits the rate of reaction. HMG-CoA reductase has been implicated as transforming 3-hydroxy-3-methylglutaryl CoA into mevalonate as shown in **Figure 1.8**.[53] The enzyme activity is regulated by phosphorylation and by cholesterol feedback regulation. Liver HMG-CoA reductase activities decrease if dietary cholesterol is increased. Some studies of short duration (4-6h) have shown that native cholesterol, not bound to lipoproteins, does not affect HMG-CoA reductase activity. However, at longer incubation times, inhibition is found. These observations suggest the hypothesis that cholesterol would be oxidized during incubation, and oxysterols would be responsible for this inhibition.

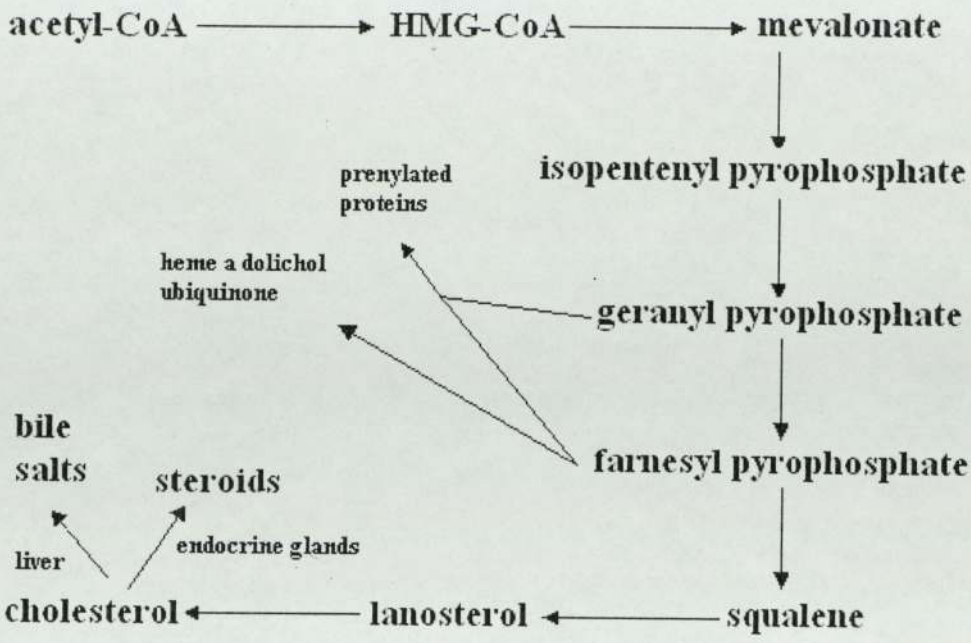


Figure 1.8 Biosynthesis of Cholesterol.

The inhibitory effect has been observed for some oxysterols, mainly those containing a free hydroxy group at C-3 position[54] because 3β-esters of these oxysterols show decreased inhibitory activity.[55] The intensity of the inhibitory effect, as a rule, increased when other oxygenated groups of the oxysterol molecule are far from the C-3 hydroxy group, thus 25-Oh-cholesterol and other derivatives oxidized in the side-chain and ring D are most active. However, a large number of derivatives oxidized in ring B show this inhibitory effect as well.[56] The presence of a third oxygenated group in the molecule does not seem to increase this effect. Therefore, cholesterol-3β, 5α, 6β-triol and 5α-cholestane-3β, 17α, 20α-triol have less inhibitory effect than 3β, 6β- and 3β, 20-diols.[57]

1.7 Oxysterols and Cell Death

Oxysterols have been suggested to be involved in apoptosis, a form of programmed cell death, interest has especially been focused on leukemic lymphocytes that appear to be very sensitive to the effects of oxysterol in the inhibition of cell growth and the lysis of the cells. These cells are known to show high rates of sterol synthesis. 25-OH-cholesterol has been reported to be a potent block of cell growth. It can prevent lymphoid cell growth, arresting the cells in the G1 phase of the cycle.[58] Bansal and his colleagues reported that 25-OH-cholesterol induced apoptosis, as measured by DNA fragmentation, in human leukemia cell lines (CEM-C7, glucocorticoid sensitive; and CEM-C1, glucocorticoid resistant).[59] The effects of 25-OH-cholesterol on c-*myc*, c-Myc, and apoptosis in CEM cells was also reported by the related work of Ayala and Thompson.[60]

Little is known about the mechanism(s) involved in oxysterol-induced cell death. Some reports on a few cell lines have correlated internucleosomal DNA fragmentation and apoptosis induced by oxysterols.[61] Some of these reports combined the detection of DNA ladders with the analysis of cellular DNA content by using flow cytometric analysis.[62] Recent study carried out in bovine aortic endothelial cells showed clearly that oxysterol evoked cell death was an apoptotic process. Apoptosis is defined fundamentally by morphology, with dying cells showing loss of cell junctions and microvilli, cell shrinkage, membrane budding, a

distinctive pattern of chromatin margination, nuclear breakdown and cell breakage into discrete membrane-bound apoptotic bodies.[63] While oncosis, a term proposed as synonym of nonapoptotic or accidental cell death, is morphologically characterized by cellular swelling, an irregular pattern of chromatin organization, and membrane disintegration.

Hwang studied the effects of two oxygenated sterols, 7-keto-cholesterol and 25-OH-cholesterol, on the cell viability in two murine cancer cell lines (EL4 lymphoma and K36 leukemia) in culture media containing 5% lipoprotein-deficient newborn calf serum. With both cell lines, the 7-keto-cholesterol was reported to have reproducibly killed more than 80-85% of the cell in culture after 48h. Similar result were observed with a 24h incubation with 25-OH-cholesterol at the same concentration.[64] Both the cycloheximide and actinomycin D were reported to increase the viability of cells incubated in the presence of 7-keto-cholseterol and 25-OH-cholsterol. This protective effect of either cycloheximide or actinomycin D on the toxic effect of oxysterol was not due to suppression of the uptake of oxysterol, since none of these agents had an effect on the uptake of oxysterol by the EL4 cells. The combined findings of Hwang strongly suggest that the oxysterol-induced reduction in cell viability involves mechanisms requiring protein synthesis. The nature of the protein(s) involved in this process has not been determined. As suggested by Hwang, the possibility that the protein synthesis required for oxysterol-induced effects on cell viability might involve the reported requirement

for the protein synthesis in the accelerated degradation of HMG-CoA reductase caused by oxysterols in cultured mammalian cells.

1.7.1 Gene Control of Oxysterols-Induced Cell Death

Oxysterols are strong regulators of gene expression and are known to regulate colesterol synthesis by transcriptionlly and prostrancriptionally inhibiting the key enzyme, 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCoAR) as introduced in 1.5. They also control other genes involved in cholesterol homeostasis. With regard to their cytotoxicity, oxysterols are polyfunctional biological effectors which can prevent growth of various cell types,[65] exerting differential cytotoxic effects in normal versus malignant cells. Oxysterols appear to induce cell death by evoking the process of apoptosis. Apoptosis can involve the activation and/or repression of certain critical genes. One such gene is the protooncogene c-*myc*, whose product c-Myc is critical for the control of cell proliferation, differentiation, and oncogenesis.[66] As a early response gene, c-*myc* plays an important role in cell progression through the cell cycle, and its regulation has been correlated with the occurrence of apoptosis in various systems. Inappropriate overexpression of c-Myc protein has been found to induce apoptosis in some cell systems.[67] In contrast to these findings, evidence has been mounting during the past years in support of a role for c-*myc* down-regualtion during apoptosis of various cell types. For example, the negative regulation of c-*myc* gene expression after anti-Ig treatment promotes

apoptosis of B lymphoma cells.[68] Studies in B lymphoma, HL-60, and skin keratinocytes cells using antisense oligonucleotides for c-*myc* further suggest a role of c-*myc* regulation in the occurrence of apoptosis. Moreover, glucocorticoids, typical inducers of lymphoid apoptosis, down-regulate c-*myc* mRNA and protein in several lymphoid systems. An important observation is that in CEM cells, the negative regulation of c-*myc* by gulcocorticoids not only precedes apoptosis but is a critical factor in the occurrence of the process, since c-*myc* antisense oligonucleotide in the absence of glucocorticoid treatment evoked typical apoptosis.[69] In addition, overexpression of c-*myc* was sufficient to protect the cells from apoptosis in the presence of the ligand.[70]

Oxysterols have shown to be potent inducers of apoptosis in various cell systems, but the mechanism by which these oxysterols induced apoptosis is still unknown. In order to test the hypothesis that oxysterols-induced apoptosis of human leukemic cells involves the regulation of the c-*myc* gene, time course and dose-response studies of c-*myc* mRNA expression were performed after exposure of CEM cells to 25-OH-cholesterol.[71] The time course studies showed that 25-OH-cholesterol negatively regulates the level of c-*myc* mRNA in the oxysterol-sensitive CEM-C7 cells and that a statistically significant reduction was observed after 24h of oxysterol treatment. The fact that the full down-regulation of the c-*myc* message is delayed suggest that regulation of c-*myc* by 25-OH-cholesterol is a secondary event and that earlier effects of the oxysterols are necessary prior to c-*myc* regulation. The role of protein synthesis studies suggested that synthesis of macromolecules is necessary for

the occurrence of apoptosis.[72] However, the protein has not been determined yet. Response studies of c-*myc* mRNA expression showed that the reduction in c-*myc* mRNA pool levels occurs in a dose-dependent fashion. The concentrations of 25-OH-cholesterol that reduce c-*myc* mRNA are similar to those that kill the cells. A conclusion could then be drawn form the studies above that c-*m*yc plays an important role in oxysterol-induced apoptosis. The reduction of the c-Myc protein that expressed by c-*m*yc gene is a main reason for the cell death.

1.8 Oxysterol and Arteriosclerosis

Several studies have indicated that products of the oxidation of cholesterol may be important in the production of atheosclerotic lesions in animals. In 1968, Cook and MacDougal reported the production of aterosclerotic lesions in aortas of male NZW rabbits after dietary administration of 5β, 6β-diOH-cholestereol.[73] Imai and his colleagues reported that administration of impure cholesterol was associated with electron microscopic lesions in aortas rabbits.[74] Toda et al reported that force feeding of 7-keto-cholesterol as an emulsion in corn oil to female chicks for 4 or 8 weeks was associated evidence of smooth muscle degeneration in the abdominal aorta. All these evidences indicate that oxysterols are involved in the development of atherosclerosis lesions with a proposed mechanism discussed below.

1.8.1 Mechanism of Oxysterol Induced Atherosclerosis

Atherosclerotic cardiovascular disease is the leading cause of mortality in industrialized nations, accounting for nearly 50% of all deaths. Risk factors for the development of atherosclerosis include both genetic and environmental factors. Numerous epidemiological studies have identified decreased high-density lipoprotein (HDL) cholesterol and increased low-density lipoprotein (LDL) cholesterols as major contributors to atherogenesis.[75] As a result, many current therapies for the treatment of atherosclerosis are aimed at raising HDL cholesterol or lowering circulated LDL cholesterol. Although the mechanism underlying the atheroprotective effects of HDL are not clearly understood, one important mechanism may include a role for HDL in reverse transport of cholesterol from arterial cells to the liver.

The earliest atherosclerotic lesion, the fatty streak, is characterized by the accumulation of lipid-laden macrophage in the arterial wall. The involvement of macrophages is associated with their ability to oxidize LDL, to take up oxidized LDL, leading to cellular accumulation of oxysterols. Foam cells develop following the uptake of large amount of oxidized LDL by scavenger receptors such as CD36, SR-A and SR-BI.[76] Unlike the LDL receptor, scavenger receptor expression is not subject to feedback inhibition by intracellular cholesterol levels. Initially, macrophage uptake of oxysterol can be viewed as a physiological reponse designed

to eliminate harmful extracellular debris, for example, apoptotic cells and oxysterols. It becomes pathological only when the cell is overwhelmed by the lipid load, and becomes trapped within the lesion.

Oxysterols involve the initiation and propagation of atherosclerols. Beside their involvement in LDL oxidation as an "oxidative stress", oxysterols also affect cellular cholesterol homeostasis. Oxysterols stimulate cellular cholesterol esterification,[77] impair cholesterol efflux,[78] inhibit cholesterol biosynthesis and affect LDL receptor expression. These effects of oxysterol on cellular cholesterol metabolism suggest that macrophage-associated oxysterols, derived from cellular lipid peroxidation, and/or from the uptake of oxidized LDL, might play a key role in foam cell formation. In the present study, the major oxysterol in arterial macrophage was 7-keto-cholesterol. Accumulation of 7-oxygenated cholesterol in arterial macrophages may indicate the involvement of the oxidative process in the arterial cells, or may possibly reflect an uptake of oxLDL (which is enriched with such oxysterols) by these cells.

1.8.2 Lipoproteins and LDL

Lipoprotein can be divided into 5 broad categories on the basis of their functional and physical properties, primarily on their densities. They are: chylomicrons, very low-density lipoprotein (VLDL), intermediate density protein (IDL), low-density

lipoprotein (LDL) and high-density lipoprotein (HDL). The LDL has a function to transport endogenous TAGs and cholesterol from the liver to the tissue, while the HDL could transport cholesterol back to the liver. The structure of LDL is shown in **Figure 1.9**.[79]

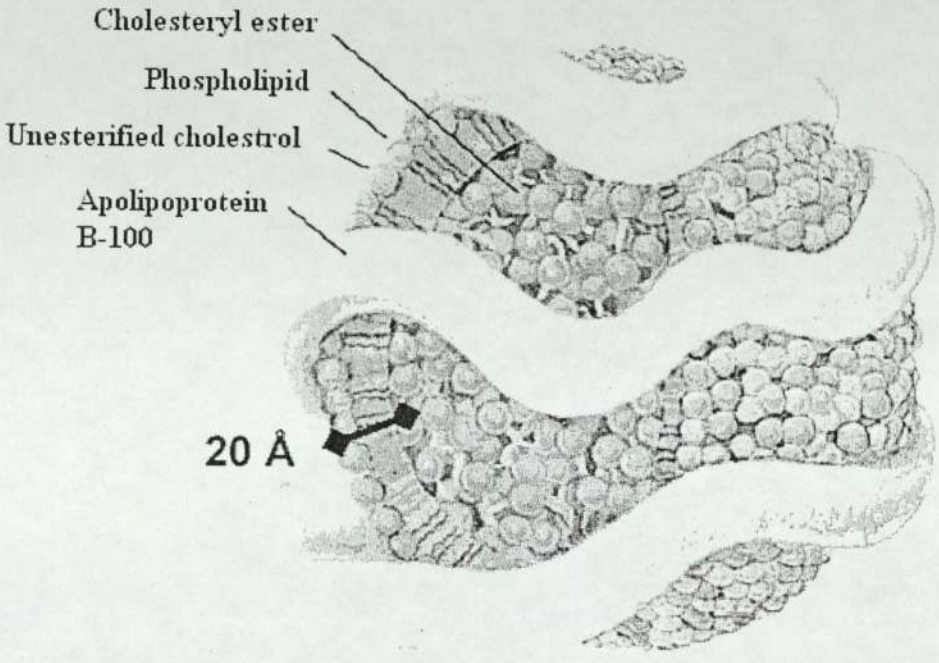


Figure 1.9 Structure of LDL.

1.9 Oxysterols Induced Mutagenic Activities

Early researches showed that some tumors, chiefly local fibrosarcomas, would happen after the injection of a number of oxygenated derivatives of cholesterol.[80] A report made by Parsons and his colleagues demonstrated that 5α, 6α-epoxide of

cholesterol could cause chromosomal aberrations in fibroblasts,[81] the frequency of which was increased by UV irradiation of the cells. This epoxide was also reported to induce DNA repair synthesis in human fibroblasts and melanoma cells. Blackburn et al. studied the interaction of 5α, 6α-epoxy-cholesterol with calf thymus DNA. They reported that "the extent of steroid association was in excess of one molecule per hundred DNA base pairs" and that "much lower levels of physical association were observed under identical conditions for cholesterol, estradiol, and progesterone."[82] Prolonged incubation was reported to give extensive covalent attachment of the sterol to DNA.

