Exploration of Stereoselective Cleavage of 4, 5-Epoxycholestane-3, 6-diols and Stereo-controlled Epoxidation of Cholest-4-en-3β, 6β-diol-6-acetate

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A thesis submitted by Li Han B.Sc. for the degree of

Master of Philosophy

Abstract: The research of oxygenated sterols, oxysterols, has been moving on from their original chemical structures and total syntheses to their diverse biological activities and their potential medical usage. However, due to the availability in amounts and in kinds of isomers with desired oxygenated groups attached at preferred positions with desired stereochemisty, the research has been hindered greatly.

The aim of the project was to explore an effective and economic synthetic method for the stereoselective cleavage of 4, 5-epoxycholestane-3, 6-diols to give desired stereoisomers of cholestane-3, 4, 6-triols. We are also interested in the effect of different benzoyl esters at position 3 on outcomes of epoxidation of cholest-4-en-3β, 6β-diol-6-acetate.

For the first part, five attempts with different combinations of reagents, $NaBH_3CN/BF_3{\cdot}Et_2O$; $BH_3/LiBH_4$; $LiAlH_4/AlCl_3$; Li/ethylenediamine and $LiAlH_4$/different Lewis acids, were made to explore the feasibility to cleave the epoxide ring to locate the hydroxyl group at position 4. However, the desired products could not be obtained in most of reactions because the hydroxyl group preferably went to the position 5 rather than position 4. When the desired cleavages occurred and gave the products with the hydroxyl group at the position 4, at the same time, the anion from Lewis acid, such as Cl^- and F^-, attached to the position 5 to give 5-chloro or fluoro products.

For the second part, 30 benzoyl esters, with different substituent(s) on benzene rings, of cholest-4-en-3β, 6β-diols-6-acetate were prepared successfully. On the epoxidation, these benzoyl groups showed some effects on the stereochemistry of epoxidation through comparison of proportions of the α and β oxide rings in the products; however, these effects were not significant enough to give stereospecific products. Therefore, no big differences were observed about the effects of 3-benzoyl esters on outcomes of epoxidation of cholest-4-en-3β, 6β-diols-6-acetate.

Keywords Oxysterol, epoxide cleavage, epoxidation stereochemistry, biological activities

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General Abbreviations

Ac	acetyl
allo	compounds differ from the natural or typical steroids with reference to configuration at C-5 (Latin)
mCPBA	3-chloroperbenzoic acid
DCC	N, N'-dicyclohexylcarbodiimide
DCM	dichloromethane
DET	(+)-diethyl tartrate
DHU	dicyclohexylurea
de novo	from the beginning (Latin)
DMAP	4-dimethylaminopyridine
DMDO	dimethyldioxirane
DMF	N, N'-dimethylformamide
DNA	deoxyribonucleic acid
ES	electrospray
Et	ethyl
HMG-CoA	3-hydroxy-3-methylglutaryl coenzyme A
in situ	in the normal, natural, original, or appropriate position (Latin)
in vacuo	in a vacuum (Latin)
in vitro	occurring or made to occur outside a living organism (Latin)
in vivo	occurring or made to occur within a living organism (Latin)
IR	infrared
LAH	lithium aluminium hydride
LDL	low density lipoprotein
LXR	liver X receptor
Me	methyl
MS	mass spectrometry

NMR	nuclear magnetic resonance
Rf	retention factor
SCAP	SREBP cleavage activating proteins
SREBP	sterol regulatory element binding protein
TBHP	t-butyl hydroperoxide
THF	tetrahydrofuran
$Ti(O\textit{i}\text{-}Pr)_4$	titanium(IV) isopropoxide
TLC	thin layer chromatography
$VO(acac)_2$	vanadyl acetylacetonate

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Chapter One: Introduction

1.1 Keys to Life

"They come from everywhere—from flower buds and insects, from yeast fungi and bacteria, from fish and fowl, from coral, toads and the tubers of the sweet potato. They are components of the cell membrane, fat metabolizers, cardiac-active poisons, hormones and therapeutic drugs. That is why they are so very important. Without them, there would be no life as we know it; without them—population explosion; without them—pain; without them—no love."

—Rupert F. Witzmann
Steroids, Keys to life

1.1.1 Steroids

Steroids (Greek, stereo=solid) represent a large group of naturally occurring family of organic molecules of biochemical and medical interest, which are extensively distributed in the animal and plant kingdoms. They form a group of lipids resistant to saponification found in an appreciable quantity in all animal and vegetal tissues. Such nonsaponifiable matter may include one or more of a variety of molecules belonging to C27-C30 crystalline alcohol.

The term steroid is generally applied to compounds containing the same fundamental four hydrocarbon-rings skeleton. Since many of these compounds are alcohols, sometimes, the name sterol is used for the whole class. However, sterol is better reserved for the substances that are actually alcohols. (Roberts et al, 1974)

The basic structure feature of steroids is that they are saturated tetra cyclic hydrocarbons. They can be considered derivatives of hydroxylated perhydrocyclopentanophenanthrene (also called sterane). The prefix perhydro means that enough hydrogen atoms have been added to phenathrene to saturate the molecule. Cyclopentano means that the structure contains a five-membered ring, which is fused at the 1, 2-position.

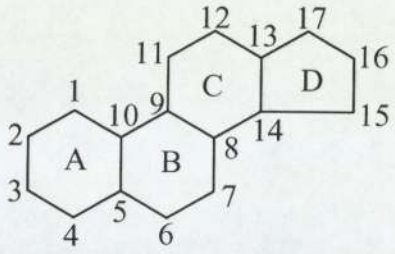


Figure 1-1 Basic structure of steroid

Many steroids have angular methyl groups attached to carbons 10 and 13, as well as an alkyl, alcohol or ketone group attached to carbon 17. The majority of the steroids conform to the generalized formula and have a hydroxyl group in position 3, although in some of them this group is missing and others are ketonic or polyhydroxyl in type.

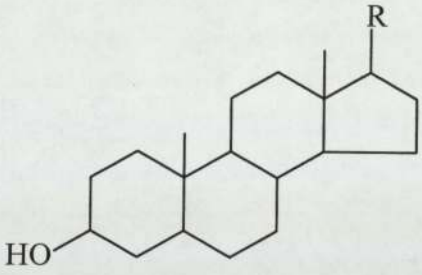


Figure 1-2 Generalized formula of sterol

Steroids differ considerably from one another in the degree of saturation in each of the four hydrocarbon rings and in the side-chain substituents as R attached to these rings. With wide variations in structure, they encompass compounds of vital importance to life, such as cholesterol, the bile acids, vitamin D, sex hormones, corticoid hormones, cardiac aglycones, antibiotics and insect moulting hormones; some of the most potent toxins are steroidal alkaloids.

Table 1-1 Vital steroids with variation of R

Steroids	R
Sterols	$-CH(CH_3)(CH_2)_3CH(CH_3)_2$

Bile acid		$-CH(CH_3)(CH_2)_3COOH$ or similar group
Sex hormones	Estrogenic	-OH or >CO
	Corpus luteum	$-COCH_3$
	Androgenic	-OH or >CO
Cardiac poisons		Lactone ring
Adrenal substances		-CO, CH_2OH etc

In the cardiac poisons, the methyl group (19) may be replaced by the CHO or CH_2OH group.

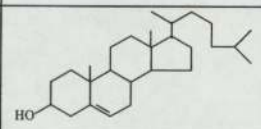
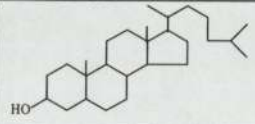
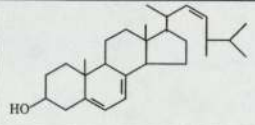
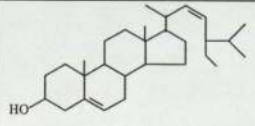
Steroids may be classified into three types: (Campbell, 1964)

Zoosterols of animal origin, e.g. cholesterol and coprostenol

Phytosterols of plant origin, e.g. stigmasterol

Mycosterols of fungal origin, e.g. ergosterol

Table 1-2 The most important sterols (Stewart et al., 1948)

Sterol	Structure	Formula	m.p. (°C)	No. of Double bonds	Occurrence
cholesterol		$C_{27}H_{45}.OH$	149	1	All animal cells, gallstones, whale oil etc
coprosterol		$C_{27}H_{27}.OH$	101	0	In faeces
ergosterol		$C_{28}H_{43}.OH$	165	3	Ergot yeast, gives vitamin D_2 when irradiated
stigmasterol		$C_{29}H_{47}.OH$	170	2	Calabar and soya beans plant sterol

Because of the vital role that some of steroids play in the regulation of physiological function, they may be regarded as fundamental constituents of all living cells except those of bacteria, and have been carefully studied by the organic chemists. Due to their academic as well as pharmacological importance, a staggering number of steroids have been prepared in the laboratory in addition to those occurring naturally.

1.1.2 Cholesterol

Among the great variety of steroids, the most abundant compound is cholesterol, a kind of unsaturated crystallized alcohol of formula $C_{27}H_{46}O$, existed in fat and cholelith. It was first isolated around 1770 by Poulletier de la Salle from gallstones. In 1815, cholesterol was found as component of human gallstones and isolated from the unsaponifiable fraction of animal fats by M. E. Chevrevl, a young French chemist, who named it cholesterine. (www.cyberlipid.org)

It takes its name from the Greek chole, bile; and stereos, solid. Cholesterol has eight asymmetric centres and therefore there are 2^8, 256 possible stereo isomers, but only cholesterol itself occurs naturally. (Goodman et al., 1973)

It has the structure—the four-ring system with an attached eight-carbon side chain at C17 and two angular methyl groups at C10 and C13 respectively.

