Synthetic Studies of Oxysterols: Methodology for Introducing Hydroxy Groups to Ring A of Steroids

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A thesis submitted by Xin Xiong BSc for the degree of
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Abstract: Oxysterols, the oxygenated derivatives of cholesterol, have shown cytotoxic effects to tumour cells. Our research interests were to improve oxysterol cytotoxicities through enhancing hydrophilicity of oxysterol molecules.

The aim of this project was to develop methodologies for stereoselectively introducing hydroxy groups to ring A of steroids.

For introducing hydroxy groups to the C1 and C4, 5α-hydroxy-6β-acetoxycholest-2-en-4-one was synthesized from selective oxidation of 5α-hydroxy-6β-acetoxycholest-2-en with chromium trioxide and 90% aqueous acetic acid. 5α-Hydroxy-6β-acetoxycholest-2-en-1-one was obtained as one of the resulting four compound mixture of the reaction of 5α-hydroxy-6β-acetoxycholest-2-en with chromium trioxide and 3,5-dimethylpyrazole. When 5α,6β-diacetoxycholest-2-en underwent allylic oxidation with chromium trioxide and 3,5-dimethylpyrazole, 5α,6β-diacetoxycholest-1-en-3-one was obtained as the single product. 6β-Acetoxycholestane-3β,5α-diol was stereoselectively obtained from the reduction of 5α-hydroxy-6β-acetoxycholest-1-en-3-one with sodium borohydride. The reduction of 5α,6β-dihydroxy-cholest-1-en-3-one with sodium borohydride gave cholest-1-en-3α,5α,6β-triol.

For introducing hydroxy groups to the C2 and C3, a number of epoxidation reagents were adopted to treat cholest-2-en-5α,6β-diol and several of its analogues, most of the resulting epoxides are with α configuration. 2β,3β-Epoxy-cholestane-5α,6β-diol-4-one was obtained as the only 2β,3β-epoxide with *trans* A/B ring fusion in my project. The rearrangement of 2α,3α-epoxy-cholest-5α,6β-diol with lithium aluminum hydride gave cholestane-3α,5α,6β-triol as the single product in high yield.

Keywords: Oxysterols, steroid, cytotoxicity.

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General Abbreviations

Ac	acetyl
cDNA	complementary deoxyribonucleic acid
COSY	correlated spectroscopy
CT	cholestane-3β,5α,6β-triol
DCM	dichloromethane
de novo	*Latin* from the beginning
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethylformamide
DMP	3,5-dimethylpyrazole
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-*N,N,N',N'-tetraacetic acid*
Et	ethyl
FCS	fetal calf serum
HMG-CoA	hydroxymethylglutaryl-coenzyme A
HPLC	high-performance liquid chromatography
IC_{50}	Inhibitory concentration to reduce uptake by 50%
in situ	*Latin* in the normal, natural, original, or appropriate position.
in vacuo	*Latin* in a vacuum
in vitro	*Latin* occurring or made to occur outside an organism
in vivo	*Latin* occurring or made to occur within a living organism
IR	infrared
KB cell	an established cell line of cells derived from a human carcinoma in 1954 and maintained in tissue culture since then
LDL	low-density lipoprotein
m-CPBA	*m*-chloroperbenzoic acid
Me	methyl
mRNA	messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazole-2yl)-2,5-diphenyl-tetrazolium bromide
NBS	*N*-bromosuccinimide

NK cell	natural killer cell
NMR	nuclear magnetic resonance
PBA	perbenzoic acid
Py	pyridine
SD	standard deviation
SRE	sterol regulatory element
SREBP	sterol regulatory element binding protein
TBHP	*t*-butyl hydroperoxide
THF	tetrahydrofuran
TLC	thin-layer chromatography
TNF	tumour necrosis factor
Ts	*p*-toluenesulphonyl
USP	United States Pharmacopeia
UV	ultraviolet

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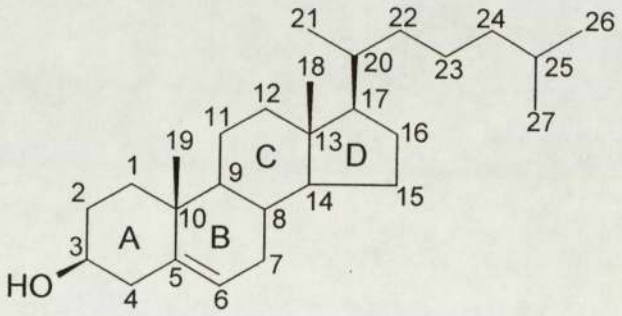
Chapter One: Introduction

1. Introduction

Oxysterols comprise the compounds formed from the oxidation of naturally occurring sterols (Table 1-1). One of the most prominent members among the natural sterol family is cholesterol (Figure 1-1). Cholesterol widely exists in nature mainly as an important component of all tissues of animals; it is also a minor sterol of plants (Coffey, 1970). Most of the oxysterols are oxygenated derivatives of cholesterol with same structure skeletons, and one or more additional oxygen functional groups such as a hydroxy group, a ketone group, or an epoxide group on the sterol nucleus or on its side chains.

Fig. 1-1 Chemical Structure and Numbering System for Cholesterol



In the human body, oxysterols are produced by endogenous oxidation and may also be derived from food. Absorption of oxysterols has been demonstrated (Bascoul et al., 1985 and 1986; Emanuel et al., 1991; Fornas et al., 1984; Osada et al., 1994), and some oxysterols have been isolated from some fresh foods and many processed foods: dairy, eggs, meat and fish products (Guardiola et al., 1995). Early research on oxysterols showed that oxysterols, from endogenous origin, were formed solely by enzymatic processes, mainly in biosynthesis of steroid hormones and bile acids. However, it is now known that non-enzymatic

processes can also occur in the body (Smith, 1990). Table 1-2 (Guardiola et al., 1996) presents the major naturally occuring oxysterols from enzymatic and non-enzymatic origins. A large number of studies demonstrated that oxysterols exist naturally in plasma, low-density lipoproteins, tissues cerebrospinal fluid, meconium, and some food products. It also revealed the potential physiological or pathophysiological importance of oxysterols as high levels of certain oxysterols have been identified in cataracts, in membranes of red blood cells from patients with sickle cell disease, and in plasma from patients with liver disorders. In last decades, several advances have been made in the technologies for the separation, identification, and quantitative analysis of oxysterols.

Due to the significance of oxysterols in biological processes, the study interests in the oxysterols have grown rapidly. A few very important biological effects of oxysterols, such as cytotoxicity, gene regulation, atherogenesis, mutagenesis, and carcinogenesis, so far have been observed. Among them, cytotoxicity can most possibly be utilized for the development of anticancer agents. That is leading the research work of oxysterols to a more scientifically direct way.

Table 1-1 Naturally-occurring Sterols. Adopted from Coffey (1970).

Name (Formula)	Structure: substituents in Cholestan-3β-ol (unless specified) (5α-H unless specified)	Source
Cholesta-5,7,24-trien-3β-ol ($C_{27}H_{42}O$)	$\Delta^{5,7,24}$	Pig tissues
Zymosterol ($C_{27}H_{44}O$)	$\Delta^{8(9),24}$	Yeast
7-Dehydrocholesterol ($C_{27}H_{44}O$)	$\Delta^{5,7}$	Molluscs Mammalian skin
22-Dehydrocholesterol ($C_{27}H_{44}O$)	$\Delta^{5,22}$	Red algae Scallops
Desmosterol ($C_{27}H_{44}O$)	$\Delta^{5,24}$	Barnacles Rat skin Chick embryo Red algae
3β-Hydroxycholest-7-en-6-one ($C_{27}H_{44}O_2$)	Δ^7; 6-oxo	Cactus
Viperidone ($C_{27}H_{44}O_3$)	Δ^7; 9α-OH; 6-oxo	Cactus
Viperidinone ($C_{27}H_{44}O_4$)	Δ^7; 9α, 14α-OH; 6-oxo	Cactus
Cholesterol ($C_{27}H_{46}O$)	Δ^5	Principal animal sterol Red algae Date palm Marine invertebrates
Lathosterol (5α-cholest-7-en-3β-ol) ($C_{27}H_{46}O$)	Δ^7	Molluscs Rat skin Spinal cord
5β-Cholest-7-en-3β-ol ($C_{27}H_{46}O$)	Δ^7; 5β-H	Rat faeces
22-Hydroxycholesterol ($C_{27}H_{46}O_2$)	Δ^5; 22α-OH	Narthecium Ossifragum
Cerebrosterol ($C_{27}H_{46}O_2$)	Δ^5; 22β-OH	Brain

(continued)

Table 1-1 (continued)

Name (Formula)	Structure: substituents in Cholestan-3β-ol (unless specified) (5α unless specified)	Source
Peniocerol ($C_{27}H_{46}O_2$)	Δ^8; 6α-OH	Cactus
Cholestanol ($C_{27}H_{48}O$)	—	Spinal cord
Coprostanol ($C_{27}H_{48}O$)	5β-H	Faeces
14-Dehydroergosterol ($C_{28}H_{42}O$)	$\Delta^{5,7,14,22}$; 24β-Me	Yeast
Ergosterol ($C_{28}H_{44}O$)	$\Delta^{5,7,22}$; 24β-Me	Yeast
5,6-Dihydroergosterol ($C_{28}H_{46}O$)	$\Delta^{7,22}$; 24β-Me	Yeast Grapeseed oil
22,23-Dihydroergosterol ($C_{28}H_{46}O$)	$\Delta^{5,7}$; 24β-Me	Fungi
4α-Methylzymosterol ($C_{28}H_{46}O$)	$\Delta^{8,24}$; 4α-Me	Yeast
24-Methylenecholesterol (Chalinasterol) ($C_{28}H_{46}O$)	Δ^5; 24-Methylene	Pollen Clam, oyster
24-Methylene-5α-cholest-7-enol ($C_{28}H_{46}O$)	Δ^7; 24-Methylene (No 3β-OH)	Starfish
Brassicasterol ($C_{28}H_{46}O$)	$\Delta^{5,22}$; 24β-Me	Rapeseed oil Mussels
Episterol ($C_{28}H_{46}O$)	Δ^7; 24-Methylene	Yeast
Fecosterol ($C_{28}H_{46}O$)	$\Delta^{8(9),24(28)}$ 24-Methylene	Yeast
Ascosterol ($C_{28}H_{46}O$)	$\Delta^{8(9),23}$; 24-Me	Yeast
Cerevisterol ($C_{28}H_{46}O_3$)	$\Delta^{7,22}$; 5α, 6β-diOH	Yeast
Campesterol ($C_{28}H_{48}O$)	Δ^5; 24α-Me	Wheat germ oil Molluscs

(continued)

Table 1-1 (continued)

Name (Formula)	Structure: substituents in Cholestan-3β-ol (unless specified) (5α unless specified)	Source
Lophenol (Methostenol) ($C_{28}H_{48}O$)	Δ^7; 4α-Me	Cactus Rat faeces, tissues
Spongesterol ($C_{28}H_{48}O$)	Δ^{22}; 24α-Me	Sponge
Pollinastanol ($C_{28}H_{48}O$)	9,19-cyclo; 14α-Me	Pollen
Macdougallin ($C_{28}H_{48}O_2$)	Δ^8; 6α-OH; 14α-Me	Cactus
7-Dehydrostigmasterol ($C_{29}H_{46}O$)	$\Delta^{5,7,22}$; 24α-Et	Phellodendron Amoeba
Stigmasterol ($C_{29}H_{48}O$)	$\Delta^{5,22}$; 24α-Et	Calabar bean Soya bean
Poriferasterol ($C_{29}H_{48}O$)	$\Delta^{5,22}$; 24β-Et	Sponge
α-Spinasterol ($C_{29}H_{48}O$)	$\Delta^{7,22}$; 24α-Et	Spinach, etc. Acacia
Chondrillasterol ($C_{29}H_{48}O$)	$\Delta^{7,22}$; 24β-Et	Sponge
Fucosterol ($C_{29}H_{48}O$)	Δ^5; 24-Ethylidene (*cis*)	Brown algae Coconut
Avenasterol ($C_{29}H_{48}O$)	Δ^5 24-Ethylidene (*trans*)	Oat seeds Marine green algae
Sargasterol ($C_{29}H_{48}O$)	Δ^5; 24-Ethylidene (*cis*) 20α-H	Sargassum
Stigmasta-5,25-dienol ($C_{29}H_{48}O$)	$\Delta^{5,25}$; 24α-Et	Momordica charantia L
24-Methylenelophenol ($C_{29}H_{48}O$)	Δ^7; 4α-Me 24-Methylene	Sugar cane
4,4-Dimethylzymosterol ($C_{29}H_{48}O$)	$\Delta^{8,24}$; 4,4-diMe	Yeast
Saringosterol ($C_{29}H_{48}O_2$)	Δ^5; 24-Vinyl; 24-OH	Sargassum
β-Sitosterol ($C_{29}H_{50}O$)	Δ^5; 24α-Et	Soya bean etc.

(continued)

Table 1-1 (continued)

Name (Formula)	Structure: substituents in Cholestan-3β-ol (unless specified) (5α unless specified)	Source
Clionasterol ($C_{29}H_{50}O$)	Δ^5; 24β-Et	Sponge
5α-Stigmast-7-enol ($C_{29}H_{50}O$)	Δ^7; 24α-Et (No 3β-OH)	Wheat germ oil Acacia
5α-Stigmast-22-enol ($C_{29}H_{50}O$)	Δ^{22}; 24α-Et (No 3β-OH)	Bupleurum falcatum L Slime mould
Stigmastanol ($C_{29}H_{52}O$)	24α-Et	Plants
Citrostadienol ($C_{30}H_{50}O$)	Δ^7; 4α-Me 24-Ethylidene (*trans*)	Grapefruit
α_1-Sitosterol (24-Ethylidenelophenol) ($C_{30}H_{50}O$)	Δ^7; 4α-Me 24-Ethylidene (*cis*)	Potato
4β-Methyl-5α-stigmasta-7,24(28)-dien-3β-ol ($C_{30}H_{50}O$)	Δ^7; 4β-Me 24-Ethylidene (*trans*)	Marigold flowers

Table 1-2 The Main Oxysterols Formed from Enzymatic and Non-enzymatic Origin.

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)-Cholest-5-en-3β,26-diol*	Cholest-5-en-3β,7β-diol
(25S)-Cholest-5-en-3β,26-diol*	5α-Cholestane-3β,5α,6β-triol

(continued)

Table 1-2 (continued)

Enzymatic origin	Non-enzymatic origin
(20S)-Cholest-5-en-3β,20-diol†	3β-Hydroxycholest-5-en-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
	(20S)-Cholest-5-en-3β,20-diol

*Bile acid biosynthesis intermediate

†Steroid hormone biosynthesis intermediate

1.1 Bioactivities of Oxysterols

Oxysterols have several *in vitro* and *in vivo* biological effects at the cellular level, illustrating potentially significant biological effects that demand further investigation. The effects that have been reported are: cytotoxicity, gene regulation, atherogenesis, mutagenesis, and carcinogenesis (Hyun et al., 1997).

1.1.1 Oxysterols cytotoxicity and mechanisms of this action

1.1.1.1 Oxysterols cytotoxicity

A number of studies have concerned the cytotoxicity of various oxygenated sterols. But their results can not necessarily be extrapolated to systemic human effects. There is frequently no clear differentiation of toxic actions due to the primary or secondary effects of 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 indicative of cytotoxicity including changes in cell growth, cell viability, cell detachment, plating efficiency of various aspects of morphology, transport of small molecules (i.e., 2-deoxyglucose, uridine, thymidine), protein synthesis, and DNA synthesis.

The conditions of study are also of particular importance. Studies with

cells incubated in chemically defined medium in the absence of added serum or serum lipoproteins provide for reduction in many variables; however, such incubation conditions cannot be considered to be "physiological." In contrast, studies with media containing serum (or even lipoproteins) present an infinitely more complex situation, including the fact that variable amounts of various oxygenated sterols may already exist in the serum (or in the lipoprotein fractions derived therefrom).

Sevanian et al. (1991) reported on the "cytotoxicity" of the 5α,6α- and 5β,6β-epoxides of cholesterol and 5α,6β-diOH-cholesterol in cultured rabbit aortic endotheliar cells. 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. However, it is difficult to interpret the experimentaion. The sterols were added in ethanol (0.5% by volume) to the culture medium. The authors noted that "after the 30 min preincubation in medium, a considerable amount of precipitable material was apparent, particularly when high concentrations of cholesterol epoxides were added."

Chisolm et al. (1994) reported on the cytotoxicity of 7β-hydroperoxycholest-5-en-3ß-ol in human fibroblasts (as measured by the release of ^{14}C into the culture medium after incubation of the cells with [^{14}C]adenine). The concentration of the 7β-hydroperoxide required to give a half-maximal effect was reported to be ~1.4 μM. In further studies from the same laboratory (Colles et al., 1996), 7β-hydroperoxy-cholesterol, under similar experimental conditions, was found to show higher cytotoxicity than 7β-OH-cholesterol, 7-keto-cholesterol, and 5α,6α-epoxy-cholesterol with human skin fibroblasts. The 7β-hydroperoxide was also reported to show higher cytotoxicity than 7β-OH-cholesterol and 7-keto-cholesterol with rabbit

and human aortic smooth muscle cells and with bovine aortic endothelial cells. In the human fibroblasts, the IC_{50} values were as follows: 7β-hydroperoxycholesterol, 1.4 ± 0.8 μM; 7β-OH-cholesterol, 16.3 ± 5.5 μM; 7-keto-cholesterol, 21.6 ± 6.5 μM; 5α,6α-epoxy-cholesterol, 24.8 ± 6.2 μM; and 25-OH-cholesterol, 59 ± 4.4 μM. Oxygenated sterols used in this work were of commercial origin except for the 7β-hydroperoxide that was prepared by chemical synthesis. Chemical characterization of the product was very limited. Under the conditions studied, the 7β-hydroperoxide was reported to show higher cytotoxicity than that caused by either lysophosphatidylcholine or 4-hydroxynonenal. These findings are of importance in considerations of the cytotoxicity induced by oxidized low density lipoprotein and its possible involvement in the arterial injury induced by oxidized low density lipoprotein. The increased release of [^{14}C]adenine induced by the 7β-hydroperoxycholesterol in skin fibroblasts was suppressed by the addition of known inhibitors of peroxidation of lipids, i.e., vitamin E, *N,N'*-diphenyl-1,4-phenylenediamine, and deferoxamine mesylate. It is noteworthy that none of these agents blocked the reported cytotoxicity induced by 7β-OH-cholesterol or 7-keto-cholesterol.

Very recently, Kölsch et al. (1999) reported neurotoxic effects of 24-OH-cholesterol (C-24 stereochemistry not specified) on SH-SY5Y human neuroblastoma cells. Cell viability was not reduced at concentrations of 0.1-1 μM 24-OH-cholesterol, but less than half the cells survived at 10-50 μM concentration. Despite the high 24-OH-cholesterol levels required for toxicity, the authors concluded that "in a physiological concentration range, 24-OH-cholesterol damages neuronal cells."

Clare et al. (1995) studied the effects of a number of oxysterols on

human monocytes-macrophages maintained in a medium (RPMI 1640) containing 10% lipoprotein-deficient FCS. Cytotoxicity (increased cell permeability) was assayed following the release of ^{3}H from cells preincubated with [^{3}H]adenine. The specific conditions used for addition of the oxysterols (from ~ 1.2 to 249 μM) to the cells were not presented. The order of potency in increasing cell permeability was 26-OH-cholesterol > 7-oxygenated sterols (7β-OH-cholesterol, 7-keto-cholesterol, 7α-OH-cholesterol) > 25-OH-Cholesterol. Simultaneous addition of cholesterol was reported to show significant protection against the cytotoxicity of 25-OH-cholesterol and 26-OH-cholesterol; however, cholesterol had little or no effect on [^{3}H]adenine release from cells treated with 7-keto-cholesterol, 7α-OH-cholesterol, or 7β-OH-cholesterol. The authors indicated that the order of potency noted above does not correspond to the order of potency of the same oxysterols in lowering the level of HMG-CoA reductase activity in mouse L cells (Taylor et al., 1984) in which 25-OH-cholesterol and 26-OH-cholesterol were considerably more potent than the 7-oxygenated sterols. It should be noted that the order of potency with regard to cytotoxicity may vary with different cell types. For example, Christ et al. (1993) reported that 25-OH-cholesterol was more potent than 7β-OH-cholesterol in mouse thymocytes and in mouse lymphoma cells in reducing cell viability as measured by Trypan blue exclusion assay or MTT assay. These studies were carried out in media containing 10% heat-inactivated FCS. Liu et al. (1997) observed that 25-OH- cholesterol at 6.2, 12.4, and 24.9 μM (but not at 2.5 μM) caused a dose-dependent increase of the leakage of lactate dehydrogenase from human monocytes incubated in RPMI medium containing delipidated FCS. In the same study a similar effect of 25-OH-cholesterol was not observed with human macrophages.

Guyton et al. (1995) described the effects of 7-keto-cholesterol and 7β-OH-cholesterol on cultured smooth muscle cells from porcine aorta. Studies were carried out with DMEM with lipoprotein-deficient serum (usually 0.4%) for 72 h. Remaining adherent cells were released (trypsin-EDTA) and counted. 7-Keto-cholesterol (added in ethanol) was studied at concentrations of 0.25 to 5 μM. 7β-OH-cholesterol was studied at 0.5 and 1.25 μM. Mevalonate (100 μM) did not reverse the "toxic" effects (reduced number of adherent cells) of the oxysterols. The authors noted that higher concentrations of mevalonate were not used since, at 1 and 20 μM, variable adverse effects on cell growth and even viability were observed. Mevalonate (100 μM) also had no effect on the "toxicity" (reduced cell number) caused by oxidized LDL. Lizard et al. (1997) studied the effects of a number of oxygenated sterols on the growth of bovine aortic endothelial cells in media containin 10% FCS. The various oxysterols caused a dose-dependent decrease in the number of adherent cells after a 48-h incubation. The results appear to reflect the same or similar experimentation as described by the same group previously (Lizard et al., 1996). Mean concentrations reported to cause a 50% reduction in the number of adherent cells at 48 h were as follows: 7β-OH-cholesterol, 23 μM; 7-keto-cholesterol, 34 μM; 19-OH-cholesterol, 72 μM; and 5α,6α-epoxy-cholesterol, 99 μM. 25-OH-cholesterol was considerably less potent than the above oxysterols and did not cause a 50% reduction at the highest concentration tested (199 μM). Cholesterol was reported to have no effect at all at the concentrations tested (including 207 μM). The lowest concentrations of the oxysterols reported to give significant reduction in the number of adherent cells were as follows: 7β-OH-cholesterol, 12 μM; 19-OH-cholesterol, 25 μM; 5α,6α-epoxy-cholesterol, 25 μM; 25-OH-cholesterol, 25 μM; and 7-keto-cholesterol, 50 μM.

Duncan and Buckingham (1980) observed that 25-OH-cholesterol (0.62 μM) or 20α-OH-cholesterol (1.24 μM), at concentrations at which very substantial inhibition of sterol synthesis from labeled acetate (-88%) was observed, showed no effect on the uptake of labeled 2-deoxy-*D*-glucose in HeLa cells. Marinovich et al. (1995) reported 26-OH-cholesterol and 25-OH-cholesterol had little or no toxic effects in mouse epidermal cells in short-term incubations in media without added serum or lipoproteins. For example, 26-OH-cholesterol and 25-OH-cholesterol had little or no effect on cell growth (as measured by cellular protein) or on leakage of lactate dehydrogenase from the cells at concentrations of the oxysterols up to and including 200 μM (with incubation times of 2, 6, and 24 h). Similarly, 25-OH-cholesterol and 26-OH-cholesterol had little effect on protein synthesis (incorporation of [^{3}H]leucine into cellular protein). Modest decreases were observed with 26-OH-cholesterol (at 100 and 200 μM) in 6-h incubations but not at 50 and 100 μM in 24-h incubations. 25-OH-cholesterol caused a modest decrease at 100 μM (but not at 50 μM) in 24-h incubations. In contrast to the 25- and 26-hydroxysterols, 26-aminocholesterol showed significant effects on cellular protein, protein synthesis, and the leakage of lactate dehydrogenase. The lack of effects of the oxygenated sterols could be a special feature of these mouse epidermal cells. More likely, the lack of effects of the 25- and 26-hydroxysterols is attributable to their lack of uptake by the cells under the conditions studied, i.e., direct addition of the oxysterols (in ethanol) to media containing no protein (serum, delipidated serum, lipoprotein-deficient serum). In the absence of protein in the media, it would seem unlikely that the oxysterols are present in other than a particulate form under such conditions. De Caprio et al. (1992) have reported the precipitation of 26-OH-cholesterol upon its addition in ethanol at a level of 6 μM in DMEM. The study of Marinovich et al.