Sevanian and Peterson reported that 5α,6α-epoxy-cholesterol is a weak direct-acting mutagen in V79 chinese hamster lung fibroblasts. The data presented showed that mutagenicity reported to be induced by the epoxide was not dependent on dose or time of treatment of the cells with the sterol. Since 5,6-diOH-cholesterol was reported to be a even weaker mutagen, the report suggested that the mutagenicity of 5α, 6α-epoxy-cholsterol may be reduced in cells active in the conversion of the epoxide to 5,6-diOH-cholsterol.[83]

1.10 Other Functions of Oxysterols

In 1975, Brown and his colleagues reported that certain oxysterols, for example 25-OH-cholesterol, 7-keto-cholesterol and 6-keto-cholesterol, stimulates the

formation of labeled cholesterol esters from endogenous (^{14}C) cholesterol in human fibroblasts incubated in lipoprotein-deficient medium.[84] Under the same condition, addition of cholesterol or β-sitosterol had no effect. Bates et al reported that different oxysterols had different effects on the incorporation of labeled oleate into cholesterol esters in human fibroblasts and in monkey arterial smooth muscle cells incubated with media containing lipoprotein-deficient serum.[85] Bhadra et al observed that incubation of epoxides of cholesterol with human fiboblasts for 24h in the presence of [^{14}C] oleate led to increased levels of incorporation of ^{14}C into cholesterol esters as judged by TLC.[86]

Studies showed that oxysterols are involved in the process of cholesterol efflux. Maor and Aviram reported results suggesting an impairment of the HDL_3-mediated efflux of cholesterol on incubation of oxidized LDL with J774A mouse macrophage. The results of studies with intact cells and lysosomal extracts indicated that incubations with the oxidized LDL led to a impairment in the degradation of its protein component but not of its sterol ester component.[87] Different oxysterols varied in their effects on cholesterol efflux from L cells. Preincubate of [^{3}H] cholesterol -loaded cells with 25-OH-cholsterol plus ovalbumin caused an inhibition of the efflux of [^{3}H] cholesterol from L cells incubated in the presence of HDL_3. Other oxysterols tested showed lesser inhibition effects under similar condition. Incubation of 7-keto-cholesterol, 7-OH-cholseterol, 22-OH-cholesterol showed no inhibitory effects.[88]

Studies showed that 25-OH-cholesterol could cause a stimulation of the uptake of Ca^{2+} into P815 mastocytoma cells. This effect could be prevented by the addition of high concentrations of either cholesterol or mevalonic acid. Different oxysterols have different effects on Ca^{2+} influx into human erythrocytes. The effects of oxysterols also appear to differ with different cell types. In contrast to results presented above for P815 cells, 25-OH-cholesterol has been reported to inhibit the influx of Ca^{2+} into human erythrocytes.[89] 22-OH-cholesterol, 5α, 6β-diol-cholesterol, 5α-cholestan-3β-ol could cause substantial increase in Ca^{2+} influx into human erythrocytes.[90] Stimulation of Ca^{2+} uptake by oxysterols could contribute to the acceleration of the degradation of HMG-CoA reductase induced by oxysterols. Roitelman and co-workers have reported that Ca^{2+} is important in the regulated degradation of HMG-CoA reductase. However, the concentrations of the oxysterols required to affect Ca^{2+} levels were, in most studies, higher than those required to lower HMG-CoA reductase activity in most cells.[91] Changes in the levels of intracellular Ca^{2+} have been implicated in the regulation of apoptosis recently.[92] The induction of the changes of apoptosis in human aortic smooth muscle cells by 25-OH-cholesterol have been inhibited by two Ca^{2+} blockers, verapamil and nifedipine.[93] 25-OH-cholesterol was also reported to induce oscillations in the levels of intracellular Ca^{2+} in aortic smooth cells, an effect that was inhibited in incubations of Ca^{2+}-free mediums.

1.11 Potential Pharmaceutical Usages of Oxysterols

Oxysterols were reported to have potential pharmaceutical usages including antibacterial activity, anti-HIV activity and anti-histamine activity et al. Halistanol, a sulfated trihydroxy sterol (**Figure 1.10**), has been isolated naturally and reported to inhibit melanin synthesis in human melanoma cells and to show hemolytic activity.[94] Other closely related compounds were reported to have antithrombin and antifungal activities and to be cytoprotective against HIV.[95]

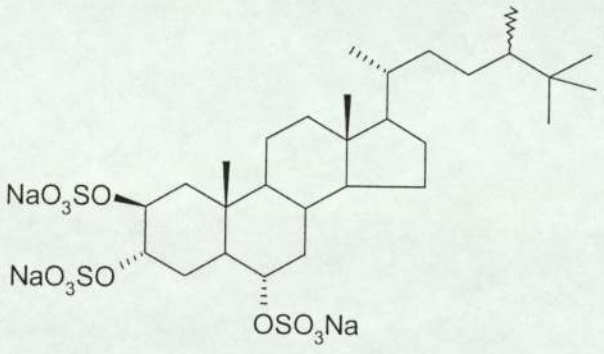


Figure 1.10 Structure of Halistanol Trisulfate.

This compound has also been reported to have inhibitory activity to the growth of gram-positive (but not gram-negative) bacteria to its detergent properties.[96]

Contignasterol, a polyoxygenated sterol isolated from a sponge, cause a dose-dependent inhibition of the release of histamine from peritoneal mast cells treated with anti-immunoglobulin E (**Figure 1.11**).[97]

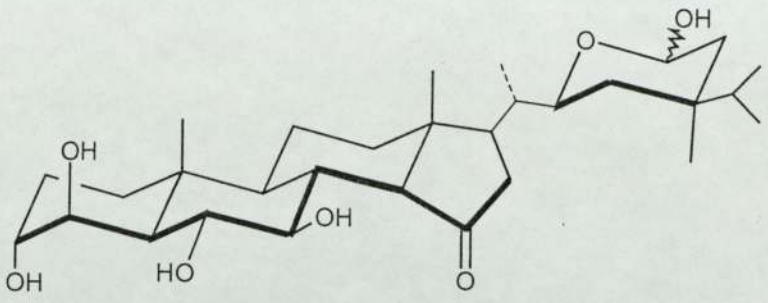


Figure1.11 Structure of Contignasterol.

In 1993, Squalamine (**Figure 1.12**), a novel derivative of an oxygenated sterol, was reported to have significant antimicrobial activity.[98]

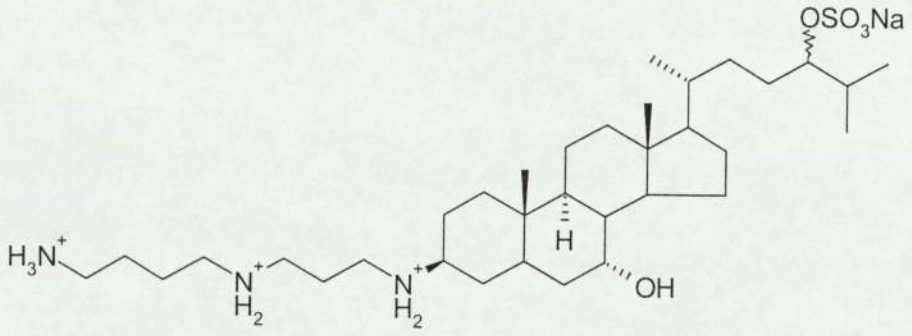


Figure 1.12 Structure of Squalamine.

Squalamine was reported to have in vitro antimicrobial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus, Streptococcus faecalis, Proteus vulgaris, Candida albicans and Paramcecium caudatum.*[98] The squalamine also caused hemolysis of human erythrocytes but did so at concentration higher than those required for antimicrobial action.

Potterat and his colleagues reported the isolation and structure determination of an oxysterol, boophiline (**Figure 1.13**), from femal ticks. This compound was reported to have antifungal and antibactierial activities.[99]

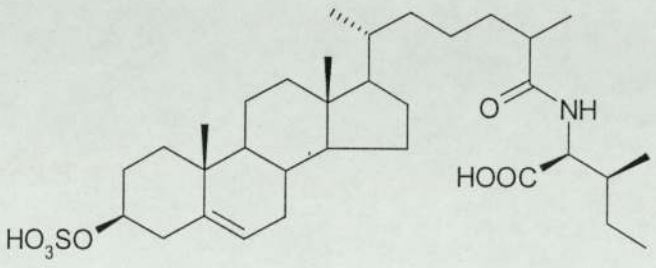
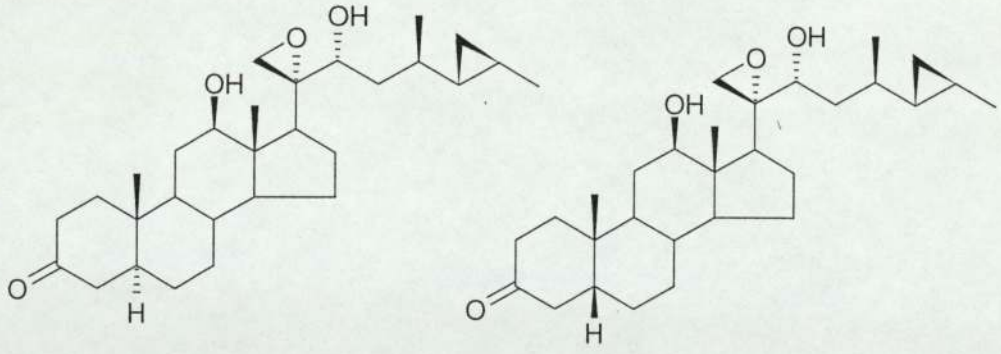


Figure 1.13 Structure of Boophiline.

1.11.1 Oxysterols as Antitumor Agents

Oxysterol can be considered as potential chemotherapeutic agents for the control of cellular growth of cancer cell. Such effect can be simply explained by the inhibition ability of oxysterols to sterol synthesis. This inhibition will then reduce the availability of cholesterol for the new membrane formation required for cellular replication. Inhibition of other mevalonate-derived products such as dolichol, ubiquinones, or isopentyl-substituted adenine moieties in tRNA, or the modified farnesyl group in hemea of cytochrome oxidase[100] can also be envisioned as affecting tumor cell growth. Furthermore, inhibition of the formation of mevalonic acid could also affect tumor cell growth by inhibition of the formation of isoprenoid pyrophosphates required for the pentyl modifications of ras and other key regulatory

proteins. In 1974, Chen and his colleagues first described the inhibitory effect of three oxysterols (25-OH-cholsterol, 20α-OH-cholsterol, and 7-keto-cholsterol) on the growth of cultured mammalian cells.[101] It was reported that 7β-OH-cholsterol exhibits some degree of selectivity in its effects on tumor cells relative to normal cells. However, the reason for this selectivity was not clear.[102] In 1993, Iguchi reported the isolation of an oxysterol, aragusterol A (shown in figure 1.14), from a marine sponge that was very active in the inhibition of growth of KB cells (IC_{50} ~0.092 nM) and showed significant in vivo antitumor activity against LI210 leukemia cells and P388 cells in mice. The chemical synthesis of its 5β-isomer, 5-epiaragusterol A (shown in **Figure 1.14**), was reported in 1997.[103] This compound was shown to be highly active in the inhibition of growth of KB cells (IC_{50} ~0,090nM).



Aragusterol A 5-Epiaragusterol A

Figure 1.14 Structure of Aragusterol A and its Isomer.

Several extraordinarily potent oxysterol glycosides were isolated from bulbs of *ornithogalum saunderside.*[104] These compounds were observed to have inhibitory

effect on the cell growth of HL-60 leukemia cells. One of the most potent of these compounds (3β, 16β, 17α-trihydroxycholest-5-en-22-one) was shown in **Figure 1.15.**

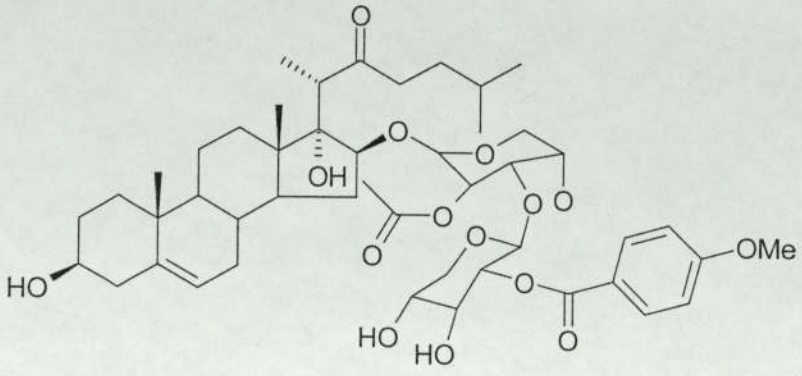


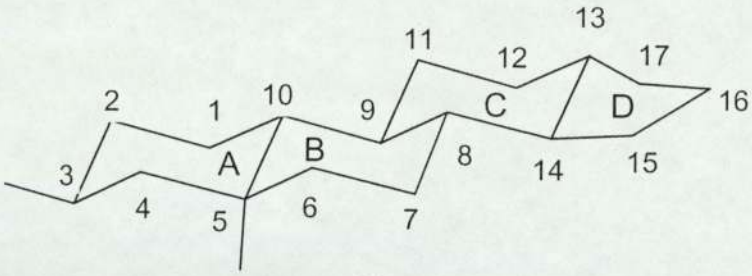
Figure 1.15 Structure of a Glycoside of 3β, 16β, 17α-Trihydroxycholest-5-En-22-One.

At a concentration of 115 μ M, this compound showed no hemolytic action with human red blood cells. In the pharmacological aspect, this oxysterol was reported to be more potent in the HL-60 than any other anticancer agents including mitomycin C, cis-platin, campothecin, and taxol. Removal of the acetyl and p-methoxybenzoyl moieties was reported to decrease potency. The sterol glycoside also showed high potency in inhibiting the growth of other tumor cells in culture including some cell lines that were resistant to other anticancer agents. Melanoma cell lines were reported to be particularly sensitive to this compound.

1.12 Aims of this Project

Synthesis of C, D ring oxygenated sterols have not been well studied, though the research of oxysterol became more and more interested. Currently, the sterols with C, D ring hydroxy groups used for the biological and pharmacological research were mainly obtained from natural source and hence were limited with regard to structure types, available quantities, and reasonable costs.

In this project, my aim is to introduce hydroxy groups onto sterol ring C and ring D, namely to synthesize C, D oxygenated sterol. Cholesterol was selected as the starting material in our experiments because of its natural abundance.



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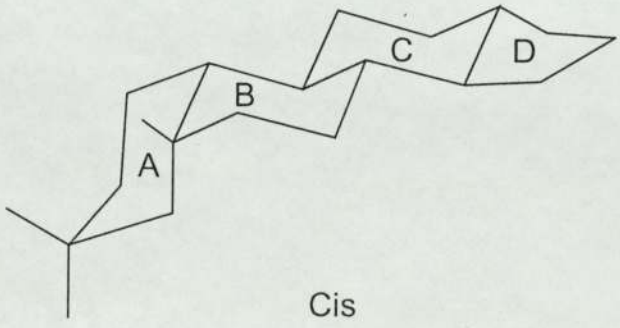

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CHAPTER TWO

CHEMISTRY AND DISCUSSION

2.1 Introduction

Oxysterols are a group of compounds with potent biological and pharmacological effects as we discussed in chapter one. Lots of researches have been done on this area during the last two decades. However, the chemistry aspect has somehow held up the research of oxysterols. Despite continuing advances in studies of the chemistry of oxysterols, most researchers rely on commercial materials that are unfortunately very limited with regard to structure types, available quantities, and reasonable costs. This situation has resulted in the acquisition of a large amount of information on the test of one oxysterol, 25-OH-cholesterol, on a wide variety of parameters in cultured mammalian cells. Unfortunately, other oxysterols may be of considerably more physiological importance than 25-OH-cholesterol, and moreover, the results of studies with 25-OH-cholsterol have been frequently generalized to other oxysterols without experimentation. The limited availability of oxysterols is also a major factor responsible for the very restricted number of studies of their in vivo effects in animals. Therefore, the chemical synthesis of oxysterols becomes a "rate-limited" step for the researches of oxysterols.

In my project, I plan to develop a method to introduce hydroxy groups onto sterol ring C and ring D, namely, to prepare C, D ring oxygenated oxysterols. Some C, D ring oxygenated oxysterols have already been well studied, for example, 15-oxygenated cholesterols were not only extraordinarily potent in suppressing sterol synthesis in cultured cells but also showed significant hypocholesterolemic

action upon administration to rodents and nonhuman primates.[105] However, there are still lots of potent oxysterols not been well investigated, simply because they have not been chemically synthesized yet and therefore not commercially available. Two examples are given in **Figure 2.1**.

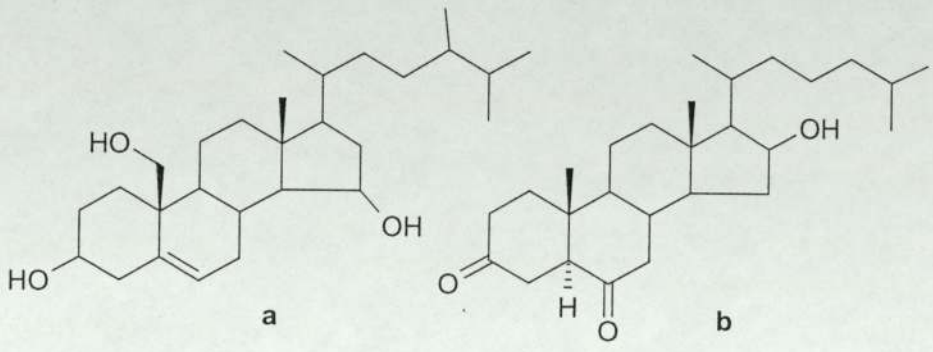


Figure 2.1 Two Examples of Potent Oxysterols.

Oxysterol **a** and **b** have been proved to have potent effects on P-388 and KB cells.[106,107] But their chemical synthesis have not been studied.

To introduce hydroxy groups onto C and D rings, the 7,8 double bond is of critical importance, because this double bond connects ring B and ring C and can not easily be bypassed. The position of 7,8 double bond and the way to prepare it are shown in **Figure 2.2**.