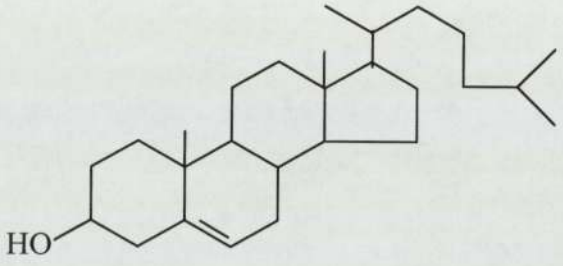


Figure 1-3 Structure of cholesterol

It occurs everywhere in all tissue of the animal body either as the free alcohol or in ester form, particularly abundantly in nerve tissue, spinal cord, brain, bile, blood, liver, kidneys and gallstones. It is also found in many oils fats and waxes and in egg yolk. It is its existence in the arteries of arteriosclerosis patients that first became clinical significant, at the same time providing a stimulus to steroid research. The fact that cholesterol is one of the principal constituents of gallstones makes it of prime medical importance. (Witzmann, 1977)

While cholesterol was considered to be nearly absent in vegetal organisms, its presence is now largely accepted in higher plants. It can be detected in vegetal oils in a small proportion (up to 5% of the total sterols) but remains frequently present in trace amounts. However, several studies have revealed the existence of cholesterol as a major component sterol in chloroplasts, shoots and pollens. Furthermore, cholesterol has been detected as one of the major sterols in the surface lipids of higher plant leaves (rape) where it may amount to about 27% of the total sterols in that fraction. (Noda et al., 1988) Sterols are ubiquitous components of cell membranes in eukaryotes, much more common than in prokaryotes. Undoubtedly, cholesterol plays an indispensable role in regulating the properties of every cell membrane selectively permeable in mammalian cells. It is an amphipathic molecule and is readily soluble in the hydrophobic region of membrane. It is involved in regulation of the fluidity of the membrane as a result of the nonpolar fused ring. In addition, it is an almost obligatory precursor of the other two major metabolically important classes of steroids: the steroid hormones and the bile acids. All their biologic roles figure prominently in human well-being. (Geoffrey, 1997)

Therefore, as one of the most important sterol with its hormonal and pharmacological effects recognized, cholesterol attracts a lot of attentions and stimulates further research commitment. It stimulated new research areas being emerged and developed and also the basic principals being further studied.

1.1.3 Oxysterol

Although many oxidized sterols can be included in the group of oxygenated derivatives of cholesterol, oxysterols especially refer to those compounds having a similar carbon skeleton structure to cholesterol but contain one or more oxygen atoms in addition to the oxygen at C-3. (www.atherognic.com) Therefore, sometimes they were also called cholesterol oxidation products.

There are many positions in the skeleton that can be oxidized. (Laurent-Meynier, 2002)

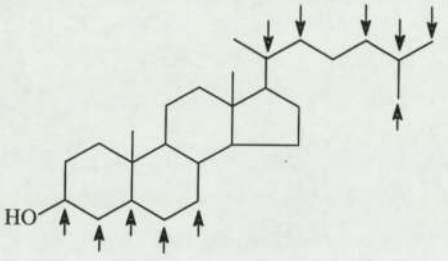
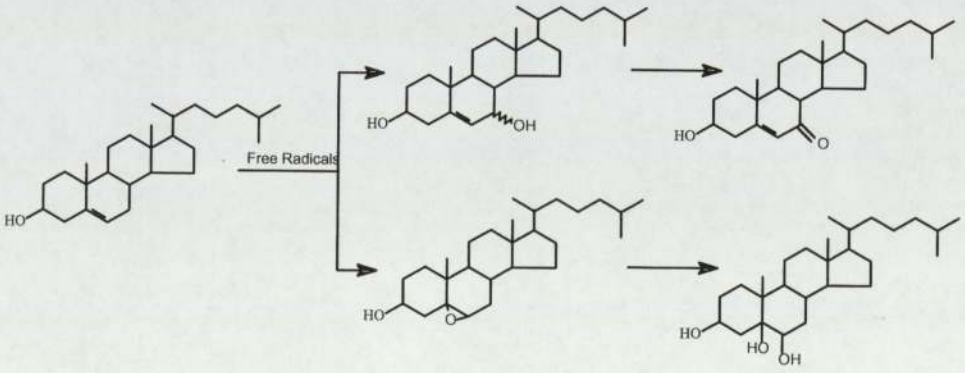


Figure 1-4 Common positions of oxidation on the steroid skeleton

Oxysterols can be obtained by cholesterol undergoing auto-oxidation, photo-oxidation, enzymatic oxidation and chemical synthesis. (www.cyberlipid.org)

(1) Via auto-oxidation



Scheme 1-1 Oxysterols formed by auto-oxidation

(2) Via photo-oxidation

Cholesterol can be peroxidated by singlet oxygen, affording mainly a C-5 oxygenated molecule.

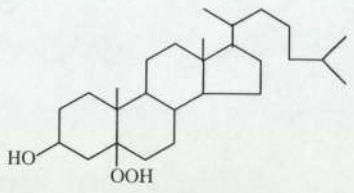


Figure 1-5 5α-Hydroperoxycholesterol

It can be further rearranged to form 7α-hydroxycholesterol that may be successively epimerised into 7β-hydroxycholesterol.

(3) Via enzymatic oxidation

Some other complicated different type oxysterols could be obtained by way of the enzymatic catalysed in vivo. These reactions mainly occur at C-7 position. Other compounds arisen from the enzymatic reactions are the derivatives with oxygenated groups on the side chain at C-20, C-22 *(R)*, C-24 *(S)*, C-25, and C-27 positions.

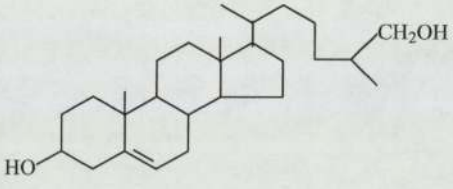
(4) Via chemical synthesis

Due to the general interest in these compounds new methods of chemical synthesis have been developed to provide them for biological investigations.

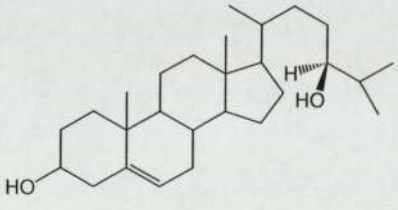
Oxysterols are also widely distributed in nature, being found in the blood and tissues of animals and men as well as in foodstuff. They can be considered as ultimate products of an oxidative stress and are chemically inert. As a class of compounds, they have exhibited a wide variety of biological activities, which are of potential physiological, pathological or pharmacological importance. (Parish et al., 1986) Many oxysterols have been found to be potent inhibitors of cholesterol biosynthesis and one or more oxysterols may play a role as the physiologic feedback regulator to repress cholesterol synthesis. Oxysterols also inhibit cell replication and have cytotoxic properties, which suggest that these sterols may participate

in the regulation of cell development as inhibiting its proliferation and differentiation and may be potentially useful as therapeutic agents for cancer. Furthermore, there is considerable evidence that oxysterols may be involved in the pathogenesis of atherosclerosis. Although the mechanism of action of oxysterols in all these instances is not well understood, the existence of cytosolic and microsomal proteins, which bind oxysterols with high affinity and specificity, suggest that this group of compounds may represent a family of intracellular regulatory molecules. (Chen, 1984)

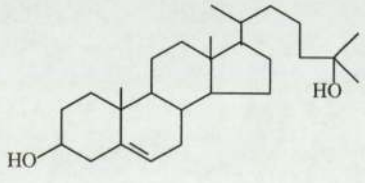
Three abundant naturally occurring oxysterols are 24-hydroxycholesteol, 25-hydroxycholesteol and 27-hydroxycholesteol. (Russell, 2000) All of them have a hydroxyl group on the side chain, playing several roles in lipid metabolism. They regulate the expression of genes that participate in both sterol and fat metabolism, serve as substrates for the synthesis of bile acids and are intermediates in the transfer of sterols from the periphery to the liver.



27-hydroxycholesterol



(S)-24-hydroxycholesterol



25-hydroxycholesterol

Figure 1-6 Naturally occurring oxysterols

1.2 Road from the past to the present

"The growth of knowledge and understanding in research is essentially dependent upon a circular intellectual process, with three components to the circle: observation, measurement and question. This is true no matter at what level of intellectual or scientific sophistication one is working. All new areas of knowledge have as their conception an observation, which at the extremes might be noted by chance or might result from a completed and expensive collaboration of theoretical and experimental advances. Further growth and development of this concept depend upon measurement. Thus far might be termed knowledge. Understanding comes when we question and subsequently receive an answer. The circularity of this process derives from the fact that each measurement and each answer become themselves further observations, which can in turn generate further measurement and stimulate further questions. The continuing progress in science just comes from it is not a closed circle. Particularly for chemistry, it contributes not only new observations but also frequently the means to make the measurements."