(1995) presents no evidence that the oxysterols entered the cells under the study conditions. For example, no direct studies of sterol uptake (or studies of effects on HMG-CoA reductase activity) were made. Marinovich et al. (1995) also reported that 25-OH-cholesterol (20 μM) reduced leakage of lactate dehydrogenase in mouse epidermal cells caused by 26-amino-cholesterol or Triton X-100 in shortterm (2h) experiments. However, the results of this experimentation should be evaluated in light of the considerations presented above as well.

Ares et al. (1997) reported on the cytotoxicity (as measured by MTT assay) of 25-OH-cholesterol (12.4 or 25 μM) in human aortic smooth muscle cells after 24 or 48 h of incubation in DMEM-F-12 medium supplemented with 5% FCS and antibiotics. Addition of the cytokines TNF-α and interferon-γ increased the toxicity of the 25-hydroxysterol.

Chang and Liu (1997) studied the effects of selected oxysterols on the viability of cultured PC12 (tumor cells originating from an adrenal pheochromocytoma) and on "neuronal PC12 cells" (PC12 cells treated with nerve growth factor). In studies carried out with medium containing high levels of FCS and horse serum, decreased viability (as measured by MTT assay) was observed in the PC12 cells with each of 25-, 7β-, 22(*R*)-, 22(*S*)-, and 19-OH-cholesterol. 25-OH-cholesterol and (22*R*)-22-OH cholesterol appeared to be most potent. The neuronal PC12 cells appeared to be less sensitive to the effects of the oxysterols on cell viability. Under the conditions studied, 25-OH-cholesterol did not appear to cause a decrease in the neurite outgrowth induced by nerve growth factor. Chang et al. (1998) also studied the cytotoxicity induced by 25-OH-cholesterol in neuronal PC12 cells; vitamin E, but not ascorbic acid or aurinetricarboxylic acid, reduced the cytotoxicity caused by the 25-OH-cholesterol (25 μM). Chang and Liu (1998) also studied the

effects of selected oxysterols on the viability (as measured by MTT assay) of cultured rat cerebellar granule cells. Oxysterols resulting in decreased viability included 7β-OH-cholesterol, 7-keto-cholesterol, 25-OH-cholestrol, 19-OH-cholesterol, and the 22*R*- and 22*S*-isomers of 22-OH-cholesterol. 25-OH-cholesterol (6.2 μM) caused a 34% decrease in viability in a 48-h experiment. 7β-OH-cholesterol (25 μM) caused a 50% decrease in viability and was more potent than 7-keto-cholesterol. The unnatural 22*S*-isomer of 22-OH-cholesterol was reported to be considerably more potent than its 22*R*-isomer. 19-OH-cholesterol showed only modest activity, with ~ 40% decrease in viability at 50 μM. Aurintricarboxylic acid and vitamin E, but not ascorbic acid, were partially effective in reduction of the decreased viability induced by 25-OH-cholesterol. Chang and liu (1998), using the same methodology, reported similar findings using rat neuroretinal cells. However, with these cells, 19-OH-cholesterol showed relatively high potency (~ 39% decrease in viability at 6.2 μM).

1.1.1.2 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 membranes, perturbating permeability, stability and other membrane properties.

HMG-CoA reductase has been implicated as regulating *de novo* cholesterol biosynthesis, transforming 3-hydroxy-3-methylglutaryl CoA

into mevalonate. The enzyme activity is regulated by phosphorylation (phosphorylated enzyme inactive) and by cholesterol feedback regulation. Liver HMG-CoA reductase activity decreases if dietary cholesterol is increased. Some studies of short duration (4-6 h) 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 agents for this inhibitory effect (Kandutsch et al., 1978).

This inhibitory effect has been observed for some oxysterols, mainly those containing a free hydroxy group at C-3 (Kandutsch and Chen, 1973); 3β-esters of these oxysterols show decreased inhibitory activity (Kandutsch and Shown, 1981; Parish et al., 1979). However, Miller et al. (1987) reported a non-C-3-hydroxysterol showing this activity [5α-cholest-8(14)-en-15-one]. The intensity of the inhibitory effect, as a rule, increases when other oxygenated groups of the oxysterol molecule are far from the C-3 hydroxyl group; 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 the ring B show this inhibitory effect (Brown and Goldstein, 1974; Kandutsch and Chen, 1973). The presence of a third oxygenated group in the molecule does not seem to increase this effect. Thus, cholestane-3β,5α,6β-triol and 5α-cholestane-3β,17α,20α-triol have less inhibitory effect than 3β,6β- and 3β,20-diols (Kandutsch and Chen, 1978). On the basis of the foregoing effects, some oxysterols have been tested as drugs that could be used in control of hypercholesterolaemia. The studies have focused on derivatives with a second oxygenated group in the ring D or side-chain. 3β-Hydroxy-5α-cholest-8(14)-en-15-one has been found to be a good

inhibitor *in vitro* and displays a powerful effect in rats and monkeys. This compound also inhibits cytosolic enzymes, acetoacetyl CoA thiolase and HMG-CoA synthase (Smith and Johnson, 1989).

The mechanisms of the action through which oxysterols inhibit HMG-CoA reductase activity is a controversial subject. Several mechanisms have been suggested, and participation of more than one is probable depending on the oxysterols and kind of cell considered. However, activity of the isolated enzyme is not inhibited by oxysterols which suggests that integrity of cellular membrane is necessary for this effect (Smith and Johnson, 1989). Four most probable mechanisms were reported to interpret the inhibition action. They were as follows: inhibition of enzyme synthesis; degradation of enzyme; modification of enzyme structure; and binding of oxysterols to cystolic proteins.

It has been demonstrated that inhibition of HMG-CoA reductase by lipoproteins occurs through a decrease in enzyme synthesis (Bell et al., 1976; Brown and Goldstein, 1974; Sinensky et al., 1979). Some mechanisms have been proposed to explain this synthesis inhibition. Oxysterols could affect transcription and translation of mRNA for HMG-CoA reductase, and consequently inhibit its activity (Smith and Johnson, 1989; Taylor, 1992). Chen et al. (1982) found that treatment with RNA synthesis inhibitors (cordycepin or actinomycin D), in Chinese hamster ovary cells, did not affect inhibition of 25-OH-cholesterol on HMG-CoA reductase. However, when these cells were incubated with the same drugs there was a decrease in inhibitory power of 25-OH-cholesterol and a similar decrease if 25-OH-cholesterol was added together with a protein synthesis inhibitor (cycloheximide or puromycin); the effect of 25-OH-cholesterol disappeared when cells were preincubated with one of these drugs. Further studies (Choi et al., 1993)

showed that cycloheximide added in conjunction with 25-OH-cholesterol to baby hamster kidney cells cultured in lipoprotein-free medium increased levels of HMG-CoA reductase mRNA, but did not block oxysterols-mediated suppression of transcription. In addition, cycloheximide added to the same cells grown in lipoprotein-containing medium increased mRNA levels, with no comparable increase in transcription. These results indicated that cycloheximide stabilizes HMG-CoA reductase mRNA in the presence of oxysterols and suggest that a protein regulator is required for oxysterols-mediated posttranscriptional suppression of HMG-CoA reductase mRNA. In addition, Larsen et al. (1994) showed that seco-oxysterols analogues and 25-OH-cholesterol reduced levels of mRNA for HMG-CoA reductase and stimulated low-density lipoprotein receptor in human hepatoma cells, demonstrating regulation of HMG-CoA reductase gene expression without concomitant attenuation of low-density lipoprotein receptor activity.

Chang et al. (1981) demonstrated, in Chinese hamster ovary cells, that changing a rich-lipid media to a poor one increased the half-life of HMG-CoA reductase. The authors explained that change by a decrease in enzyme degradation. There are also some studies showing that this half-life is different from inhibition produced by lipoproteins or by oxysterols. (Bell et al., 1976; Brown and Goldstein, 1974). Taylor (1992) observed, in Chinese hamster ovary cells, oxysterols-activated degradation of HMG-CoA reductase and that the effect correlated with inhibition of HMG-CoA synthase.

Beirne et al. (1977) observed that 25-OH-cholesterol was able to reduce HMG-CoA reductase activity to 60% in hepatoma tissue culture cells, similar to the proportion of catalytically inactive form of the enzyme

(50%). The authors suggested that an enzyme structural change could be responsible. Different structural modifications were proposed. Firstly, a change in active site; secondly, a more extensive modification involving synthesis leading to production of an inactive form. On the other hand, there is an important controversy concerning the mechanism that involves action of oxysterols on enzyme phosphorylation. Three enzymatic systems are involved in this phosphorylation: a bicyclic cascade system consisting of reductase kinase and reductase kinase kinase, protein kinase C and Ca^{2+}/calmodulin-dependent reductase kinase. It appears that oxysterols could participate in regulation of the bicyclic system and Ca^{2+}/calmodulin complex formation. Regulation of this complex formation could be due to influence of oxysterols on Ca^{2+} flux through cellular membrane.

It is probable that oxysterol binding to some cytosolic proteins could be one of the initial steps in regulation of cholesterol biosynthesis. The formation of the oxysterols/protein complex has been observed by several authors in different cellular models. It was observed that these proteins showed a high affinity for oxysterols which correlated with their inhibitory potency on HMG-CoA reductase (Astruc et al., 1985; Kandutsch and Thompson, 1980; Sinensky and Mueller, 1981). However, its function in maintaining cholesterol homeostasis has not been determined. One of the most interesting studies in this field is that of Taylor et al. (1984) in which 47 oxysterols were studied to check correlations existing between their cytosol protein-binding activity and inhibition of enzyme. A very good correlation was obtained for 35 of these oxysterols. However, 12 of the studied oxysterols showed poor binding activity in relation to their high inhibitory potency. Other works reported that this binding process is specific, reversible, and saturable

(Gibbons, 1983; Smith and Johnson, 1989). These proteins present low affinity for cholesterol and are different from proteins binding steroid hormones. Therefore, Patel and Thompson (1990) isolated and characterized one of those proteins from human liver cells and found that its binding to oxysterols was competitively inhibited by other oxysterols but not by cholesterol and steroid hormones. Other authors characterized, purified, and cloned these proteins in different cellular models (Srinivasan et al., 1993). Similar binding was found between cytosolic proteins of different cells (human lymphocytes and rat embryo fibroblasts) and oxysterols that are active in inhibiting DNA biosynthesis (Smith and Johnson, 1989).

Endogenous and exogenous cholesterol is incorporated into membranes and is essential. In *in vitro* assays, oxysterols can also be incorporated into membranes, substituting for cholesterol (Mahfouz et al., 1995). As a consequence, a change is observed in fluidity, permeability and stability of the cellular membrane (Figure 1-2), as well as in cellular growth, morphology and viability. Oxysterols in membranes affect protein and phospholipid structure. 7α-OH-cholesterol in human erythrocytes increases protein helical structure (Smith and Johnson, 1989). In liposomes, C-7 oxygenated sterols decrease packing ability of phospholipid non-polar chains (Benga et al., 1983; Rooney et al., 1986). However, this effect has not been observed for 20-OH-cholesterol, 25-OH-cholesterol, 22(*S*)-OH-cholesterol, and epimers of 23-OH-cholesterol (Rooney et al., 1986).

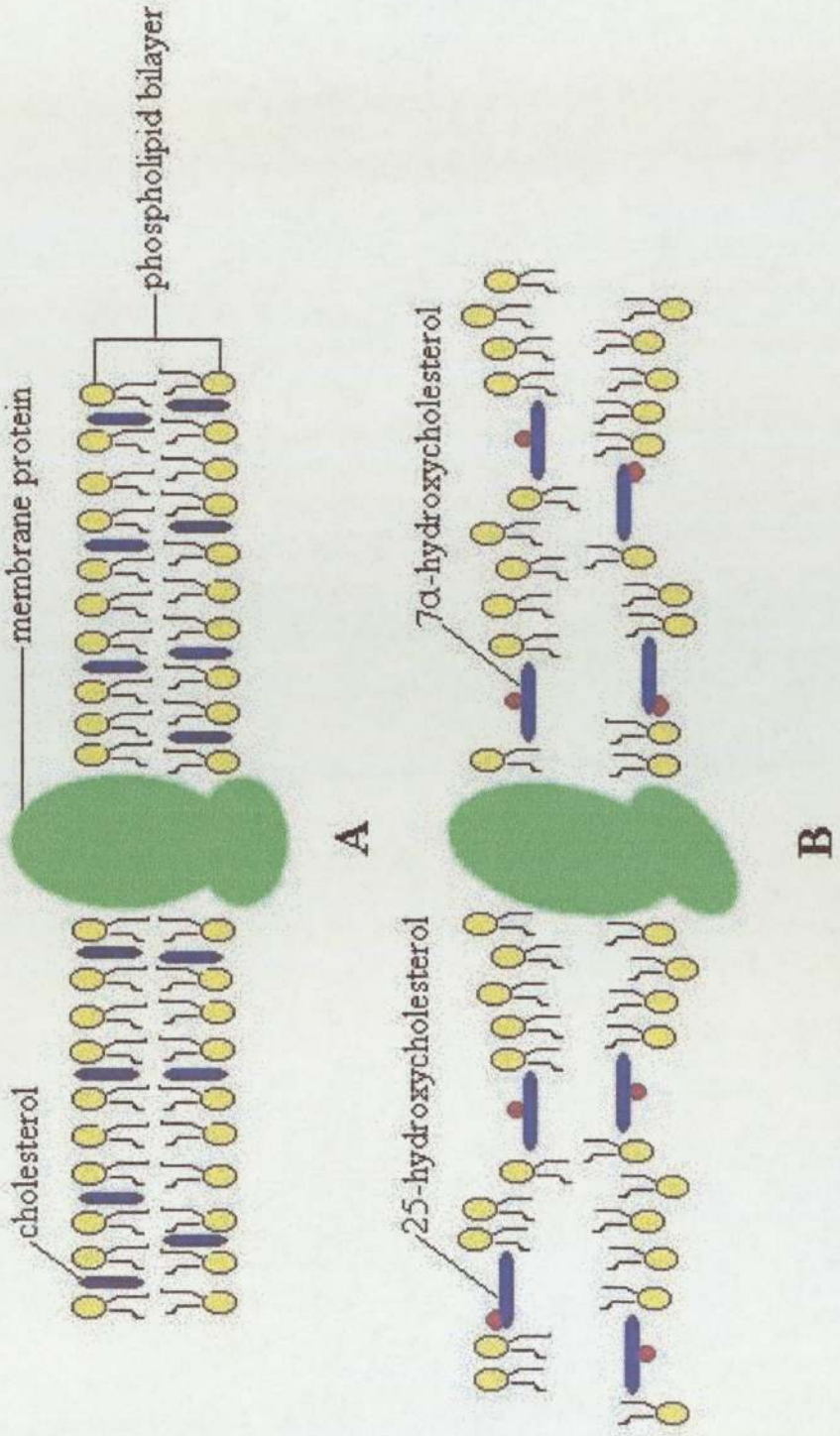
Oxysterols affect hexose uptake and sodium/potassium adenosine triphosphatase (Na/K-ATPase) activity. Cholestane-3β,5α,6β-triol, but not 25-OH-cholesterol, affects hexose uptake; both affect Na/K-ATPase activity. To explain these different effects it was suggested that

cholestane-3β,5α,6β-triol is incorporated into membrane, while 25-OH-cholesterol acts only by inhibiting cholesterol synthesis (Peng and Morin, 1987).

Oxysterols also modify calcium ion (Ca^{2+}) flux. It is well known that the cholesterol/phospholipid ratio determines Ca^{2+} flux and, therefore, it appears likely that oxysterols can modify it. It has been observed that the influx of Ca^{2+} is increased in human erythrocytes by cholestane-3β,5α,6β-triol, 22(*S*)-OH-cholesterol, and 26-OH-cholesterol and decreased by 7β-OH-cholesterol, 7-keto-cholesterol, 20-OH-cholesterol, and 25-OH-cholesterol (Neyses et al., 1985; Stimpel et al., 1985). However, in rat hepatocytes and platelets, Ca^{2+} influx is increased by 7-keto-cholesterol, cholestane-3β,5α,6β-triol and 26-OH-cholesterol, while its efflux is increased by 5α,6α- and 5β,6β-epoxy-cholesterol (Holmes et al., 1986; Sevanian and Peterson, 1986). The suggested machanism for this effect is that permeability change induced by oxysterols also affects Ca^{2+} channels. Studies developed on liposomes revealed that oxysterols incorporation could alter the potential energy barrier to inorganic ion conduction (Lau and Das, 1995).

The immunosuppressive effects of oxysterols are postulated due to their replacing of cholesterol in membrane, such as the inhibition of cytolytic T lymphocyte activity and natural killer cell-mediated cytotoxicity (Kucuk et al., 1992), in these cases oxidation in the cholesterol nucleus which is situated closer to the phospholipid headgroups at the lipid bilayer-aqueous interface results in a more profound effect on the plasma membrane physical structure or protein activity (Moog et al., 1991).

Fig. 1-2 Structural changes induced by oxysterol incorporation into the membrane.



With respect to morphological changes, electron microscopy studies show morphological changes in bovine plateles and erythrocytes induced by 7β-OH-cholesterol (Saito et al., 1985) and in human glioma cells by 25-OH-cholesterol (Maltese et al., 1981).

1.1.2 Other bioactivities of oxysterols

1.1.2.1 Gene regulation

Cholesterol is an essential constituent of all mammalian cell membranes, and its availability is a prerequisite for celluar growth and other functions. *De novo* cholesterol synthesis is required for DNA synthesis, cell growth and cell proliferation. Quiescent cells synthesize little cholesterol. When cells are stimulated to proliferate, a cycle of sterol synthesis can be detected in the G1 phase of the cell cycle, total blockage of HMG-CoA reductase by oxysterols such as 25-hydroxycholesterol leads to inhibition of DNA synthesis and of cellular prolifc..tion (Kandutsch et al., 1978; Chen, 1984). Most cells can take in exogenous cholesterol from low-density lipoproteins through its receptor and suppress the endogenous cholesterol synthesis to some extent; however, it can not totally replace endogenous cholesterol to support cell growth and other function (Quesney-Huneeus et al., 1983; Reimann et al 1991).

It was often found that the cholesterol feedback inhibition mechanism which regulates cholesterol synthesis (Brown and Goldstein, 1980) is lost in malignant transformation, such as human colon tumour cells, unlike

normal colon cells and fibroblasts, exhibit a high endogenous cholesterol synthesis which low-density lipoproeins can not regulate (Cerda et al., 1995). Cancer cells seem to require an increase in the concentrations of cholesterol and of cholesterol precursors, so cholesterol synthesis inhibition maybe a selectivity approach to inhibit tumor cell growth (Labit-Le Bouteiller et al., 1998; Kishinaka et al., 1998).

Balanced cholesterol metabolism in mammalian cells is maintained through the feedback regulation of key proteins involved in its biosynthesis and cellular uptake. A major control point is at the level of transcription for genes that encode important proteins, HMG-CoA reductase and low-density lipoprotein receptors, of both processes (Goldstein and Brown, 1990; Osborne, 1995). Oxysterols are regulators of *de novo* cholesterol synthesis and metabolism. The regulation of gene expression by oxysterols is involved in cholesterol and lipid metabolism (Wolf, 1999). Recent studies showed that oxysterols are both positive and negative regulators of gene expression. As positive effectors, they bind to and activate the nuclear receptors liver X receptors (Janowski et al., 1996), which increase transcription of the cholesterol 7α-hydroxylase gene, the rate-limiting enzyme in the formation of bile acids (Lehmann et al., 1997). This activation stimulates the conversion of cholesterol into bile acids (Russell and Setchell, 1992). Excessive dietary cholesterol leads to increased oxysterol formation. Oxysterol binds to liver X receptors and thereby induces transcription of cholesterol 7α-hydroxylase, thus increasing the removal of cholesterol as bile acids (Brown and Goldstein, 1997). As negative regulators, oxysterols suppress the cleavage of two transcription factors known as sterol regulatory element binding protein-1 and sterol regulatory element binding protein-2 (SREBP-1 and SREBP-2) (Brown and Goldstein, 1997). These proteins

are synthesized as inactive precursors in the membrane compartment of the cell. When intracellular cholesterol levels decline, SREBPs are proteolytically cleaved to release amino-terminal fragments that migrate to the nucleus where they bind with a high affinity to sterol regulatory element (SRE), thus activating the transcription of a network of genes involved in cholesterol synthesis and supply (Brown and Goldstein, 1997). This activation restores intracellular cholesterol levels (Horton and Shimomura, 1999). An interaction between SREBPs and another membrane-embedded protein, SREBP cleavage activating protein, is required for the cleavage to occur. Recent studies showed that sterols such as 25-hydroxycholesterol could inhibit the cycling of SREBP cleavage activating protein between endoplasmic reticulum and Golgi (Nohturfft et al., 1999).

The SREBPs are thought to ensure the "concert regulation of sterol-sensitive genes, particularly that of HMG-CoA reductase and low-density lipoprotein receptor, and that of HMG-CoA reductase, squalene synthase (Kisseleva et al., 1999, Vallett et al., 1996)." The HMG-CoA reductase is mediated by oxysterols with ring D and side chain hydroxy groups as the most potent, and the 6,7 oxygenated sterols (Guardiola et al., 1996).

Some reports show that cells independently regulate cholesterol uptake from its biosynthesis, and different oxysterols can repress HMG-CoA reductase gene expression *via* different mechanisms, the 25-hydroxycholesterol also can decrease the gene expression of the low-density lipoprotein receptor by a dissociated way (Kisseleva et al., 1999), but now, the regulation of low-density lipoprotein receptor is far from clear.

The enzymatically synthesized oxysterols that are secreted from tissues represent a mechanism of reverse cholesterol transport by which excess sterol is returned from the periphery to the liver for catabolism. They transport through high-density lipoproteins and low-density lipoproteins (Babiker and Diczfalusy 1998). These oxysterols include 24(S)-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol. The sterol 27-hydroxylase is a mitochondrial cytochrome P-450s enzyme (Cali et al., 1991). Brain, which can not transfer excess cholesterol from cells circulating lipoprotein particles due to blood-brain barrier, uses cholesterol 24-hydroxylase as a mediator of cholesterol homeostasis, and the 24(S)-hydroxycholesterol is readily secreted from the central nervous system into the plasma (Lund et al., 1999; Lütiohann et al., 1996).

These side chain oxygenated sterols generated *in vivo* serve as a gene regulator. 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol and 24(*S*)-hydroxycholesterol show significant activation on liver X receptors, while 25-hydroxycholesterol and 27-hydroxycholesterol have no effect on them. The (24*S*),25-epoxycholesterol may function as endogenous activators of liver X receptors in liver and 24(*S*)-hydroxycholesterol do the same in brain (Lehmann et al., 1997). Potent gene-regulate properties through SREBPs are ascribed to 25-hydroxycholesterol. Unlike other sterol hydroxylases, cholesterol 25-hydroxylase is not a cytochrome P-450, but it is a member of a small family of microsomal enzymes that utilize diironcofactors to catalyze the hydroxylation of hydrophobic substrates (Lund et al., 1998). 25-Hydroxycholesterol represses cholesterol synthesis and the provision of transcription factor that is required for the expression of lipid metabolizing genes. These different hydroxylases can be modulated separately (souidi et al., 1999).