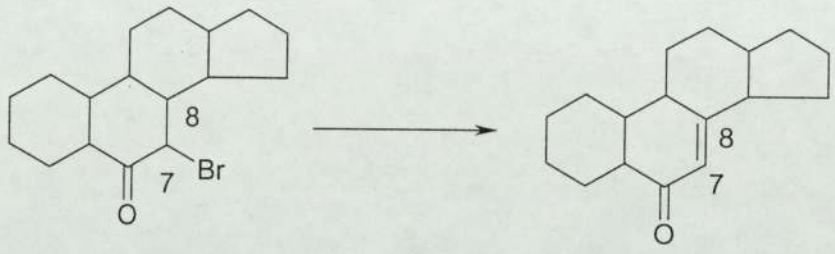


Figure 2.2 Preparation of 7-En-Sterol

However, The yield of 7-en-sterol was affected by the hydroxy groups on ring A and ring B, and sometimes the effects were so much that the yield of 7-en-sterol was not high enough to carry out the following steps.

To study these effects of hydroxy groups on ring A and B, and to develop a method to prepare C, D ring oxygenated oxysterols in high yield, several different synthetic routes were then tried in my project.

2.2 Preparation of 7,8-en Sterol with 3β, 5α-Diol on Sterol Ring

Attempts were made to prepare 5α-cholestan-3β, 5α-diol-7-en-6-one-3-acetate, which have 3β-OH and 5α-OH on ring A and ring B. The structure of this compound is shown in **Figure 2.3**.

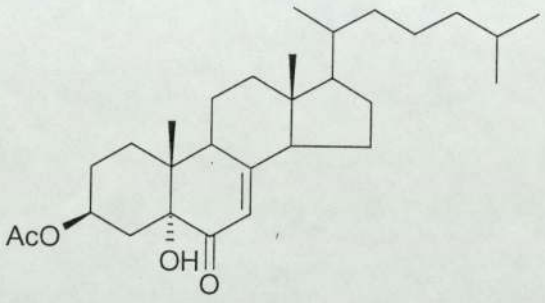


Figure 2.3 Structure of 5α-Cholestan-3β, 5α-Diol-7-en-6-One-3-Acetate.

2.2.1 Preparation of 5α-Cholestan-3β, 5α-Diol-6-One 3-Acetate (3)

To introduce the 3β, and 5α hydroxy groups onto ring A, cholesterol was first treated with formic acid at 70~80 °C to form an ester on 3-OH. Then H_2O_2 was added this ester to give an epoxide, which was then hydrolysed by NaOH to achieve the product **1** in 93% yield (**Figure 2.4**). The 6-OH in product **1** was then selectively oxidized by NBS. The selectivity in this experiment was 100%. No 3β-OH oxidation and 5α-OH oxidation were observed. Product **2** was a white solid in over 90% yield. 5α-Cholestan-3β, 5α-diol-6-one 3-acetate **3** was then given by refluxed Product **2** with acetic acid anhydride in toluene in 100% yield.

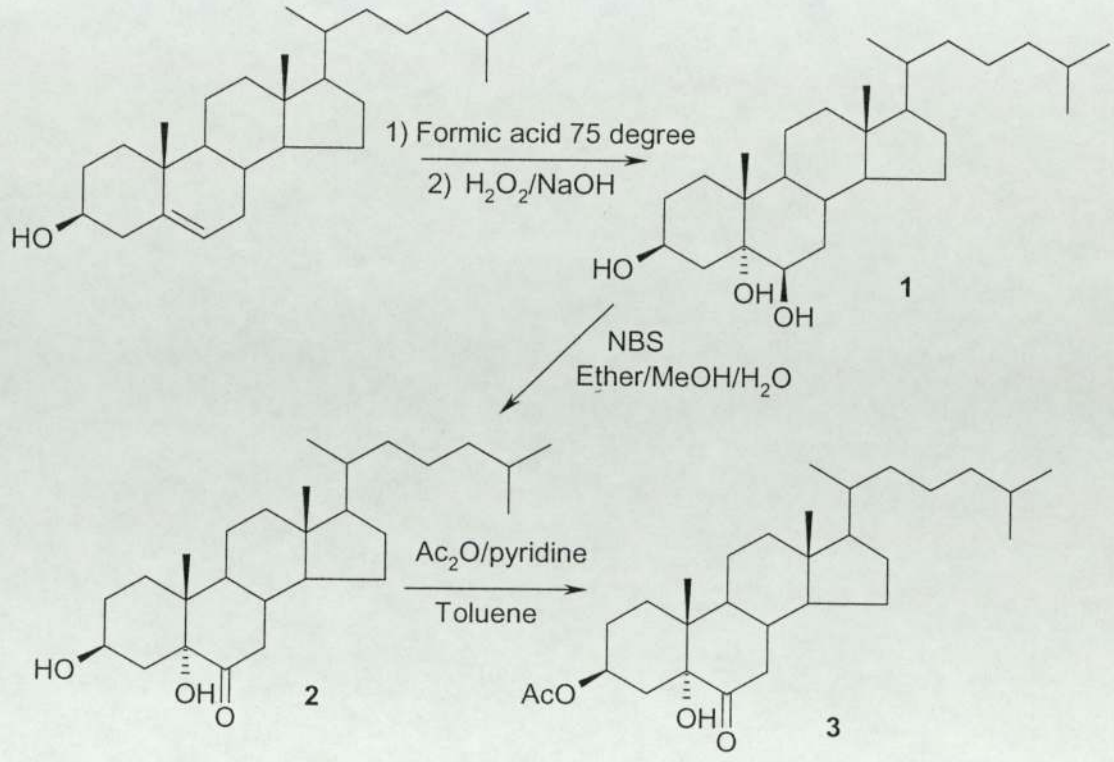


Figure 2.4 Synthesis of 5α-Cholestan-3β, 5α-Diol-6-One 3-Acetate (**3**).

Compound **2** could be achieved by NBS oxidation with high yield and high selectivity. The mechanism for this reaction however has not been clearly established till now. However, it is generally accepted that a positive halogen is the attacking species[108] and a hypobromite, which readily loses hydrogen bromide to achieve the carbonyl product, was formed during the reaction.

A proposed mechanism of selective oxidation on 6β-OH by NBS is shown in **Figure 2.5**. As to the selectivity, it could be explained that the presence of angular methyl group somehow increases the electron density of the environment, which could then

attract the positive halogen to attack the hydroxy group.

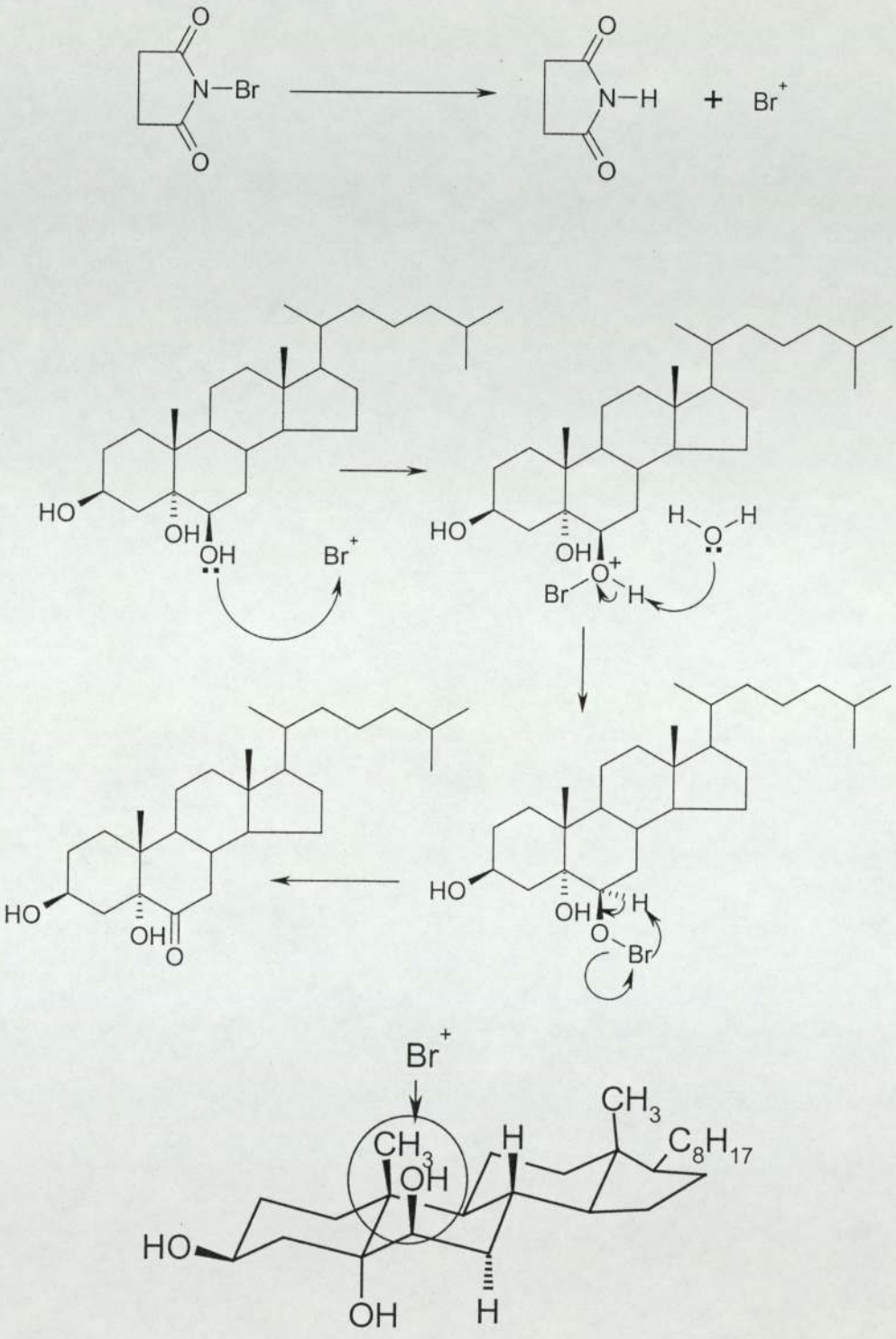


Figure 2.5 A Proposed Mechanism of NBS Oxidation.

2.2.2 Preparation of 7-Bromo-Cholestan-3β, 5α-Diol-6-One-3-Acetate (4)

5α-cholestan-3β, 5α-diol-6-one 3-acetate **3** was treated with Br_2 and $BF_3.Et_2O$ in acetic acid at 60 °C for 2h to give the 7-bromo product **4** in 79% yield (**Figure 2.6**).

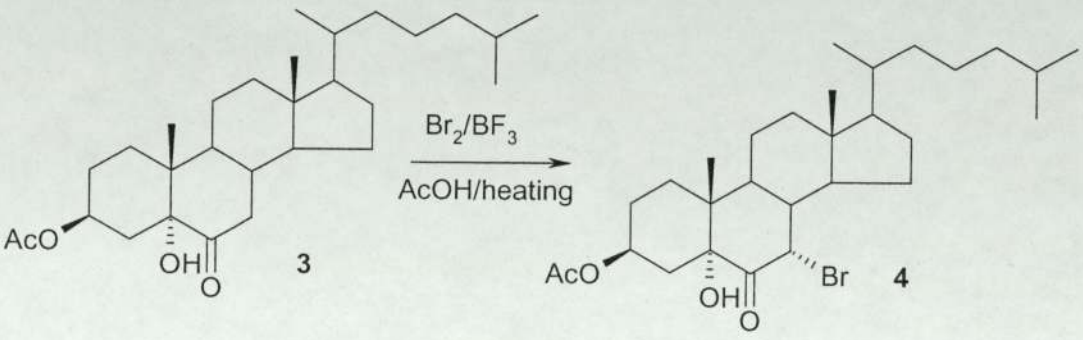


Figure 2.6 Preparation of 7-Bromo-Cholestan-3β, 5α-diol-6-One-3-Acetate (**4**).

According to Louis and Fieser, the configuration of bromo group should be 7β when use this bromination method.[109] However, in our experiment, the proton NMR (**Figure 2.7**) of this product suggested that the bromo group should be at 7α position as is evidenced by the coupling constant between 7-H and 8-H was 4.54. This value is not big enough for a 7β-bromo configuration, because in 7β-bromo structures, the coupling between 7-H and 8-H should be "aa" (**Figure 2.8**) and the coupling constant is expected to be much more than 5. This suggestion could be further proved by the fact that bromination of **3** with other methods[110,111] which were reported to generate 7α-bromo sterol has given the same product **4** (same Rf value on TLC plate and same coupling constant in proton NMR). Hence it is concluded

that bromination with BF_3 and Br_2 in acetic acid gave 7α product in our experiment.

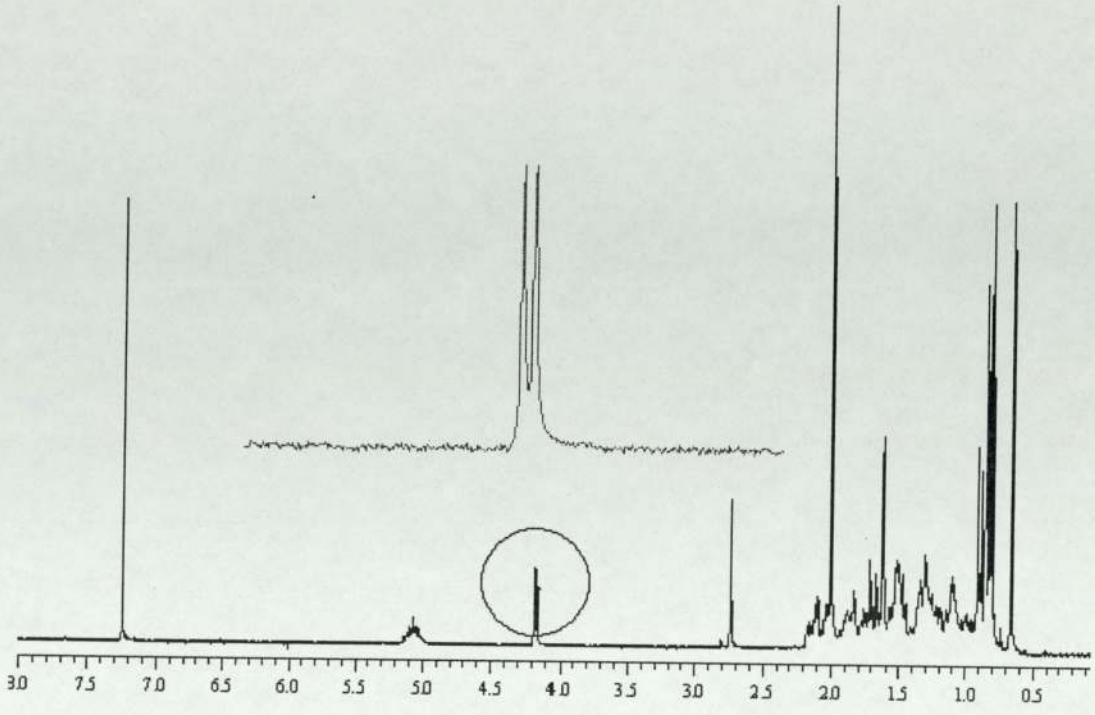


Figure 2.7 ^{1}H NMR of 7-Bromo-Cholestan-3β, 5α-Diol-6-One-3-Acetate (**4**).

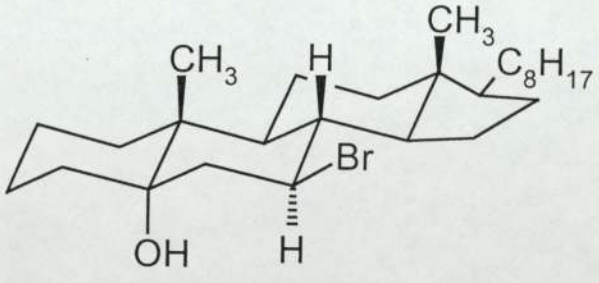


Figure 2.8 3D Structure of 7β-Bromo-Sterol.

2.2.3 Preparation of 5α-Cholestan-3β, 5α-Diol-7-En-6-One-3-Acetate (5)

The 5α-cholestan-3β, 5α-diol-7-en-6-one-3-acetate **5** (**Figure 2.9**) was synthesized by refluxing 7-bromo product **4** with Li_2CO_3 and/or LiBr in DMF.[112]

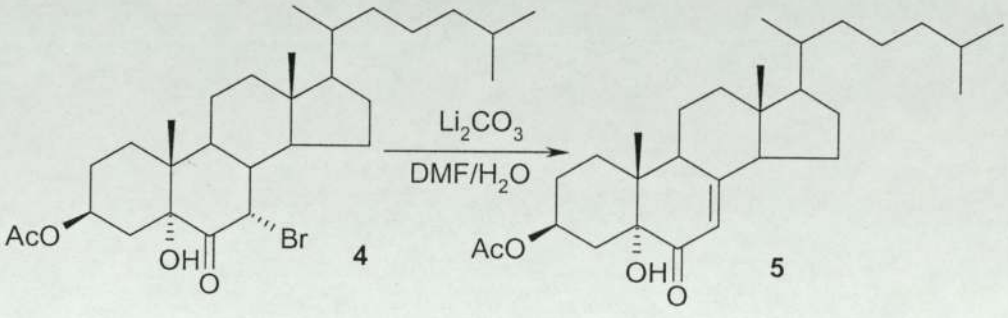


Figure 2.9 Synthesis of 7-En (**5**).

This method was widely used for the elimination of 7- Br group in sterol structures. However, this method didn't work well in the presence of 3- and 5- OH in our experiments. Various attempts were made to optimize the reaction conditions. Despite all the efforts, only 20% yield of the compound **5** could be achieved. From our results, it is concluded that water plays an important role in this reaction. If water was not added to the reaction system, the presence of the product **5** could not be seen from the proton NMR of the reaction mixture. The major product under this condition was suggested to be the structure in **Figure 2.10**.[112](this compound was not purified in our experiment).