John R. Sabine

Cholesterol

1.2.1 Historic development

The road from the first discovery of the steroids, via their isolation to their magnificent application in medicine, as well as the insights gained along the way, makes for an extraordinary story. Steroid related research started in ancient times and has been one of the key areas of scientific investigation in the 20^{th} century. The compound now known as cholesterol was first described towards the end of the eighteenth century by the French chemist de Fourcroy, who isolated a laminated and crystalline substance from the alcohol soluble fraction of human gallstones, a substance that he said was first mentioned by Poulletier de la Salle more than 20 years earlier. The nineteenth century saw significant advances in knowledge of both the chemistry and biology of the new

substance. F. Reinitzer also isolated it and partially characterized it as the correct formula ($C_{27}H_{46}O$) in 1888. (www.cyberlipid.org)

At the beginning of the century, little was known about its structure until the pioneering research of Adolf Windaus and Heinrich Wieland completely elucidated its correct formulated structure in 1932. It was an achievement that concluded a brilliant chapter in structural organic chemistry. Without NMR, only with the crude techniques and methodologies, H. Wieland and A. Windaus worked out the exact steric representation of cholesterol. H. Wieland was awarded the Nobel Prize for Chemistry in 1927 for his research on bile acids and related substances and A. Windaus, the father of steroid chemistry was awarded the Nobel Prize for Chemistry in 1928 for his studies on the constitution of the sterols and their connection with vitamins that he had done during a quarter of a century. This revision was based on X-ray studies by J. D. Bernal, and chemical studies by Rosenheim, King, Wieland and Dane. And the final proof of its correctness has been provided by the total synthesis of equlenin, by subsequent syntheses of estrone, epiandrosterone, cholesterol and other steroids and by further X-ray investigation. In 1936, Callow and Young have designated all steroids compounds chemically related to cholesterol. With its completion, the work on the biosynthesis of this complex molecule could begin. (Witzmann, 1977; Sabine, 1977)

The relative configurations at the chiral centres of key steroids were established in 1947, while the absolute configuration was not known until that of glyceraldehydes was determined by X-ray analysis in 1952. The complexity of the problem is apparent from the fact that cholic acid, for example, contains 11 centres of asymmetry, and thus $2^{11}(2048)$ modifications are theoretically possible. (Nakanishi, 1974)

In 1956, the main elements of the biosynthetic pathway became known when isopentenyl pyrophosphate was discovered as a precursor through the research of Folkers and associates. K. Bloch was awarded Nobel Prize in Physiology and Medicine in 1964 for his work on cholesterol biosynthesis. In 1966, J. Cornforth and G. Popjak predicted

that there were 16234 possible stereo chemical pathways by which mevalonate could be converted into squalene. They subsequently showed which of these pathways were correct. In the 1970s and 1980s K. Bloch was able to provide intriguing evidence for an evolutionary advantage of cholesterol over lanosterol or some of the intermediates in the conversion of lanosterol to cholesterol. The last quarter of the 20th century was when M. Brown and J. Goldstein showed that the low-density lipoprotein receptor was a key regulator of cholesterol homeostasis. They have also demonstrated that cholesterol balance in the cell is transcriptionally regulated via the sterol regulatory element binding protein. In the later part of the 20th century, drugs were developed that effectively lower plasma cholesterol and lessen the risk of atherosclerosis and cardiovascular disease. (Vance, 2000)

1.2.1.1 Stereochemistry of steroids

The seven nuclear asymmetric centres (C-5, C-8, C-9, C-10, C-13, C-14 and C-17) formally permit 128 possible diastereoisomers. Many of these, however, could not exist because of steric limitations. (Brooks, 1970) Theoretically, the cyclic nucleus of four rings present in the steroids may exist in a number of isomeric forms, since the component rings (if hydrogenated) may be joined together by either cis or trans linkages as in the cis and trans decalins.

The problem of the configuration of the steroid nucleus is somewhat simplified by the fact that rings B & C, and rings C & D appear in most case to be fused together by trans linkages in the natural steroids. There are therefore two fundamental steroid nuclei: a) nuclei with trans A/B rings and described as allo structures and b) nuclei with cis A/B rings known as normal structures. Included in the allo series are cholestane, androstane, 5-allopregnane, and 5-allochilane; while in the other series are coprostane, testane, pregnane and cholane. (Campbell, 1964)

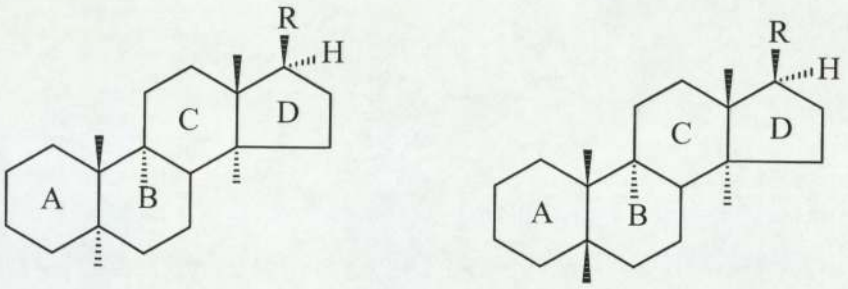


Figure 1-7 Allo-A/B trans rings and Normal-A/B cis rings

Position is specified by the number of the nuclear carbon atom bearing the substituent and configuration by the suffixes α and β. These suffixes denote a definitive stereo chemical orientation: α denotes that the group lies below the general plane of the ring system and is shown in formula by a broken line, while β denotes a group lying above general plane of the ring system and is often shown in formula by a heavy unbroken line. The absolute configuration of the angular methyl groups attached to C-10 and C-13 in both types of steroid nuclei are similarly β orientated, that is the groups lie above the plane of the ring. Only these with absolute β configuration are biologically active. Most of the steroids have a hydroxyl group at C-3 and many have others in different parts of the molecule. These hydroxyl groups may be attached either *cis* or *trans* to any given reference point such as the nearest angular group. For example, the hydroxyl group in cholesterol, cholestanol and coprostanol is denoted by 3β signifying that it is situated at C-3 and is *cis* to the C-10 methyl group; the epicholesterol, epicholestanol and epicoprostanol, epi—denoting the epimeride of the normal or common form—it has a 3α configuration showing that it is likewise situated as C-3, but is *trans* to the C-10 methyl group.

A. Rings A & B (Shoppee, 1964)

Fusion of rings A and B may be *trans* or *cis*, affording steroids of the 5α and 5β (allo and normal) series, respectively. The stereochemistry of rings A and B in the cholestane series was established by the work of Windaus on the pyrolytic behaviour of the four isomeric lithobilianic

acids, which was assigned the more stable *cis*-configuration, indicating the A/B *cis*-fusion for the parent compound. Similar results have been obtained with the analogous acids from 5α and 5β-cholestan-3β-ol. The correctness of the above assignment was confirmed by the isolation of bile acid derivatives containing a 3α, 9α-oxide bridge, the formation of which is impossible with ring A and B *trans*. The relative configurations of the angular methyl group and hydrogen atom were indicated by the observed stability of *trans*-decalins compare to that of the *cis*-isomers. In bile acids the ring junction is *cis* while in the cholestane series it is *trans*.

B. Rings B & C (Brooks, 1970)

The *trans*-fusion of these rings in all naturally occurring steroids was shown by X-ray crystallographic studies and by dipole moments. Synthetic confirmation of the B/C *trans*-fusion was provided by the total synthesis of the four diketones by J. W. Cornforth and R. Robinson.

C. Rings C & D (Campbell, 1964)

Rings C and D form an indane system and in such cases, the *cis*-isomer is more stable. Other factors, however, are encountered in the steroids most of which contain a *trans* C/D union. The fusion of these rings is *trans* in most of the sterols. K. Dimroth and H. Jonsson obtained a *trans*-ketone by oxidation of Vitamin D_2 (from ergosterol) with permanganate, which was isomerised with acid or alkali to the *cis*-ketone. The configurations, assigned by analogy with the *trans*- and *cis*-α-hydrindanones, indicate a C/D *trans* fusion. Since other steroids have been correlated with ergosterol (e.g. by conversion to 3β-acetoxynor-5α-cholanoic acid), the result applies widely. The cardiac, glycosides and aglycones and toad poisons, however, have a cis C/D fusion.

D. The side chain (Brooks, 1970)

For steroids in which the side chain is replaced by heterocyclic structures, geometrical isomerism may occur, notably at C-20, C-22 and C-25. Isomerism is generally possible at C-20, also at C-24 in 24-alkylated steroids and at any possible rendered asymmetric by substitution. Many natural steroids have a C-20 methyl group: cholesterol, 5α-

cholestanol, ergosterol, stigmasterol, campesteol, campertanol and the bile acids etc. is usually β-oriented; the algal sterol, sargasterol, stellasterol and stellastanol has a 20α-methyl group (20-isocholestane skeleton). Stereoisomerism at C-24 arises in the higher sterols and derived stanols. Ergosterol, brassicasterol and poriferasterol belong to the 24β series whereas campesterol, stellasterol, stigmasterol, β-sitosterol and α-spinasterol belong to the 24α series.

E. The hydroxyl group at C-3

The steric orientation of the secondary alcoholic hydroxyl group at C-3 is β in cholesterol, cholestanol, coprostanol and in most natural sterols.

The most stable conformation of the form rings in sterols is shown in the Figure 1-8.

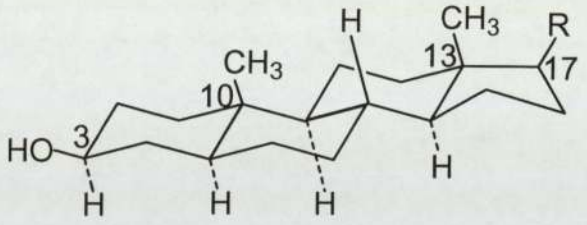


Figure 1-8 The most stable conformation of sterol

It contains three chair-form cyclohexane rings. The 3β-hydroxyl group is equatorial and hence is thermodynamically more stable than an axial 3α-hydroxyl. Experiments also show that if hydroxyl group presents at C-4, C-6, C-7, the corresponding stability of α and β is: 4α(e)>4β(a); 6α(e)>6β(a); 7β(e)>7α(a) and that would explain the more accessible hydroxyl groups (as at equatorial position) are, the easier they can be acylated. (Stewart et al., 1948) The axial or equatorial orientation of groups determines the relative stability of the structures under equilibrium conditions, but it does not control the orientation assumed in addition reactions.

A characteristic property of the 3β-hydroxyl steroids is their precipitation with digitonin, a rare glycoside of the saponin group found in digitalis seeds along with digitalis drugs. The digitonin contains a pentasacharide unit linked to a steroid sapogenin nucleus. The α- or epi-compounds do not form such complexes and this specific reaction, discovered by Windaus, has proved very useful in steroid chemistry for determining the configuration of the hydroxyl group in position 3. Cholesterol, cholestanol, coprostanol and ergosterol are among the steroids precipitated by digitonin; while the α-compounds not precipitated include the epi-derivatives, the bile acid and androsterone. (Campbell, 1964)

1.2.1.2 Conformation of steroid nuclei

The cyclohexane ring can adopt two principal conformations, the chair, which is rigid, and the boat, which is flexible. The boat form is of higher energy than the chair (~5Kal/mole) and the latter conformation is more thermodynamically stable than the boat conformation but can undergo conversion into the boat conformation by passage over the small energy barrier unless special features, e.g. substituent interactions; modify the difference in stability. With this exception, the most stable conformations of fused cyclohexane ring systems are those containing the greatest number of chair forms. (Shoppee, 1964)

The steroid nucleus is built up of three cyclohexane rings and one cyclopentane fused in a *trans* sense at each ring junction. Each ring then includes bonds that are equatorially related to the adjoining ring or rings. The only common exception is in 5β steroids where rings A and B are fused in the *cis* sense. These rings then each include one bond axial to the other, making 5β steroids generally less stable than the 5α series.