Binding to liver X receptors is structural specific. Position-specific

mono-oxidation of the sterol side chain is requisite for liver X receptor high-affinity binding and activation. Enhanced binding and activation can also be achieved through the use of 24-oxo ligands that act as hydrogen bond acceptors in the side chain. In addition, introduction of an oxygen atom on the sterol molecule ring B results in a ligand with liver X receptor-α-subtype selectivity. These results support the hypothesis that naturally occurring oxysterols are physiological ligands for liver X receptors and show that a rational, structure-based approach can be used to design potent liver X receptor ligands for pharmacological use (Janowski et al., 1999).

Another receptor, the steroidogenic factor-1, which was originally thought to be an orphan receptor, is postulated using oxysterols as activators (Christenson et al., 1998; Lala et al., 1997). The steroidogenic factor-1 is a key transcription factor controlling the expression of steroidogenic enzymes. The steroidogenic acute regulatory protein plays an essential role in steroid hormone synthesis by enhancing the delivery of cholesterol to the inner mitochondrial membrane, where the cholesterol side-chain cleavage enzyme system resides. 22-Hydroxycholesterol, 25-hydroxycholesterol, 27-hydroxycholesterol can modulate the steroidogenic factor-1 mediated enzymes like the steroidogenic acute regulatory protein.

The details of the gene regulation by oxysterols are far from clear. Besides the liver X receptors and SREBPs, a variety of cholesterol derivatives, including steroid hormones and vitamin D, exert effects on gene expression through interactions with members of the nuclear receptor superfamily (Mangelsdorf et al., 1995). Members of this family function as ligand-activated transcription factors by binding to short stretches of DNA, termed hormone response elements, present in the

regulatory regions of target genes. In addition to the nuclear receptors with known ligands, this superfamily includes a large number of structurally related mumbers that contain DNA binding domains and putative ligand binding domains but lack identified ligands, the so-called "orphan receptors". Other biological effects of oxysterols such as effects on DNA synthesis, cell growth and cell proliferation maybe mediated by liver X receptors and other orphan receptors, related to cholesterol metabolism or not (Lehmann et al., 1997).

1.1.2.2 Atherogenesis

Some studies have indicated that oxysterols may be important in the production of atherosclerotic lesions in animals. However, other studies have claimed that oxysterols formed from cholesterol are less atherogenic than cholesterol. Studies of this important matter are actually quite limited, both in number and experimental design.

In 1968, Cook and MacDougall (1968) reported the production of atherosclerotic lesions in aortas of male NZW rabbits after dietary administration of 5α,6β-diOH-cholesterol. The sterol composition of the basal diet was not given. However, it was stated that the sterols present in the diet were "mainly phytosterols". This frequently cited study was limited by the absence of data regarding the characterization and purity of the triol and an experimental design that involved variable lengths of administration (27-350 days) of the triol (0.1% in basal diet or ~30 mg/kg body weight/day) to the individual experimental animals and a very small number of control animals (which also varied in the duration of

consumption of the basal diet). Imai et al. (1976) reported that administration of a mixture containing cholesterol enriched with contaminants was associated with electron microscopic lesions (evidence of smooth muscle injury) in aortas of rabbits (at 24 h after the administration by gavage of the sterol mixture in an aqueous gelatin suspension). The authors also carried out long-term studies of the effects of gavage administration of the above mixture as a suspension in aqueous gelatin at a frequency of three times per week. The concentrate (or purified cholesterol) was administered initially at a single dose of 100 mg/kg and later at 25 mg/kg. The different groups of rabbits were given different dose ranges or different lengths of the administration period. In all of these studies, serum levels of cholesterol (and of total protein, calcium, and phosphorus) were reported to have been within normal range with no differences between the various groups. A subsequent publication by the same group (Taylor et al., 1979) reviewed and extended their previous study. In this report, the authors made the following statements relative to the atherogenicity of cholesterol: "Our work strongly suggested that pure cholesterol (either endogenously synthesized or chemically isolated pure cholesterol) is not atherogenic. However, when cholesterol becomes a mixture of cholesterol plus spontaneously produced toxic derivatives which develop in pure cholesterol and dehydrated cholesterol containing foods stored in air at room temperature (the compositions are as following: cholesterol, 38.0%; 25-OH-cholesterol, 13.4%; 7-keto-cholesterol, 12.9%; 7α- and 7β-OH-cholesterol, 6.7%; 5 and 7 hydroperoxides of cholesterol, 3.8%; 5α,6β-diOH-cholesterol, 5.1%; and others, 21.1%) it may be highly atherogenic."

Toda et al. (1981) reported that "force feeding" of 7-keto-cholesterol (10

mg/day for 2 wk, 20 mg/day thereafter) as an emulsion in corn oil to female chicks for 4 or 8 wk was associated with evidence of smooth muscle degeneration in the abdominal aorta. Little changes were observed in ascending aorta. Feeding of a high-cholesterol (1% in diet) diet did not result in changes in the abdominal aorta similar to those in the 7-keto-cholesterol-treated birds. Matthias et al. (1987) reported that intragastric administration of 5α,6β-diOH-cholesterol in olive oil to male Wistar rats led to the development of microscopic changes in the aorta. In contrast to the above studies, Higley et al. (1986) reported that a mixture of oxidation products of cholesterol was markedly less atherogenic in rabbits than was cholesterol. The oxygenated sterol mixture was reported to have the following composition (based on HPLC analysis): cholesterol, 0.06%; 25-OH-cholesterol, 2.0%; 7-keto-cholesterol, 26.0%; 7α-OH-cholesterol, 4.5%; 7β-OH-cholesterol, 5.3%; 7α-hydroperoxycholest-5-en-3β-ol, 18.2%; 5α,6α-epoxy-cholesterol, 24.0%; 5β,6β-epoxy-cholesterol, 17.0%; and unknown, 2.9%. The purified cholesterol and the oxygenated cholesterol mixture were, after heating in corn oil, added to a semipurified rabbit diet to give a diet containing 2% corn oil by weight. Control animals received the diet containing added corn oil (2%). Experimental animals received the diets corresponding to a dosage of 166 mg/kg/day of either purified cholesterol or the oxygenated cholesterol mixture. After administration of the diets for 11 wk, the animals (n = 5 in each group except for one death in the control group) were evaluated with regard to severity of atherosclerosis. The cholesterol-fed animals were reported to show a much larger number (6-fold) of arterial lesions than the groups receiving the oxysterol mixture. In addition, the results of microscopic examination of the arterial lesions were reported to demonstrate a significantly greater magnitude of lesions in the cholesterol-fed group relative to the animals treated with the

oxygenated cholesterol mixture.

In 1998, Staprans et al. (1998) reported that oxidized cholesterol accelerated the development of atherosclerotic lesions in rabbits fed a cholesterol-enriched diet. NZW rabbits were fed (for 12 wk) either a diet containing 0.33% cholesterol (n = 13) or the same diet in which ~5% of the cholesterol was oxidized (n = 13). The cholesterol oxidation products were listed as containing the following: 7-keto-cholesterol, 42%; 7β-OH-cholesterol, 20%; 7α-OH-cholesterol, 7%; 5β,6β-epoxy-cholesterol, 16%; 5α,6α-epoxy-cholesterol, 12%; 25-OH-cholesterol, 3%; and unidentified, 48%. After feeding the two diets for 12 wk, the percentage lesion areas in aorta were 28.5±4.9% and 57.1±4.8% for the control and oxidized cholesterol groups, respectively. At the end of the experiments there were no significant differences in the levels of total serum cholesterol, β-very low-density lipoprotein cholesterol, low-density lipoprotein cholesterol, and high-density lipoprotein cholesterol in the two groups. The levels of 7α-OH-cholesterol, 7β-OH-cholesterol, and 5α,6α-epoxy-cholesterol (but not 5β,6β-epoxy-cholesterol) in fasting serum β-very low-density lipoprotein were reported to be significantly higher in the oxidized cholesterol group. No significant differences were reported for 7β-OH-cholesterol, 5α,6α-epoxy-cholesterol, 5β,6β-epoxy-cholesterol, and 7-keto-cholesterol in fasting serum low-density lipoprotein in the two groups. The levels of 7β-OH-cholesterol, 5β,6β-epoxy-cholesterol, and 7-keto-cholesterol (but not 7α-OH-cholesterol and 5α,6α-epoxy-cholesterol) in liver were reported to be significantly higher in the oxidized cholesterol group.

The atherosclerosis process begins by a modification of the barrier function of the vascular endothelium, leading to penetration of low

density lipoproteins and platelets. Growth factors are released, which induce proliferation and migration of subintimal smooth muscle cells to affected endothelium area. Monocytes invade this area and become macrophages. Smooth muscle cells and macrophages ingest and degrade low-density lipoprotein, resulting in foam cells. The cholesterol coming from low-density lipoprotein is stored inside and between these foam cells. Further accumulation of cholesterol and its esters, proliferation of smooth muscle cells, collagen and elastin synthesis and calcium deposites lead to formation of fibrous atheromatous plaques (Ross, 1986). The start of this complex process is one of the most controversial points, especially in relation to agents that produce endothelium lesion and modify, as a result, selectivity of the endothelium as a barrier. This injury can be produced by chemical or mechanical action and may include viruses, hypertension and increased plasma low-density lipoproteins. Nicotine, bacterial toxins, lipases, proteasea, kinins, histamine and oxidized lipids (including oxysterols) are also reported as harmful agents to the endothelium (Hubbard et al., 1989). Various oxysterols activities have been checked *in vitro* (Peng et al., 1991), namely: their effect on vascular permeability, and on prostaglandin synthesis and platelet aggregation; their cytotoxicity on smooth muscle cells; their ability to modify low-density lipoprotein receptor functionality; the effect on accumulation of cholesteryl ester and formation of foam cells; and their involvement in advanced phases of atherosclerosis. All these activities could be related to oxysterol atherogenic properties *in vivo*. The oxysterols that were reported to have these activities are as follows: 5α,6α-epoxy-cholesterol, 5β,6β-epoxy-cholesterol, 7α-OH-cholesterol, 7β-OH-cholesterol, 7-keto-cholesterol, cholestane-3β,5α,6β-triol, 25-OH-cholesterol, 25(*R*)-26-OH-cholesterol, 25(*S*)-26-OH-cholesterol. Among them, cholestane-3β,5α,6β-triol and 25-OH-cholesterol are the

most powerful oxysterols.

1.1.2.3 Mutagenesis

Oxysterols are mutagenic, although some have been studied as antitumour agents based on their cytotoxic properties. Early research on the production of tumors, chiefly local fibrosarcomas, in animals after the injection of a number of oxygenated derivatives of cholesterol were presented by Bischoff (1963 and 1969), and a review by morin et al. (1991) summarizes more recent research in this area. Kelsey and Pienta (1979) reported that the 5α,6α-epoxide of cholesterol, but not cholesterol, was active in the transformation of hamster embryo cells. The authors suggested that the bioassay system, which they used, might be more valuable than the Ames test in detection of potential mutagenicity of steroids. Parsons and Goss (1978) were unsuccessful in detecting transformation of human fibroblasts by the 5α,6α-epoxide of cholesterol. However, the epoxide was reported to cause chromosomal aberrations in fibroblasts, the frequency of which was increased by ultraviolet irradiation of the cells. The epoxide, but not cholesterol, was also reported to induce DNA repair synthesis in human fibroblasts and melanoma cells. However (and in contrast to ultraviolet-treated cells), DNA profiles from human fibroblasts and melanoma cells treated with the epoxide did not reveal breaks in DNA upon sucrose gradient ultracentrifugation, Reddy and Watanabe (1979) found that intrarectal administration of the 5α,6α-epoxide of cholesterol or 5α,6β-diOH-cholesterol (20 mg/animal, 3 times/week for 46 weeks) to germ-free rats did not result in colon tumor formation, nor did these

compounds act as tumor promoters after intrarectal administration of the known carcinogen *N*-methyl-*N*-nitrosoguanidine.

Blackburn et al. (1979) studied the interaction of the [4-^{14}C]5α,6α-epoxy-cholesterol with calf thymus DNA. The labeled complex was analyzed by cesium chloride density centrifugation and gel filtration on Sephadex G-200. It was reported that "the extent of steroid association was in excess of one molecular per hundred DNA base pairs" and that "much lower levels of physical association were observed under identical conditions for cholesterol, estradiol, and progesterone." Prolonged incubation was reported to give extensive convalent attachment of the sterol to DNA and that "this binding servived enzymatic degradation of the DNA" giving "a principal radioactive peak on LH-20 gel chromatograms similar in elution profile to fragments resulting from the covalent binding of benzopyrene epoxides to DNA" observed by others.

Air-aged USP cholesterol, purified cholesterol heated at 70℃ for several weeks, or purified cholesterol oxidized by 60℃ irradiation for several days have been reported to contain mutagenic species as evaluated by the Ames test, whereas purified cholesterol had no mutagenic activity (Ansari et al., 1982; Smith et al., 1979). In an attempt to identify the mutagenic species in the oxidized cholesterol preparations, a large number of purified oxygenated derivatives of cholesterol were studied and found to be nonmutagenic (Ansari et al., 1982; Smith et al., 1979). Among the sterols tested were the 5α,6α- and 5β,6β-epoxides of cholesterol. It was suggested that the mutagenic species in the oxidized cholesterol preparations were sterols that were more extensively oxygenated than the previously established products of the autoxidation of cholesterol (Ansari et al., 1982). These studies assayed direct

mutagenicity in various *Salmonella typhimurium* strains and did not include prior incubation of the compounds with liver enzyme preparations. El-Bayoumy et al. (1996) reported that injection of either 5α,6α-epoxy-cholesterol or 5β,6β-epoxy-cholesterol (total dose, 12.3 μmol in dimethyl sulfoxide) into mammary tissue underneath thoracic and inguinal nipples of rats had no significant tumorigenic action, whereas administration of *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydrobenzo[c]phenanthrene (total dose, 1.2 μmol) produced mammary tumors in each of 20 rats studied.

Sevanian and Peterson (1984) reported that 5α,6α-epoxy-cholesterol is a weak direct-acting mutagen in V79 Chinese hamster lung fibroblasts. Mutagenicity was assayed by determining the frequency of 8-azaguanine-resistant mutants. The data presented indicated that mutagenicity reported to be induced by the epoxide was not dependent on dosage or time of treatment of the cells with the epoxysterol. 5α,6β-diOH-cholesterol was reported to be not significantly mutagenic. The author suggested that the mutagenicity of the 5α,6α-epoxide may be reduced in cells active in the conversion of the epoxide to 5α,6β-diOH-cholesterol. Raaphorst et al. (1987) also studied the mutagenicity of the 5α,6α- and 5β,6β-epoxides of cholesterol. Both epoxides were reported to be mutagenic in a strain of mouse embryo cells. In contrast to the study of Sevanian and Peterson (1984), the frequency of transformation in the mouse cells was found to be dependent on the concentration of the epoxide and the time of exposure. The 5β,6β-epoxide was reported to result in a higher frequency of transformation than the 5α,6α-epoxide. The possible mutagenicity of 5α,6α-epoxy-cholesterol, 7α-hydroperoxy-cholesterol (Smith et al., 1986), 5-hydroperoxy-5α-cholest-6-en-3β-ol (Smith et al., 1986), and other

sterols are deserving of further attention as well as their interaction with DNA. At the present time, the state of knowledge on these important subjects is very considerably less than that for epoxy derivatives of aflatoxin B_1 (Johnson and Guengerich, 1997) and of benzopyrene (Feng et al., 1997).

1.1.2.4 Carcinogenesis

Carcinogenic activity has been detected for some oxysterols, of which only cholesterol-5α,6α-epoxide is found in foods and biological samples (Morin et al., 1991). In addition, cholesterol-5α,6α-epoxide is the only oxysterols that has induced significant carcinogenesis without an oily vehicle. Bischoff (1969) showed that this oxysterols-induced formation of local sarcoma in the mouse after its subcutaneous administration in aqueous suspension. Cholesterol-5α,6α-epoxide was isolated from human skin (Black and Lo, 1971; Chan and Black, 1974) and from mouse skin (Black and Douglas, 1972) after UV irradiation. Further, the increase in cholesterol-5α,6α-epoxide concentration in skin was accompanied by a higher incidence of skin cancer in the mouse. This causal relationship was confirmed by the same authors in a further study (Black and Chan, 1976). However, the presence of other possible carcinogenic agents in skin should be checked to affirm that cholesterol-5α,6α-epoxide was the only responsible agent. In fact, other oxysterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-keto-cholesterol, and cholestanetriol) without demonstrated carcinogenic effects have been isolated from irradiated human skin (Lo and Black, 1972). Parsons and Goss (1978) found that cholesterol-5α,6α-epoxide in human skin fibroblasts induced similar

degrees of chromosome damage and similar stimulation of DNA repair synthesis as did low doses of UV radiation.

On the other hand, cholesterol-5α,6α-epoxide and cholestanetriol-induced transformation of syrian hamster embryo cells (Kelsey and Pienta, 1981). Furthermore, cholesterol-5α,6α-epoxide and cholesterol-5β,6β-epoxide were able to transform cultures of Chinese hamster V79 lung cells and of C3H-10T1/2 mouse embryo cells (Raaphorst et al., 1987). Moreover, high amounts of cholesterol-5α,6α-epoxide, cholesterol-5β,6β-epoxide, and cholestanetriol have been isolated from secretions and excretions from people with hyperplastic problems. Thus, three oxysterols have been isolated from breast fluids (Gruenke et al., 1987; Petrakis et al., 1981; wrecsch et al., 1989), cholesterol-5α,6α-epoxide and cholesterol-5β,6β-epoxide from prostatic gland secretions (Sporer et al., 1982) and cholestanetriol from faeces (Reddy and Wynder, 1977).

1.2 Exploitation of Oxysterol Cytotoxicity

1.2.1 Oxysterols as potential cancer chemotherapeutic agents

Attempts have been made to capitalize on cytotoxicity of oxysterols and use these compounds as chemotherapeutic agents for the control of cellular growth of both normal and cancer cells.

In 1974, Chen et al. first described the inhibitory action of three oxygenated sterols (25-OH-cholesterol, 20α-OH-cholesterol, and

7-keto-cholesterol) on the growth of cultured mammalian cells. In studies with mouse L cells, grown in chemically defined sterol-free medium, the 25-hydroxy-, 20α-hydroxy-, and 7-ketosterols inhibited cell growth by >90% at concentrations of 2.5, 6.2, and 25 μM, respectively. The inhibitory action of these oxygenated sterols was reported to be reversed by the addition of desmosterol (65 μM), the major sterol of L cells, or by mevalonic acid (~1 mg/ml). 7α-OH-cholesterol, at 25 μM, showed no inhibitory action on cell growth; however, at a much higher concentration (124 μM), the 7α-hydroxysterol was reported to show 50% inhibition of cell growth. A large number of oxygenated sterols have been shown to suppress the growth of both normal and transformed cells in culture.

Many *in vitro* and *in vivo* studies of the antitumour effect of oxysterols showed that several compounds of oxysterols exhibited higher cytotoxicity on highly proliferative cells. That means these compounds have some degree of selectivity in their effects on tumor cells relative to normal cells. In recent years, some groups have started to design oxysterol derivatives, which can be applied as antitumor drugs (Smith and Johnson, 1989).

Hietter et al. (1986), Luu (1986) and Rong et al. (1985) reported a toxic effect of 7β-OH-cholesterol and its sodium dihemisuccinate on rat Morris hepatoma cells (tissue culture). These compounds also exhibited greater potency *in vivo* than other antitumour drugs (methotrexate, cyclophosphamide and 5-fluorouracil) against Krebs II tumours in mice (Luu, 1986; Rong et al., 1985). 7β-OH-cholesterol is more toxic on hepatoma cells than on normal hepatocytes (Nordman et al., 1989). It is also more toxic on cultured mouse lymphoma cells than on normal lymphocytes (Hietter et al., 1986). However, toxic concentrations of this oxysterol are much higher (3 – 12 μM) than concentrations usually found

in plasma. Therefore, a possible protective effect of endogenous oxysterols against tumor proliferation is unlikely. Ji et al. (1990) prepared two monophosphonic acid diesters of 7β-OH-cholesterol and of pyrimidine nucleosides to provide water-soluble derivatives of 7β-OH-cholesterol that could be used in *in vivo* studies with animals. The compounds were esters at C-3 of the sterol with nucleosides (5-fluoro-2'-deoxyuridine or 2'-deoxyuridine). Both compounds were reported to show high solubility in water (>30 g/100 ml) and high antitumor activity upon intraperitoneal administration to mice with ascites tumors. The dosage used was 80 μmol/Kg/day, which was given for 2 or 3 days after administration of ascites cells. Ji et al. (1990) reported that the 3β-deoxyuridine monophosphate esters of 7β-OH-cholesterol and 7β,25-diOH-cholesterol showed antiproliferative action against murine and human tumor cells. However, the new derivatives were less cytotoxic to the cells than were the parent 7β-hydroxy- and 7β,25-dihydroxysterols. Christ et al. (1991) studied the effects of two water-soluble phosphoric acid diesters, 3β-hydroxy conjugates of 7β-OH-cholesterol with 2-deoxyuridine (JB69) or thymidine (XA29). The latter two derivatives of 7β-OH-cholesterol were reported to be less potent than 7β-OH-cholesterol itself in an *in vitro* assay of antiproliferative activity with cultured mastocytoma cells (P815). Christ et al. (1991) reported that daily intraperitoneal administration of XA29 (at 20mg/Kg/day) and JB69 (at 20 or 40 mg/Kg/day) to mice, in which mastocytoma cells (P815) were implanted subcutaneously, showed longer survival times than controls (saline vehicle alone). The results were not analyzed statistically. XA29 (0.5 mg/day; ~20 mg/Kg/day for 15 days) was reported to cause a modest decrease in body weight. At doses of the analogs "exceeding 40 mg/Kg/day, mice began to lose weight, become weak, and died if we prolonged the treatment." At doses between

20 and 40 mg/Kg/day, "in all the cases, injection of the phosphodiesters induced abdominal cramps."

Iguchi et al. (1993) reported the isolation of an oxygenated sterol, aragusterol A, 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* antitumour activity against L1210 leukemia cells and P388 cells in mice. The chemical synthesis of its 5β-isomer, 5-epiaragusterol A, was reported (Mitome et al., 1997), and the compound was shown to be highly active in the inhibition of growth of KB cells (IC_{50} ~0.090 nM). The isolation of other related analogs of aragusterol A from the Okinawan sponge has recently been reported (Miyaoka et al., 1997).

Mimaki et al. (1997) observed the extraordinarily high potency of glycosides of an oxygenated sterol (3β, 16β, 17α-trihydroxycholest-5-en-22-one), isolated from bulbs of *Ornithogalum saundersiae,* in the inhibition of cell growth of HL-60 leukemia cells. At a concentration of 115 μM, one of the most potent of these compounds (IC_{50} = 0.25 nM) showed no hemolytic action with human red blood cells. The sterol was reported to be more potent in the HL-60 cells than other anticancer agents including mitomycin C, cisplatin, campothecin, and taxol. 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 the compound. The compound was also reported to show favorable *in vivo* effects against a mouse P388 tumor.

Aoki et al. (1998) reported that a polyhydroxylated sterol, agosterol A, from a marine sponge reverses the multidrug resistance of human carcinoma cells. The structure of the basic sterol was shown to be

5α-cholest-7-ene-3β,4β,6α,11α,22(*R*)-pentol, which occurs in the sponge as acetate esters of the 3-, 4-, and 6-hydroxyl functions.