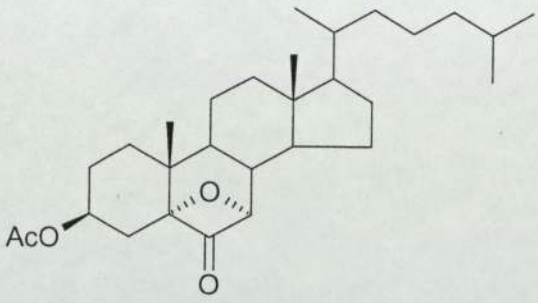
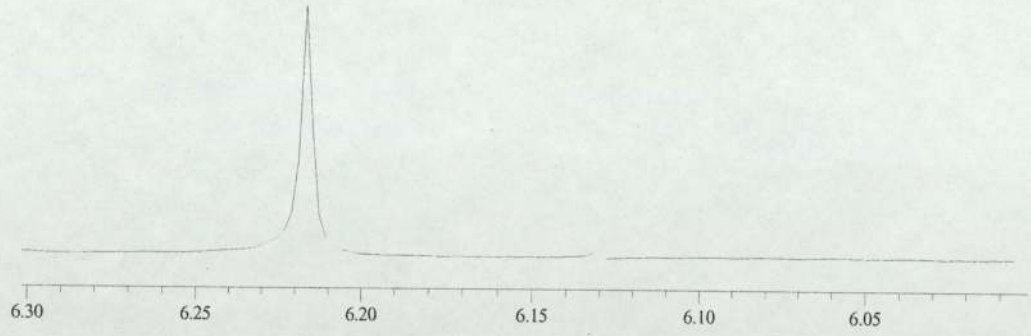


Figure 2.10 Structure of Byproduct.

If water was added to the reaction system, a singlet at 6.22 ppm could be seen in the ^{1}H NMR of the reaction mixture. The 7,8 double bond product **5** could then be isolated by column chromatography and confirmed by ^{1}H NMR and Cosy spectrum (**Figure 2.11**). In this experiment, less than 5% of 3β -OH elimination products were also isolated, which indicated that the ester protecting at position 3 could also be removed under this condition.



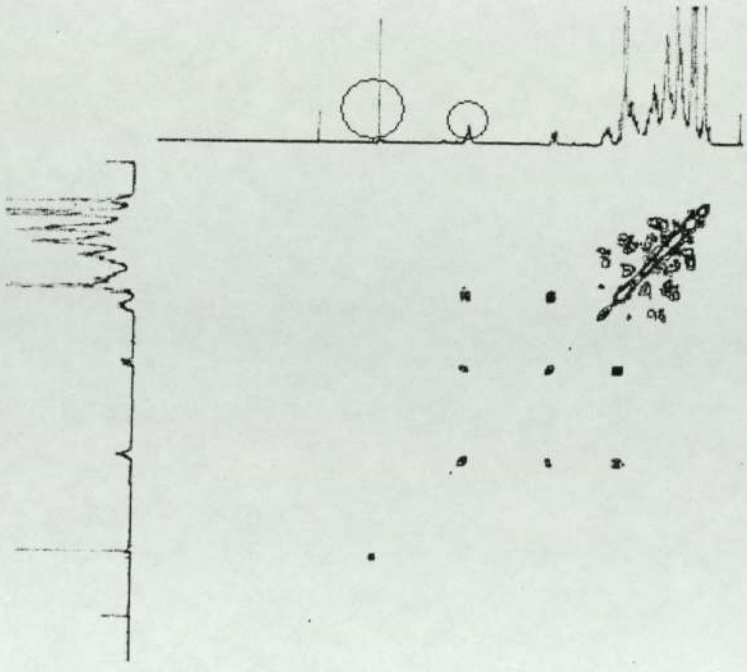


Figure 2.11 Spectrum of 5α-Cholestan-3β, 5α-Diol-7-en-6-One-3-Acetate (**5**)

The role of water in this reaction is not clear. It probably because that water could break the four-membered ring of the compound shown in figure 2.10 and then increase the yield of the desired compound **5**.

2.3 Preparation of 7-en-Sterol with 3β, 4α-Diol on Ring A

Since the presence of 5α-OH could reduce the yield of 7-en sterol, attempt was made to prepare a compound without 5-OH (shown in **Figure 2.12**) in our experiments.

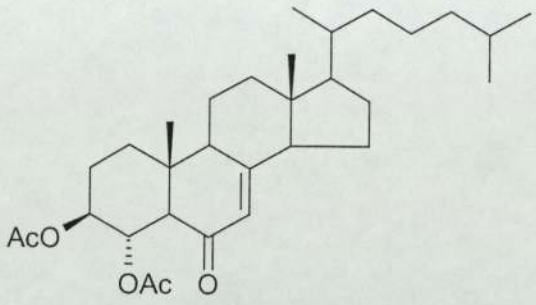


Figure 2.12 Structure of 3β, 4α-Diol-Cholestan-7-En-6-one-3, 4-Diacetate.

The reason to choose the structure with 4α-OH is that many naturally occurred sterols have this 4α hydroxy group. The chemical synthesis has never been reported before, and hence this reaction route is worth a try.

2.3.1 Preparation of Cholest-4-En-3β, 6β-Diol-3, 6-Diacetate (7)

The Cholest-4-en-3β, 6β-diol-3, 6-diacetate **7** could be prepared from 5α-cholestane-3β, 5, 6β- triol **1** (**Figure 2.13**). The 3β, 6β hydroxy groups of **1** were first protected by refluxing with Ac_2O and pyridine in toluene. The yield of the following ester **6** was 94%. Under this condition, the 5α-OH would not been protected because of the steric hindrance at C-5 position. This ethyl ester **6** was then treated with thionyl chloride and pyridine to give the product cholest-4-en-3β, 6β-diol-3, 6-diacetate **7** in over 90% yield.

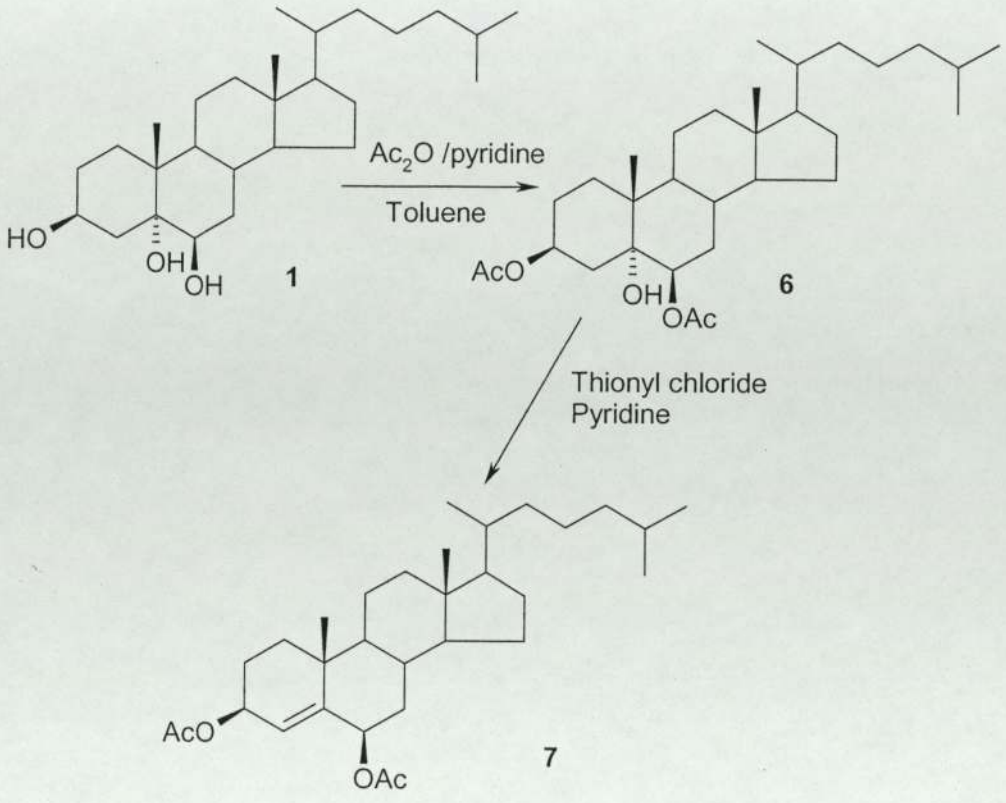


Figure 2.13 Preparation of Cholest-4-En-3β, 6β-Diol-3, 6-Diacetate (**7**).

The mechanism of the elimination reaction from **6** to **7** is shown in **Figure 2.14**. Pyridine first reacted with thionyl chloride to form a good leaving group, which was then attacked by the 5-OH. Experimental evidence indicates that the five atoms involved in this kind of elimination reaction must lie in the same plane and the anti-periplanar conformation is preferred, because this anti-periplanar conformation is necessary for the orbital overlap that must occur for the π bond to be generated in the alkenes.[113] The predominant product, in our experiment, is 4-en, the anti-periplanar product, due to this stereoselectivity of the reaction and the rigidity of sterol ring.

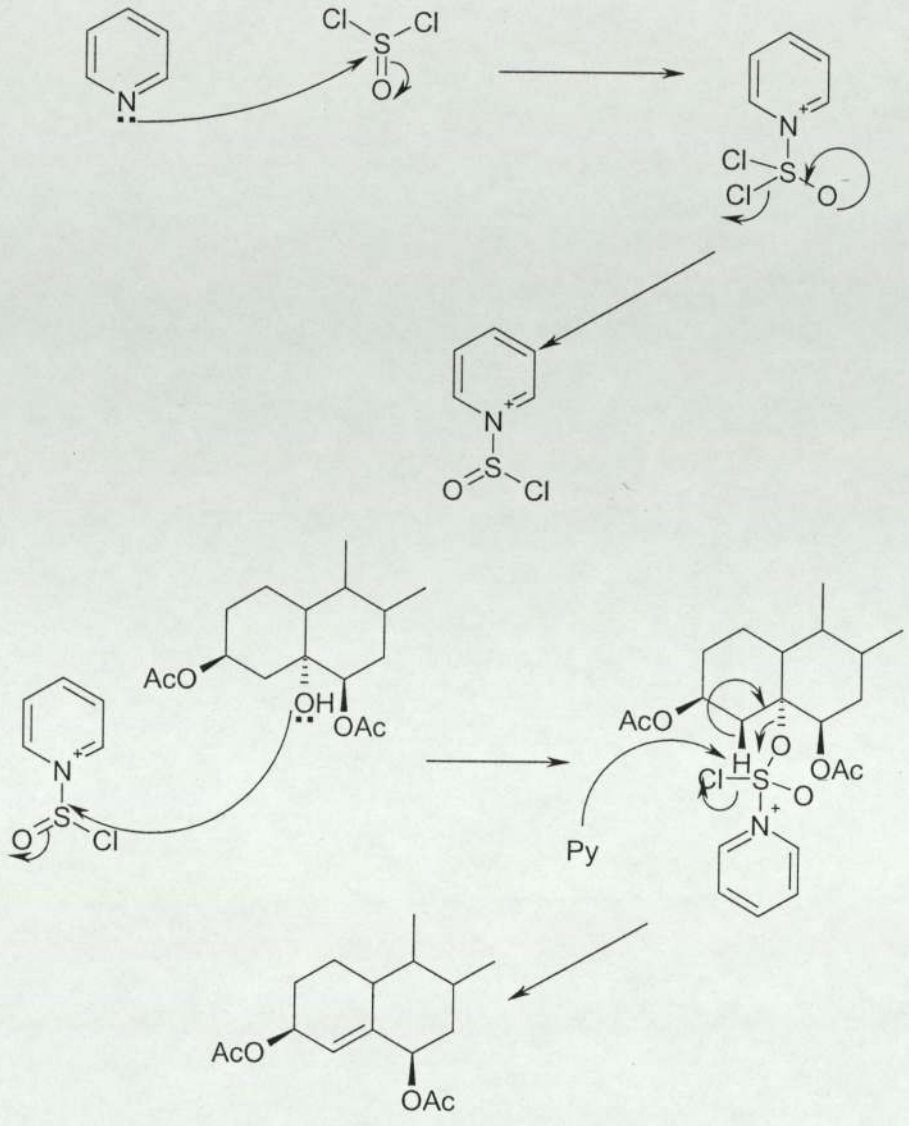


Figure 2.14 Mechanism of Elimination Reaction.

2.3.2 Hydrolysis of Cholest-4-En-3β, 6β-Diol-3, 6-Diacetate (7)

The ester **7** could be hydrolysed by refluxing with NaOH in ethanol. The yield of the product cholest-4-en-3β, 6β-diol **8** was 90%. In our experiment, the 3β-OAc was

found to be slightly more sensitive to the hydrolysis condition, because when 1 equivalent of NaOH was used at room temperature, the major product is proved to be cholest -4-en-3β, 6β-diol 6-acetate **9** (**Figure 2.15**) and only less than 10% of **8** was isolated.

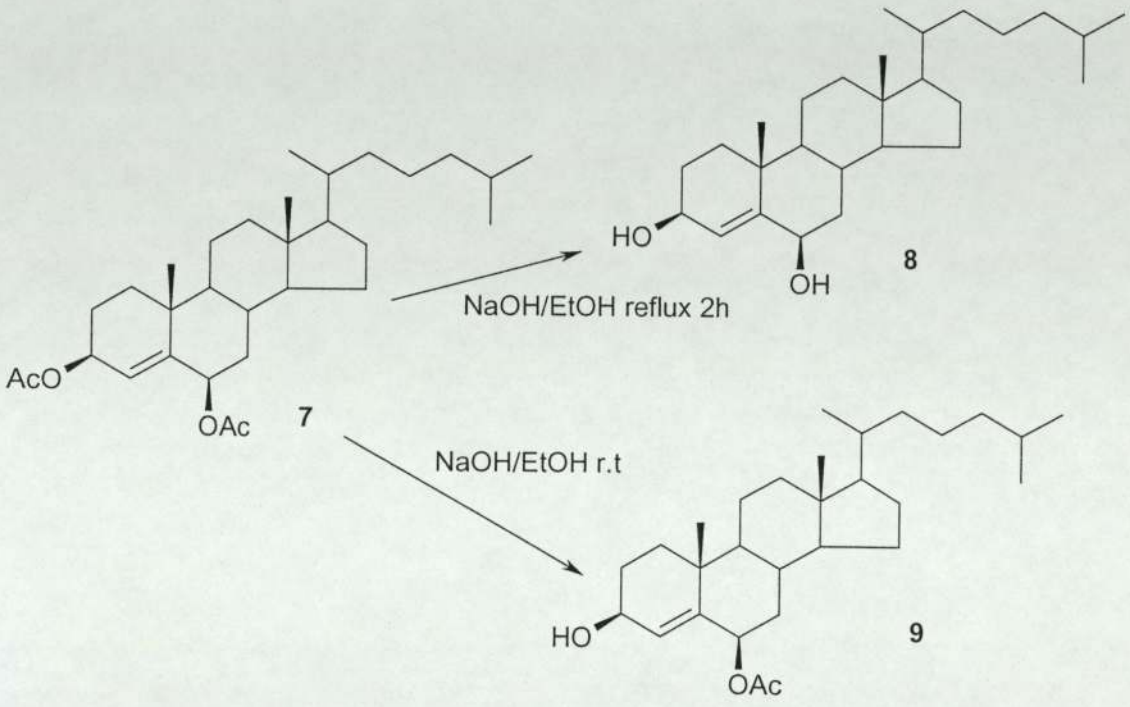


Figure 2.15 Hydrolysis of Cholest-4-En-3β, 6β-Diol-3, 6-Diacetate (**7**).

2.3.3 Preparation of Cholestane-3β, 4α, 6β-Diol (10)

Preparation of cholestane-3β, 4α, 6β-diol **10** from **8** was achieved by using hydroboration reaction (**Figure 2.16**). In this reaction, 4α-OH product was found to be the major product. The ratio between 4α product and 4β product was 7.4:1

(judged by proton NMR of the reaction mixture). The reason for this selectivity is that when a hydroboration reaction starts, a trialkl borate forms (**Figure 2.17**) with the boron added from less substituted side of the double bond,[114] and then the reaction will carry on from this trialkyl borate by reacting with H_2O_2 and NaOH. In the case of my experiment with starting material **8**, the presence of C-19 angular methyl group and 3β, 6β hydroxy groups at the β side of the molecule prevented the access of the boron from β side, and hence the formation of 4α product was of great priority.

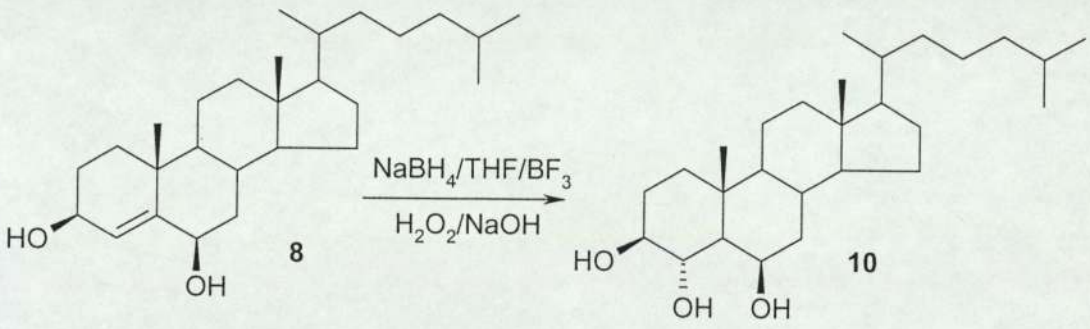


Figure 2.16 Preparation of Cholestane-3β, 4α, 6β-Diol (**10**).