The steroid nucleus includes the three fused cyclohexane rings A, B and C. With a *trans* C/D fusion, ring C is rigidly fixed in the chair conformation because the C-13 angular methyl group has the β-conformation. Due to the double locking by rings B and D, with a *cis* C/D

fusion, ring C is more flexible and can adopt a boat form with ends at C-8 and C-12 ring D planar, and the molecule L-shaped. Similarity, a *trans* A/B fusion tends to fix ring B in the chair conformation, while ring A will normally also be a chair. With a *cis* A/B fusion, the preferred conformation normally corresponds to the two-chair form of *cis*-decalin. The conformation of ring D is not well defined, but appears to be dependent on the nature of the substituents in the nucleus: thus X-ray studies have shown that bulky substituents in ring A affect the conformation of the entire steroid nucleus. Because of the rigidity of the chair form of cyclohexane the 12 C-H bonds may be divided into a set of six parallel "axial" (verticality) bonds and a set of "equatorial" (horizontality) bonds, comprising three parallel pairs.

In the steroid nucleus: a) a substituent linked by an equatorial bond is thermodynamically more stable than its axially-bonded epimer; b) ionic elimination reactions involving trans-substituents on adjacent carbon atoms proceed most readily between axial bonds which permit a four-centre planar transition state; c) a substituent linked by an equatorial bond is less sterically hindered than its axial epimer, and ionic elimination may lead to ring contraction since the formation of the four-centre planar transition state involves the nuclear bonds.

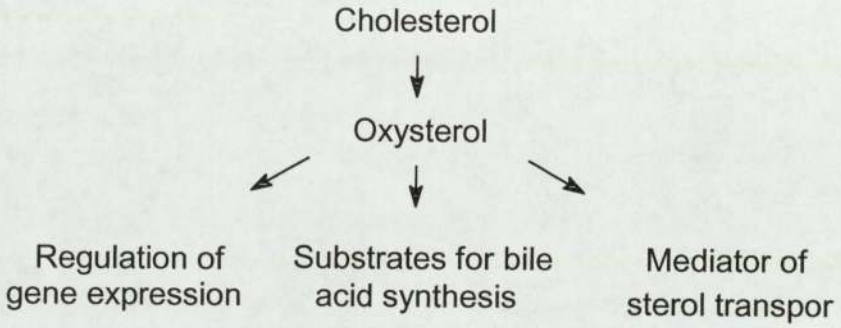
With the studies of chemistry of steroids, a number of measurement methods targeted on determining the physical properties were also developed, Such as 1. Optical rotation data, which has long been recognized as important characteristics of steroids; 2. Optical rotatory dispersion and circular dichroism; 3. Infrared absorption spectrophotometry; 4. Ultraviolet absorption spectrophotometry; 5. Nuclear magnetic resonance spectroscopy; 6. Mass spectrometry; 7. X-ray crystallography; 8. Chromatography with a liquid mobile phase; 9. Gas-liquid chromatography.

Further progress in determination of sterol chemical structure, stereochemistry of nuclear substituents and all the analytical methods' development, especially their application in the steroid field were reviewed in Rodd's series books. (Brooks, 1970)

1.2.2 The current research activities

Since the last quarter of the 20th century, steroid research has continued but turns its focus to biochemistry with stronger emphasis. Especially for oxysterols, oxygenated sterol, play more and more important roles in chemical, biochemical, pharmacological, toxicological and medical research.

From1970s, research showed that oxysterols participate in several different aspects of lipid metabolism.



Scheme 1-2 Physiological roles of oxysterols

Numerous studies about their biological effects were found, such as mutagenicity, (Sevanianm et al., 1986) carcinogenicity, (Morin et al., 1991) angiotoxicity, (Dorset et al., 1992; Peng et al., 1991) cytotoxicity, atherogenicity, cell membrane damage, (Peng et al., 1991; Higley et al., 1984; Smith et al., 1989; Guardiola et al., 1996) and inhibition of cholesterol biosynthesis. (Addis et al., 1990; Addis et al., 1991) In addition, they may induce atherosclerosis. (Peng et al., 1991; Imai et al., 1976; Morin et al., 1989; Peng et al., 1990)

Current progress on oxysterol chemical and biological activities research is discussed here.

1.2.2.1 Research in preparation of oxysterols (Smith, 1996)

The fundamental oxysterol chemistry research is concerned the different ways of oxysterol generation, and also their analysis. It can be

formed in the presence of heat or/and radiation, by enzyme catalysing or by defined oxygen species.

A. Oxysterol generation

1. The initial oxygenated position by cholesterol auto oxidation is at the carbon-centred allylic C-7 radical, which can be altered by subsequent reaction with molecular oxygen to generate a series of oxysterols with different oxygenated groups such as peroxide, polyhydroxyl, ketone or epoxide ring attached at position 3, 5, 6, 7, 25. Radical generation at these and other sites leads to the full panoply of recognized auto oxidation products. It is from these initial hydroperoxides that the complex array of cholesterol auto oxidation products arises. Although the products of cholesterol auto oxidation vary with the conditions such as temperature, time, concentration, physical state of cholesterol, pH, buffer, moisture, phospholipids, dispersant or other system components, the same set of oxysterols would be gained if the reaction carried out in solid state, solution of dispersion, in particulate systems such as liposome, plasma, lipoprotein and in foods. The effect of other system components is particularly important, because polyunsaturated fatty acid derivatives are expected to be auto oxidized more rapidly than cholesterol, and per oxidants, antioxidants present may alter the processes.

Since sterol hydroperoxides are the first isolable oxidation products, many sterol hydro peroxides were found either in vitro or in marine animal and plant issues. The positions that can be hydroperoxidized vary from C-5, C-6, C-7, C-20, C-22, C-24, C-25 and C-29. Cholesterol esters of per oxidized polyunsaturated fatty acids (particularly linoleic acid) found decades ago in human aortal plaques are now under active investigation in human blood plasma as measures of oxidative stress, possibly related to chronic health disorders.

Singlet molecular oxygen attacks $\Delta^{5,7}$-sterols to yield not only the well-known Δ^{6}-5α, 8α-peroxides, but also newly discovered $\Delta^{5,8}$-7-hydroperoxides. (Albro et al., 1994) Sterol 5α, 8α-peroxides are found in

marine creatures and in fungi, all apparently formed from parent $\Delta^{5,7}$-sterols. The $\Delta^{5,7}$-sterol 5α, 8α-peroxides are regarded as possibly protective against microbial agents or predators harmful to the organism. Oxidations of cholesterol induced by ionising radiation yield the usual oxysterols but in different proportions, depending on conditions and are sometimes accompanied by rearrangements.

2. Oxysterols in vivo are usually generated via enzymatic catalytic biosynthesis. There are at least three types of enzymatic oxidations that may contribute to mammalian tissue oxysterols. 1) Monohydroxylations, directed to bile acid and steroid hormone biosynthesis, to lanosterol (5α-lanosta-8, 24-dien-3β-ol) transformation to cholesterol, and perhaps to other as yet unrecognised products and purposes. 2) Oxidation of the 3β-hydroxyl to the corresponding cholest-4-en-3-one, implicated in 5α and 5β-cholestan-3β-ol biosynthesis and 3) perhaps biosynthesis of lanosterol 24, 25-epoxide via squalene 2, 3; 22, 23-bis-epoxide cyclization and/or via lanosterol epoxidation. There are mainly two enzymes participated in oxysterols biosynthesis: 7α-hydroxylase and (25*R*)-26-hydroxylase. They function in separate pathways of bile acid biosynthesis.

3. Oxysterol can also be obtained via reactive oxygen species ozone (O_3), electronically excited (singlet) molecular oxygen ($^1O_2$), di-oxygen cation (O_2^+), peroxyl ($ROO^·$) and oxyl ($RO^·$) radicals, hydroxyl radical ($HO^·$), HOCl, and nitrogen oxides NO/NO_2, but not by super oxide ($O_2^{·-}$). While the differential stability of different oxysterols may distort their proportions in product mixtures, and different oxysterols may also be yielded depending on conditions, such as different solvent system.

B. Oxysterol analysis

Crucial to most studies of oxysterols formed under controlled circumstances, in metabolic disposition and in tissues and foods, is the matter of analysis and availability of reference oxysterol samples.

For less complex samples, thin-layer and high-performance liquid chromatography serves suitably for isolation of individual oxysterols from

foods and using solid-phase extraction techniques is also suitable. Oxysterol identities may then be established by physical properties, including mass spectra and more recently ^{1}H and ^{13}C nuclear magnetic resonance data. Both spectral methods also serve for quantitative estimation.

Liquid chromatography with ultraviolet light absorption, flame ionisation, differential index of refraction, colorimetric or mass spectrometry detection are standard methods, but oxysterol light-absorbing esters and oxysterol Δ^4-3-ketone derivatives formed by action of cholesterol oxidase may also be used. However, it is capillary gas chromatography of oxysterol silyl ethers coupled with electron impact or chemical ionisation mass spectrometry that provides the most effective resolution, identification and quantitative (by selected ion monitoring) for serious analyses.

Readily available simple chromatography method with spectrometry and more recently with colorimetry serves to verify the purity of cholesterol employed in experimental work.

The amounts of oxysterols formed during analysis procedures may be estimated by adding isotopically labelled cholesterol as a control. Improved specificity and accuracy of measurement is gained by addition of isotopically labelled individual oxysterols. Nevertheless, operation avoiding air and light with added antioxidant and no storage (or minimal storage at low temperature) are necessary.