1.2.2 Mechanisms of antitumor effect

Inhibition of endogenous cholesterol synthesis and incorporation of oxysterols to membrane structure are the mechanisms of antitumour effect. Some other mechanisms have been reported as relevant. HMG-CoA reductase, as well as being the key enzyme in cholesterol synthesis, is also the key enzyme in the synthesis of prenyl alcohols, activitors of some oncogenes (Glomset et al., 1990; Schafer et al., 1989). Some studies suggest a possible mechanism related to immune system alteration induced by oxysterols, since attraction of macrophages and neutrophils by oxysterols has been observed (Christ et al., 1991; Luu and Moog, 1991). Other authors suggest that oxysterols affect genesis and functionality of cytotoxic lymphocytes by inhibiting proliferation and transformation of lymphocytes (blastogenesis), mixed lymphocyte response and activity of NK cells (Smith and Johnson, 1989). This mechanism has been demonstrated for 25-hydroxycholesterols, 7-hydroxycholesterols and 7-keto-cholesterol. Hwang (1992) observed that 7-keto-cholesterol and 25-hydroxycholesterol showed ability to decrease viability of murine cells (EL4 lymphoma and K36 leukaemia cell lines). However, the presence of inhibitors of protein or RNA synthesis increases this viability. This finding was confirmed later by Chirst et al. (1993), who showed that cycloheximide and actinomycin D increase viability of murine lymphoma cells (RDM4) in the presence of 25-hydroxycholesterol and 7β,25-dihydroxycholesterol, which have

antiproliferative activity. This suggests that oxysterols toxicity could be mediated by mechanisms involving protein or RNA synthesis. It would be interesting to find what proteins are key factors in cellular death induced by these oxysterols. Furthermore, Bakos et al. (1993) identified an oxysterols-binding protein and showed that 25-hydroxycholesterol-induced cell death of different human leukaemic T-lymphocyte clones correlates with oxysterols affinity for this cytosolic-binding protein, which has been related to the inhibition mechanism of HMG-CoA reductase. A recent study suggested a role for a DNA-binding protein in oxysterols-induced regulation of lymphoid cell viability and growth (Ayala-Torres et al., 1994).

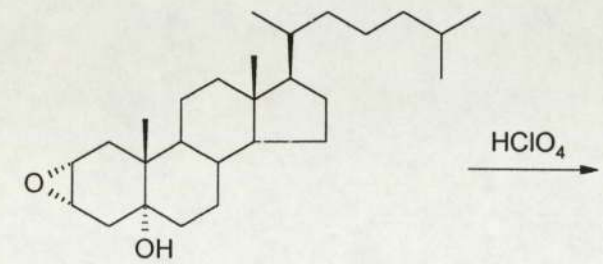
1.3 Studies of The Chemistry Relevant to Oxysterols in Literatures

Most of chemical synthesis of oxysterols derived from cholesterol, and most of chemical studies focused on the A and B rings. Two main reasons for that are, first, most of the natural oxysterols with one or more hydroxy groups on A and B rings showed much better bioactivities; second, although chemical features of oxysterols are reported most on their rings A and B, no standard methodology is available to obtain a desired stereoisomer. This project focuses on developing methods to synthesize the ring A polyoxygenated sterols with defined stereo features.

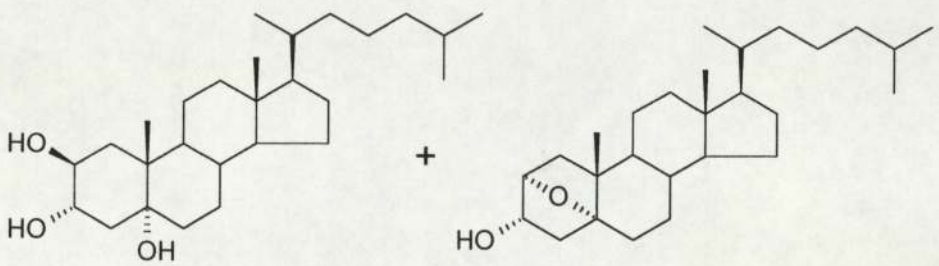
1.3.1 Chemistry of Ring A on Steroidal Skeleton

The chemical studies of oxysterols have a long history. A few reports described chemical studies on the ring A of oxysterols, but little work has been done on the polyoxygenation. It is obvious that the more oxygenated groups on the molecules, the more complicated interactions among the chemical groups. Furthermore, the relative rigid ring system intensifies interactions between adjacent or distanced groups. For instance, the epoxide **1.3.1** gave not only the compound **1.3.2** but also **1.3.3** (Scheme 1-1) when treated with perchloric acid (Komeno and Itani, 1970).

Scheme 1-1 The Epoxide Cleavage of 1.3.1



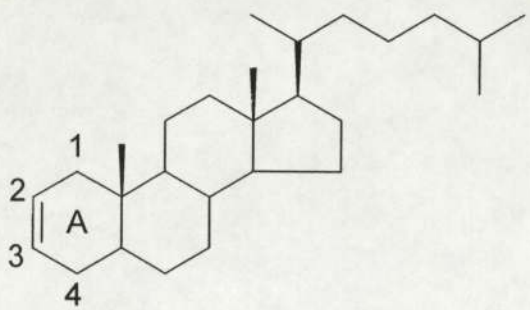
1.3.1



1.3.2 **1.3.3**

There are more reports on the reactions about C2 and C3 than C1 and C4. The literature methods of the oxidation reactions on the allylic carbons are very limited (Figure 1-3). The most common method for the oxidation of allylic position is chromium trioxide reagent. Normally this method has little selectivity on the oxidation of C1 or C4. However, it was claimed that chromium trioxide oxidation tended to happen on the position C4 (Kohout et al., 1973, Baldwin et al., 1973, Glotter et al., 1980, Flaih et al., 1999). Another selective allylic oxidation reagent is potassium acetate with palladium(II) chloride and copper(II) chloride. The cholest-2-enes were treated with this reagent to give the 1α-hydroxy-cholest-2-enes (Horiuchi et al., 1982). Some other C1 or C4 oxidized cholest-2-ene compounds in the literatures were obtained from diverse approaches. Such as the conversion from 1α,2α-epoxy-6β-acetoxycholest-3-one to the conjugated ketone 6β-acetoxycholest-2-en-1-one, by reduction with hydrazine, followed by oxidation with Jone's reagent (Ishiguro et al., 1974), or oxidation of cholestane-1-one (Ortar and Romeo, 1976)

Fig. 1-3 The Structure of Steroidal Compound for the Review of Ring A Chemistry



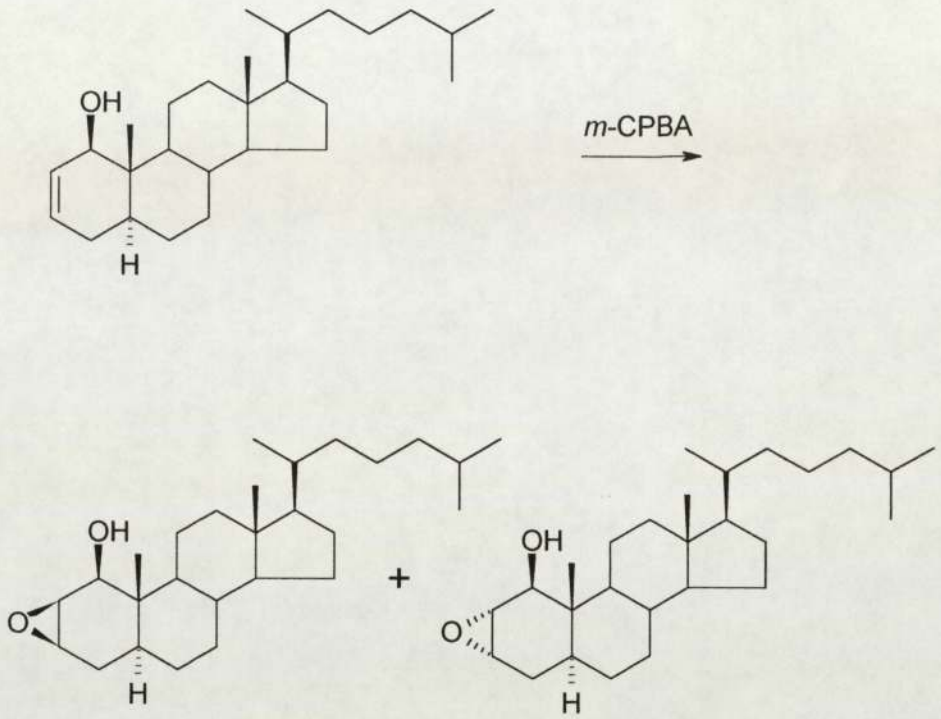
The reductions of the carbonyl group on C1 or C4 are mainly carried out with three different reagents, sodium borohydride, lithium aluminum hydride and sodium metal. Normally none of them is stereoselective reagent for producing the α or β configuration of hydroxy group from carbonyl group. However, in some cases of reduction of carbonyl group on C1 or C4 of oxysterols, it was observed that $NaBH_4$ tended to afford axial hydroxy group (Noam et al., 1981); metal Na tended to get the equatorial hydroxy group (Striebel and Tamm, 1954); $LiAlH_4$ tended to get a mixture of 1α- and 1β-hydroxyl products or 4α- and 4β-hydroxyl structures (Henbest and Wilson, 1956).

The reported reactions of the C2=C3 double bond were mainly two kinds. One is addition reaction; the other is epoxidation. In addition reactions, hydrogenation with Pd/C or PtO_2 as the catalysts is very common (Takatsuto and Ikekawa, 1987). The reaction of hydroxylation with either osmium tetroxide or the reagent iodine silver acetate occurs with syn addition, and the former always gives a 2α,3α-diol product, while, the latter gives a 2β,3β-diol (Shoppee et al., 1957). When the reagents aqueous hydrogen peroxide and acetic acid are used, the reaction occurs with anti addition and gives a 2β,3α-diol (Shoppee et al., 1957). Hydroboration/oxidation can introduce a hydroxy group from the less hindered face of steroidal molecules. From cholest-2-en or its derivatives one can obtain the mixture of 2α-hydroxy and 3α-hydroxy compounds (Nussim et al., 1964). The reactions with halogens or halogens-H_2O always occur with anti addition and yield 2α,3β and 2β,3α-dihalogen or 2α-ol, 3β-halogen and 2β-ol, 3α-halogen products (Alt and Barton, 1954). The reagents NBS (or AcNHBr) and aqueous $HClO_4$ can give 2β-ol(halogen) and 3α-halogen(ol) (King et al., 1963; Tsui and Just, 1973).

The other kind of reaction of alkenes is epoxidation. In the laboratory, epoxides are normally prepared by treatment of an alkene with a peroxyacid. Many different peroxyacids were reported to be used to accomplish epoxidation, among them *m*-chloroperoxybenzoic acid (*m*-CPBA) is the most common choice (Scheme 1-2). The literature methods for the epoxidation of this kind of compounds were mainly peroxyacids and tert-Butyl hydroperoxide (TBHP) $Vo(acac)_2$ method. The *trans*-steroid structures gave only 2α,3α-epoxide (Boto et al., 1994; Kocovsky, 1994) and the *cis*-steroids yielded only 2β,3β-epoxide (Glotter and Zviely, 1986). Only one report claimed that the 1β-hydroxy-cholest-2-en was treated with *m*-CPBA to give a mixture of 2,3α and 2,3β-epoxide (Kocovsky, 1994) (Scheme 1-2).

The cleavage or rearrangement reactions of steroidal epoxide afford dihydroxy or hydroxy group that tends to become an axial stereoconfiguration. More details on these reactions will be described in the next section.

Scheme 1-2 The Epoxidation of 5α-cholest-2-en-1β-ol



1.3.2 Chemistry of The Positions Other Than Ring A on Steroidal Skeleton

Most reports about oxidation reactions that took place on the positions other than ring A of oxysterols focus on C5, C6, and C7. The main reactions are epoxidation on C5 = C6 double bond and oxidation of C7.

There are many kinds of reagents that are used to synthesize 5,6-epoxycholestan-3β-ol from cholesterol. However, few of them can

make single product of 5α,6α- or 5β,6β-epoxide. Some literatures reported that when the 3-hydroxy group was protected by certain acyl group, 5β,6β-epoxide was synthesized with some reagents (*m*-CPBA, PBA, or perfluoro-*cis*-2-n-butyl-3-n-propyloxaziridine) as the main product. On the other hand, when the 3-hydroxy group was free, 5α,6α-epoxide was obtained (Baxter and Spring, 1943; Arnone et al., 1996). Two reagents reported specifically stereoselective for 5α,6α-epoxide syntheses are urea-hydrogen peroxide complex, maleic anhydride (Astudillo et al., 1993) and magnesium monoperphthalate hexahydrate, methyltrioctylammonium chloride (Brougham et al., 1987). Meanwhile, according to the literatures, 5β,6β-epoxide was obtained principally with two kinds of reagents, they are $KMnO_4$, $CuSO_4 \cdot 5H_2O$ (Parish and Li, 1996) and Br_2, Ag_2O, 10% KOH (Barone et al., 1984).

Most of the reactions on C7 of steroidal skeleton in literatures are allylic oxidation reactions because the reactants that were reported are always with the C5 = C6 double bond (Keller and Weiss, 1950; Wintersteiner and Ruigh, 1942; Sobel et al., 1949). 7-Ketocholesterol was synthesized with some chromium-mediated reagents or non-chromium containing reagents (Miller et al., 1996). Reduction of 7-ketocholesterol with isopropyl alcohol and aluminium isopropylate gave the epimeric 7-hydroxycholesterols' mixture. 7β-Hydroxycholesterol was separated and purified by crystallization from the product mixture and 7α-epimer was obtained as the sole product from hydrolysis of 7α-benzoxy-cholesteryl benzoate that was prepared from benzoylation of the epimeric 7-hydroxycholesterols' mixture (Wintersteiner and Ruigh, 1942).

1.4 The Research Objectives of The Project

The early history of the research of oxysterols was marked by controversy and this persists today. From the view of medicinal chemistry, more stereospecific derivatives of oxysterols are needed for studies of bioactivity mechanisms and for establishment of possible structure-activity relationships. The range and the contents about the current study on oxysterols are too far from enough. There are few reports about the synthesis and chemistry of the series of compounds which have 5α,6β-dioxygenated groups on the ring B and polyoxygenative groups on the ring A. The chemical behaviour of this kind of compounds is complicated. We are interested in this challenging work.

Chapter Two: Syntheses and Chemistery of Oxysterols

2. Syntheses and Chemistry of Oxysterols

2.1 Cholest-2-en-5α,6β-diol

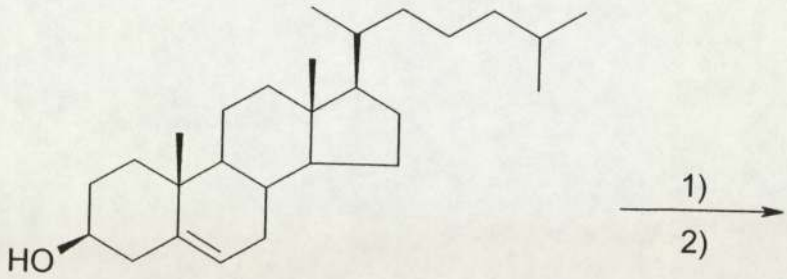
In 1949, Fieser and Rajagopalan reviewed that the best previous methods for preparation of cholestane-3β,5α,6β-triol were by reaction of cholesterol 3-acetate with hydrogen peroxide in acetic acid over a period of four days and saponification of the resulting acetate mixture. Of more importance was the development of a reliable procedure for hydroxylating the C5=C6 double bond of cholesterol with hydrogen peroxide and formic acid (Swern et al., 1945). Brief heating of cholesterol with 88% formic acid produced the 3-formyl derivative, and on addition of hydrogen peroxide to the resulting suspension a clear solution was obtained quickly and precipitation with water gave cholestane-3β,5α,6β-triol 3,6-diformate. Brief saponification of the resulting diformate afforded the pure triol in 91% yield. Komeno et al. (1970) reported the synthesis from cholestane-3β,5α,6β-triol 3-monotosylate to cholest-2-en-5α,6β-diol by heating to 140 ℃ for 1 hr in 2,4,6-collidine solution. Then, the cholest-2-en-5α,6β-diol was converted to 5α,6β-diacetoxycholest-2-en by forced acetylation in the presence of *p*-toluenesulphonic acid catalyst. We employed most of the above literature methods to synthesize our starting material (**1**) and its derivatives except the elimination from cholestane-3β,5α,6β-triol 3-monotosylate to cholest-2-en-5α,6β-diol. According to the report (Komeno et al., 1970), the yield of this dehydration reaction with 2,4,6-collidine was only 49%. The yield rose to 99% when we used lithium bromide and lithium carbonate as catalyst and reacted in the

DMF solution.

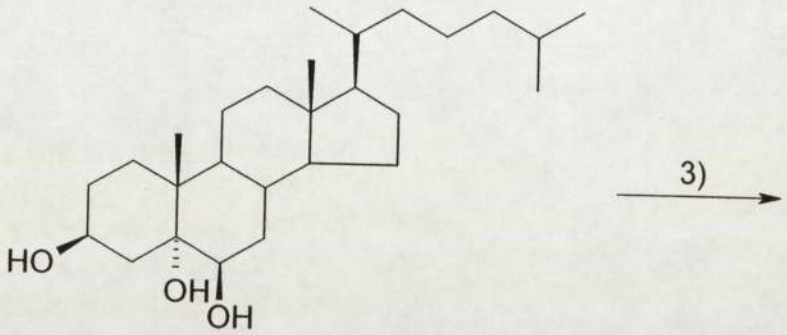
To obtain ring A polyoxygenated oxysterols, we designed cholest-2-en-5α,6β-diol (**1**) as the initial starting material. An obvious precursor for this compound is cholestane-3β,5α,6β-triol (**CT**), which can be synthesized by oxidation of **cholesterol** with hydrogen peroxide in formic acid, followed by hydrolysis. **CT** was *p*-toluenesulphonyled with p-toluenesulphonyl chloride to form **3β-tosyloxycholestane-5α,6β-diol**, which underwent the elimination reaction to give the starting material (**1**) (Scheme 2-1).

To retain the 5α- and 6β-hydroxyl group and introduce hydroxy groups on the ring A of **1**, we selectively acetylated the more reactive 6β-hydroxy group with acetic anhydride and pyridine to give **1a** in 96% yield. Then, the monoacetate **1a** was converted to diacetate **1b** by stirring with acetic anhydride and *p*-toluenesulphonic acid in glacial acetic acid solution. Another monoacetate **1c** was obtained by hydrolysis of **1b** with sodium hydroxide and ethanol (Scheme 2-2). Another important purpose to synthesize the compounds **1a, 1b,** and **1c** is to study whether or not and how the 5- and 6-acetyl groups affect the reactions that occur on ring A.

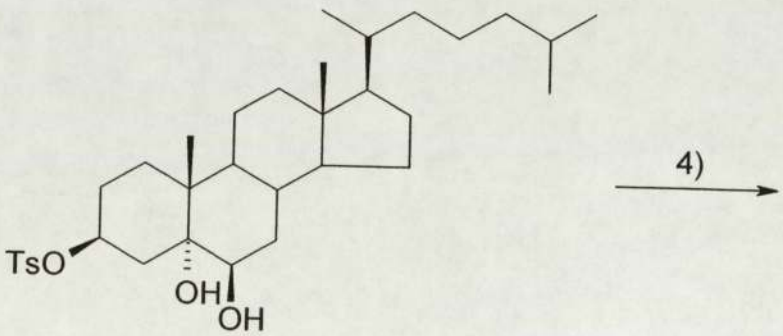
Scheme 2-1 Synthesis of Cholest-2-en-5α,6β-diol



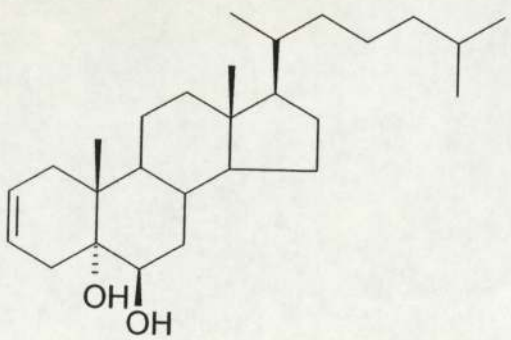
cholesterol



cholestane-3 β ,5 α ,6 β -triol (CT)



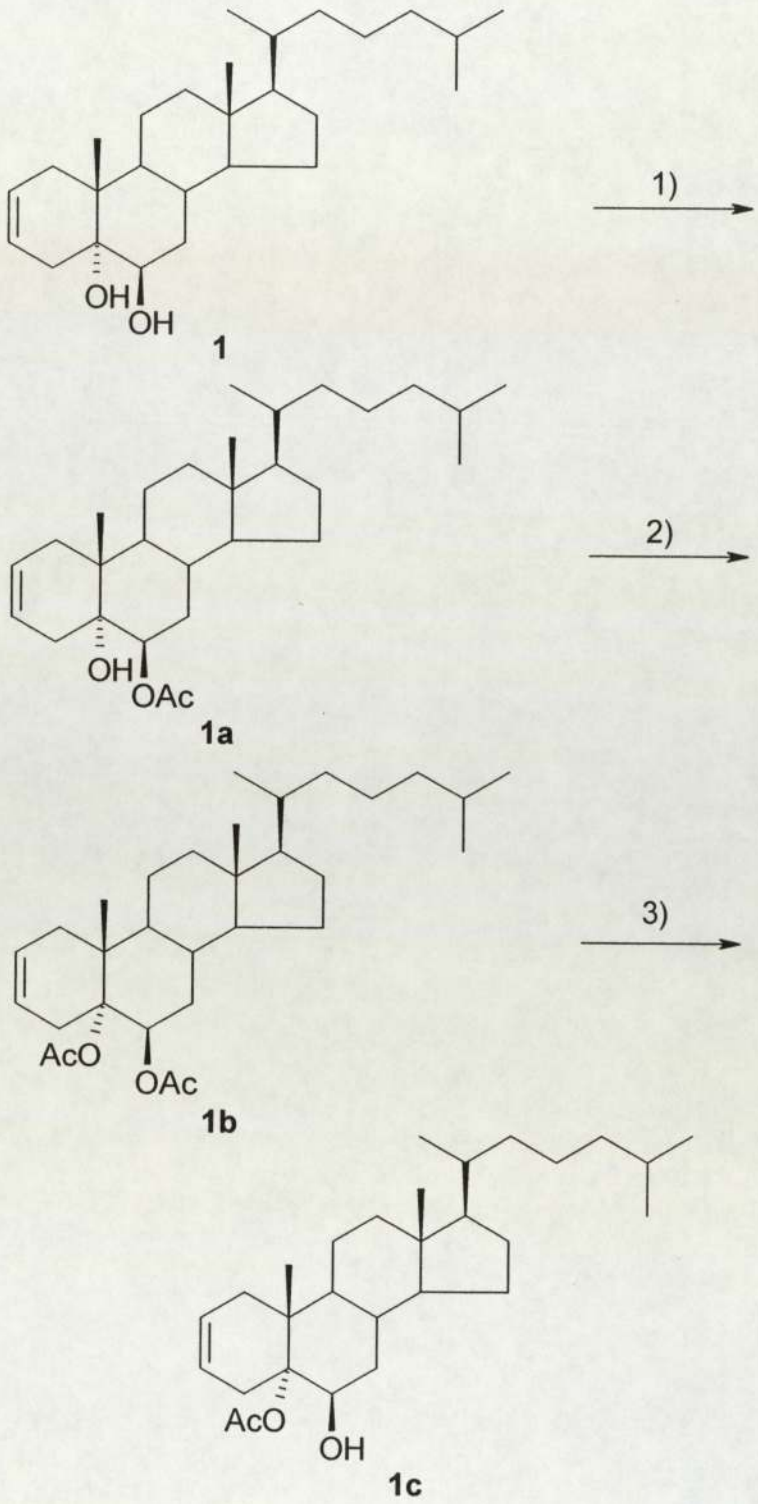
3 β -tosyloxy-cholestan-5 α ,6 β -diol



1

1) HCOOH, 30%H_2O_2; 2) NaOH; 3) TsCl, Py; 4) LiBr, Li_2CO_3

Scheme 2-2 Protection of 5α- and 6β-hydroxy groups



1) Ac_2O, Py; 2)Ac_2O, AcOH, *p*-TsOH; 3) NaOH, EtOH

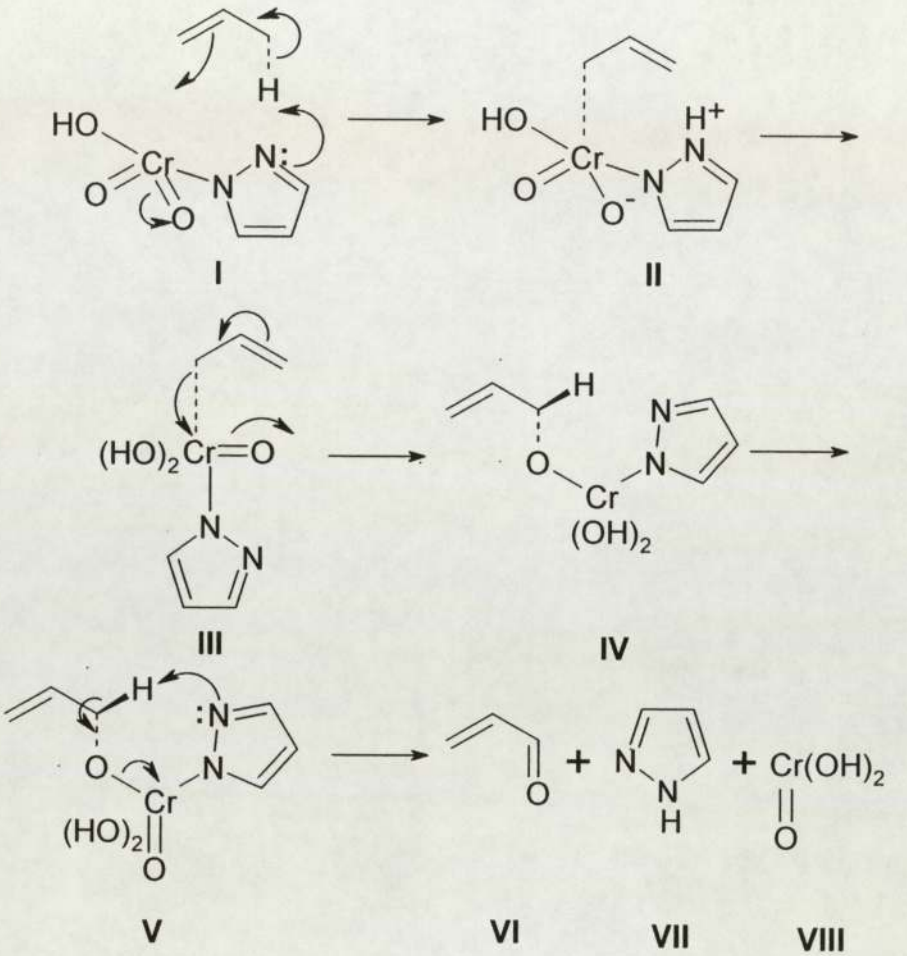
2.2 The Allylic Oxidation Reactions with Chromium Trioxide Reagents