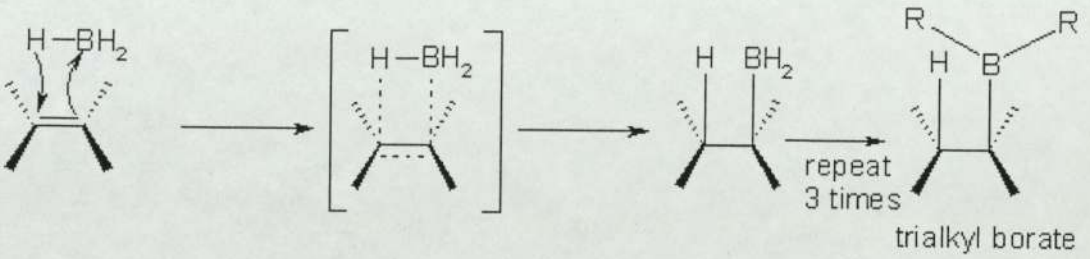


Figure 2.17 Formation of Trialkyl Borate.

2.3.4 Oxidation of Cholestane-3β, 4α, 6β-Diol (10) by NBS

The cholestane-3β, 4α, 6β-diol **10** was oxidized by NBS to give the product **11**, 5α-cholestan-3β, 4α-diol-6-one-3, 4-diacetate (**Figure 2.18**), in 90% yield. This NBS oxidation was done under different conditions for optimization. Different solvents were compared in order to achieve a better reaction rate. The detail was shown in **Table 2.1** (the results were judged by TLC).

Cholestane-3β, 4α, 6β-diol **10** Heated to 40 °C				
Solvent system	After 0.5h	After 2h	After 2.5h	After 3h
Dioxane 4ml, water 0.6 ml, methanol 2.5 ml	Reaction started	Reaction finished		
THF 4ml, water 0.6 ml methanol 2.5 ml	Reaction started	Not finished	Reaction finished	
Ether 4ml, water 0.6 ml, methanol 2.5 ml	Not reacted	Not finished	Not finished	Not finished

Table 2.1 Optimization of the Reaction Conditions for NBS Oxidation.

According to the above study, dioxane was therefore chosen as the best solvent for the NBS oxidation.

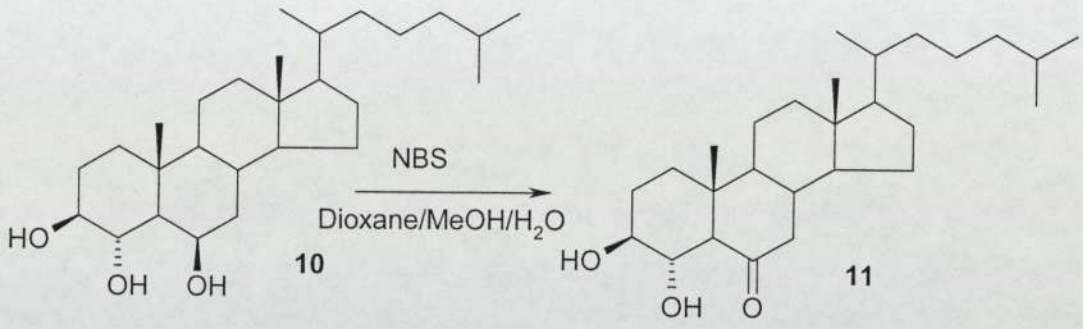


Figure 2.18 Formation of 5α-Cholestan-3β, 4α-Diol-6-One (**11**).

In chapter 2.2, we demonstrated that the selectivity of this reaction was 100%. However, in the presence of 4α hydroxy group, the selectivity was somehow reduced and a minor amount of **12** (shown in **Figure 2.19**) was isolated in 10% yield. This byproduct was then identified to be 4α-Hydroxy-5α-cholestane-3, 6-dione by NMR spectrum. Moreover, the reaction rate was also slowed down due to the presence of 4α-OH. The function of 4α hydroxy group however in this case was not clear.

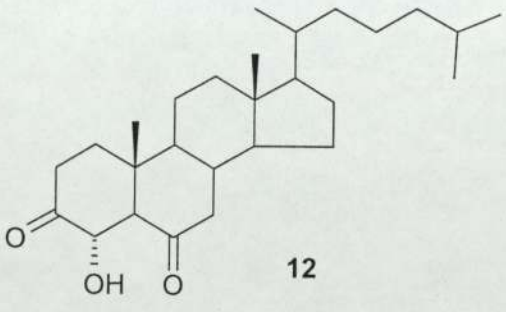


Figure 2.19 Structure of 4α-Hydroxy-5α-Cholestane-3, 6-Dione (**12**).

2.3.5 Attempt to Prepare 7-Bromo Product from (13)

5α-cholestan-3β, 4α-diol-6-one **11** was first protected by refluxing with pyridine and acetic acid anhydride in toluene (**Figure 2.20**). The yield of following ester **13** was 96%. Compound **13** was then treated with $BF_3.Et_2O$ and Br_2 in acetic acid. However, with the starting material **11**, this method didn't work as expected. No major products could be identified from the TLC and the occurrence of 7 bromination reaction could only be told from the proton NMR of reaction mixture. We assume that the reason for this inefficiency of the method relies on the fact that a 5-bromo-product or even a 5, 7 – dibromo-compound could easily be formed since the absence of 5-OH. The presence of 4α-OH also increased the complexity of this reaction.

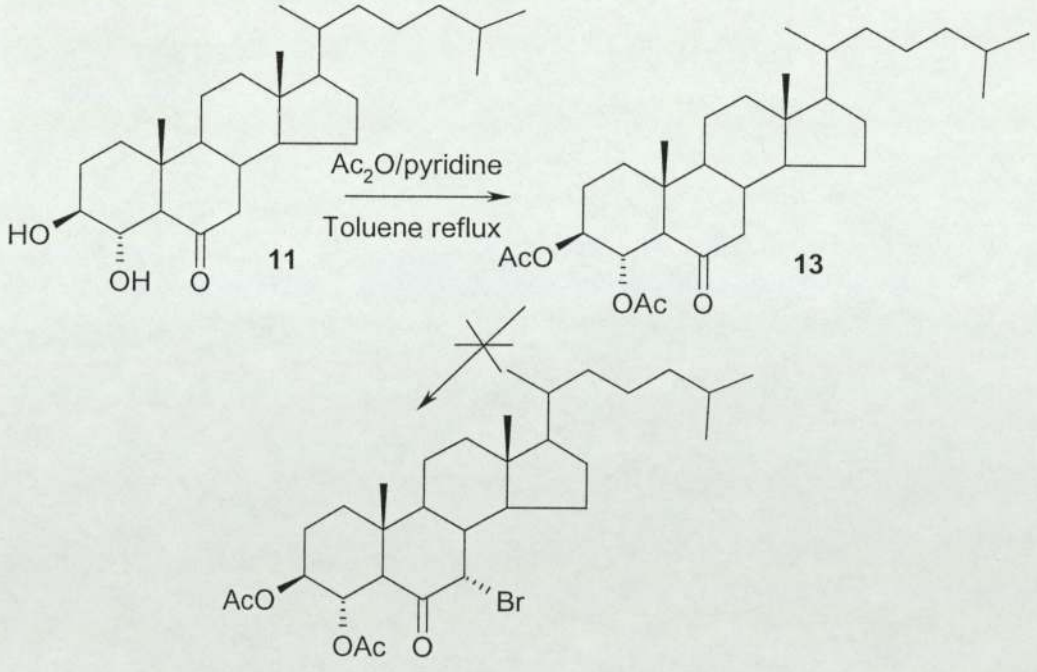


Figure 2.20 Synthesis of 7-Bromo Product from (**11**)

Since the bromination in this experiment could not give decent yield, the following elimination reaction, which would lead to the 7,8 double bond was therefore not attempted.

2.4 Preparation of 7-en-Sterol with no Hydroxy Groups on Sterol Rings

In chapter 2.2 and 2.3, we proved that both 5-OH and 4-OH could hinder the formation of 7,8 double bond in some way, even the 3-OH could add to the complexity of the elimination reaction as we observed in our experiment. To get rid of the affects of hydroxy groups, a structure with no hydroxy groups on ring A and ring B was then considered. In our experiment, such a compound was prepared successfully with a yield 76% which was good enough to carry out next steps (**Figure 2.21**).

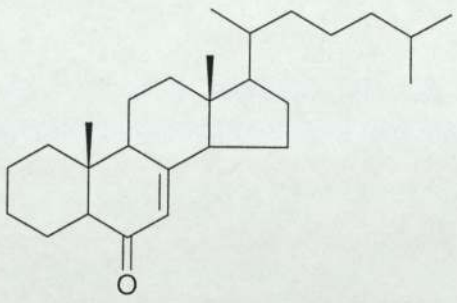


Figure 2.21 A Successful 7-en Sterol.

2.4.1 Preparation of Cholest-5-ene (15)

Cholesterol was reacted with thionyl chloride at room temperature for 20h to give 3β -chlorocholest-5-ene 14 in 91% yields. And then compound **14** was treated with sodium to form Cholest-5-ene **15** with a yield 99% (**Figure 2.22**).

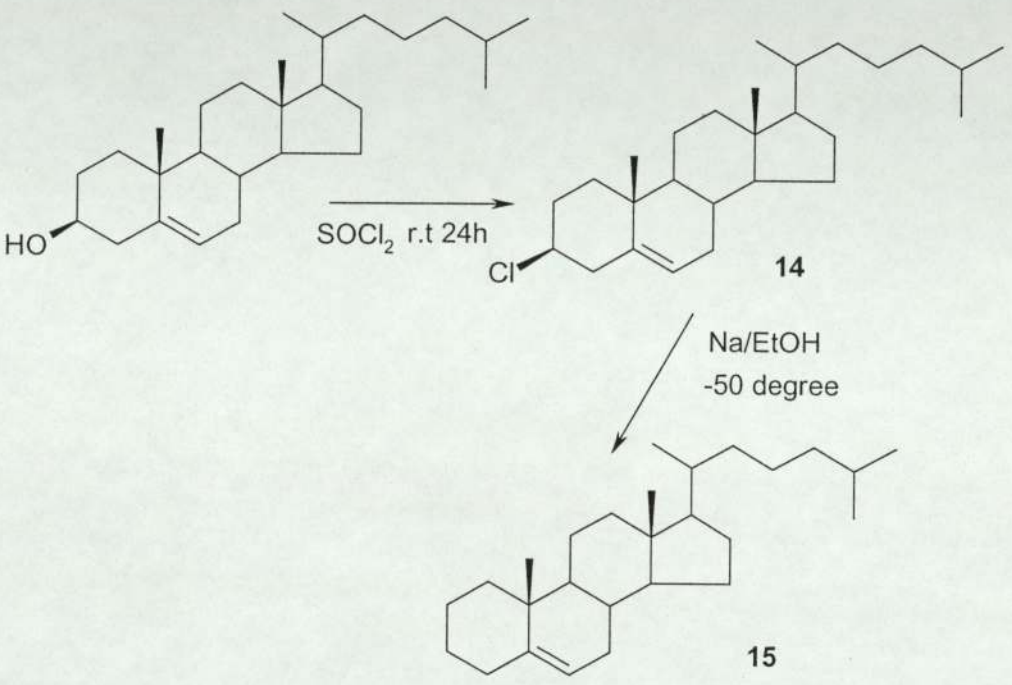


Figure 2.22 Preparation of Cholest-5-Ene (**15**).

This reaction was accomplished by stirring sodium in ethanol at –50 °C for 5h. However, if the reaction was carried out at room temperature, it might take 24h or longer and much more excess of sodium was needed. The reason for the need of low temperature is that when the temperature rose, large amount of sodium is consumed to generate hydrogen gas, which will then leave the reaction system, therefore the

chance for this reduction reaction become small.

2.4.2 Introduction 6-OH by Hydroboration Reaction

The 6-OH could be introduced to the molecule by hydroboration reaction (**Figure 2.23**). As we discussed in chapter 2.3, 6α-OH could be formed prior to the 6β one. In this case, the ratio between 6α-hydroxy-cholestane **16** and 6β-hydroxy-cholestane **17** was 4:1.

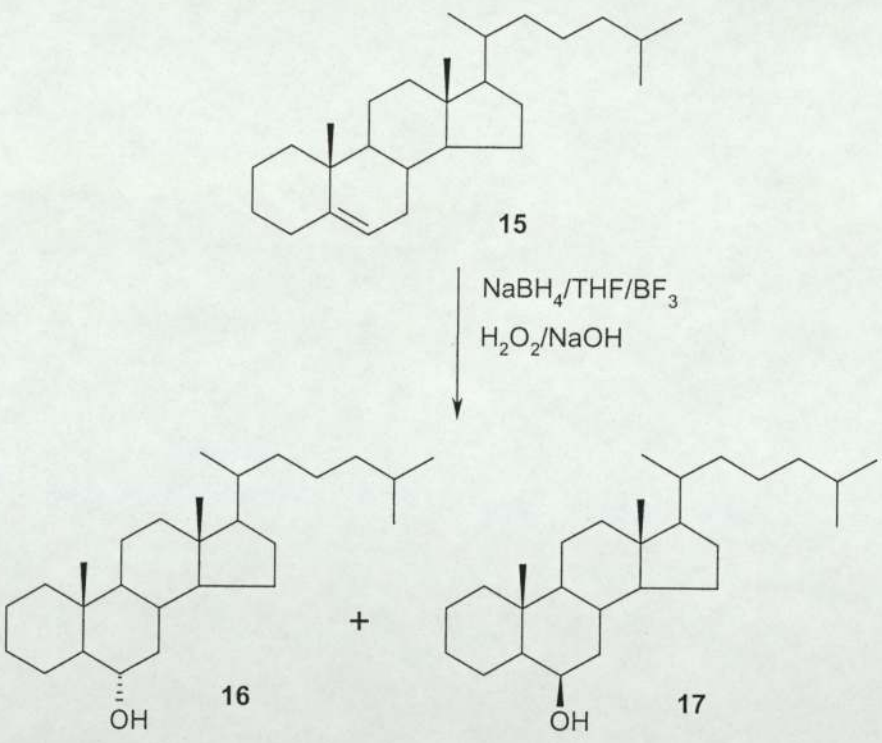


Figure 2.23 Hydroboration Reaction of (**15**).

2.4.3 Separation and Oxidation of 6α-Hydroxy-Cholestane (16)

Because 6α-hydroxy-cholestane **16** and 6β-hydroxy-cholestane **17** are quite close in their properties, and hence very hard to be separated. In our experiment, the reaction mixture was first oxidized by NBS. The 6β-hydroxy-cholestane **17** was then converted to cholestan-6-one **18**, since it is highly reactive under this reaction condition. After the oxidation, the 6α-hydroxy-cholestane **16** could be easily separated from the reaction mixture. Oxidation of 6α-hydroxy-cholestane **16** was accomplished by treatment **16** with CrO_3 and water at room temperature. This reaction could then yield 80% of compound **18** in ten min (**Figure 2.24**).

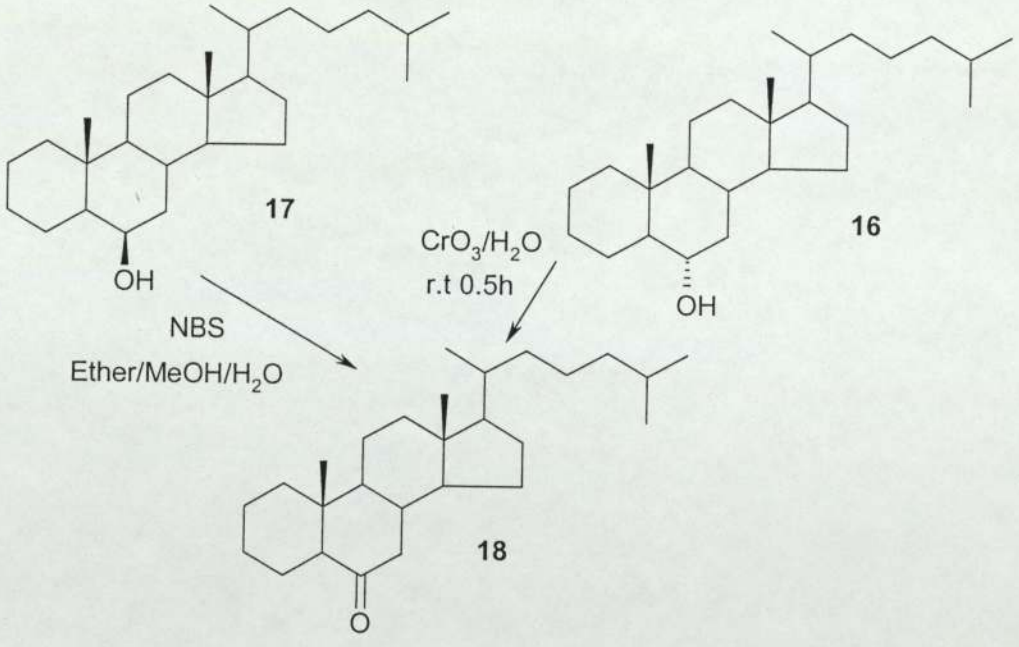


Figure 2.24 Oxidation of 6- OH-Cholestane.