1.2.2.2 Oxysterols' biological activities

Oxysterols have a wide spectrum of biological activities, including inhibition of DNA and cholesterol synthesis, immune response modulation and others. (Hwang, 1991; Kraemaer et al., 1993; Zwijsen et al., 1992; Henning et al., 1987) Oxysterols have been shown to be toxic to lymphocytes, (Bakos et al., 1993) smooth muscle cells and endothelial cells. (Hughes et al., 1994; Ramasamy et al., 1992)

Much more work has been carried out in applied biomedical research dealing with the relationship of oxysterols and human health. Because these matters are quite complex, none have thus far been resolved. They have been known to exhibit a number of biological activities. (Kandutsch et al., 1978; Parish et al., 1986; Ji et al., 1990)

The presence of a second or a third oxygenated chemical group in the skeleton of the sterols makes these oxysterols less likely to interact with the lipid part of the cell membrane and subsequently renders their incorporation more difficult. Thus, the nature of the interactions leading to the different biological activities of oxysterols should be the subject of a major study. Oxysterols modify membrane dynamic properties that consequently trigger several biological effects. Despite the presence of at least one oxygenated group in addition to the C-3β-hydroxyl, oxysterols insert perfectly into the lipidic bilayer of the membrane including a condensing effect similar to, but less potent than that of cholesterol. In biological membranes, oxysterols probably interact with membrane components, as they are not easily exchanged after their incorporation into the cell membrane. These lipid-protein interactions are probably crucial for the expression of the biological activities of the oxysterols. (Luu et al., 1991)

1. Cytotoxicity of oxysterols

The lack of toxicity of cholesterol itself suggests that the introduction of an additional oxygen function into the cholesterol molecule dramatically alters its biological properties. (Tipton et al., 1987) Oxysterols' cytotoxicity have led much recent work establishing the presence of oxysterols in human tissues and in foods, as well as their bioassay toxicities. It is still obscure that excessive oxysterols in diseased tissues may affect the disease process.

It has been known for some time that oxygenated derivatives of cholesterol or of similar sterols (oxysterols) are cytotoxic to many different cell types both in vitro and in vivo, (Smith et al., 1989) and pose a potential threat to human health, particularly with respect to such chronic

conditions as atherosclerosis, cancer and aging. They are cytotoxic to a wide variety of cells at micro molar concentrations. Although many elements in support have been adduced, direct evidence of causation is unavailable. (Smith, 1996)

Oxysterols show their properties in many vitro as well as in vivo bioassays. A variety of oxysterols are contained in human plasma at nano molar levels and also contained in human erythrocytes, all variously toxic in acute *in vivo* test or cytotoxic in bioassays *in vitro*. They are carried by plasma lipoprotein and appear to be formatted in lipoproteins oxidized by Cu^{2+}, peroxynitrile, $^1O_2$, macrophages and neutrophils. Oxidized plasma lipoprotein containing these oxysterols affects metabolic process *in vitro* and *in vivo* and is variously toxic in bioassays. The 7β-hydroperoxide cholesterol is considered to be a major toxic agent in oxidized plasma lipoprotein. Additionally, hydroperoxides of cholesterol fatty acyl esters occur in oxidized plasma lipoprotein. (Smith, 1996)

There are two main factors that lead to the oxysterol cytotoxicity. One is due to their ability of inhibiting HMG-CoA (3-hydroxy-3-methyglutaryl Coenzyme A) reductase activity, which result in the reduction of endogenous cholesterol synthesis. Another one is the substitution of oxysterol for cholesterol in membranes, which can interfere their permeability, stability and other membrane properties. (Smith, 1996) Although there were concerns that oxysterols maybe implicated in cancer and aging, it is their possible involvement in human atherosclerosis that dominates the literature. Obviously, chronic disorders of this sort pose serious problems of study design, and only indirect evidence at best is available. Generally, in vitro bioassay studies, acute *in vivo* studies, and evidence of oxysterol accumulation in affected tissues support the concept.

The mechanisms of oxysterol toxicity are not known, but have been suggested to involve such effects as reduced cholesterol availability through inhibition of HMG-CoA reductase, replacement of cholesterol in membranes by oxysterols with associated perturbations to their vital properties or stimulation of apoptosis. (Aupeix et al., 1995; Christ et al.,

1993; Nishio et al., 1996; Ayala et al., 1997; Harada et al., 1997; Hynn et al., 1997; Johnson et al., 1997)

2. Oxysterol and atherosclerosis

Oxysterols have shown to possess many potent and diverse biological activities in vitro, several of which may implicate them in the initiation and/or development of atherosclerosis. Some of them (Cholesterol, 25-OH-cholesterol, 26-OH-cholesterol etc.) have been identified in human atherosclerotic lesions. (Gilbert et al., 1972; Jacobson et al., 1981; Smith and Van Lier, 1970; Taylor et al., 1979; van Lier and Smith, 1967) But whether oxysterols are pro-atherogenic, anti-atherogenic or begin in vivo remains unresolved. Various oxysterols have been detected in appreciable quantities in human tissue and fluids, including human plasma, atherogenic lipoproteins and atherosclerotic plaque. How these oxysterols originate in vivo is not fully known.

The tendency of the oxysterols to influence the molecular order as compared to pure cholesterol may contribute to cell membrane permeability changes affecting crucial cell functions and vents leading to vascular cell injury. Increased low-density lipoprotein (LDL) oxysterol levels may account for some of the structural changes noted for oxidatively modified LDL as well as its toxicity to vascular cells. (Verhagen et al., 1996)

Oxysterols have a variety of atherogenic effects when administered to animals including damage to endothelial and smooth muscle cells and the development of atherosclerotic lesions. (Smith, 1989; Addis, 1986) They also have a variety of effects on cultured endothelial and smooth muscle cells that may be related to atherosclerosis including cytotoxicity, inhibition of cholesterol uptake and decreased high-density lipoprotein binding. (Smith, 1989) They are present in human lesions too. (Carpenter et al., 1995; Jacobson, 1987; Carpenter et al., 1993)

Early atherosclerotic lesions are characterised by the accumulation of foam cells laden with lipid droplets containing cholesterol and

cholesterol esters. There is evidence indicating that all the oxysterols showed time- and concentration-dependent toxicity. (Clare et al., 1995)

The suspicion that oxidized cholesterol derivatives influence human aortal atherosclerosis developed in earlier accounts continues to be restated. There is evidence that increase plasma concentrations of certain oxysterols (7β-OH-cholestane, in particular) may be associated with an increased risk of atherosclerosis, overall the literature remains equivocal. Several studies have reported the distribution of particular oxysterols between the various lipoprotein classes, but the literature awaits a comprehensive analysis on a range of oxysterols in a large number of samples and then standardization to cholesterol. (Brown et al., 1999)

A variety of oxysterols are present in human atherosclerotic plaque and are suggested to play an active role in plaque development. Moreover, the oxysterol : cholesterol ratio in plaque is much higher than in normal tissue or plasma. (Brown et al., 1999) In vitro oxysterols perturb several aspects of cellular cholesterol homeostasis (including cholesterol biosynthesis, esterification, and efflux), impair vascular reactivity and are cytotoxic and/or induce apoptosis. Injection of relatively large doses of oxysterols in animals causes acute angiotoxicity; whereas oxysterol-feeding experiments have yielded contrary result as far as their atherogenicity is concerned. There is no direct evidence in humans that oxysterols contribute to the development of atherosclerosis. However, oxysterol levels in human low-density lipoprotein are considered potentially atherogensis and raised plasma level of a specific oxysterol (7β-hydroxy cholesterol) may be associated with an increased risk of atherosclerosis. (Smith et al., 1970; Carpenter et al., 1993; Carpenter et al., 1995) Atherogensis may be stimulated by dead vascular cells, which die of oxysterols cytotoxicity, such as endothelial cells, (Ramasamy et al., 1992) macrophages, (Clare et al., 1995; Aupeix et al., 1995) smooth muscle cells (Hughes et al., 1994; Peng et al., 1979) and lymphocytes. (Christ et al., 1993)

Oxysterols have an injurious effect on arterial cells, and that the injurious effect could not be altered by cholesterol, which was present at a serum concentration 12 times higher than that of oxysterols. (Zhou et al., 1993)

The cell injury caused by oxidized lipoproteins was among the first findings that led to the theory that it is the oxidation of low-density lipoprotein, not just LDL concentration, that leads to arterial disease. Cell injury and death appear to play multiple roles in lesion development and the toxic lipid constituents of oxidized lipoproteins, including a variety of oxysterols, which are candidates for the *in vivo* effectors of this cytotoxicity. (Coll et al., 2001) There is evidence that the higher level of oxysterol in plasma of patients suffering from severe atherosclerosis could increase the concentration of sphingomyelin in the arterial cell membrane and thereby increase calcium influx require in producing the calcific type VII lesions in the coronary arteries. (Kummerow et al., 2001)

High dietary cholesterol also leads to elevations in plasma and tissue oxysterols, aortic accumulations of cholesterol hydroperoxy esters, morphological effects on arteries, and increased lipid per oxidation and liver lysosome membrane damage. Notably, dietary antioxidants and certain drugs diminish these effects.