In the following research, we needed a substantial amount of 5α-hydroxy-6β-acetoxycholest-2-en-4-one (**2a**) and 5α-hydroxy-6β-acetoxycholest-2-en-1-one (**4a**). Since in the literature (Salmond et al., 1978), the allylic oxidation of some alkenes with DMP•CrO_3 is much faster with higher yield than other oxidants based on chromium. We adopted DMP and chromium trioxide complex as the first choice of the allylic oxidation reagents. The outcome was not as we expected one major product, but a mixture. After separation and purification by silica gel column with petroleum ether/ether (1:1) as eluant, the four major products were identified as **2a, 3a, 4a,** and **5a** (9:3:2:1) in 69% yield in total (Scheme 2-4). The configuration of the four products was determined on the basis of the results of the following reactions. **5a** is the same compound as the product of epoxidation reaction shown in scheme 2-15 (P87). **CT** was obtained after **3a** was reduced by sodium borohydride followed by hydrolysis with sodium hydroxide (Scheme 2-8). **2a** was converted to 2β,3β-epoxy-cholestane-4β,5α,6β-triol (**7**) (the standard sample in our laboratory) by three steps (Scheme 2-17). The alkaline hydrolysis product (**4**) (Scheme 2-5) of **4a** has the same NMR data as the literature report on 5α,6β-hydroxy-cholest- 2-en-1-one (Ishiguro et al., 1974). Due to complexity of oxidation of **1a** with chromium trioxide and DMP (Scheme 2-4), synthesis of **2a** and **4a** became a difficult task.

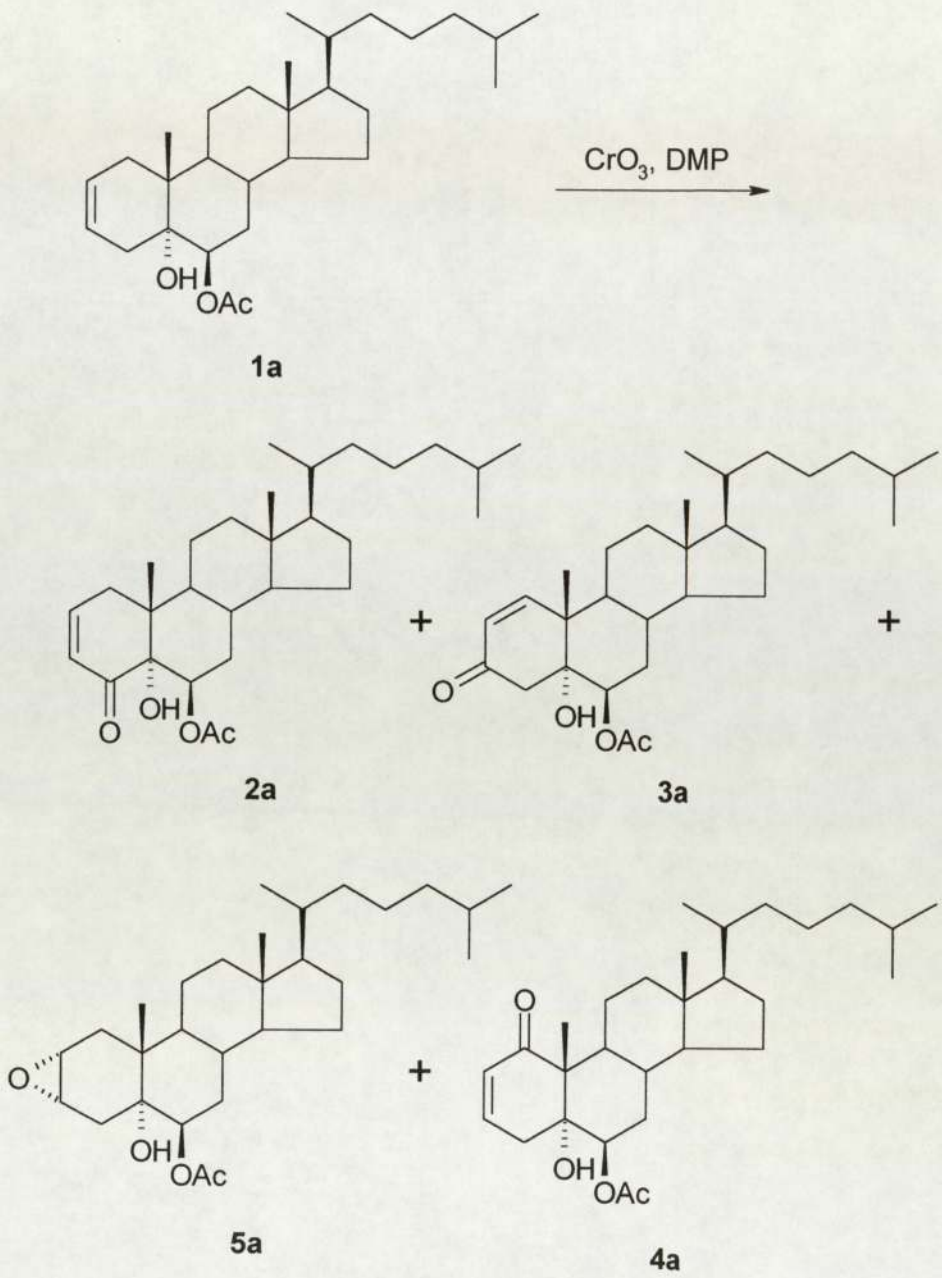
In 1978, Salmond et al. reported the reaction mechanisms of allylic oxidation with 3,5-dimethylpyrazole chromium trioxide complex (DMP•CrO_3). The scheme 2-3 assumed a one-to-one addition of DMP

and CrO_3 to give the complex (the 3,5-methyl groups are omitted for the sake of clarity), in which one ligand site remaied free on the chromium atom allowing facile attack by the π electrons of the double bond. In the scheme 2-3, a two-electron transfer took place. In passing from **I** to **II**, no reduction of the Cr^{VI} was involved. A proton shift from nitrogen to oxygen gave **III**. During the 2,3-sigmatropic shift of the chromium alkyl (**III**) to give the intermediate **IV**, oxidation of the alkene took place and reduction of Cr^{VI} to Cr^{IV} occurred. Oxidation of Cr^{IV} to Cr^{VI} or Cr^{V} must take place to make **IV** into **V**, because collapse of **IV** directly to ketone **VI** and a Cr^{II} (**IX**) was not considered likely (Corey and Melvin, 1975). The decomposition of the resulting chromate ester **V** to the conjugated α,β-unsaturated ketone was aided by an intramolecular cyclic mechanism as shown. Finally, the α,β-unsaturated ketone **VI**, the catalyst DMP **VII**, and Cr^{IV} (**VIII**) were given.

Scheme 2-3 Mechanism of Allylic Oxidation Reaction



Scheme 2-4 Allylic Oxidation Reaction of 1a with DMP•CrO_3



1a

2a

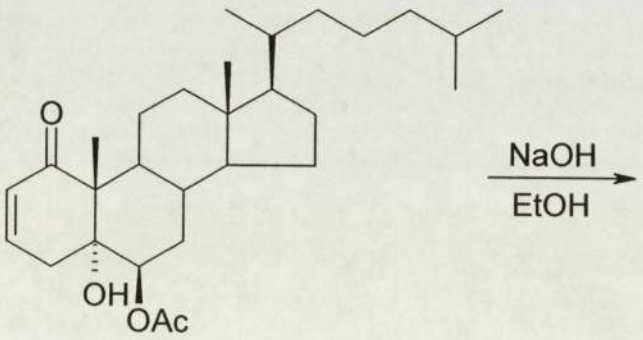
3a

5a

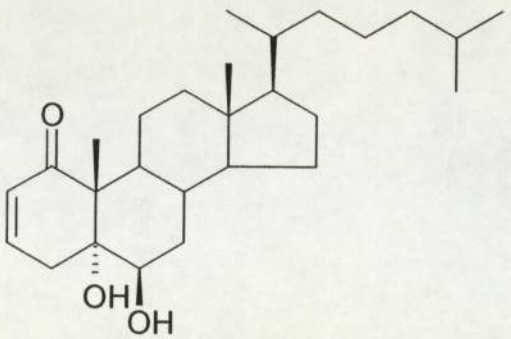
4a

To find a better method for preparing allylic oxidation compounds, firstly, we tried oxidation of **1b** with chromium and DMP, which gave **3b** as the single product (Scheme 2-6). Secondly, Erwin et al. (1980) reported 5α-hydroxy-cholest-2-en-4-one was obtained as the pure product by allylic oxidation with chromium trioxide in aqueous acetic acid from 5α-hydroxy-cholest-2-en. We applied the same condition to **1a**, and the corresponding 4-one (**2a**) was produced (Scheme 2-7).

Scheme 2-5 Hydrolysis of 4a

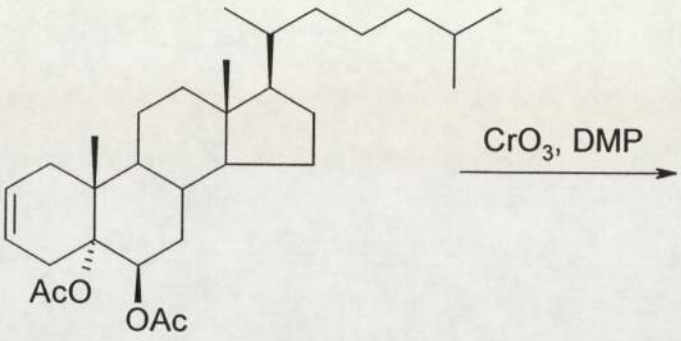


4a

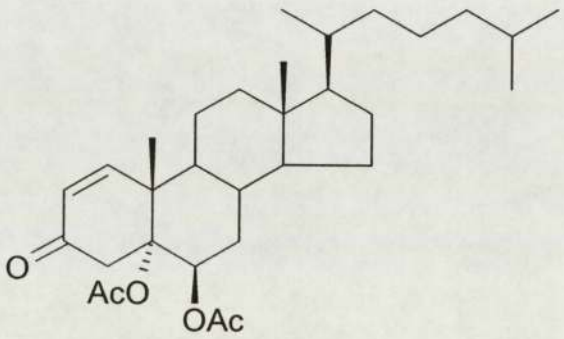


4

Scheme 2-6 Allylic Oxidation Reaction of 1b with DMP•CrO_3

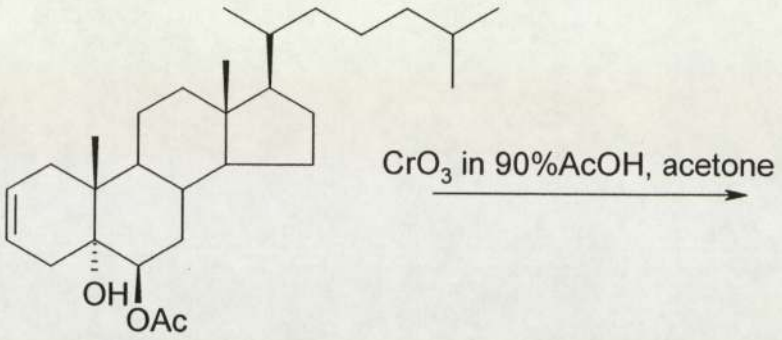


1b

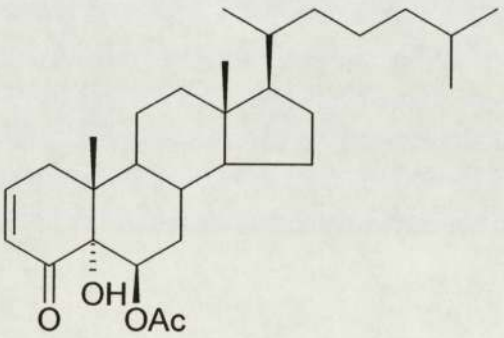


3b

Scheme 2-7 Allylic Oxidation Reaction of 1a with DMP in Aqueous Acetic Acid Solution



1a

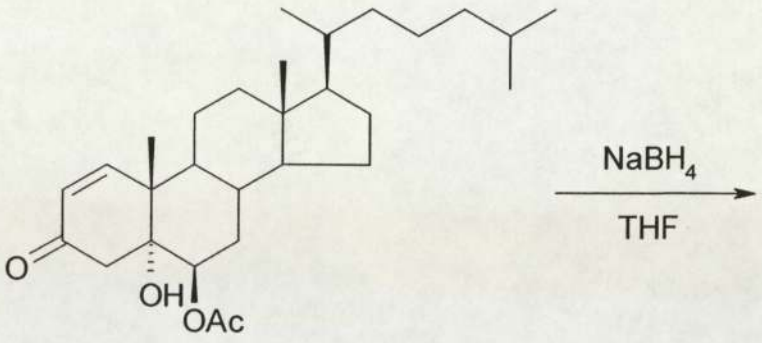


2a

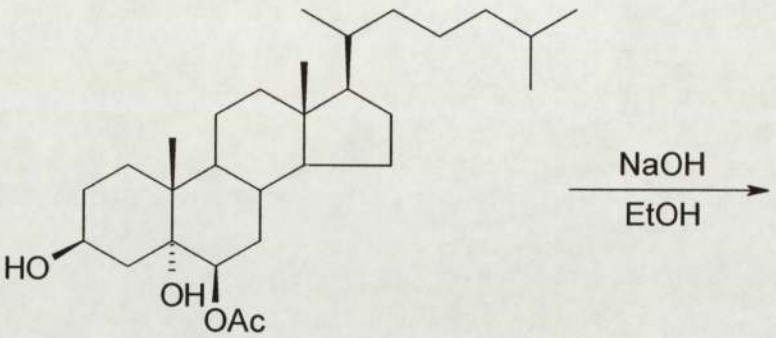
2.3 The Reduction Reactions of Allylic Carbonyl Groups

To study the characteristics of sodium borohydride reduction of our ketone compounds (**2a, 3a, 4a**), we dissolved the reactant **3a** in THF and water, followed by adding large excess $NaBH_4$ into the solution. However, it is surprising that we did not obtain the expected cholest-1-en-3α(β),5α,6β-triol but the 6β-acetoxycholestane-3β,5α-diol (Scheme 2-8). The NMR spectrum of the product showed that the double bond of C1=C2 had been reduced during the reaction. The stereochemistry of the 3β-hydroxy group was determined when we got **CT** as the hydrolysis product of 6β-acetoxycholestane-3β,5α-diol (Scheme 2-8). Hydrogenation of **3a** with Pd/C as catalyst gave the ketone **3ak**, which was finally converted to **CT** under $NaBH_4$ reduction followed by alkaline hydrolysis (Scheme 2-9).

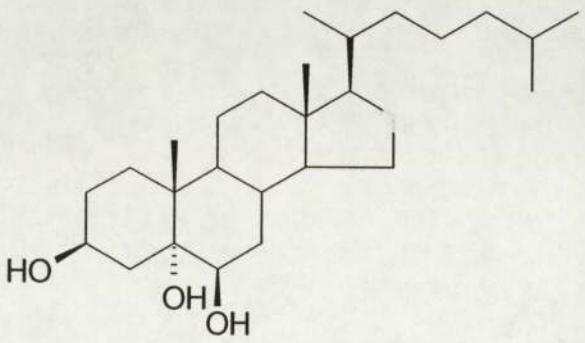
Scheme 2-8 Reduction of the Carbonyl Group and Conjugated Double Bond of 3a



3a

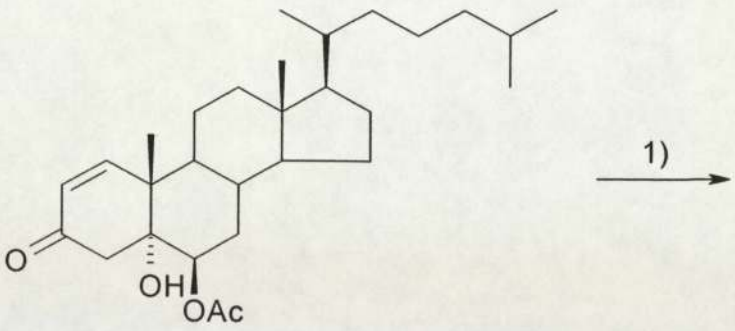


6 β -acetoxycholestane-3 β ,5 α -diol

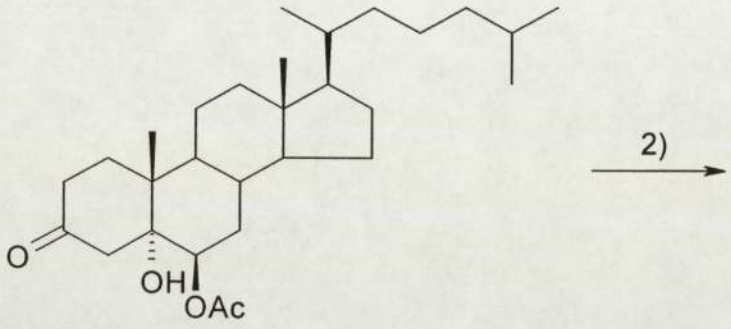


CT

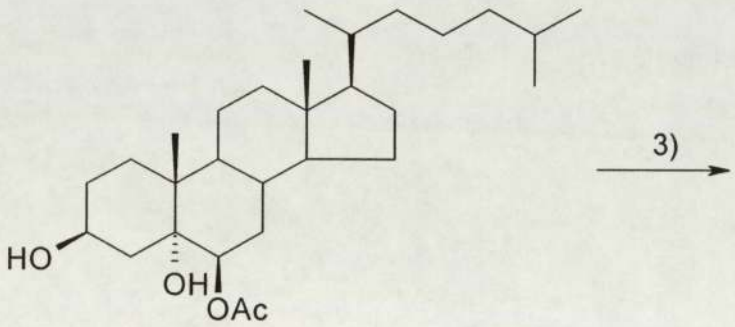
Scheme 2-9 Reduction of the Carbonyl Group of 3a



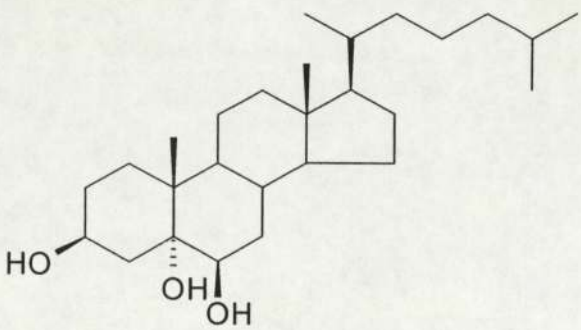
3a



3ak



6 β -acetoxycholestane-3 β ,5 α -diol



CT

1) 5% Pd/C, H_2; 2) $NaBH_4$, THF; 3) NaOH, EtOH

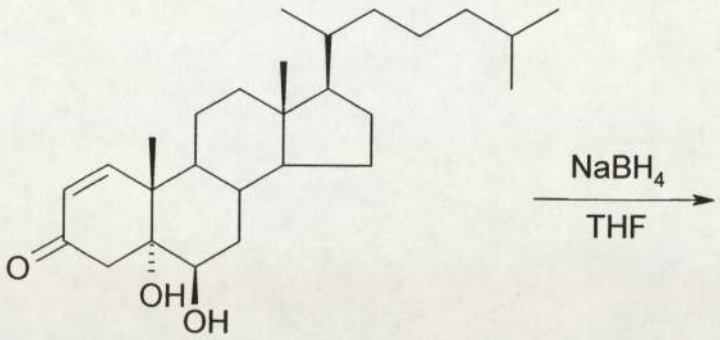
In order to find out whether or not the acetyl group on the 6-hydroxy group was able to affect the reduction reaction with sodium borohydride, Cholest-1-en-3-one-5α,6β-diol (**3**) was synthesized from **3a** by alkaline hydrolysis with sodium hydroxide. **3** reacted with a large excess of sodium borohydride at room temperature in THF to give cholest-1-en-3α,5α,6β- triol (**10**) as the main product in 76% yield. Then, **10** was hydrogenated to give **11** (Scheme 2-10). The assignment of the structure of **10** is supported by its NMR spectum and comparison between the resulting compound (**11**) and the standard sample cholestane-3α,5α,6β-triol in our lab. The two vinyl protons are at 5.55 p.p.m., and a singlet at 3.84 p.p.m. was assigned to the 6α-H, a multiplet at 4.28 p.p.m. to the 3β-H.

From the comparison between the two $NaBH_4$ reduction reactions of 5α,6β-dihydroxy-cholest-1-en-3-one with or without acetyl protecting group on the 6β-hydroxy group, it is very interesting to be noticed that not only the 3-carbonyl group but also the C1=C2 double bond were reduced when the acetoxyl group was as the substituent on the C6. If the 6-hydroxy group was free, the reduction reaction with sodium borohydride took place only on the 3-carbonyl group.