2.4.4 Formation of Cholest-7-En-6-One (20)

Cholestan-6-one **18** was reacted with Br_2 and HBr in AcOH at 50 °C for 5h to form 7α-bromo-cholestane **19** in 80% yield. Though in this reaction, 5-bromination was also possible, the 5-bromo products remained minor because of the migration of bromo group from 5 position to 7 position for some unknown reasons.[115] Therefore the compound **19** could be achieved as a major product in good yield. Cholest-7-en-6-one **20** was prepared by refluxing compound **19** with Li_2CO_3 in DMF/H_2O as we discussed in chapter 2.2. As there were no hydroxy groups on the steroid ring, the elimination reaction went quite well this time with a major spot on the TLC plate. The 7-en **20** was then purified by column chromatography in 76% yield (**Figure 2.25**).

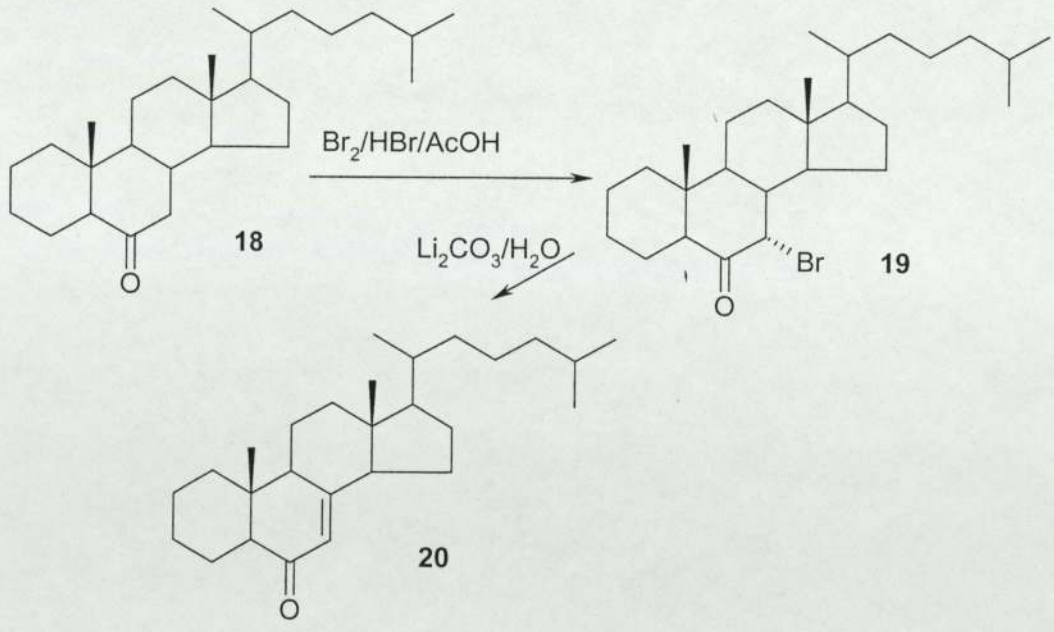


Figure 2.25 Synthesis of Cholest-7-en-6-one (**20**).

In this experiment, addition of water to the reaction system or not didn't affect the yield of 7-en **20**, because there were no hydroxy groups on ring A and ring B. Some other bases were also tried in my experiments. LiBr/DMF could do the same work as $LiCO_3$/DMF to achieve cholest-7-en as major product. The yield for this reaction is 70%. Triethylamine however couldn't give the desired 7-en (judged by TLC), and no other products was detected either. CH_3CH_2ONa/ethanol was the last base tried in my experiment. It could give cholest-7-en at a yield of 40%.

2.4.5 Introduction of Hydroxy Group to C-14

Since we have achieved the introduction of 7,8 double bond successfully, further attempt was then made to introduce the hydroxy groups to ring C and ring D. For this aim, an allylic oxidation[116] was first considered, because it would introduce 14-OH into the molecule in one step (**Figure 2.26**).

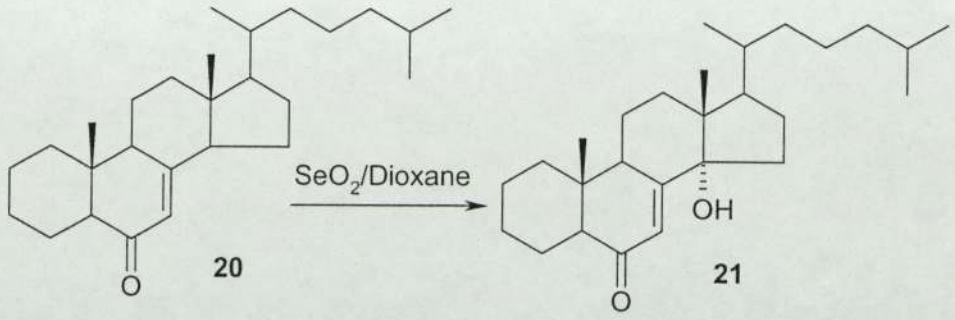


Figure 2.26 Introduce 14-OH to Sterol Ring.

Cholest-7-en-6-one **20** was heated with SeO_2 in dioxane at 50 °C for 0.5h to give 14α-hydroxy-5α-cholest-7-en-6-one **21** in 93%yield. The mechanism for this reaction is shown in **Figure 2.27**.

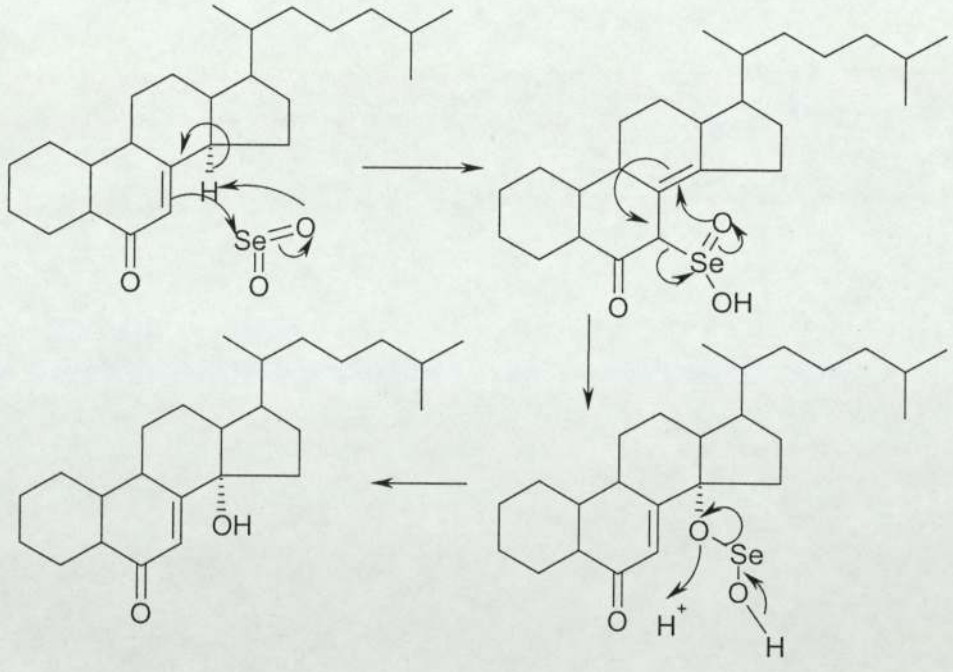


Figure 2.27 Mechanism of Allylic Oxidation.

2.4.7 Synthesis of Cholest -7, 14-Dien-6-One (22)

Synthesis of 14α-hydroxy-5α-cholest-7-en-6-one **21** somehow paved the way to introduce hydroxy groups to ring D. After this compound, a 7, 14-dien was successfully synthesized in our experiment. Cholest -7, 14-dien-6-one **22** was achieved by reacting 14α-hydroxy-5α-cholest-7-en-6-one **21** with thionyl chloride and pyridine at room temperature. 60% yield was obtained after 1 hour reaction (**Figure 2.28**).

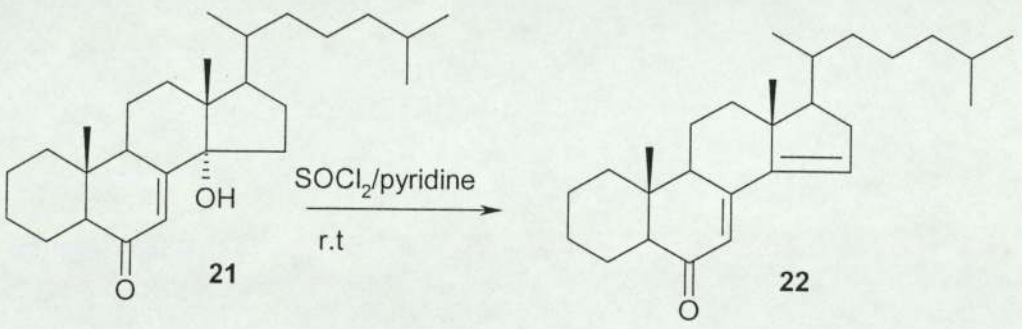


Figure 2.28 Synthesis of Cholest -7, 14-Dien-6-One (**22**).

The reaction condition was not optimized further due to lack of time.

2.5 Studies for Future Work

After the successful synthesis of cholest -7, 14-dien-6-one **22**, the way to prepare C, D ring hydroxy groups became clear. We can either introduce 16-OH by allylic

oxidation using SeO_2 or add hydroxy groups directly to the double bond by addition reaction (catalyzed by H_2SO_4) or hydroboration reaction. Among these methods, epoxidation reaction is of great interest because the following ring opening or rearrangement could be a good way to add hydroxy groups. Some studies on the stereochemistry of epoxide were made in my experiment based on the fact that sometimes different configuration of epoxy ring would affect the yield of hydroxy compounds dramatically.

*m*CPBA (*meta*-Chloroperbenaoic acid) was reported to be a potent reagent to generate epoxide. As the reactions with *m*CPBA were dominantly controlled by facial selectivity, the less steric hindrant α side was therefore more favored than the steric hindered β side, namely the α-epoxides were the major products in the *m*CPBA reactions. Li[117] proved this stereoselectivity by reacting *m*CPBA with 40 different sterols. The results showed that all of these reactions gave α-epoxides predominantly. An example 5, 6α-epoxy-cholestane **23** was made in my experiment by using mCPBA as the reacting agent (shown in **Figure 2.29**). The β epoxide could be achieved by a new method first introduced in 1992. In this method, mixture of $KMnO_4$-$CuSO_4.5H_2O$ in DCM was used as reacting reagent. Trace of water and tert-butyl alcohol was presented as phase tranfer catalyst. It is believed that water and tert-butanol form the third phase over the surface of the inorganic solid in which the reaction takes place.[118] In my experiment, 5, 6β-epoxy-cholestane **24** was formed with a high degree of stereoselectivty, although the β face is the sterically more

hindered face of the molecule. The mechanism for this reaction, however, was not clearly explained by now.

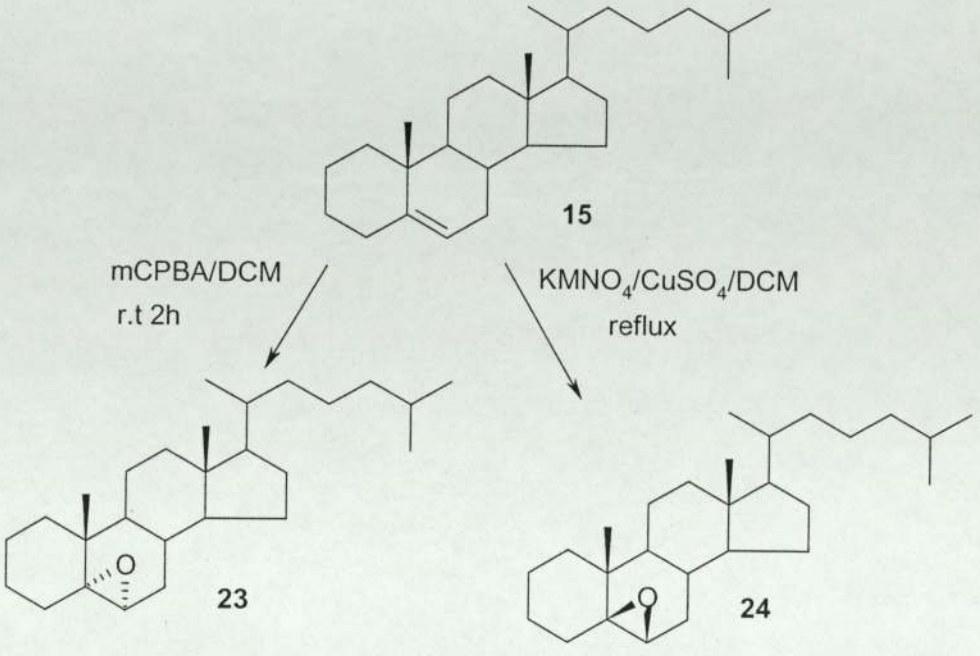
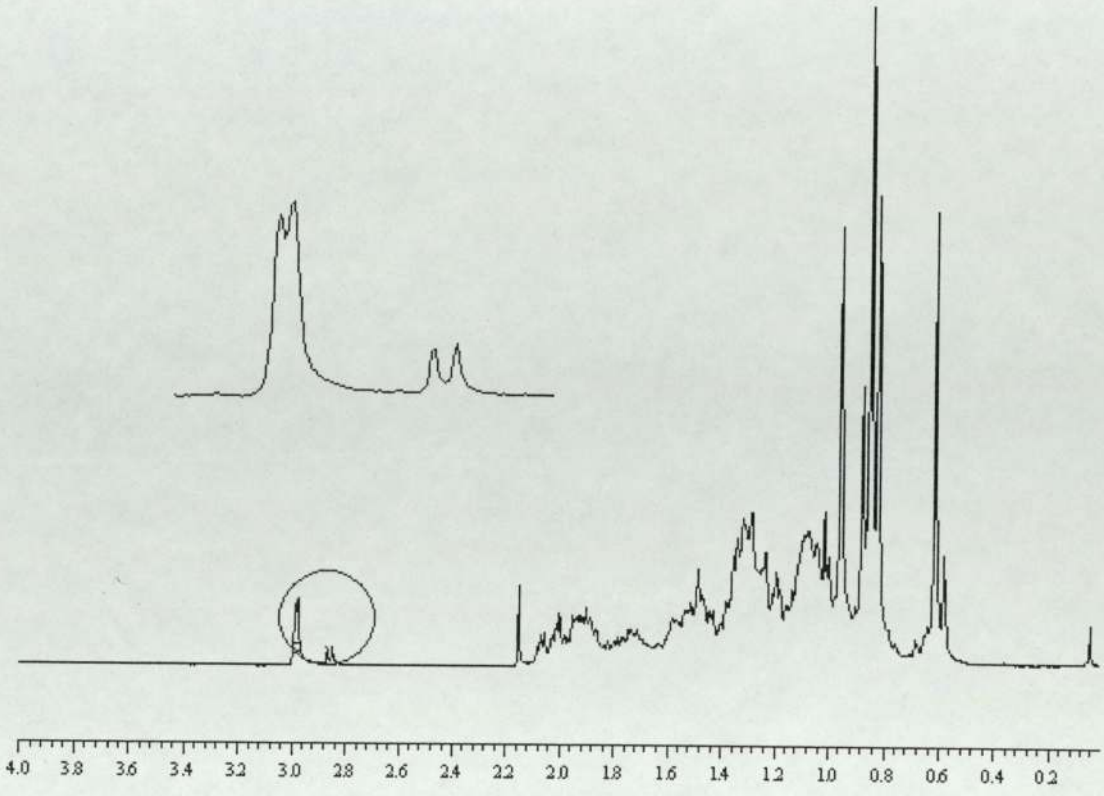
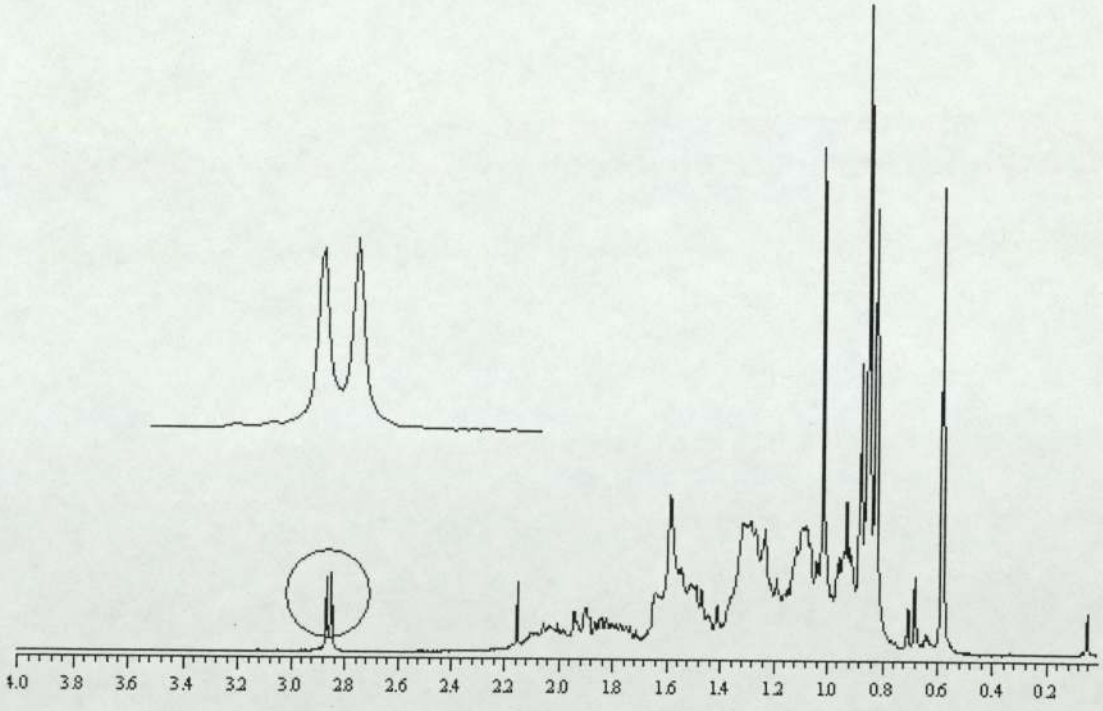


Figure 2.29 Synthesis of Epoxide.