Oxysterols produced by enzymatic or non-enzymatic reactions are accumulated in large quantity in vascular endothelium and atherosclerotic plaques. A variety of 7-hydroperoxycholesterol and its products were found in human atherosclerotic plaque (Brown et al., 1997). Increased plasma 7β-hydroxycholesterol concentration was found significantly associated with progression of carotid atherosclerosis (Salonen et al., 1997) and also found in a population with a high risk for cardiovascular disease. (Zieden et al., 1999)

3. Oxysterol and Cancer

Due to the cytotoxicity of oxysterols, they have the potential to be used as antitumour agents. Many natural occurring oxysterols with

antitumour activities were found from plant and marine resources. Since 1974, quite a lot reports were involved in the oxysterol inhibition of certain malignant cell lines, such as 7-Keto-cholesterol, 25-OH-cholesterol and 20α-OH-cholesterol on the growth of mouse Lymphoma cells; 7β-hydroxyl cholesterol on rat Morris hepatoma cells (tissue culture), mice Krebs II tumours *in vitro* and rat lymphomas; cholestane-triol on murine Lymphoma cells, macrophages and pig arterial smooth muscle cells; aragusterol A on the growth of KB cells and in vivo antitumour activity against L1210 Leukaemia cells and P388 cells in mice; 3β, 16β, 17α-trihydroxycholest-5-en-22-one on the HL-60 Leukaemia cells; agosterol A on reversing the multi-drug resistance of human carcinoma cells etc. (Guardiola et al., 1996)

Such an effect could be obtained by simple inhibition of endogenous cholesterol synthesis and incorporation of oxysterol to membrane structure, thereby reducing the availability of cholesterol for the new membrane formation required for cellular replication and attending alternations of membrane fluidity, stability and function leading to cell destruction and consequently, changing cellular growth, morphology and viability. Inhibition of the formation of other mevalonate-derived products, such as dolicholesterol, ubiquinones, or isopentyl-substituted adenine moieties in tRNA, or the modified farnesyl group in cytochrome oxidase, can also be envisioned as affecting tumour cell growth. Furthermore, inhibition of the formation of mevalonic acid could also affect tumour cell growth by inhibition of the formation of isoprenoid pyrophosphates required for the key regulatory proteins. (Guardiola et al., 1996) 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, activators of some oncogenes. (Glomset et al., 1990; Schafer et al., 1989) Some studies suggest a possible mechanism related to immune system alteration induced by oxysterol, since attraction of macrophages and neutrophils by oxysterol has been observed in vivo. (Moog et al., 1990) Further, an inhibition

effect on the first steps of lymphocyte division, induced by mitogens or alloantigens, was observed. (Christ et al., 1991; Moog et al., 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 natural killer cells. (Smith et al., 1989) Christ et al 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. (Christ et al., 1993) It suggests that oxysterol toxicity could be mediated by mechanisms involving protein or RNA synthesis. It is obvious that structural features of these oxysterols are so different to each other that structure-activity analysis is difficult.

Many oxysterols, particularly those obtained from the oxidations of phytosterols and tetra cyclic triterpenes are potent cytotoxic agents. They are selectively cytotoxic against tumour cells. This cytotoxic, while their stereoisomers are inactive. The activity depends on the tumour cells which are used in the assay system: some compound display inhibitory activity towards hepatoma cells but are inactive against lymphoma cells while others act in the opposite manner. Free oxysterols do not depress tumour growth in living animals. However, several water-soluble prodrugs of oxysterols are able to depress different type of tumours in vivo. Clinical trial studies are presently conduced in order to learn the therapeutic values of these oxysterols. (Luu, 1995)

According to the inhibition of Cytolytic T lymphocytes activity upon short-term incubation with oxysterol, it shows that it is not due to the inhibition of cholesterol synthesis, but may be due to the insertion of oxysterol into the plasma membrane to replace cholesterol and alteration of membrane physical properties. (Kucuk, 1994)

4. Oxysterol in regulations

Most mammalian cells are able to satisfy their requirements for cholesterol from either external (lipoprotein) or internal (biosynthetic)

sources. Cholesterol homeostasis at the cellular level is the product of a delicate balance between its uptake and endogenous synthesis on the one hand and metabolism and export on the other. While cholesterol is essential for many cellular functions, excess cholesterol must be avoided because it forms insoluble crystals that are cytotoxic. (Brown et al., 1999) Oxysterols are known to repress sterol synthesis and inhibit cellular proliferation and differentiation. Total blockage of the enzyme activity by treatment of cells with potent oxysterols or compactin (competitive inhibitor of the enzyme) leads to inhibition of DNA synthesis and of cellular proliferation. (Chen, 1984)

Oxysterol are important degradation products of cholesterol and are intermediates in the biosynthesis of steroid hormones and bile acids. These compounds have a broad spectrum of biological effects including regulation of genes for enzymes in cholesterol homeostasis. The most notable oxysterol bioactivities centre around the regulation of cholesterol homeostasis, which appears to be controlled in part by a complex series of interactions of oxysterol ligands with various receptors, such as the oxysterol binding protein, the cellular nucleic acid binding protein, the sterol regulatory element binding protein, the LXR nuclear orphan receptors and the low-density lipoprotein receptor. Identification of the endogenous oxysterol ligands and elucidation of their enzymatic origins are topics of active investigation. (Schroepfer et al., 2000)

Oxysterols play several roles in lipid metabolism. Members of this class regulate the expression of genes that participate in both sterol and fat metabolism, serve as substrates for the synthesis of bile acids, and are intermediates in the transfer of sterols from the periphery to the liver. (Russell, 2000) According to a recent exhaustive review, (Schroepfer, 2000) research during the ensuing 26 years revealed that oxysterols participate in several different aspects of lipid metabolism. They are regulators of gene expression, substrates for bile acid synthesis, and mediators of sterol transport. This demonstration suggested a regulatory role for oxysterols in lipid metabolism and it has since served as a stimulus

to explore their physiological actions. (Russell, 2000) As regulatory molecules, they inhibit the production of transcription factors required for the expression of genes in the cholesterol supply pathways, (Brown et al., 1997) and they are ligands that activate members of the nuclear hormone receptor gene family. (Janowski et al., 1996; Forman et al., 1997; Lehmenn et al., 1997)

Oxysterols are inactivated by conversion into bile acids, and in some instances the essential need for bile acids can be met solely by the metabolism of oxysterols. (Schwarz et al., 1996) They also may be substrates for steroid hormone synthesis. (Nes et al., 2000) Tissues such as the lung and brain secrete measurable amounts of oxysterols into the circulation, which are then transported to the liver and converted into bile acids. (Lutjohann et al., 1996; Babiker et al., 1999) This secretion represents a form of reverse cholesterol transport, (Bruce et al, 1998) a mechanism that peripheral tissues use to return cholesterol to the liver and thus to maintain homeostasis.

Although the rate of cellular cholesterol biosynthesis appears to bear an inverse relationship to the availability of external lipoprotein cholesterol, the exact nature of the regulatory molecules remains to be unequivocally established. Several oxygenated derivatives of cholesterol (oxysterols) are powerful inhibitors of cholesterol biosynthesis, an effect that appears to be due primarily, but not exclusively, to a decrease in the activity of HMG-CoA reductase. (Gibbons, 1983) Introduction of a second oxygen function onto the molecule in addition to that at C-3 usually gives rise to a large increase in inhibitory activity. The position of the second oxygen function is of some importance in determining the inhibitory power of the derivative. Although each sterol type shows a rather wide range of activity, selection of the most potent sterol in each group shows that, in general, the greater the molecular distance between C-3 and the second oxygen group, the greater the inhibitory activity. An intact (i.e. an iso-octyl) side-chain is also required for full activity, a gradual decrease in the length of the side-chain resulting in a gradual

diminution in biological effectiveness. (Kandutsch et al., 1974) Within some groups of steroids there appears to be an inverse relationship between inhibitory effectiveness and the extent to which the oxygen function is sterically hindered. (Gibbons G. F., 1983)

In addition, introduction of axial hydroxyl groups at the 3α, 6β, 7α, or 15β-positions produces steroids, which are less inhibitory than those in which each hydroxyl group is in the corresponding, less hindered equatorial conformation. Oxygen functions in the conformationally flexible positions such as those in Ring D and in the side-chain also appear to produce more inhibitory steroids and it has previously been suggested that the biological potency of steroid hormones is dependent upon conformationally flexible functional groups which permit effective hydrogen-bonding or hydrophilic interactions with receptor molecules. (Romers et al., 1974; Duax et al., 1990) Oxysterols may regulate sterol biosynthesis at the cellular level. (Parish, 1986)

Studies in the 1960s and 1970s demonstrated that addition of cholesterol to either cells in culture or animal diets inhibited the expression of HMG-CoA reductase, HMG-CoA synthease and the LDL receptor. (Reviewed in Brown et al., 1997 and Edwards et al., 1999) Detailed studies by Kandutsch and colleagues demonstrated that various oxysterols were far more potent repressors than cholesterol. (Kandutsch et al., 1978) A major breakthrough for understanding the mechanism involved in feedback repression came when the gene encoding the LDL receptor was isolated; the promoter of this gene was shown to contain a 10 bp cis element that was named sterol regulatory element 1 (SRE-1). (Reviewed in Brown et al., 1997 and Edwards et al., 1999; Goldstein et al., 1990; Flier et al., 1999; Osborne, 2000)

Oxysterols are regulators of de novo cholesterol synthesis and metabolism. The regulation of gene expression by oxysterol is involved in cholesterol and lipid metabolism. (Wolf, 1999) Oxysterols can be both positive and negative regulators of gene expression. As positive effectors,

they bind to and activate the nuclear receptor LXR, (Janowski et al., 1996) which in turn increases 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 et al., 1992) As negative regulators, oxysterols suppress the cleavage of two transcription factors known as sterol regulatory element binding proteins-1 and -2 (SREBP-1 and -2). (Brown et al., 1997) Recent studies show that sterols such as 25-OH-cholesterol can inhibit the cycling of SREBP cleavage activating protein between endoplasmic reticulum and Golgi apparatus. (Nohturfft et al., 1999)

These side chain oxygenated sterols generated *in vivo* serve as gene regulators. 20S-HC, 22R-HC and 24S-HC show significant activation of LXRα and LXRβ, while 25- and 27-HC have no effect. (Lehmann et al., 1997; Lund et al., 1998) The gene regulation properties of other A, B ring OS to LXR and SREBP, together with the synthetic-bioactivity study are totally scarce.

Current interest in regulation of cellular processes showed that certain endogenous oxysterols are natural regulatory agents involved in de novo sterol biosynthesis, particularly in regulation of HMG-CoA reductase. Regulation of other enzymes (such as cholesterol 7α-hydroxylase) by endogenous oxysterols is also suspected.