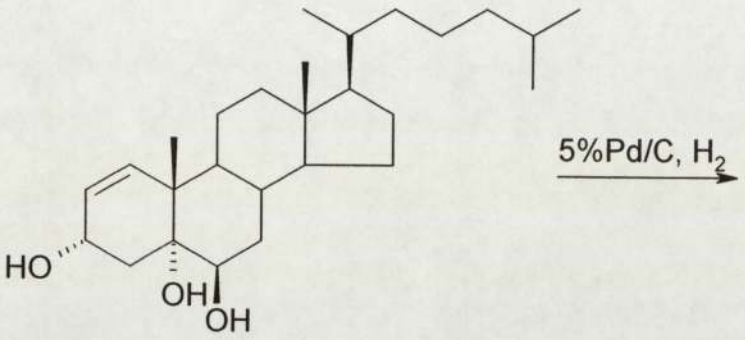
Sodium borohydride is a well-established and widely utilized reagent for the reduction of aldehydes and ketones. Its application in large excess to the preparation of alcohols from carboxylic acid esters was reported as well (Brown and Rapoport, 1963). However, there are only limited reports in the literature on the employment of sodium borohydride to reduce carbon-carbon double bond. In 1966, Kadin reviewed some relevant work and postulated that the delocation of the π electrons of the ehtylenic linkage, which occurs through conjugation of the carbon-carbon double bond with carbonyl group, and resulted in the

creation of an electrophilic center which was capable of being attacked by a nucleophile, in this case the borohydride anion. The failure of the reduction of conjugated double bonds with sodium borohydride was probably due to the lack of a relatively sufficiently strong and localized electrophilic center brought about by conjugation of the double bonds. From the reaction result and the above discussion, we can only hypothesize the mechanisms of sodium borohydride reduction of 3a approximately. We suppose that the contribution of the acetyl group on the 6-hydroxy group to the reduction reaction was to obstruct the β vinyl carbon (C1) to get the great enough electron-withdrawing ability.

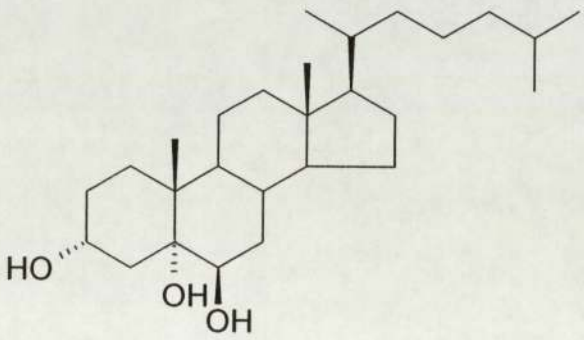
Scheme 2-10 Reduction of the Carbonyl Group of 3



3



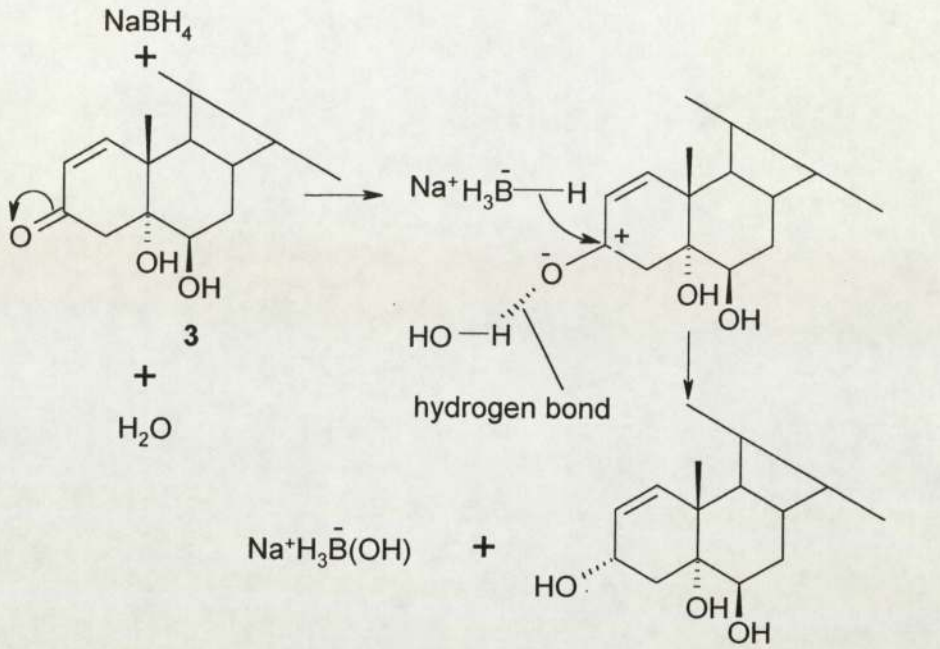
10



11

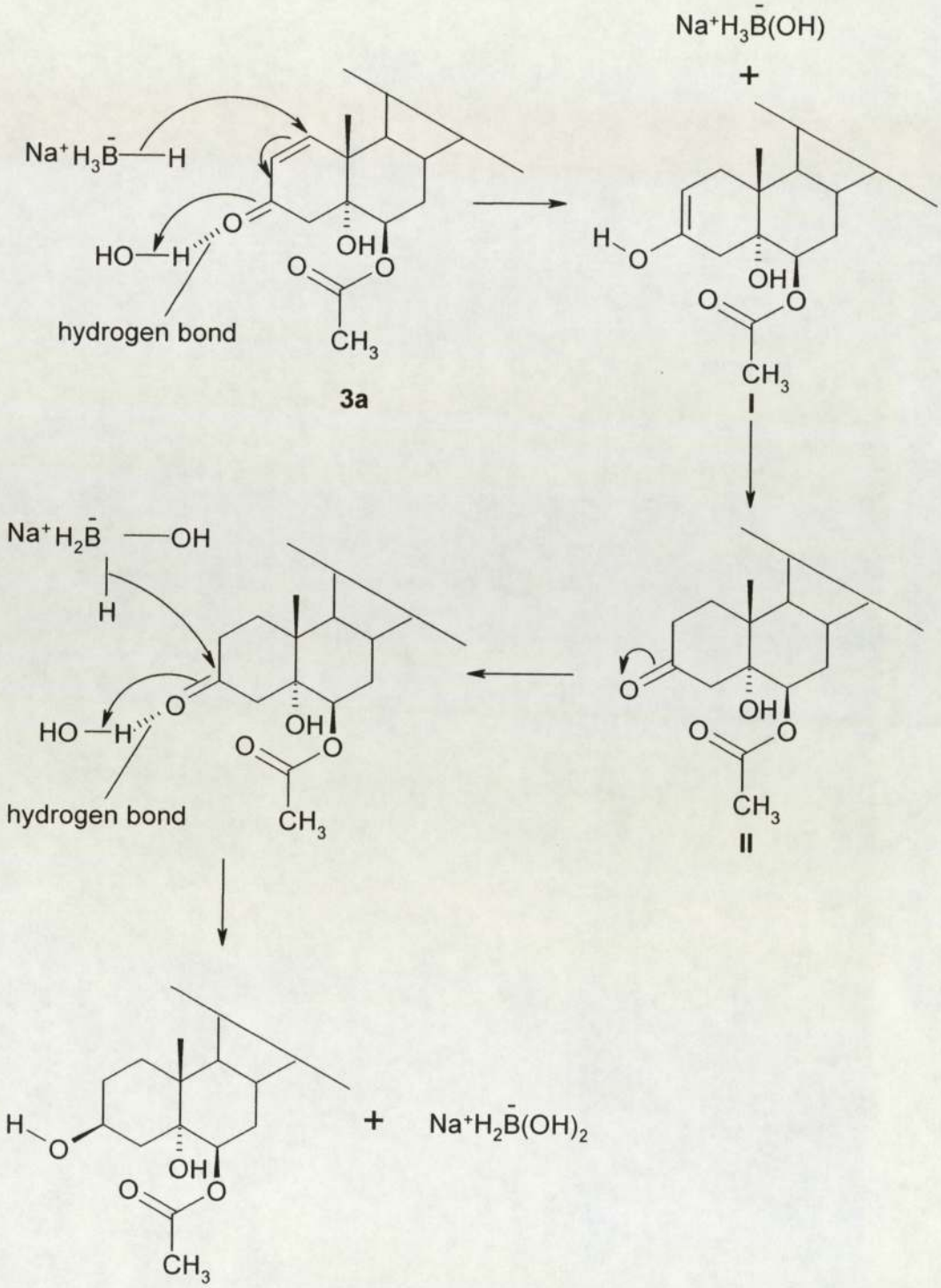
The possible mechanisms of reduction of **3** and **3a** with sodium borohydride are shown in the scheme 2-11 and 2-12, respectively. The reduction of the compound **3** with free 6β-hydroxy group follows the general mechanisms of the reaction of sodium borohydride with ketones. The reduction of the compound **3a** follows the different mechanisms. Firstly, the β-carbon (C1) on the α,β-unsaturated carbonyl compound became the strong enough electrophilic center under the participation of 6-acetoxyl group. Secondly, the nucleophilic borohydride attacked the electrophilic center. Hydrogen bonding between water and the carbonyl group served as a weak acid catalysis that activated the carbonyl group. The addition, which is similar to Michael addition, happened, followed by the automatic conversion from the intermediate enol (**I**) to the intermediate ketone (**II**). Then, the reaction carried on following the same mechanisms as shown on the scheme 2-11 (Scheme 2-12). However, according to the present considerations, no possible interaction between 6-acetyl group and the β vinyl carbon (C1) can be observed from the molecular model that we set up to study the reaction mechanisms. Therefore, the exact mechanisms of the two $NaBH_4$ reduction reactions are still unclear. Further research is needed to discover whether or not the 6-acetyl group affects the reaction result. If yes, how it works; if not, what are the real mechanisms in the two reactions.

Scheme 2-11 The Mechanism of Reduction of the Carbonyl Group of 3



cholest-1-en-3 α ,5 α ,6 β -triol

Scheme 2-12 The Mechanism of Reduction of the Carbonyl Group and Conjugated Double Bond of 3a



6 β -acetoxycholestane-3 β ,5 α -diol

2.4 The Oxidation at the Double Bond

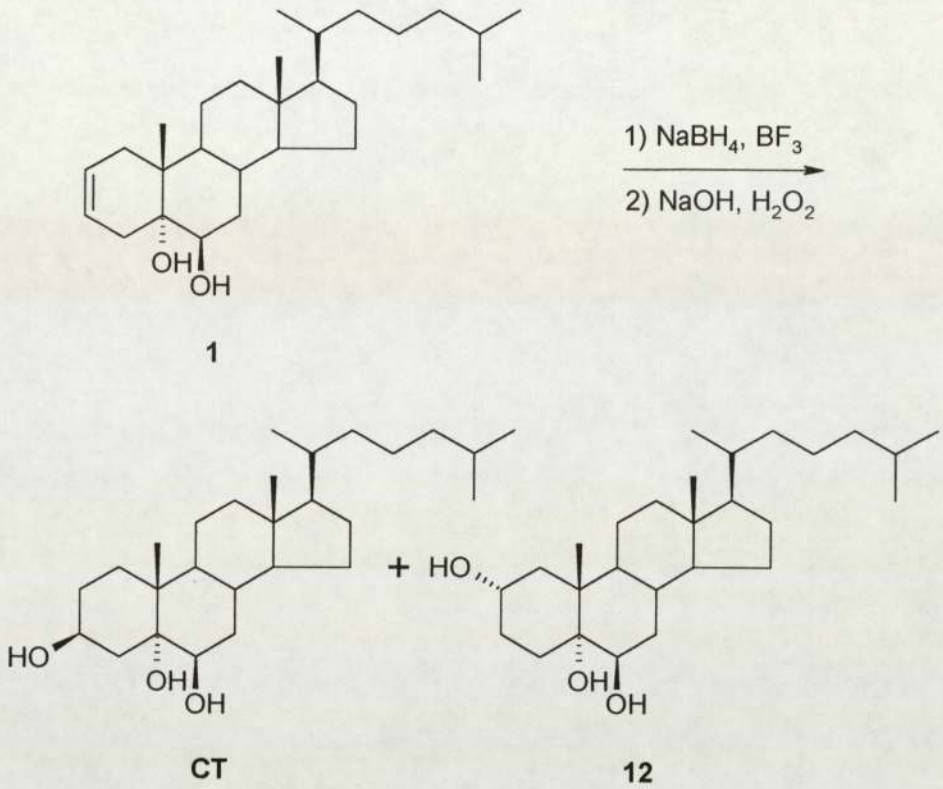
Oxidative hydroboration can introduce a hydroxyl group from the less hindered face of steroidal molecules. Pure borane (B_2H_6) gas is often used in the reaction. In our study, generation of the B_2H_6 gas *in situ via* addition of boron trifloride etherate to sodium borohydride suspended in THF was found to be a convenient method and the result of the reaction is the same to that using pure borane gas. The cholestane-3β,5α,6β-triol (**CT**) and cholestane-2α,5α,6β-triol (**12**) were prepared by using this method from the starting material **1** (Scheme 2-13).

Unlike the epoxide ring open reactions, the direct hydroxylation by borohydration gives the equatorial 2α and 3β-hydroxy group products in 36% and 42.7% yield separately. The structure of the 3β-hydroxy isomer (**CT**) was determined by the standard sample in our laboratory. After comparing the other product with the standard sample cholestane-3α,5α,6β-triol in our laboratory, we deduced that the hydroxy substituent is on C2. The 2-hydroxy isomer was oxidaised by DMP•CrO_3 to afford the 2,6-dione and reduced by $NaBH_4$ to give two products, the 2α and 2β-hydroxy mixture, the starting 2-hydroxy isomer **(12)** and the its epimer **(13)** (mole ratio 69:31) (Scheme 2-14). The stereochemistry was determined by the two big coupling effect of the 2β-hydrogen in the 2α-hydroxy isomer with the 1β and 3β hydrogen atoms on the HHCOSY spectrum. The 2β-hydroxy isomer gave a single peak and weak coupling peaks in the HHCOSY spectrum.

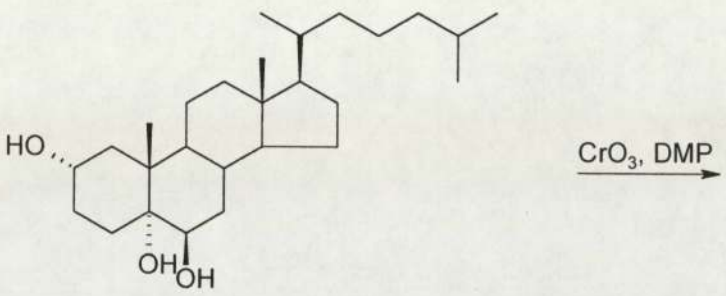
According to the literature, hydroboration–oxidation reaction has its special feature. First of all, it provides a highly convenient procedure for the anti-Markownikoff hydration of double bond (Brown and Sabba Rao,

1956). Secondly, the hydroboration reaction must involve a *cis* addition of the hydrogen-boron bond to the double bond. Then, it always takes place from the less hindered side of a double bond. At last, the alkaline hydrogen peroxide oxidation evidently proceeds with retention of configuration (Brown and Zweifel, 1959). The result of our reaction (Scheme 2-13) can be explained from the above characters of hydroboration-oxidation reaction. There is no significant discrimination between the two positions of C2=C3 double bond. So the boron could be added on any of the two carbons. When the boron was added to C2, the addition took place on the less hindered α side of the steroid structure. Because of the effect of 5α-hydroxy group, the β side of the steroid structure was comparatively less hindered than α side. When the boron was added to C3, the addition took place on the β side. Thus, we get the 2α- and 3β-hydroxy compounds as the products in 36% and 42.7% yield, respectively.

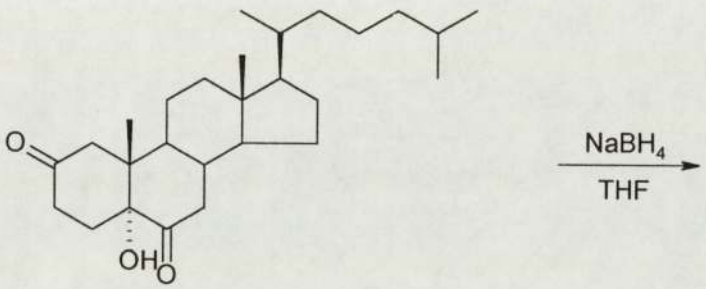
Scheme 2-13 Hydroboration-Oxidation of Cholest-2-en-5α,6β-diol



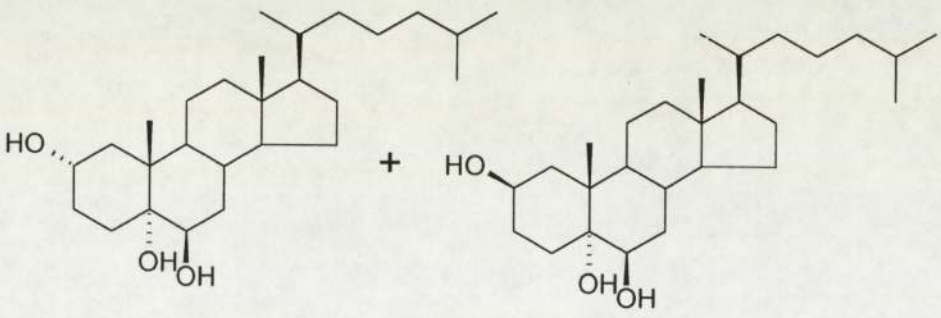
Scheme 2-14 Identification of the Stereochemistry of Cholestane-2α,5α,6β-triol



12



5 α -hydroxy-cholestane-2,6-dione



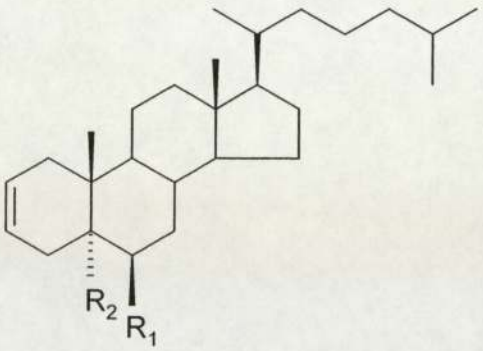
12 **13**

2.5 The Epoxidation of the Alkene Group of Cholesten

There are few reports about the 2β,3β-epoxide as the product of the reactions, when 2-en sterols with *trans* A/B ring fusion and without substituents on C1 and C4 were treated with various epoxidation reagents. We tried the epoxidation on our starting materials (**1, 1a, 1b, 1c, 1d, 1e**) with all available oxidants, only pure 2α,3α-epoxide was obtained (Scheme 2-15). The stereochemistry of these epoxides was deduced from the literature report (P. Tsui et al 1973). Firstly, the melting point and all the spectral data of **5b** coincide with the data of 2α,3α-epoxy-5α,6β-diacetoxycholestane in this report. Secondly, the chemical shifts of the C-19 methyl groups on all these epoxides remained at the same position as in the 2-en reactants. On treatment of 2-en-4-one or 1-en-3-one compounds with *m*-CPBA, there was no reaction. On the other hand, when TBHP/$Vo(acac)_2$ was used as the epoxidation reagent to treat **2a** and **3a**, **2a** gave the β-epoxide which can be crystallized from ethanol directly without chromatography; nevertheless, with **3a,** there was no reaction (Scheme 2-16).

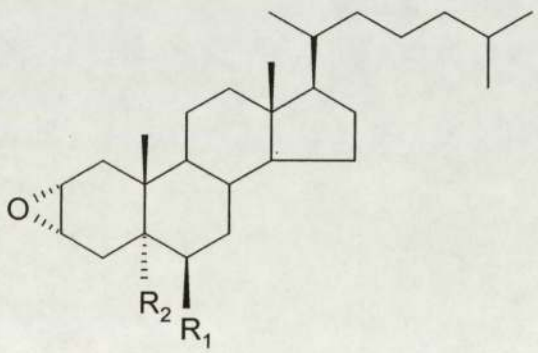
At the same time, we tried to treat the hydrolysis product (**2**) of 5α-hydroxy-6β-acetoxycholest-2-en-4-one (**2a**) with a large excess of hydrogen peroxide in methanol at –20 ℃ with addition of 1.0-equiv portion of 3 M sodium hydroxide. The same product (**6**) was obtained as shown in the scheme 2-16 and 2-17. Reduction of the 4-carbonyl group of **6** with sodium borohydride gave the product **7**, the spectra data of which are just the same as those of the standard sample (2β,3β-epoxy-cholest-4β,5α,6β- triol) in our laboratory. As a result, we discovered a efficient procedure to introduce β-epoxide on the C2 and C3 of steroids with *trans* A/B ring fusion.