The assignment of the stereochemistry to the epoxide and ratio of the isomers are readily inferred from their proton NMR spectrum.[119] The 6β-H appears in the range 2.82-2.86, while the 6α-H signal is found in the range 3.05-3.10. The proton NMR of compound **23** and **24** is shown in **Figure 2.30**.



Proton NMR of **24**



Proton NMR of **23**

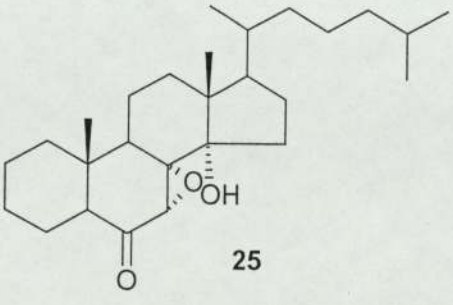
Figure 2.30 ^{1}H NMR of **23** and **24**.

2.6 Conclusions

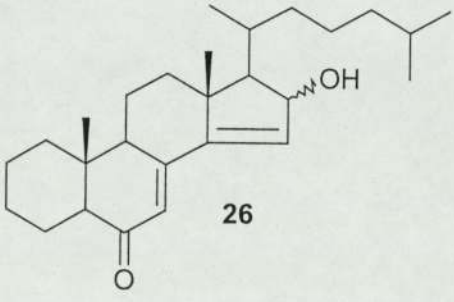
In this project, we have developed a synthetic route to prepare C, D ring oxygenated cholesterol in an acceptable yield. Two useful intermediates, Cholest -7, 14-dien-6-one **22** and cholest-7-en **20** were also synthesized in our experiment, from which introduction of hydroxy groups to ring C and D could be achieved in one or two steps. However, this chemistry route is just a simplified model because the effects of hydroxy groups on sterol ring A and ring B were eliminated. These hydroxy groups, however, are very important to the biological and pharmacological effects of oxysterols, therefore further studies will be needed to make this route more generalized.

2.7 Future Work

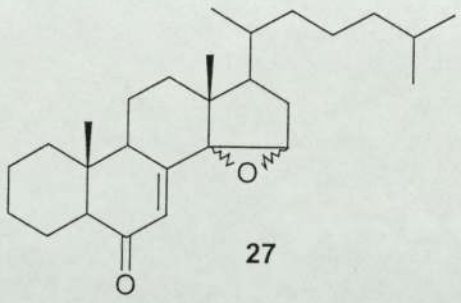
Based on the progresses I have made, some structures are of great interest to prepare in the future (**Figure 2.31**).



25



26



27

Figure 2.31 The Possible Work in the Future.

Structure **25** will be tried by reacting **21** with $Vo(acac)_2$/TBHP,[120] and structure **26** will possibly be achieved by using allylic oxidation as we discussed in chapter 2.4.6. As for the last structure, Reagents like *m*CPBA or $KMnO_4/CuSO_4$ will be a good start to try.

CHAPTER THREE

EXPERIMENTAL DETAILS

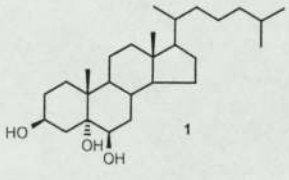
3.1 Materials and Methods

Melting points were taken on Reichert-Jung Microthermal, uncorrected. I.R. spectra were recorded on a Mattson 3000 instrument and N.M.R. spectra were taken on a Bruker AC-250 at 250 MHz. Mass spectra were obtained on a VG Quatro II or VG Autospec instruments by using atmospheric chemical onization (ACPI) and electrospray (ES) method.

Solvents and chemicals used for reactions were purchased from commercial suppliers and used without further purification. All column chromatographic purifications were accomplished on silica gel 60 (200-400 mesh) with the appropriate solvent gradients. Thin-layer-chromatography (TLC) was performed using 0.25 mm Merck Kieselgel 60 F254 precoated silica gel plates.

3.2 Reaction Details

5α-Cholestane-3β, 5, 6β-Triol (1)

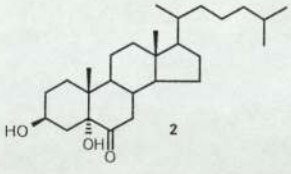


1

This compound was prepared according to the published method.[109] Formic acid (98% 350ml) was added to cholesterol (50g 129mmol). The mixture was heated to 75 °C with stirring and standed at this temperature for 5 min. After cooling to room temperature, aqueous

hydrogen peroxide (30%, 70ml) was dropped in during 15 min with constant stirring. The mixture was then stirred at room temperature overnight. The resulting foam product was diluted with hot water (760ml) and the solid was filtered after cooling to room temperature, washed with saturated sodium carbonate and dried. These solid was dissolved in methanol (1500ml) and then sodium hydroxide (25%, 50ml) was added and the resulting solution was refluxed for 1h. Water was added slowly to make the precipitate complete. The mixture was cooled to room temperature and the solid product was filtered and dried to give a white solid in 93% yield. IR: ν_{max}: 3500-3300, 2937, 2865, 1465, 1375 and 1039 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.08 (m, 1H, H-3), 3.52 (s, 1H, H-6), 1.16 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); MS (ES^+): m/z 419 (M^+-H, 100).

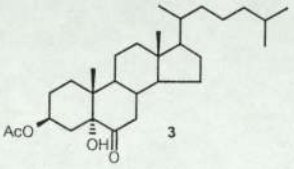
5α-Cholestan-3β, 5α-Diol-6-One (2)



5α-Cholestane-3β, 5, 6β-triol **1** (10g, 25mmol) was dissolved in ether (140ml), methanol (50ml) and water (20ml). This reaction mixture was then stirred at 28-30 °C for 10 min. To this solution, NBS (3.0g, 30mmol) was added and then this mixture was kept at 28-30° C for 70 min. A white precipitate was formed (sometimes not) during this period. 5% of $NaHSO_3$ (60ml) was added to the reaction system to quench the reaction (disappear of yellow color could be observed). The white solid was then filtered and

dried at room temperature. The filtrate was extracted with ether (30ml×3) and washed with water thoroughly. After removal of ether, a further part of product would be given. The total yield of this reaction was 91%. IR: $\nu_{max:}$ 3500-3300, 2948, 2871, 1706, 1465, 1375, 1248 and 975 cm^{-1}; ^1H NMR ($CDCl_3$): 3.95 (m, 1H, H-3), 0.78 (s, 3H, CH_3-19), 0.62 (s, 3H, CH_3-18); MS (ES^+): m/z 441 (M^++Na, 100).

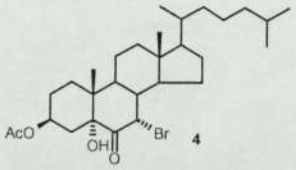
5α-Cholestan-3β, 5α-Diol-6-one 3-Acetate (3)



3

5α-Cholestan-3β, 5α-diol-6-one **2** (5g, 25mmol) was dissolved in toluene (10ml). To this solution acetic acid anhydride (10ml) and pyridine (1ml) were added. This mixture was then refluxed for 1.5h. After that, the tolene was removed by evaperation in *vacuo*. To this crude product, DCM (100ml) was added and this solution was washed with 5% HCl thoroughly and dried over Na_2SO_4. Removal of the solvent gave compound **3** as white solid in 100% yield. IR ν_{max}: 3500-3300, 2951, 2869, 1735, 1711, 1362, 1276, 1040 and 962 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 5.01 (m, 1H, H-3), 2.01 (s, 3H, CH_3 of acetate), 0.79 (s, 3H, CH_3-19), 0.62 (s, 3H, CH_3-18); MS ($ACPI^+$): m/z 461 (M^++1), 401 (M^+- AcOH).

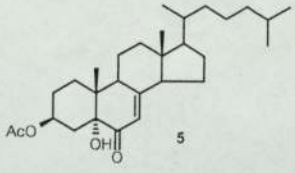
7-Bromo-Cholestan-3β, 5α-Diol-6-One-3-Acetate (4)



4

A solution of 5α-cholestan-3β, 5α-diol-6-one 3-acetate **3** (3.3g, 6.7mmol) in acetic acid (70ml) was treated at 60° C with a solution of bromine (1.23g, 7.7mmol) in acetic acid (35ml) and boron fluoride etherate (1ml). The reaction mixture was kept at 60 °C for 15 min. When the solution became light yellow, it was stirred at room temperature for a further 1.5h. Then this reaction mixture was diluted with water (100ml) and the participate that followed was filtered and dried in vacuum. The dried precipitate was grounded in a mortar with methanol (2ml). Part of this precipitate dissolved and part became a gum. Further trituration could produce a thick paste with pale yellow color. This paste was then dissolved in ether (10ml), washed with water (20ml) and dried over $MgSO_4$. Removal of the solvent gave a yellow solid which was then purifid by silica gel column with hexane: ethyl acetate 10:1 as solvent system. The pure product was obtained as a white solid in 79% yield. IR ν_{max}: 2941, 2805, 1738, 1711, 1460, 1356, 1246 and 1028cm^{-1}; ^1H NMR ($CDCl_3$): 5.07 (m, 1H, H-3), 4.16 (d, J = 4.5Hz, 1H, H-7), 2.00 (s, 3H, CH_3 of 3-acetate), 1.13 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 12.4, 13.9,18.5, 22.7, 21.3, 23.0; (CH_2): 39.4, 38.5, 35.9, 33.0, 29.0, 27.8, 26.0, 23.7, 23.0, 20.7; (CH): 69.8, 55.4, 53.3, 52.4, 38.9, 37.5, 35.6, 28.0; (C): 205.0, 170.5, 42.7, 42.1, 81.8; MS ($ACPI^+$): m/z 496 (M^++1).

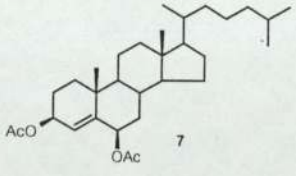
5α-Cholestan-3β, 5α-Diol-7-En-6-One 3-Acetate (5)



5

To 7-bromo-cholestan-3β, 5α-diol-6-one-3-acetate **4** (100mg, 0.25mmol) and Li_2CO_3 (50mg, 0.5mmol) were added DMF (4ml) and water (3ml). This reaction mixture was then heated under reflux for 4.5h. After cooling down to room temperature, the solvent was removed and the product that followed was dissolved in DCM (20ml). The solid that didn't dissolve was filtered out (inorganic salt). The crude product left in the filtrate was then subjected to silica gel column, elution with Hexane: Ethyl Acetate 5: 1. White solid was given in 20% yield. IR ν_{max}: 2939, 2864, 2363, 1662, 1464, 1378, 1311, 1193 and 864 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 6.20 (s, 1H, H-7), 4.69 (m, 1H, H-3), 2.11 (s, 3H, acetyl- CH_3), 1.15 (s, 3H, CH_3-19), 0.67 (s, 3H, CH_3-18).

Cholest-4-En-3β,6β-Diol-3,6-Diacetate (7)

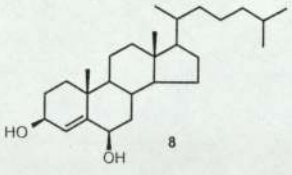


7

5α-Cholestane-3β, 5, 6β-triol **1** (5g, 12mmol) was dissolved in toluene (50ml). To this solution acetic acid anhydride (10ml) and pyridine (1ml) were added. This mixture was then refluxed for 1.5h. After that, the toluene was removed by evaperation in *vacuo*. To this crude product, DCM (20ml) was added and this solution was washed with 5% HCl thoroughly and dried over Na_2SO_4. Removal of the solvent will give white solid product cholestan-3β, 5, 6β-triol-3, 6-diacetate (**6**) in 94% yield. The crude product

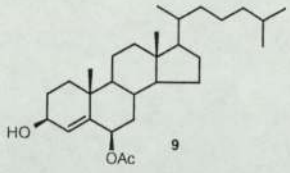
(**6**) was dissolved in pyridine (18ml) and cooled to –20 °C. To this solution was added thionyl chloride (0.4ml, 5.5mmol) dropwisely with constant sritrring. The mixture was kept at –20 °C for 5 min and at 0 °C for 10 min. and then it was poured into ice-water (100ml). The precipitae was filtered out and dried in vaccum. Pale yellow product was obtained in 91% yield. IR: ν_{max}: 3500-3300, 2952, 2871,1753, 1471,1383, 1240, 1008, 705 and 609 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 5.58 (s, 1H, H-4), 5.26 (s, 1H, H-6), 5.19(m, 1H, H-3), 1.17 (s, 3H, CH_3-19), 0.69 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 11.8, 18.4, 20.2, 20.9, 21.2, 22.3, 22.6; (CH_2): 20.6, 23.6, 23.9, 24.5, 27.9, 35,9, 36.3, 36.9, 39.3, 39.5; (CH): 27.7, 30.5, 35.6, 53.6, 55.7, 55.5, 69.9, 74.6, 127.4; (C): 36.3, 42.2, 143,5, 169.1, 169.9; MS (ES^+): m/z 509 (M^++Na).

Cholest-4-En-3β, 6β-Diol (8)



Cholest-4-en-3β,6β-diol-3,6-diacetate **7** (800mg, 2.0mmol) was dissolved in ethanol (20ml). To this was added a solution of sodium hydroxide (0.4g, 10mmol) in water (4ml) and this mixture was heated at reflux for 1h. After cooling, the mixture was poured into water and the solid that followed was collected by filtration and then dried in vaccum. White solid was given in 90% yield. IR: ν_{max}: 3500-3300, 2935, 2867, 1635, 1645, 1378 and 1037 cm^{-1}; ^{1}HNMR ($CDCl_3$): δ_H 5.52 (s, 1H, H -4), 4.21 (s, 1H, H-6), 4.08 (m, 1H, H-3), 1.24 (s, 3H, CH_3-19), 0.69 (s, 3H, CH_3-18); MS (ES^+): m/z 425 (M^++Na).

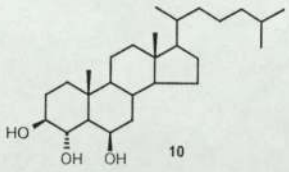
Cholest -4-En-3β, 6β-Diol 6-Acetate (9)



9

Cholest-4-en-3β,6β-diol-3,6-diacetate **7** (804mg, 2.0mmol) was dissolved in DCM (5ml) and ethanol (2.5ml). To this solution was added a solution of sodium hydroxide (80mg, 2.0mmol) and this mixture was kept at room temperature for 1h. After treatment with water (20ml), the solid that followed was filtered out and dried. White solid was obtained in 90% yield. M.p. 136.4-137.6°C; IR: ν_{max} 3523, 2930, 2862, 2843, 1718, 1470, 1374, 1259, 1016 cm^{-1}, ^1H NMR ($CDCl_3$): δ_H 5.64 (s, 1H, H-4), 5.22 (m, 1H, H-6), 4.08 (m, 1H, H-3), 1.10 (s, 3H, CH_3-19), 0.67 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C(CH_3): 12.0, 18.5, 20.7, 21.6, 23.7, 24.0; (CH_2): 20.7, 23.7, 24.0, 28.1, 28.8, 36.0, 36.6, 37.1, 39.4, 39.6; (CH) : 28.0, 30.8, 35.7, 53.9, 55.9, 56.0, 67.7, 75.6, 131.8; (C): 36.7, 42.4, 142.2, 170.2; MS (ES^+): m/z 467 (M^++Na).