Several endogenous oxysterols have been advanced as regulatory agents. Chief among these are the commercially available 3β, 25-diol and (25*R*)- 3β, 26-diol implicated in bile acid biosynthesis, but also lanosterol and desmosterol 24, 25-epoxides and other 32-hydroxy- and 32-oxo-derivatives of lanosterol, these being biosynthesis intermediates liking lanosterol and cholesterol.

Different mechanisms of oxysterol action have been proposed, including repression of gene transcription, post transcriptional effects, protein synthesis, and accelerated enzyme degradation, all processes

requiring oxysterol receptor or carrier. A rather complicated balance among all influences on de novo sterol biosynthesis may be the case.

Ring C oxygenated sterols or their derivatives are known natural products, and may serve as regulators of isoprenoid biosynthesis in the organisms or tissues in which they occur. (Schroepfer et al., 1988)

5. Other bioactivities of oxysterol

A. Effects of oxysterols on membrane

Cholesterol plays an important role in biological membranes of eucaryotic cells. Inclusion of cholesterol into lipid bilayer reduces the permeability of membranes, inverses molecular order and produces a condensing effect. (For review see McMullen et al., 1996) It is believed that these effects arise from the rigidity of the steroid ring system, which restricts the notion of lipid chains next to it. At the same time it was observed that small modifications in the sterol molecular structure could significantly alter the properties of membranes. Oxygenate sterols provide another example of compounds that alter phospholipids membrane in a different way compared to cholesterol. (Smondyrev et al., 2001)

Endogenous and exogenous cholesterol is incorporated into membranes and is essential. In vitro assays, oxysterol can be also incorporated into membranes, substituting for cholesterol (Crastes de Paulet et al., 1988). Oxysterols, especially the A, B ring oxygenated one, have more potent affinity with cell membranes than cholesterol. (Mahfouz et al., 1995) As a consequence, a change is observed in fluidity, permeability a stability of the cellular membrane, as well as in cellular growth, morphology and viability. Oxysterol in membranes can also derive from oxidation of cholesterol of the membrane by means of some extra cellular agents; it was hypothesized that cholesterol may act as a membrane antioxidant. (Guardiola et al., 1995; Smith, 1990 and 1991)

The membrane effects of oxysterol can vary a lot due to their chemical structures. Depending on their molecular structural in particular, oxysterols may exert cholesterol-like homogenizing effect in membranes (Li et al., 1994). The A, B ring oxygenated oxysterols, to a certain extent,

have some structure effects similar to cholesterol such as an increase in molecular order or their condensing effect, which are often less than that of cholesterol and may cause the functional change of the membrane.

The modulation effects of membrane properties are different with individual oxysterol, as the activities of enzymes and receptors embedded in membrane may be mediated differently (Lau et al., 1995).

In liposomes, 7-ketocholesterol, 7β-hydroxycholesterol, 7α-hydroxycholesterol reduce glucose permeability and in the same order as their condensing effect. They have a cholesterol like effect, although less efficient than cholesterol, whereas 25-hydroxycholesterol showing no condensing effect acts as a spacer molecule. Packing defects in the hydrophobic core of the bilayer due to the presence of the C-25 hydroxyl group are believed to cause the permeability increase. (Theunissen, 1986) Depending on their molecular structural particulars, oxysterols may exert cholesterol-like homogenising effect in membranes. (Li, 1994)

B. Oxysterols and gene expression

Intracellular and extra cellular cholesterol levels are tightly maintained within a narrow concentration range by an intricate transcriptional control mechanism. Excess cholesterol can be converted into oxysterols, signalling molecules, which modulate the activity of a number of transcription factors, as to limit accumulation of excess of cholesterol. Two key regulatory pathways are affected by oxysterols. The first pathway involves the uptake and de novo synthesis of cholesterol and is controlled by the family of sterol response element binding proteins, whose activity is regulated by a sterol-dependent feedback mechanism. The second pathway, which only recently has become a topic of interest, involves the activation by a feed forward mechanism of cholesterol utilization for either bile acid or steroid hormone synthesis by oxysterol-activated nuclear receptors, such as liver X receptor and steroidogenic factoe-1. Furthermore, biosynthesis and enterohepatic reabsorption of bile acids are regulated by the farnesol X receptor, a receptor activated by bile acids. Both the feedback inhibition of cholesterol uptake and production

and the stimulation of cholesterol utilization will ultimately result in a lowering of the intracellular cholesterol concentration and allow for a fine-tuned regulation of the cholesterol concentration. (Schoonjans, 2000)

C. Oxysterols and apoptosis

The apoptosis induced by oxysterol are attributed to their anti-proliferative, immunosuppressive activities. 25-OH-cholesterol can kill murine lymphoma cells in vitro as well as thymocytes (Christ et al., 1993), human leukaemic CEM cells (Ayala-Torres et al., 1997) and microglia cells (Chang et al., 1998) by apoptosis.

The general mechanism clarified for oxysterol induced apoptosis cell death are down regulation of c-myc (Thompson et al., 1999 b) and bcl-2 protein (Nishio et al., 1996, Lizard et al., 1997), through activating membrane sphingomyelinase (Harada-Shiba et al., 1998) and Ca++ influx (Ares et al., 1997). In some cases, the results show mixed-mechanical tendency of single compound (Chang et al., 1998, Harada et al., 1997).

Oxysterols different in structure such as the configuration and position of oxygenated groups give different results and may act through various mechanisms (Aupeix et al 1995, Lemaire et al 1998).

Above all, the studies of apoptosis are limited by severe shortage of oxysterols; so far most studies were done only by use commercial available 7-hydroxycholesterol and 25- hydroxycholesterol. Few reports described structure-activity relationship studies (Zhang et al 1997) with a limited number of oxysterols.

D. Oxysterols and inflammation

Oxysterols, and 25-hydroxycholesterol in particular, may modulate the inflammatory response in human macrophages. Consequently the presence of oxysterols in atherosclerotic tissue may dramatically influence the effect of inflammation. (Rosklint, 2002)

The oxysterol in inflammatory disease and atherosclerosis are linked together by the notion of vascular eicosanoid production induced by oxysterols through COX-2 induction. (Wohlfeil et al 1997) Oxysterols

that found in high concentrations in atherosclerotic lesions are potent immuno-suppressive agents inhibiting T-cell responses to different stimuli. Some inflammatory functions of macrophages and lymphocytes may be modified by such environmental conditions as the presence of oxysterols. (Dushkin et al., 1998)

E. Antiviral effects of oxysterols

7β-hydroxyl-cholesterol, 25- hydroxyl-cholesterol and 7β, 25-di hydroxyl-cholesterol have been tested in vitro on the replication of HIV virus (Moog et al 1998), yielding inhibition with modest but reproducible selectivity index. It has been also reported that sulphated polyhydroxysterols isolated from marine organisms have shown antiviral effects on herpes simplex virus (Roccatagliata, et al 1996). Brassinosteroid analogs also have weak to moderate antiviral activity in vitro in Vero cells infected with herpes simplex virus HSV-1 (Ramy rez et al., 2000).

F. Oxysterols and 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 oxysterol that has induced significant carcinogenesis without an oily vehicle.

G. Oxysterols and mutation

Oxysterols are mutagenic, although some have been studied as antitumour agents based on their cytotoxic properties. So far it is known only epoxide form of oxysterol (cholesterol-5, 6-epoxide, cholesterols) are mutagenic due to their electrophilic reactivity, (Sevanian et al 1986) and a recent report by Woods and O'Brien denied the possibility of genotoxicity that may be caused by oxysterol. (Wood and O'Brien 1998)

6. The roles of oxysterol in medical research and drug development

Oxysterols exhibit many biological activities that are of potential physiological, pathological or pharmacological importance. One marine

oxysterol, the contignasterol, is under preclinical trials as an anti-inflammatory agent (Figure 1-9). (Jaspars, 1999)

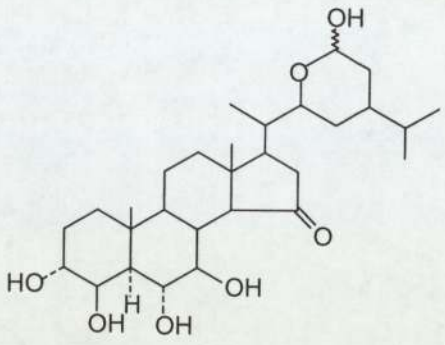


Figure 1-9 Contignasterol

Recent studies have been focused on the mechanisms of oxidized lipoprotein-induced cell death, whether the cell die by apoptosis or necrosis, and the identities of the toxins that induce injury. Understanding the roles of theses agents in lesion development could lead to therapies that modulate cell death and inhibit lesion formation. (Colles et al., 2001)

Many oxysterols have been found to be potential inhibitors of cholesterol biosynthesis and one or more oxysterols may play a role as the physiologic feedback regulator of cholesterol synthesis. Oxysterols also inhibit cell replication and have cytotoxic properties, effects that suggest that these sterols may participate in the regulation of cell proliferation and may be potentially useful as therapeutic agents for cancer. (Hwang, 1991)

Aside from steroid hormones/antagonists and bile acids selected oxysterol derivatives have been advanced as potential chemotherapeutic agents in two instances. The 3β, 7β-diol and derivatives, specifically choles-5-ene-3β, 7β, 25-triol, have been evaluated as immunosuppressive agents potentially inhibiting malignant tumour growth, and 3β-hydroxy-5α-cholest-8 (14)-en-15-one and analogs have been examined as inhibitors of HMG-CoA reductase and de novo sterol biosynthesis in relation to concern over atherosclerosis. The cytotoxic effects are thought to follow incorporation of oxysterol into the plasma membrane, and function leading to cell destruction. (Smith, 1996) Oxysterols are chemically inert and

some of them (7β-hydroxycholesterol, 25-hydroxycholesterol and 7, 25-dihydroxycholesterol) are cytotoxic at micro molar concentrations towards normal and tumours in culture, particularly lymphocytes, and reduce the growth of murine transplanted tumours. In addition, they markedly modify immune cell responses. (Christ et al., 1991) Thus possible applications of oxysterols in medicine as immunosuppressants or as anticancer agents may be considered. Because they trigger apoptosis via both common and distinct mechanisms, oxysterols provide unique tools for exploring the initiation of the phenomenon in lymphoid cells, and may help design novel pharmacological approaches based on apoptotic modulation of these cells. (Bischoff et al., 2000)