Scheme 2-15 Epoxidation Reactions



1) →
2) →
3) →
4) →
5) →

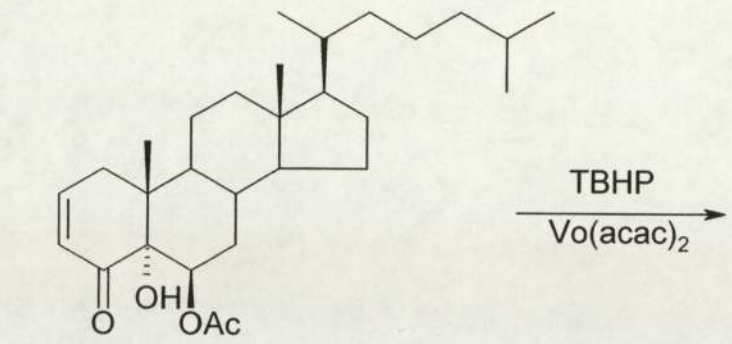
1 R_1 = OH R_2 = OH
1a R_1 = OAc R_2 = OH
1b R_1 = OAc R_2 = OAc
1c R_1 = OH R_2 = OAc
1d R_1 = H R_2 = H
1e R_1 = H R_2 = OH



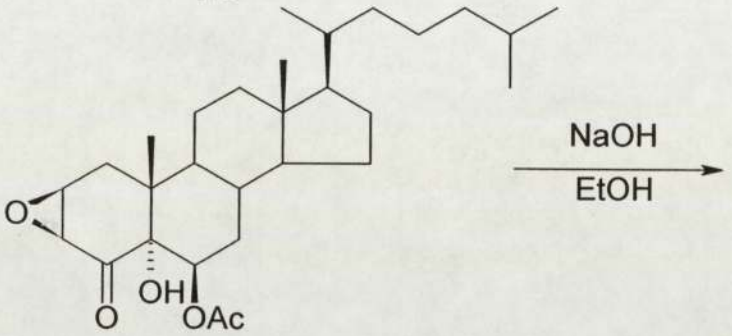
5 R_1 = OH R_2 = OH
5a R_1 = OAc R_2 = OH
5b R_1 = OAc R_2 = OAc
5c R_1 = OH R_2 = OAc
5d R_1 = H R_2 = H
5e R_1 = H R_2 = OH

1) *m*-CPBA; 2) TBHP, $Vo(acac)_2$; 3) 30% H_2O_2, $Vo(acac)_2$;
4) Sharpless Reagents; 5) Oxone, acetone

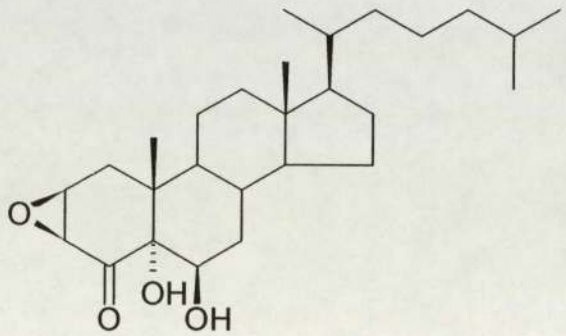
Scheme 2-16 The Attempted Epoxidation with TBHP/Vo(acac)$_2$



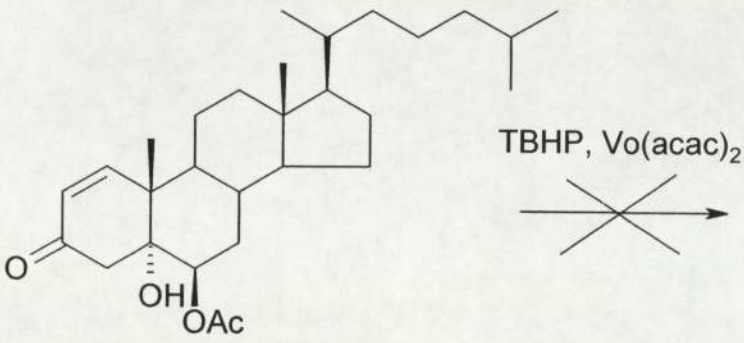
2a



6a

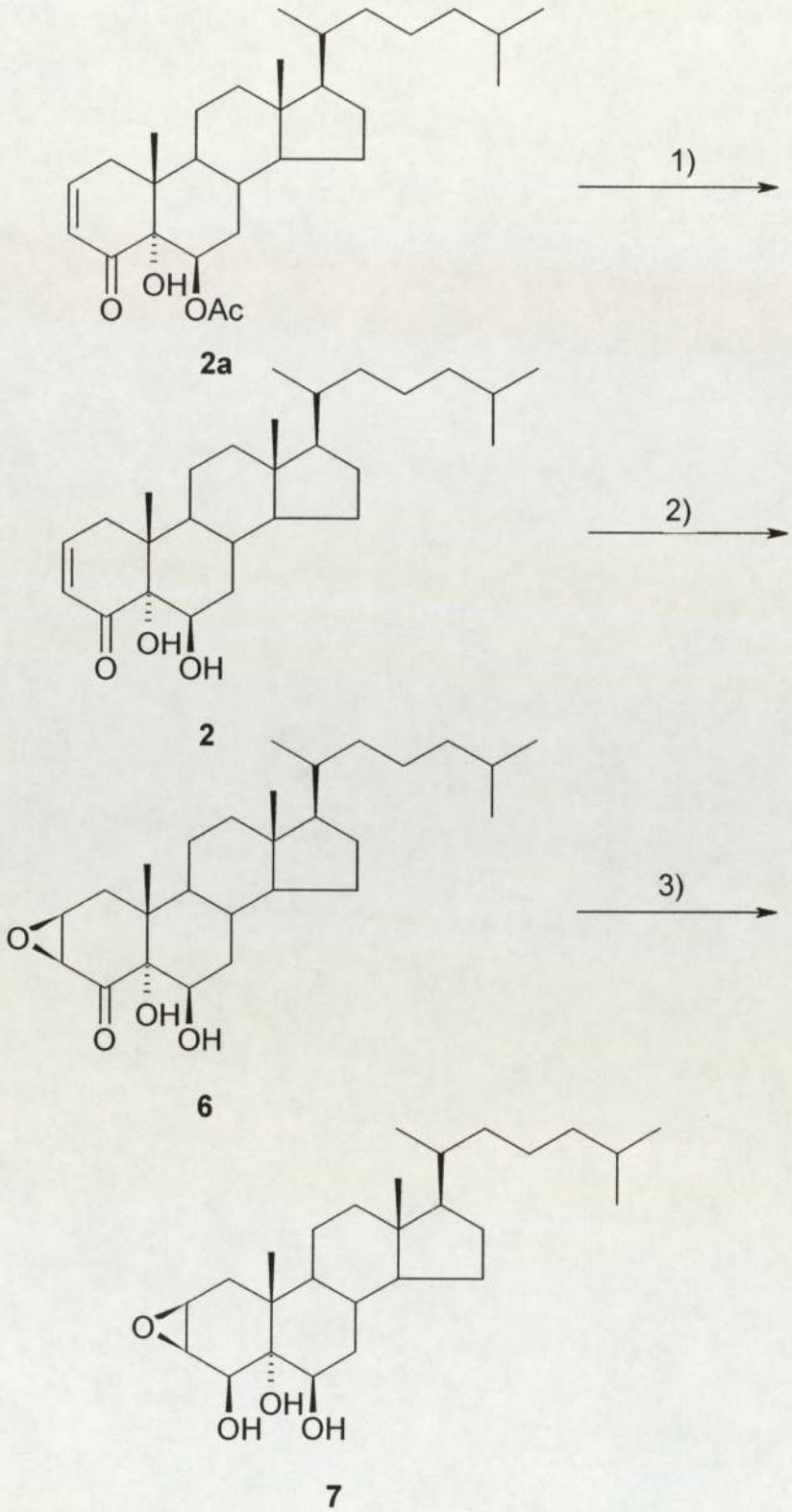


6



3a

Scheme 2-17 Synthesis of β-Epoxide



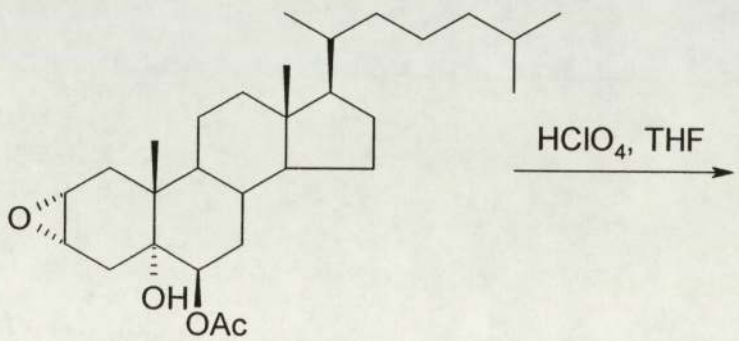
1) NaOH, EtOH; 2) NaOH, H_2O_2; 3) $NaBH_4$, THF

2.6 The Reactions of the Epoxides

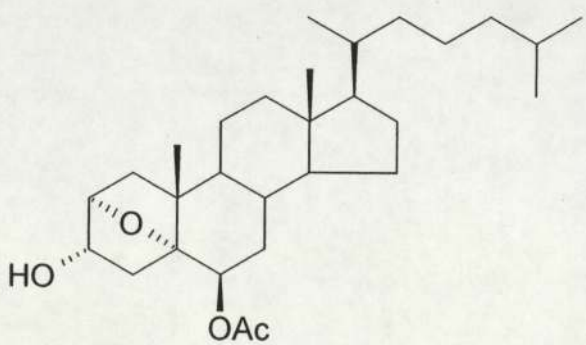
2.6.1 The Cleavage of the Epoxides

The ring open of 2,3-epoxides is a complicated process and affords not only 2,3,5,6-tetrols with variable stereo-structures but also can give the product such as **8a** (Tsui P et al 1973) (Scheme 2-18). This work is still in progress, and some obtained results are shown in scheme 2-19.

Scheme 2-18 The Epoxide Cleavage of 5a

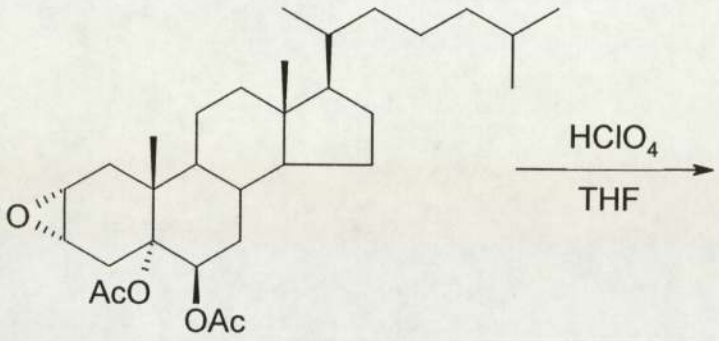


5a

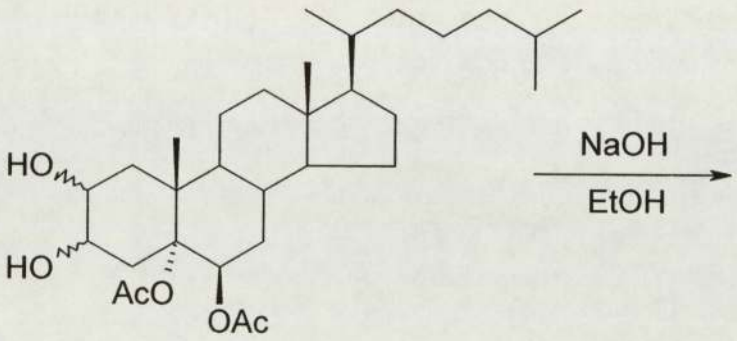


8a

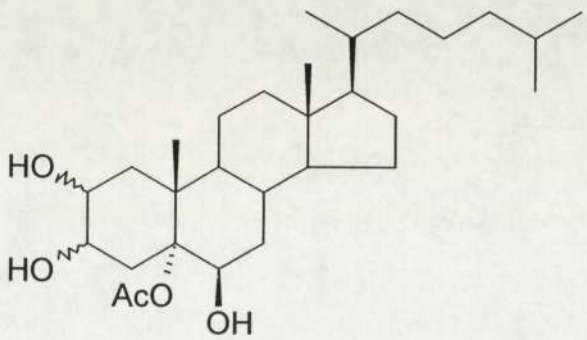
Scheme 2-19 The Epoxide Cleavage of 5b



5b



9b

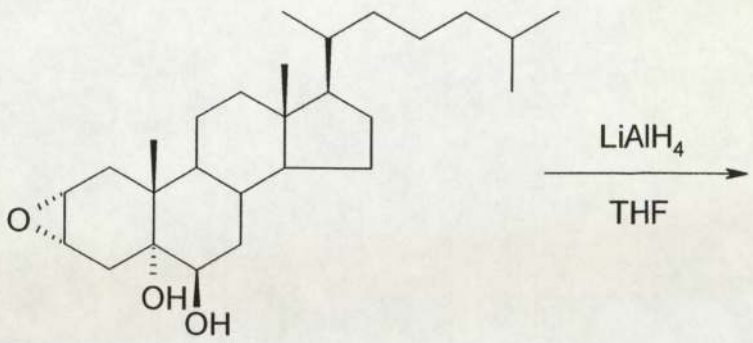


9c

2.6.2 The Rearrangement of the Epoxides

The literature method (Fürst and Plattner, 1949) was used to do the rearrangement of the epoxide with lithium aluminium hydride. As we expected, reductive cleavage of 2α,3α-epoxy-cholest-5α,6β-diol (**5**) with lithium aluminum hydride gave cholestane-3α,5α,6β-triol (**11**) in high yield (Scheme 2-20). The structure assignment of the product (**11**) was rationalized by inspection of the ^{1}H NMR spectrum where a multiplet signal as the equatorial 3β-proton was observed. The characteristic of stereochemistry of this reaction is the same as the report of Rickborn and Quartucci in 1964 (Rickborn and Quartucci, 1964). They illustrated that epoxides were reductively cleavaged in the presence of $LiAlH_4$ with attack generally occurring at the less hindered side and formed axial alcohol.

Scheme 2-20 The Epoxide Rearrangement of 5



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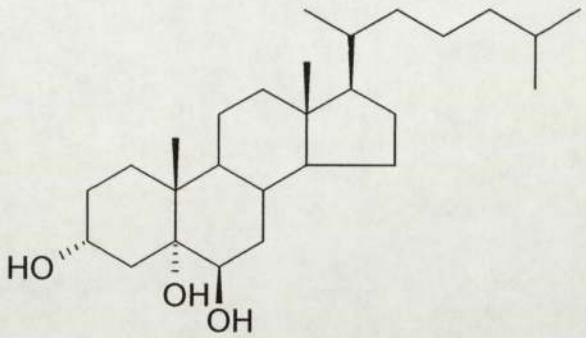

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Chapter Three: Experimental

3. Experimental

3.1 General Methods

All melting points were measured on Reichert-Jung Micrthermal, and are uncorrected. Infrared spectra were recorded on a Mattson 3000 FTIR spectrometer using potassium bromide pellets, unless otherwise stated. NMR spectra were measured on a Burker AC-250 Spectrometer at ^{1}H (250.1 MHz) and ^{13}C (62.9 MHz). Deuteriochloroform was as solvent unless stated otherwise. Chemical shifts are reported in p.p.m. Due to the low working frequency of ^{1}H NMR, only the key protons' ^{1}H NMR data are given in every compound's specta data. Mass spectroscopic analysis was carried out on a Hewlett Packard 5989B MS engine with an HP 59987A API Electrospray LC/MS interface using atmospheric chemical ionisation method.

Solvents and chemicals used for reactions were purchased from commercial suppliers and used without further purification. Solutions in organic solvents were dried with anhydrous sodium sulphate or magnesium sulphate.

TLC was carried out using aluminium backed Merck Silica Gel 60 F_{254} plates and 50% sulphuric acid water solution was used to develop TLC plates.

All column chromatographic purifications were accomplished on silica gel 60 (200-400 mesh) with the appropriate solvent gradients.

3.2 General Procedures for Some Reactions

1. General procedure of cholestan-3β,5α,6β-triol (CT) synthesis from cholesterol

Cholesterol (50.0 g, 129.0 mmol) was added into formic acid (88%, 500 ml), and the mixture was heated to 75 ℃ with stirring, then, standing at this temperature for 5 mins. After being cooled to room temperature, aqueous hydrogen peroxide (30%, 70 ml, 620.0 mmol) was dropped in during 15 mins with constant stirring. The mixture was stirred at 28 ℃ for 5 hrs and at 40 ℃ for 10 mins. The clear light blue solution was poured into boiling water (760 ml) and the solid was filtered out after being cooled to room temperature, washed with saturated sodium carbonate and dried. These solid was dissolved in methanol (1500 ml), sodium hydroxide (25%, 50 ml) was added and the resulting solution was refluxed for 1 hr. The solution was cooled to room temperature and diluted with water (500 ml). After standing overnight, the solid product was filtered out and dried to give a white solid.

2. General procedure of the sterols *p*-toluenesulfonation at room temperature

The oxysterol (47.5 mmol) was dissolved in pyridine (35 ml), *p*-toluenesulfonyl chloride (12.2 g, 64 mmol) was added and the mixture was kept at room temperature for 16 hrs. The resultant mixture was poured into ice-water (150 ml) slowly and the ice-water mixture was stirred for 2 hrs to get a well dispersed suspension, the suspension was filtered and the solid was washed with 5% hydrochloric acid followed by water, after dried in air a light brown solid product was given.

3. General procedure of elimination of *p*-toluenesulfonyl substituent from oxysterol

The sterol *p*-toluenesulfonate (45.5 mmol) was dissolved in DMF (130 ml), lithium bromide (13.0 g, 150.0 mmol) and lithium carbonate (10.0 g, 135.0 mmol) were added, and the mixture was refluxed for 1 hr. After being cooled to room temperature, the solid was filtered out with Celite, the solution was poured into ice-water (200 ml), the precipitate was collected by filteration and dried in the air, to obtain pure product.

4. General procedure of acetylating of sterols

4.1 The steroid (10.0 mmol) was dissolved in toluene (80 ml), pyridine (3 ml) and acetic anhydride (12 ml) was added, the mixture was stirred under reflux for 5 hrs, after cooling down, removal of solvents *in vacuo* give the product, using appropriate solvent to do recrystallization if necessary.

4.2 The steroid (4.0 mmol) was dissolved in glacial acetic acid (75 ml), acetic anhydride (27 ml) was added, *p*-TsOH (9.0 mmol) was added in portionwise, the mixture was stirred at room temperature for 15 hrs, the resulting mixture was poured into ice-water (500 ml) and extracted with dichloromethane, dried and evaporated under reduced pressure, to give the product.

5. General procedure for the alkaline hydrolysis of steroid acetates

The oxysterol acetate (2.0 mmol) was dissolved in ethanol (20 ml) (if it was not dissolved at room temperature, the mixture was heated to 40-50 ℃), a solution of sodium hydroxide (0.4 g, 10.0 mmol) in water (4 ml) was added and the mixture was heated at reflux (for substance not stable under heating lower temperatures are applied) for 1 hr. After being cooled down to room temperature the mixture was poured into water and the solid was collected by filtration, if the product is an oil then extracted

with a suitable solvent and normally treated.

6. General procedure for allylic oxidation

6.1 3,5-Dimethylpyrazole (DMP) (2.1 g, 21.0 mmol) was dissolved in DCM (30 ml), then the solution was cooled to –20 ℃. Chromium trioxide (2.1 g, 21.0 mmol) was added by small portion, the mixture was stirred at this temperature for 30 mins. Steroidal alkyene (4 mmol) was added and the solution was kept at –18~28 ℃ for 10 hrs. The resulted dark mixture was stirred with 10% HCl (20 ml) in an ice-water bath for 30 mins, the organic layer was separated and washed with half saturated sodium chloride solution (10 ml), dried. The product was absorbed on silica gel (4.0 g), and then the mixture was fed to a column of dry silica (10.0 g) and washed thoroughly with diethyl ether. The ether was evaporated and the residue solidified to give the product, using appropriate solvent (commonly methanol or ethanol) to do recrystallization if necessary.

6.2 The steroid (2.0 mmol) in acetone (50 ml) was treated with chromium trioxide reagent (4 ml) (dissolving chromium trioxide (1.0 g) in 90% acetic acid (5 ml)) at room temperature for 50 mins. Methanol was added to discharge the orange colour and then solid sodium hydrogen carbonate was added. The mixture was filtered through Celite. The filtrate was evaporated to near dryness and extracted into ether. The extract was washed with water and dried (Na_2SO_4). The solvent was evaporated to give the product.

7. General procedure for the reduction of steroidal carbonyl group to hydroxyl group

The steroidal ketone (10.0 mmol) was dissolved in THF (40 ml) and

water (4 ml), sodium borohydride (1.6 g, 42.3 mmol) was added and the mixture was stirred at room temperature for 1 hr, TLC was used to check the end of the reaction. The reaction was quenched by adding acetic acid (or 10% HCl) till the gas release ceased. The resultant mixture was poured into water; the solid was filtered out, dried to give the crude product (Oil product was extracted with appropriate solvent).

8. General procedure for the addition of hydrogen on cholestene

The cholestene (2.1 mmol) was dissolved in 50 ml of ethanol and hydrogenated in a Parr apparatus under 50 psi of pressure in the presence of 5% Pd/C (0.1 g) for 3 hrs. After filtration with Celite, the filtrate was evaporated under reduced pressure to give the product.

9. General procedure for the epoxidation of cholestene

9.1 Olefin (0.6 mmol) in 10 ml dichloromethane was stirred at room temperature for 5 hrs with 0.3 g of 70% *m*-chloroperbenzoic acid. After addition of 10% aqueous sodium hydroxide, the organic layer was washed with water, and dried. Evaporation gave the crude epoxide, recrystallized from ethanol if necessary.

9.2 To the steroid (10.0 mmol) and vanadyl acetyl acetonate (0.1 g, 0.2mmol) in 50 ml of DCM was added dropwise the TBHP/toluene solution (45.0 mol), the homogeneous solution was stirred at room temperature for 16 hrs. The reaction was monitored by TLC. When the reaction finished, the resulting mixture was washed with saturated sodium bicarbonate (20 ml x 2) and saturated sodium chloride (20 ml x 2), dried over $MgSO_4$, and concentrated to give the crude epoxide.

9.3 To a solution of olefin (20.0 mmol) in THF (100 ml) was added 30% aqueous hydrogen peroxide (34.0 mmol, 3 ml), followed by vanadyl acetyl acetonate (0.1 g, 0.4 mmol). The mixture was stirred at room

temperature. The reaction was monitored by TLC. After the reaction was complete as shown by TLC, adequate amount of water was added in the reaction mixture, and the mixture was extracted with diethyl ether, the ether layer was washed with saturated sodium bicarbonate and saturated sodium chloride, and dried with anhydrous sodium sulfate. After removal of the solvent under reduced pressure, the residue was shown to be the epoxide, recrystallizated from ethanol if necessary.

9.4 Freshly distilled titanium tetraisopropoxide (4.0 g, 15.0 mmol) was added to DCM (20 ml) (place appropriate amount of preactivated and powdered 3 or 4 Å molecular sieves into the reaction flask in advance). The resulting solution was cooled to –20 ℃ (dry ice/acetone). Freshly distilled (*S,S*)-(-)-diisopropyl tartrate (DIPT) or (*R,R*)-(+)-DIPT (3.0 g, 15.0 mmol) was then added. The resulting mixture was stirred for 15 mins, and then a solution of the steroid (10.0 mmol) in DCM (100 ml) was added. Ten minutes later *tert*-butyl hydroperoxide solution (6 ml of a 4.7 M solution in toluene, 30.0 mmol) was added in dropwise, and the reaction mixture was then stirred at a temperature between –10 and 0 ℃ for 48 hrs. The resulting mixture was poured into a precooled (0 ℃) aqueous solution of tartaric acid (10% w/v) (20 ml), this mixture was vigorously stirred for 30 mins, then, diluted with diethyl ether (50 ml), washing with 10% aqueous tartaric acid (2 x 25 ml) and saturated brine solution (2 x 40 ml), drying (MgSO4), and concentration under reduced pressure afforded a crude epoxide.

9.5 To an acetonitrile solution (15 ml) of olefin (2.0 mmol) was added an aqueous Na_2 • EDTA solution (10 ml, 4×10^{-4}M). The resulting homogeneous solution was cooled to 0-1 ℃, followed by addition of acetone (2 ml) *via* a precooled syringe. To this solution was added a mixture of sodium bicarbonate (1.3 g, 15.5 mmol) and Oxone (3.1 g, 10.0 mmol) over a period of 1 hr (PH ~ 7). The reaction was complete as

shown by TLC. The reaction mixture was then poured into water (200 ml), extracted with dichloromethane (3×200 ml), and dried with anhydrous sodium sulfate. After removal of the solvent under reduced pressure, the residue was shown to be the epoxide.

9.6 To a solution of 2.4 g (6.0 mmol) of 5α,6β-dihydroxy-cholest-2-en-4-one dissolved in 120 ml of methanol at –20 ℃ was added 3 ml of 2 *N* NaOH and 7.5 ml (30.0 mmol) of 30% H_2O_2. The homogeneous mixture was stirred at –20 ℃ for 2 hrs. The solution was poured into water and extracted with diethyl ether, dried and evaporated under reduced pressure, gave 2β,3β-epoxy-5α,6β-dihydroxy-cholestane-4-one.

10. General procedure for the hydroboration-oxidation reaction

A solution of 2.0 g (5.0 mmol) of cholest-2-en-5α,6β-diol and 0.1 g (2.6 mmol) sodium borohydride in 5 ml ether at 0 ℃ was treated over a period of 1 hr with 0.5 g (3.5 mmol) of boron trifluoride etherate. After a second hour at 0 ℃, 0.5 ml of water was added to destroy residual hydride, 2.1 ml of 3 *N* sodium hydroxide added, and the product oxidized with 2.1 ml of 30% hydrogen peroxide added slowly over a period of 1 hr through the dropping funnel. The solution was washed with water, the ether distilled to recover the product.

11. General procedure for the cleavage of the epoxide ring in oxysterols with perchloric acid in THF

Epoxide (5.0 mmol) in 100 ml of THF was stirred with perchloric acid (10.0 mmol) and 1 ml of water for 3.5 hrs at room temperature. The mixture was poured into water and extracted with diethyl ether, dried and evaporated under reduced pressure, gave the product.

12. General procedure for the reduction of the epoxide with lithium aluminum hydride

2α,3α-Epoxy-cholestane-5α-6β-diol (2.0 mmol) was dissolved in dried THF (20 ml), and lithium aluminum hydride (0.2 g, 6.0 mmol) was mixed with dried THF (3 ml) under efficient stirring in advance. Put the mixture of $LiAlH_4$ and THF into the oxysterol acetate THF solution in dropwise. The mixture was stirred for 1 hr. The reaction was quenched by adding water slowly till the gas exhaustion ceased. Appropriate quantity of 10% HCl was added afterwards. The product was extracted with appropriate solvent, followed by the usual procedure to get the product.