Cholestane-3β, 4α, 6β-Triol (10)

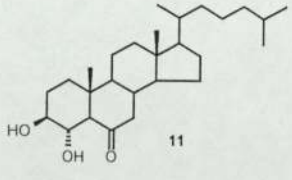


10

Cholest-4-en-3β, 6β-diol **8** (1g, 2.5mmol) was added dry THF (15ml), followed by $NaBH_4$ (0.4g, 10.7mmol). The mixture was then stirred in a ice-water bath under N_2 atmosphere. To this solution, $BF_3.Et_2O$ (1.2ml, 10.6mmol) was added dropwise during 1h, and then the mixture was stirred at room temperature for a further 2.5h. Water was added slowly to destroy the excess borane. After the gas evolution ceased, 10% NaOH (15ml) was

added and the mixture was returned to the ice-water bath with stirring. Hydrogen peroxide (30%, 5ml) was added dropwise and the mixture was stirred for a futher hour. Then the reaction mixture was diluted with water (20ml) and ether (20ml) and the organic layer was washed with sodium sulfate (20%, 20ml) and water. The crude product was then subjected to silica gel column, elution with hexane: ehtyl acetate 15:1. white solid was given after the column in 60% yield. Mp: 218-220°C (Hexane/CH_2Cl_2); IR: ν_{max}: 2937, 2865, 2848, 1469, 1463, 1380, 1363 1060 and 1032 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.32 (brs, 1H, H-6), 3.73 (m, 1H, H-4), 3.41 (m, 1H, H-3), 1.02 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); ^1H NMR (DMSO-d_6): δ_H 4.42 (brs, 1H, OH-3), 4.13 (m, 2H, OH-4 and H-6), 3.91 (brs, 1H, OH-6), 3.35 (m, 1H, H-4), 3.09 (m, 1H, H-3), 0.92 (s, 3H, CH_3-19), 0.64 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 12.0, 16.5, 18.6, 22.5, 22.7, (CH_2): 20.7, 23.8, 24.1, 28.1, 28.4, 36.1, 37.7, 39.1, 39.4, 39.7, (CH): 27.9, 29.8, 35.7, 52.4, 54.1, 56.1, 56.2, 65.0, 72.0, 76.5, (C): 36.9, 42.5; MS(ES^+): m/z 438 (M^++NH_4, 100).

5α-Cholestan-3β, 4α-Diol-6-One (11)

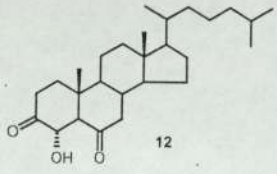


11

To cholestane-3β, 4α, 6β-triol **10** (1g, 2.4mmol) was added NBS (300mg, 3.0mmol) in dioxane (14ml), methanol (5ml) and water (2ml) at 35° C. This reaction mixture was then stirred at this temperature for further 1.5h. 5% $NaHSO_4$ was added to quench the reaction and the precipitate that followed was filtered out and dried. White solid was given in a yield of 90%. IR:

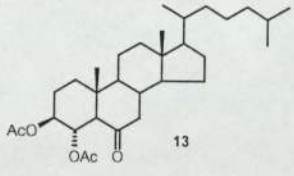
$\nu_{max.}$ 3500-3300, 2956, 2865, 2850, 1710, 1465, 1373 and 1076; ^{1}H NMR ($CDCl_3$): 3.84 (dd, J=9.7 and 9.5Hz, 1H, H-4), 3.43 (m, 1H, H-3), 0.77 (s, 3H, CH_3-19), 0.63 (s, 3H, CH_3-18); MS (ES^+): m/z 419 (M^++H, 100).

4α-Hydroxy-5α-Cholestane-3, 6-Dione (12)



4α-Hydroxy-5α-cholestane-3, 6-dione **12** was a byproduct of NBS oxidation of **10**. M.p. 186 °C; IR: ν_{max} 3470, 2962, 2865, 1712, 1465, 1427, 1380, 1282, 1257, 1232, 1111 and 1056 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ_H4.55 (d, J=11.0Hz, 1H, H-4), 1.02 (s, 3H, CH_3-19), 0.65 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 11.9, 13.7, 18.5, 22.4, 22.7; (CH_2): 21.3, 23.7, 23.9, 27.9, 35.3, 35.9, 38.5, 39.2, 39.3, 47.1; (CH): 27.9, 35.5, 38.5, 53.6, 56.0, 56.4, 65.9, 70.5; (C): 42.9, 43.1, 208.0, 210.5; MS (ES^+): m/z 434 (M^++H_2O, 100).

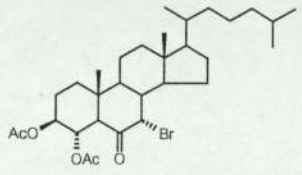
5α-Cholestan-3β, 4α-Diol-6-One-3, 4-Diacetate (13)



5α-Cholestan-3β, 4α-diol-6-one **11** (800mg, 2.0mmol) was dissolved in toluene (10ml). To this solution was added a solution of acetic acid andydride (1.5ml) and pyridine (0.1ml). This mixture was then refluxed for 1.5h. After that, the toluene was removed in vaccum. To this crude product, DCM (20ml) was added and this solution was washed with 5% HCl thoroughly and dried over Na_2SO_4. Removal of the solvent gave white solid product

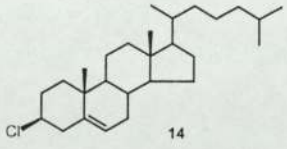
in 96% yield. IR: ν_{max}: 2960, 2810, 1738, 1356, 1247, 1219 and 1028 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 5.36 (m, 1H, H-4), 4.73 (m, 1H, H-3), 1.13 (s, 3H, CH_3-19), 0.62 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 11.9, 13.8, 18.5, 20.8, 21.0, 23.6, 23.8; (CH_2): 47.1, 39.3, 39.2, 35.9, 35.4, 25.4, 23.8, 23.7, 21.3; CH: 74.8, 68.3, 60.8, 56.5, 56.0, 54.0, 38.5, 35.6, 27.9, 25.4; (C): 206.7, 170.7, 169.7, 43.4, 42.9; MS ($ACPI^+$): m/z 503 (M^++1), 443 (M^+-HAc).

Attempt to Prepare 7-Bromo-Product from (13)



5α-cholestan-3β, 4α-diol-6-one-3, 4-diacetate **13** (800mg, 2mmol) was dissolved in acetic acid (50ml). To this was added few drops of $BF_3.Et_2O$ and then dropwise a 1M solution of Br_2 in acetic acid (2.9ml, 2.9mmol). The reaction mixture was stirred at 50 °C for 3h. No major products could be identified from TLC.

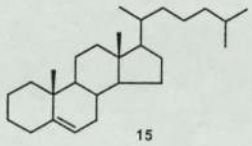
Cholesteryl Chloride (3β-chlorocholest-5-ene) (14)



To colesterol (38.7g, 100mmol) was added thionyl chloride (35ml). This mixture was then kept at room temperaure for 20h. The resulting dark colored liquid was diluted with acetone (400ml) and then added water to destroy the excess reagent. The solid that followed was filtered and washed with acetone which gave yellow crystal in 90% yield. ^1H NMR ($CDCl_3$): δ_H 5.35 (d, J=5.0Hz, 1H, H-6), 3.73 (m, 1H, H-3), 0.91 (s, 3H, CH_3-19), 0.66 (s, 3H,

CH_3-18).

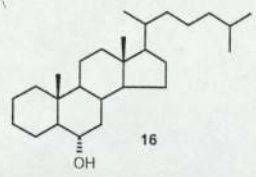
Cholest-5-Ene (15)



15

3β-Chlorocholest-5-ene **14** (4.0g, 10mmol) was dissolved in ethanol (40ml) and THF (55ml). This solution was then cooled to –50 °C with constant stirring. Sodium chips (3.0g, 130mmol) were added and this mixture was stirred at –50 °C for 5h. After this, the sodium chips were filtered out and the filtrate was poured into ice-water (200ml). Filtration and drying in vacuum gave white solid in 99% yield. ^{1}H NMR ($CDCl_3$): δ_H 5.25 (m, 1H, H-6), 0.66 (s, 3H, CH3-18), 1.01 (s, 3H, CH_3-19).

Cholestan-6α-Ol (16)

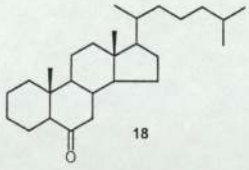


16

To cholest-5-ene **15** (3g, 7.5mmol) was added dry THF (45ml), followed by $NaBH_4$ (1.2g, 34mmol). The mixture was then stirred in a ice-water bath under N_2 atmosphere. To this solution, BF_3 (3.6ml, 33mmol) was added dropwise during 1h, and then the mixture was stirred at room temperature for a further 2.5h. Water was added slowly to destroy the excess borane. After the gas evolution ceased, 10% NaOH (45ml) was added and the mixture was returned to the ice-water bath with stirring. Hydrogen peroxide (30%, 15ml) was added dropwise and the mixture was stirred for a futher hour. Then the

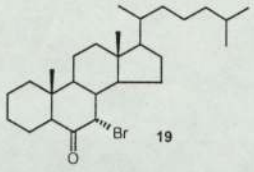
reaction mixture was diluted with water (70ml) and ether (60ml) and the organic layer was washed with sodium sulfate (20%, 40ml) and water (20ml). The crude product was dissolved in ether (50ml), methanol (15ml) and water (6ml) and this reaction mixture was stirred at 28-30 °C for 10 min. To this solution, NBS (1g, 9mmol) was added and then this mixture was kept at 28-30 °C for 70 min. A white precipitation was formed during this period. 5% of $NaHSO_3$ (60ml) was added to the reaction system to quench the reaction. The crude product was then subjected to silica column, elution with hexane: ethyl acetate 11: 1. White solid was given in a yield 63%. IR: ν_{max}: 3442, 2928, 2865, 1464, 1441, 1378, 1166, 1040, 974 and 612 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H3.35 (m, 1H, H-6), 0.77 (s, 3H, CH_3-19), 0.62 (s, 3H, CH_3-18); MS ($ACPI^+$): m/z 389 (M^++1).

5α-Cholestan-6-One (18)



Cholestan-6α-ol **16** (2g, 4.0mmol) was dissolved in acetic acid (5ml). To this solution was added a solution of CrO_3 (4g, 8.0mmol) in H_2O (0.5ml) at 5 °C. The reaction mixture was stirred at room temperature for 10 min. TLC plate showed one spot. The reaction mixture was then poured in to ice water and then extracted with DCM (20ml), washed with water and dried. Yellow solid was given after removal of the solvent. Yield 92%. ^1H NMR ($CDCl_3$): δ_H 2.24-2.30 (m, 1H, H-5), 0.71 (s, 3H, CH_3-19), 0.63 (s, 3H, CH_3-18), 0.84 (d, J=6.4Hz, 6H, H26/27).

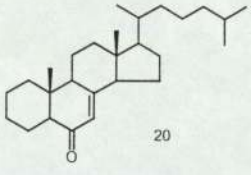
7α-Bromo-Cholestan-6-One (19)



19

To a solution of 5α-cholestan-6-one **18** (1g, 2.4 mmol) in acetic acid (50 ml) was added a few drops of hydrogen bromide and then dropwise a 1M solution of bromine in acetic acid (2.9 ml 2,9 mmol). The reaction mixture was stirred for 3 h at 50 °C and poured into ice water. The product was extracted with DCM to give yellow oil and then purified by silica gel column, elution with Hexane: acetone 20: 1. Colorless oil was given with a yield 80%. ^{1}H NMR ($CDCl_3$): δ_H 4.15 (d, J= 4.2Hz, 1H, H-7), 3.14 (m, 1H, H-5), 0.70 (s, 3H, CH_3-19), 0.65 (s, 3H, CH_3-18).

Cholest -7-En-6-One (20)

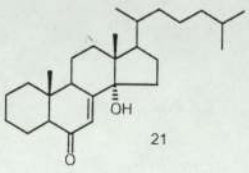


20

7α-Bromo-cholestan-6-one **19** (800mg, 2.0mmol) and Li_2CO_3 (50mg, 0.5mmol) were added DMF (30ml) and water (20ml). This reaction mixture was then heated under reflux for 4h. After cooling down to room temperature, the solvent was removed and the product that followed was dissolved in DCM (20ml). The solid that didn't dissolve was filtered (inorganic salt). The filtrate was then subjected to silica gel column, elution with Hexane: Ethyl Acetate 15: 1. White solid was given in 76% yield. IR: ν_{max}: 2900-2800, 2361, 2330, 1661, 1465, 1365, and 1196 cm^{-1}; ^{1}H NMR ($CDCl_3$): δ_H 5.67 (m, 1H, H-7), 1.00 (s, 3H, CH_3-19), 0.88 (d, J= 6.1Hz, 6H, H26/27), 0.94 (d, J= 6.3Hz, 3H, H21), 0.58 (s,

3H, CH3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 12.2, 13.1, 18.7, 22.7, 22.5; (CH_2): 39.3, 38.8, 38.5, 36.0, 27.6, 25.2, 23.7, 22.2, 21.4, 21.2, 20.5; (CH): 122.9, 56.2, 55.6, 54.7, 50.4, 35.9, 27.9; (C): 201.2, 163.4, 44.4, 38.9; MS ($ACPI^+$): m/z 385 (M^++1). 367 (M^+-H_2O).

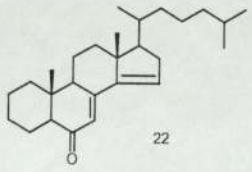
14α-Hydroxy-5α-Cholest-7-En-6-One (21)



21

Cholest-7-en-6-one **20** (400mg, 1mmol) was dissolved in dioxane (5ml), followed by selenium dioxide (428mg, 3.86mmol). The reaction mixture was heated at 50 °C for 0.5h. After cooling down, the reaction mixture was poured into ice water and then extracted with DCM (10ml ×3). The crude product was then purified by silica gel column. Elution with hexane: acetone 10:1.White powder was given in 93% yield. IR: ν_{max}: 3469, 2861, 2940, 2363, 2339, 1652, 1464, 1362, 1197, 864 and 667 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 5.83 (d, J= 3.27Hz, 1H, H-7), 2.68 (m, 1H, H-9), 2.18 (dd, J=3.24 and 4.25Hz, 1H, H-5), 0.65 (s 3H, CH_3-18), 1.11 (s, 3H, CH_3-19); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 22.7, 22.4, 18.8, 15.7, 12.8; (CH_2): 39.3, 38.4, 36.1, 31.7, 30.5, 26.4, 25.1, 23.7, 21.1, 20.5, 20.0; (CH): 122.7, 54.9, 50.5, 46.3, 35.5, 27.9; (C): 202.1, 163.0, 85.1, 46.2, 46.3; MS ($ACPI^+$): m/z 401 (M^++1).

Cholest -7, 14-Dien-6-One (22)

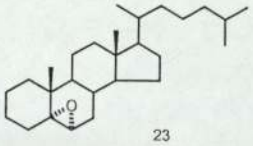


22

14α-Hydroxy-5α-cholest-7-en-6-one **21** (200mg, 0.5mmol)

was dissolved in pyridine (1ml) and the solution was cooled to –20 °C. Thionyl chloride (0.1ml, 1.4mmol) was added in several portions within 1 min under shaking. The mixture was kept at –20 °C for 5 min, at 0 °C for 10 min, and then poured into ice water. The product was filtered and washed with water. The crude product was purified by column (hexane: acetone 20: 1). White solid was given in 60% yield. IR: ν_{max}: 2900-2800, 2361, 2330, 1665, 1465, 1365, and 1196 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 6.09 (d, J= 2.8Hz, 1H, H-7), 5.92 (m, 1H, H-14), 1.12 (s, 1H, CH_3-19), 0.82 (s, 1H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 12.6, 17.0, 18.8, 22.7, 22.5; (CH_2): 39.3, 38.9, 38.3, 35.9, 35.9, 25.3, 23.6, 21.3, 20.5, 20.2; (CH): 128.1, 121.5, 58.6, 55.2, 50.9, 33.9, 27.9; (C): 201.1, 154.4, 149.8, 46.6, 39.7; MS (ACPI$^+$): m/z 401 (M^++1).

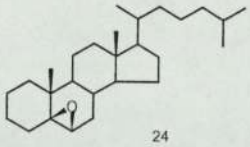
5α, 6α-Epoxy-Cholestane (23)



23

Cholest-5-ene **15** (400mg, 1.0mmol) and *m*CPBA (207mg, 1.2mmol) were dissolved in DCM (10ml) and the mixture was stirred at room temperature for 2h. The resulting mixture was washed with 10% aquous sodium hydroxide (10ml) and water (10ml) and then dried over Na_2SO_4. The crude product was subjected to silica gel column, elution with hexane: ethyl acetate 10: 1. White solid was given in 90% yield. IR: ν_{max}: 2932, 2857, 1463, 1369, 1326, 1142, 931cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 2.86 (d, J= 4.75Hz, 1H, H-6), 1.02 (s, 3H, CH_3-19), 0.58 (s, 3H, CH_3-18); MS (ACPI$^+$): m/z 387(M^++1).

5β, 6β-Epoxy-Cholestane (24)



24

Copper sulfate pentahydrate (250mg, 1mmol) and potassium permanganate (500mg, 3.14mmol) were ground together to a fine power, to which water (0.04ml) was added. This paste was transferred to a reaction flask containing a solution of Cholest-5-ene **15** (80mg, 0.144mmol) in DCM (10ml), followed by addition of tert-butanol (0.1ml). This reaction mixture was heated to reflux for 10 min and stirred at room temperature overnight. After removal of the solvent, the crude product was purified by column chromatography with hexane: acetone 10: 1. White solid was given in 80% yield. IR: ν_{max}: 2933, 2859, 1467, 1369, 1328, 1166, 1142, 929, 776 and 558 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 2.99 (d, J=2.75Hz, 1H, H-6), 1.08 (s, 3H CH_3-19), 0.88 (d, J=6.2Hz, 6H, H26/27), 0.62 (s, 3H, CH_3-18); MS ($ACPI^+$): m/z 387(M^++1), 369 (M^+-H_2O).

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