3.3 Synthetic Preparations

Cholestane-3β,5α,6β-triol (CT) (Fieser and Rajagopalan, 1949) This compound was prepared by the general procedure 1 from **cholesterol** in 93% yield; mp 239-241 ℃; IR (KBr disc): ν_{max} 3420, 2970, 1470, 1380, 1295, 1160, 1040, 950, 860 cm^{-1}; ^1H NMR δ 3.47 (s, 1H, 6α-H), 3.55 (m, 1H, 3α-H), 0.64 (s, 3H, 18-Me), 1.09 (s, 3H, 19-Me); m/z 420 [M^+].

Cholest-2-en-5α,6β-diol (1) (McMichael and Selter, 1965) This compound was obtained from **3β-tosyloxy-cholestane-5α,6β-diol** by the general procedure 3 in 99% yield; mp 134-135 ℃; IR (KBr disc) ν_{max} 3509, 2974, 1629, 1469, 1365, 1290, 1018, 867, 667 cm^{-1}; ^1H NMR δ 3.65 (s, 1H, 6α-H), 5.60 (2H,vinyl protons), 0.66 (s, 3H, 18-Me), 1.03 (s, 3H, 19-Me); ^{13}C NMR δ 11.9, 16.2, 18.6, 20.7, 22.5, 22.7, 23.8, 24.1, 27.9, 28.1, 29.8, 34.0, 35.1, 35.7, 36.1, 37.3, 38.0, 39.4, 39.8, 42.4, 46.0, 55.7, 56.1, 73.1, 73.8, 123.0, 126.5; m/z 402 [M^+].

5α-Hydroxy-6β-acetoxycholest-2-ene (1a) (Tsui and Just, 1973) This compound was obtained from **1** by the general procedure 4.1 as an oil in

96% yield; IR (KBr disc) ν_{max} 3500, 3030, 1740, 1660, 1470, 1365, 1240, 1017, 870 cm^{-1}; ^1H NMR δ 4.82 (s, 1H, 6α-H), 5.54 (2H, vinyl protons), 0.66 (s, 3H, 18-Me), 0.99 (s, 3H, 19-Me), 2.08 (s, 3H, 6-acetate); ^{13}C NMR 12.0, 15.8, 18.7, 20.9, 21.3, 22.6, 22.8, 24.0, 24.1, 27.8, 28.0, 30.5, 31.9, 33.9, 35.8, 36.0, 37.0, 38.0, 39.4, 39.9, 42.7, 45.7, 55.9, 56.2, 72.3, 75.0, 122.4, 126.8, 170.0; m/z 444 [M^+].

5α,6β-Diacetoxycholest-2-ene (1b) (Tsui and Just, 1973) This compound was obtained from **1a** by the general procedure 4.2 in 98% yield; mp 136-137 ℃; IR (KBr disc) ν_{max} 3030, 1725, 1625, 1250, 1230, 1210, 1035, 915, 850 cm^{-1}; ^1H NMR δ 5.84 (s, 1H, 6α-H), 5.43 (2H, vinyl protons), 0.65 (s, 3H, 18-Me), 1.01 (s, 3H, 19-Me), 2.03 and 1.92 (2 x s, 2 x 3H, 2 acetates); ^{13}C NMR δ 12.0, 15.9, 18.6, 20.7, 21.3, 22.2, 22.4, 22.7, 23.6, 23.9, 26.1, 27.9, 28.0, 29.9, 31.7, 35.6, 36.0, 37.5, 39.0, 39.4, 39.8, 42.4, 45.9, 55.8, 56.0, 69.1, 84.4, 121.7, 125.8, 169.5, 169.7; m/z 487 [M+H]$^+$.

6β-Hydroxy-5α-acetoxycholest-2-ene (1c) (Kocovsky and Cerny, 1977) This compound was prepared by the general procedure 5 from **1b** as an oil in 99% yield; IR (KBr disc) ν_{max} 3486, 3027, 1740, 1659, 1465, 1358, 1241, 1020, 887 cm^{-1}; ^1H NMR δ 4.72 (s, 1H, 6α-H), 5.50 (2H, vinyl protons), 0.68 (s, 3H, 18-Me), 1.06 (s, 3H, 19-Me), 1.93 (s, 3H, 5-acetate); ^{13}C NMR δ 12.0, 16.2, 18.6, 20.8, 22.4, 22.5, 22.7, 23.7, 24.0, 26.2, 27.9, 28.1, 29.3, 34.9, 35.7, 36.1, 37.6, 38.8, 39.4, 39.9, 42.4, 46.2, 56.0, 56.1, 66.9, 86.6, 122.1, 125.8, 170.6; m/z 445 [M+H]$^+$.

Cholest-2-ene (1d) (Striebel and Tamm, 1954) This compound was obtained from **3β-cholestanol** by two reaction steps of general procedure 2 and 3 in 97% over-all yield; mp 72-74 ℃; IR (KBr disc) ν_{max} 3446, 3022, 2942, 2870, 1652, 1471, 1376, 1182, 650 cm^{-1}; ^1H NMR δ 5.57 (s, 2H, vinyl protons), 0.64 (s, 3H, 18-Me), 0.73 (s, 3H, 19-Me); m/z 370

$[M^+]$.

5α-Hydroxy-cholest-2-ene (1e) (Clayton et al., 1957) This compound was obtained from **cholestane-3β,5α-diol** by two reaction steps of general procedure 2 and 3 in 96% over-all yield; mp 93-95 ℃; IR (KBr disc) ν_{max} 3560, 3050, 2870, 1740, 1379, 1250, 1128, 1030, 950 cm^{-1}; ^1H NMR δ 5.56 (2H, vinyl protons), 0.64 (s, 3H, 18-Me), 0.97 (s, 3H, 19-Me); m/z 386 $[M^+]$.

5α,6β-Dihydroxy-cholest-2-en-4-one (2) This compound was obtained from **2a** by the general procedure 5 in 98% yield; mp 188-190 ℃; IR (KBr disc) ν_{max} 3431, 2947, 2358, 1666, 1469, 1390, 1248, 1022, 815 cm^{-1}; ^1H NMR δ 4.10 (s, 1H, 6α-H), 5.93 (dd, 1H, 3-H), 6.86 (m, 1H, 2-H), 0.66 (s, 3H, 18-Me), 1.15 (s, 3H, 19-Me); m/z 416 $[M^+]$.

5α-Hydroxy-6β-acetoxycholest-2-en-4-one (2a) This compound was prepared from **1a** by the general procedure 6.2, then following the general procedure 6.2, the resulting solid was chromatographed on silica gel, eluted by diethyl ether in 59% yield; mp 72-74 ℃; IR (KBr disc) ν_{max} 3460, 2935, 1790, 1685, 1460, 1375, 1205, 1030, 790 cm^{-1}; ^1H NMR δ 5.24 (s, 1H, 6α-H), 5.90 (dd, 1H, 3-H), 6.87 (m, 1H, 2-H), 0.67 (s, 3H, 18-Me), 1.12 (s, 3H, 19-Me), 1.99 (s, 3H, 6-acetate); ^{13}C NMR δ 12.0, 15.8, 18.6, 20.3, 21.5, 22.4, 22.7, 23.7, 23.9, 27.8, 28.1, 30.2, 31.2, 35.7, 36.0, 37.9, 39.4, 39.6, 41.7, 42.5, 44.8, 55.3, 56.0, 69.4, 75.3, 126.0, 148.4, 169.9, 197.0; m/z 459 $[M+H]^+$.

5α,6β-Dihydroxy-cholest-1-en-3-one (3) This compound was obtained from **3a** by the general procedure 5 in 99% yield; mp 144-146 ℃; IR (KBr disc) ν_{max} 3459, 2937, 1754, 1660, 1482, 1399, 1230, 1070, 851 cm^{-1}; ^1H NMR δ 4.51 (s, 1H, 6α-H), 6.15 (d, 1H, 1-H), 7.01 (d, 1H, 2-H), 0.75 (s, 3H, 18-Me), 1.40 (s, 3H, 19-Me); m/z 416 $[M^+]$.

5α-Hydroxy-6β-acetoxycholest-1-en-3-one (3a) This compound was

prepared from **1a** by the general procedure 6.1, following the general procedure 6.1, the resulting solid was chromatographed on silica gel, eluted by 1:1 diethyl ether-petroleum spirit in 23% yield; mp 136-138 ℃; IR (KBr disc) ν_{max} 3477, 2954, 1766, 1675, 1459, 1391, 1215, 1019, 760 cm^{-1}; ^1H NMR δ 4.82 (s, 1H, 6α-H), 5.91 (d, 1H, 1-H), 6.98 (d, 1H, 2-H), 0.70 (s, 3H, 18-Me), 1.27 (s, 3H, 19-Me), 2.07 (s, 3H, 6-acetate); ^{13}C NMR δ 12.1, 12.5, 18.4, 19.1, 21.0, 21.3, 21.4, 22.4, 22.7, 23.7, 27.9, 28.1, 30.5, 30.7, 35.7, 36.0, 39.4, 39.6, 41.7, 42.6, 43.4, 45.5, 55.6, 56.0, 74.6, 75.4, 155.1, 169.9, 197.8; m/z 459 $[M+H]^+$.

5α,6β-Diacetoxycholest-1-en-3-one (3b) This compound was obtained from **1b** by the general procedure 6.1 in 46% yield; mp 163-165 ℃; IR (KBr disc) ν_{max} 3444, 2959, 1742, 1684, 1463, 1381, 1205, 1026, 779 cm^{-1}; ^1H NMR δ 5.89 (s, 1H, 6α-H), 5.85 (d, 1H, 1-H), 6.93 (d, 1H, 2-H), 0.71 (s, 3H, 18-Me), 1.29 (s, 3H, 19-Me), 2.07 and 1.92 (2 x s, 2 x 3H, 2 acetates); ^{13}C NMR δ 12.2, 18.5, 18.6, 21.1, 21.9, 22.4, 22.7, 23.6, 23.8, 27.8, 28.0, 30.0, 30.4, 35.6, 35.9, 39.3, 39.4, 39.5, 42.1, 42.5, 42.6, 43.7, 55.7, 55.9, 68.8, 86.5, 102.1, 126.0, 154.3, 169.2, 196.1; m/z 501 $[M+H]^+$.

5α,6β-Dihydroxy-cholest-2-en-1-one (4) (Ishiguro et al., 1974) This compound was prepared from **4a** by the general procedure 5 in 98% yield; mp 141-143 ℃; IR (KBr disc) ν_{max} 3436, 2954, 2364, 1735, 1680, 1463, 1380, 1250, 1020 cm^{-1}; ^1H NMR δ 3.64 (s, 1H, 6α-H), 5.85 (d, 1H, 2-H), 6.57 (d, 1H, 3-H), 0.68 (s, 3H, 18-Me), 1.30 (s, 3H, 19-Me); m/z 416 $[M^+]$.

5α-Hydroxy-6β-acetoxycholest-2-en-1-one (4a) This compound was prepared from **1a** by the general procedure 6.1, following the general procedure, the resulting solid was chromatographed on silica gel, eluted by 1:1 diethyl ether-petroleum spirit in 18% yield; mp 149-151 ℃; IR

(KBr disc) ν_{max} 3463, 2965, 2901, 2357, 1739, 1677, 1447, 1389, 1251 cm^{-1}; ^1H NMR δ 4.78 (s, 1H, 6α-H), 5.86 (dd, 1H, 3-H), 6.50 (m, 1H, 2-H), 0.68 (s, 3H, 18-Me), 1.27 (s, 3H, 19-Me), 2.11 (s, 3H, 6-acetate); ^{13}C NMR δ 11.3, 12.2, 15.3, 18.9, 19.8, 21.3, 22.5, 22.7, 23.1, 23.9, 24.0, 28.0, 28.9, 30.2, 30.5, 35.1, 35.8, 36.0, 39.4, 39.8, 40.8, 42.6, 51.7, 55.6, 56.2, 75.2, 75.5, 128.5, 140.7, 170.1, 204.4; m/z 459 $[M+H]^+$.

2α,3α-Epoxy-cholest-5α,6β-diol (5) This compound was obtained by the general procedure 9.1 from **1** in 98% yield; mp 89-91 ℃; IR (KBr disc) ν_{max} 3436, 2931, 2358, 1715, 1635, 1469, 1385, 1035, 805 cm^{-1}; ^1H NMR δ 3.55 (s, 1H, 6α-H), 3.28 (t, 1H, 3β-H), 3.43 (s, 1H, 2β-H), 0.64 (s, 3H, 18-Me), 1.07 (s, 3H, 19-Me); m/z 418 $[M^+]$.

2α,3α-Epoxy-5α-hydroxy-6β-acetoxycholestane (5a) This compound was obtained by the general procedure 9.1 from **1a** in 98% yield; mp 114.5-116.5 ℃; IR (KBr disc) ν_{max} 3450, 2949, 1733, 1463, 1369, 1243, 1024, 931, 806 cm^{-1}; ^1H NMR δ 4.72 (s, 1H, 6α-H), 3.28 (t, 1H, 3β-H), 3.39 (s, 1H, 2β-H), 0.65 (s, 3H, 18-Me), 1.04 (s, 3H, 19-Me), 2.06 (s, 3H, 6-acetate); ^{13}C NMR δ 11.9, 16.5, 18.5, 20.4, 21.3, 22.4, 22.7, 23.7, 24.0, 27.8, 28.0, 29.7, 30.6, 30.9, 35.4, 35.7, 36.0, 37.8, 39.4, 39.6, 42.3, 45.6, 51.9, 54.3, 55.4, 56.0, 72.8, 75.0, 169.6; m/z 460 $[M^+]$.

2α,3α-Epoxy-5α,6β-diacetoxycholestane (5b) (Tsui and Just, 1973) This compound was obtained by the general procedure 9.1 from **1b** in 97% yield; mp 188-191 ℃; IR (KBr disc) ν_{max} 3458, 2946, 1731, 1725, 1470, 1365, 1243, 1220, 1033 cm^{-1}; ^1H NMR δ 5.66 (s, 1H, 6α-H), 3.10 (t, 1H, 3β-H), 3.17 (s, 1H, 2β-H), 0.65 (s, 3H, 18-Me), 1.08 (s, 3H, 19-Me), 2.06 and 1.97 (2 x s, 2 x 3H, 2 acetates); ^{13}C NMR δ 11.9, 16.4, 18.5, 20.6, 21.2, 22.4, 22.6, 22.7, 23.6, 23.9, 27.8, 28.0, 30.0, 30.9, 34.8, 35.6, 36.0, 38.5, 39.3, 39.6, 42.3, 42.4, 45.6, 49.2, 51.2, 55.5, 55.9, 69.4, 83.2, 169.3, 170.1; m/z 502 $[M^+]$.

2α,3α-Epoxy-6β-hydroxy-5α-acetoxycholestane (5c) This compound was obtained by the general procedure 9.1 from **1c** in 98% yield; mp 97-99 ℃; IR (KBr disc) ν_{max} 3449, 2950, 2869, 1740, 1460, 1365, 1250, 1025, 810 cm^{-1}; ^1H NMR δ 4.60 (s, 1H, 6α-H), 3.37 (t, 1H, 3β-H), 3.46 (s, 1H, 2β-H), 0.62 (s, 3H, 18-Me), 1.01 (s, 3H, 19-Me), 2.06 (s, 3H, 5-acetate); ^{13}C NMR δ 11.6, 15.9, 19.5, 20.6, 21.9, 22.5, 22.9, 24.3, 24.9, 28.2, 28.8, 29.9, 30.7, 31.0, 35.4, 35.9, 36.3, 37.8, 39.4, 39.6, 43.3, 45.5, 52.6, 55.1, 55.9, 57.0, 73.6, 75.9, 168.6; m/z 460 [M^+].

2α,3α-Epoxy-cholestane (5d) (Jones and Grayshan, 1972) This compound was obtained by the general procedure 9.1 from **1d** in 98% yield; mp 104-105 ℃; IR (KBr disc) ν_{max} 3450, 2933, 1728, 1635, 1469, 1385, 1164, 1010, 806 cm^{-1}; ^1H NMR δ 3.07 (t, 1H, 3β-H), 3.12 (s, 1H, 2β-H), 0.61 (s, 3H, 18-Me), 0.72 (s, 3H, 19-Me); m/z 386 [M^+].

2α,3α-Epoxy-5α-hydroxy-cholestane (5e) (Nambara et al., 1974) This compound was prepared from **1e** by the general procedure 9.1 in 97% yield; mp 142-143 ℃; IR (KBr disc) ν_{max} 3434, 2956, 2858, 1723, 1438, 1336, 1239, 1020, 818; ^1H NMR δ 3.38 (m, 2H, 2β- and 3β-H), 0.65 (s, 3H, 18-Me), 0.81 (s, 3H, 19-Me); m/z 402 [M^+].

2β,3β-Epoxy-5α,6β-dihydroxy-cholestane-4-one (6) This compound was obtained by the general procedure 9.6 from **2** in 86% yield; mp 120-122 ℃; IR (KBr disc) ν_{max} 3421, 2922, 2364, 1728, 1469, 1385, 1205, 1030, 580 cm^{-1}; ^1H NMR δ 4.07 (s, 1H, 6α-H), 3.46 (d, 1H, 3α-H), 3.70 (t, 1H, 2α-H), 0.64 (s, 3H, 18-Me), 1.13 (s, 3H, 19-Me); m/z 432 [M^+].

2β,3β-Epoxy-5α-hydroxy-6β-acetoxycholest-4-one (6a) This compound was obtained by the general procedure 9.2 from **2a** in 67% yield; mp 178-180 ℃; IR (KBr disc) ν_{max} 3450, 2944, 2865, 2358, 1745, 1463, 1370, 1242, 1032 cm^{-1}; ^1H NMR δ 5.19 (s, 1H, 6α-H), 3.38 (d, 1H,

3α-H), 3.65 (t, 1H, 2α-H), 0.65 (s, 3H, 18-Me), 1.10 (s, 3H, 19-Me), 2.01 (s, 3H, 6-acetate); ^{13}C NMR δ 11.9, 16.0, 17.5, 18.5, 20.8, 21.3, 22.4, 22.7, 23.7, 23.9, 27.9, 28.0, 30.3, 30.8, 35.1, 35.7, 36.0, 39.4, 39.6, 42.4, 45.3, 45.8, 55.1, 55.6, 56.0, 56.9, 69.5, 169.5, 200.5; m/z 474 [M^+].

2β,3β-Epoxy-cholestane-4β,5α,6β-triol (7) This compound was obtained from **6** by the general procedure 7 in 95% yield; mp 97-99 ℃; IR (KBr disc) ν_{max} 3471, 2939, 2865, 1718, 1419, 1328, 1255, 1020, 808 cm^{-1}; ^{1}H NMR δ 4.00 (s, 1H, 6α-H), 4.21 (d, 1H, 4α-H), 3.36 (2H, 2α-H and 3α-H), 0.65 (s, 3H, 18-Me), 1.28 (s, 3H, 19-Me); m/z 434 [M^+].

2α,5α-Oxy-3α-hydroxy-6β-acetoxycholestane (8a) (Tsui and Just, 1973) This compound was prepared from **5a** by the general procedure 11 as an oil in 98% yield. IR (KBr disc) ν_{max} 3400, 2952, 2865, 1745, 1469, 1371, 1238, 1026, 970 cm^{-1}; ^{1}H NMR δ 5.29 (s, 1H, 6α-H), 3.84 (d, 1H, 3β-H), 4.25 (d, 1H, 2β-H), 2.53 (dd, 1H, 4β-H), 0.67 (s, 3H, 18-Me), 0.99 (s, 3H, 19-Me), 2.05 (s, 3H, 6-acetate) ^{13}C NMR δ 12.2, 16.4, 18.5, 21.0, 21.1, 21.4, 22.4, 22.7, 23.8, 24.0, 25.4, 26.3, 27.8, 28.1, 30.2, 33.2, 34.0, 35.7, 36.0, 39.4, 39.8, 40.8, 41.8, 42.6, 42.9, 48.3, 55.7, 56.0, 83.5, 86.0, 170.1; m/z 460 [M^+].

5α,6β-Diacetoxycholestane-2,3(*trans*)-diol (9b) This compound was obtained from **5b** by the general procedure 11 in 93% yield; mp 238-239 ℃; IR (KBr disc) ν_{max} 3449, 2927, 2856, 1738, 1480, 1365, 1239, 1010, 850 cm^{-1}; ^{1}H NMR δ 4.77 (s, 1H, 6α-H), 4.20 and 5.03 (2-H and 3-H), 0.67 (s, 3H, 18-Me), 0.99 (s, 3H, 19-Me), 2.02 and 2.07 (2 x s, 2 x 3H, 2 acetates); m/z 520 [M^+].

5α-Acetoxycholestane-2,3(*trans*),6β-triol (9c) This compound was prepared from **9b** by the general procedure 5 in 97% yield; mp 189-191 ℃; IR (KBr disc) ν_{max} 3425, 2939, 2865, 1739, 1469, 1373, 1240, 1037, 530 cm^{-1}; ^{1}H NMR δ 4.76 (s, 1H, 6α-H), 3.86 and 4.06 (2-H and 3-H),

0.65 (s, 3H, 18-Me), 1.06 (s, 3H, 19-Me), 2.02 (s, 3H, 6-acetate); m/z 478 [M^+].

Cholest-1-en-3α,5α,6β-triol (10) This compound was prepared by the general proceduce 7 from **3** in 86% yield; mp 205-207 ℃; IR (KBr disc) ν_{max} 3444, 2950, 2360, 1737, 1463, 1365, 1250, 1030, 965 cm^{-1}; ^1H NMR δ 3.84 (s, 1H, 6α-H), 4.28 (m, 1H, 3β-H), 5.55 (2H, vinyl protons), 0.67 (s, 3H, 18-Me), 1.18 (s, 3H, 19-Me); m/z 418 [M^+].

Cholestane-3α,5α,6β-triol (11) (Coxon et al., 1970) This compound was obtained from **5** by the general procedure 12 in 98% yield; mp 205-206 ℃; IR (KBr disc) ν_{max} 3400, 2942, 2871, 1469, 1376, 1042, 960, 870, 667 cm^{-1}; ^1H NMR δ 4.24 (s, 1H, 3β-H), 3.56 (s, 1H, 6α-H), 0.66 (s, 3H, 18-Me), 1.09 (s, 3H, 19-Me); m/z 420 [M^+].

Cholestane-2α,5α,6β-triol (12) This compound was obtained from **1** by the general procedure 10, then following the general procedure 10, the resulting mixture was chromatographed on silica gel, eluted by 2:1 diethyl ether-petroleum spirit in 36% yield; mp 195-196 ℃; IR (KBr disc) ν_{max} 3428, 2949, 2883, 1469, 1382, 1260, 1125, 1035, 952 cm^{-1}; ^1H NMR δ 3.85 (m, 1H, 2β-H), 3.57 (s, 1H, 6α-H), 0.67 (s, 3H, 18-Me), 1.12 (s, 3H, 19-Me); m/z 420 [M^+].

Cholestane-2β,5α,6β-triol (13) This compound was prepared by the general procedure 7 from **5α-hydroxycholestane-2,6-dione**, which was obtained from **12** by the general procedure 6.1 and used as the reactant of the next reaction without further purification. Then following the general procedure 7, the resulting mixture was chromatographed on silica gel, eluted by 50:1 dichloromethane-acetone in 39% yield; mp 223-225 ℃; IR (KBr disc) ν_{max} 3411, 2933, 2865, 1720, 1465, 1385, 1295, 1190, 995 cm^{-1}; ^1H NMR δ 4.14 (s, 1H, 2α-H), 3.52 (s, 1H, 6α-H), 0.65 (s, 3H, 18-Me), 1.35 (s, 3H, 19-Me); m/z 420 [M^+].

Chapter Four: References

4. References

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