Recent progress in the understanding of molecular and biochemical pathways involved in apoptotic cell death offer novel perspectives for therapeutic interventions, in particular in immunochemotherapeutic and anti-cancer therapies. It is believed that oxysterols may form a new class of antitumour agents. The mechanisms involved in the synthesis and regulation of cholesterol have attracted many scientists over the last half-century. The results of many of their studies have been spectacular, often unpredictable and have led to the development of drugs (e. g. stains) that have greatly influenced medical practice. (Edwards et al. 2000)

1.2.3 Why is stereochemistry in oxysterols important?

Since the structure of the representative members of the important class of natural products were first elucidated in the early 1930's, steroid chemistry as introduction, replacement and interchange of functional groups and modification of the steroid skeleton has been a rapidly expanding subject which later went far beyond the "Natural Products" aspect to which it was originally limited. In the following years, steroid research accelerated at an almost feverish pace as the utility of some of these compounds as hormonal and pharmacological agents was recognized and implemented in clinical practice. A very significant trend in 1960's has been towards the selection of steroids as model compounds for the study of organic reaction mechanisms; this was the consequence of their unique combination of ready availability and clearly defined and fairly rigid stereo chemical features.

The original reason for the study of steroids and the accumulation of so large a body of steroid related data, lay in their biological origin and activities. Natural resources usually seem too impractical as a source of specific steroids for research. Once the correct structure of cholesterol and the principal sterol hormones had been discovered, there was a fresh impetus to the study of steroids from the need to develop practicable synthetic routes to the steroid hormones and to study the effects of structural changes on their biological properties. With the arrival of conformational ideas, however, steroids were no longer to be the concern only of biochemists and "natural product" chemists, lying near what many workers in organic chemistry considered to be the outlying fringes of their subject. The crystallinity of most steroid and the very convenient range of their melting points, were of immense value to early workers and are still among the most attractive features of steroids for reaction studies.

As a result of the tremendous effort, highly selective synthetic procedures for the transformation of complex polyfunctional steroid compounds were developed. Further, it can be fairly stated that almost every new synthetic method was tested in the steroid area. These studies

led not only to a better understanding of the mechanistic and stereo chemical factors involved in the reaction, but also often afforded milder and more selective experimental conditions. All those knowledge obtained was consulted not only by the steroid chemist but also nonsteroid chemist to carry out similar transformations with other classes of compounds. (Fried et al., 1972)

The steroid molecule provides each carbon atom in its structure a unique steric environment. With simple functional groups (hydroxyl, carbonyl, olefin etc.) available at virtually every one of these positions it is possible to test hypotheses concerning reaction mechanisms in a great variety of situations. The steroids are ideally suited for chemical and physico-chemical studies and have invariably played vital roles in the development of new reactions, concepts and physical measurements. This arises from 1) their relatively flat and rigid molecular framework, 2) their highly crystalline properties and 3) the enormous number of derivatives that have been prepared. Thus they have played in dispensable roles in the development practically every phase of organic chemistry, i.e. isolation (chromatographic methods, such as column, thin-layer adsorption and gas-liquid chromatography), organic reactions, total syntheses of complex natural products, conformational analysis, ultraviolet spectroscopy, infrared spectroscopy optical rotatory dispersion and circular dichroism, nuclear magnetic resonance spectroscopy, mass spectroscopy, biosynthesis. The first successful application of X-ray crystallography to natural products was to cholesterol in 1932. (Rosenheim, 1937)

All these, at the same time, also stimulate the studies of steroids, such as we can prepare on purpose a series isomers of a specific formula, therefore, must be helpful for further biological studies of those isomers and hopefully one or some of them could be a potential medicine for cancer or other diseases.

1.3 Future ahead

As a class of compounds of increasing interest and importance in biology and medicine, a large number of important areas in oxysterol research can be regarded as in only preliminary stages and can be anticipated to expand very substantially and rapidly. There are many problems remain unclear concerning the synthesis and biology of oxysterol. For example, although various levels of oxysterol toxicity were known and many elements in support have been adduced, the direct evidence of causation is still unavailable. Oxidized grain sterols appear to accumulate in fried foods in the same manner as do cholesterol oxidation products. However, the intestinal absorption, metabolism and biological activities of these grain sterol oxidation products remain wholly uninvestigated. Common oxysterol are readily metabolised by a variety of pathways, but whether metabolic accumulations occur in particular tissues with attendant harmful effects remains unexamined.

Major advances have been made in the technologies for the separation, identification and quantitation of oxysterols. Unfortunately, a good deal of the information currently available on the levels (and even identity) of oxysterols is of dubious validity due to lack of appreciation and/or lack of attention to critical aspects in the application of existing methodologies.

Oxygenated sterols have significant potential for applications in medicine because of their extraordinarily potent regulation effects on cholesterol metabolism, cell growth (both of normal and transformed cells), and other processes. Major advances in research on the activities, mechanisms of actions and potential applications in medicine can be anticipated in the coming decade. Current information points to the challenging complexities of mechanisms about some oxysterols action and the existence of multiple mechanisms.

Oxysterols isolated from natural sources have provided novel compounds with unanticipated actions and exceptionally high potency. Some areas of research, most notably studies of the levels of oxysterols in

blood plasma and tissues, and other biological materials, are in need of not only increased attention and effort but also of much higher standards of research. Valid information on these matters, coupled with an expansion of knowledge on the biosynthesis and metabolism of the various oxysterols, is critical to a continuing assessment of the physiological significance of the actions of the various oxysterols. (Schroepfer, 2000)

Not all oxysterols are equal in regard to their potency and this can differ between the in vitro and in vivo models employed. This highlights the potential importance of the choice of individual oxysterols for study and the impact that their methods of delivery to cells and tissues (with the consequent oxysterol: cholesterol loading achieved) can have. Equally importantly, the doses of oxysterols used in vitro and in vivo have for the most part been pharmacological and their cell loadings, in the rare instances where these have been measured, are generally orders of magnitude greater than ever encountered in vivo. Consequently, their relevance to human health and disease is uncertain. (Brown et al., 1999) Despite much work essentially confirming prior understanding, none of the several aspects of biomedical interest is a settled matter. Oxysterols accumulate in some diseased human tissues, but the accumulations may be cause, effect or an epiphenomenon and the means of accumulation is unstudied. Whether any oxysterol will become a useful medical therapy is uncertain, because conclusive evaluations have not advanced beyond animal studies. (Smith, 1996) The potential pathological importance of oxysterols in atherosclerosis, and the known therapeutic utility of antiestrogens in breast cancer, suggest that studies on the mechanisms of cytotoxicity of these compounds and the cellular basis of resistance against them should be of considerable interest. (Low et al., 1995) Future studies will also hopefully delineate the roles of oxysterols in disease processes and the relative importance of dietary, per oxidation or enzymatically derived oxysterols. (Morel et al., 1996)

1.4 Obstacles in the way

As the bioactivities of oxysterols are varied with delicate changes of their structures, studies with more structural diverted and structurally more related oxysterol analogues should warrant a better understanding of the structure-activity relationships and biological mechanism.

In spite of continuing advances in studies of the chemistry of oxysterols, commercial materials still remain the main source of the study, which are unfortunately very limited with regard to structural types, available quantities, and reasonable cost. Therefore, it has resulted in the acquisition of quite a lot of information on the effects of one oxysterol, 25-OH-cholestane, on a wide variety of parameters in cultured mammalian cells. Unfortunately other oxysterols may possess considerably physiological importance. Moreover, the results of studies with 25-OH-cholestane (or the combination of 25-OH-cholestane and cholesterol) have been frequently generalized to other oxysterols without experimentation. The limited availability of oxysterols is also a major factor responsible for the very restricted number of studies of their in vivo effects in animals. (Schroepfer Jr., 2000) Therefore, increased efforts in the chemical synthesis of oxysterols and their analogues are anticipated to provide critical materials leading to expansion of studies of the activities and mechanisms of actions of oxysterols not only in cultured cells but also in whole animals.

Synthesis methods for the common oxysterol are now readily available; but only a few new oxidation procedures have been reported. (Smith, 1996) Since late 1930s after the correct cholesterol structure was elucidated, there has not been a unified and established synthetic strategy in tackling stereoselective syntheses of a defined oxysterol. The regioselective and stereoselective introduction of oxygenated functional groups are not yet studied for structural related compounds, such as the stereoisomers of certain diols, triols or tertols. Therefore if we would like to do a proper study of cholestan-3, 5, 6-triols, a better synthetic way has to be developed with easy control of the stereochemistry in order to obtain

all the isomers. On the other hand, the Structure-Activity Relationship (SAR) studies are very limited even with known compounds. The number of synthetic polyhydroxyl sterols reported is limited. As the polyhydroxyl sterols occurred in natural source bearing more oxygenated groups are often found with potent bioactivities, a systematic research is necessary on gradual introduction of the oxygenated functional groups on to the steroid molecules with the stereochemistry control. Above all, reported methods for the introduction of oxygenated functional groups to the steroidal skeleton could not satisfy the needs for preparation a big number of stereo defined OS in low costs. Therefore in order to start a proper study of the bioactivities of oxysterols, a systematic synthetic methodology needs to be developed. The antitumour study of oxysterol, on the one hand, is hampered by limits of its biological studies on mechanisms that seem to be a common problem today. On the other hand, the structure-activity relationship studies are very limited due to the material's availability. More stereospecific derivatives should help us to understand further details of inside about how oxysterol exert their bioactivity. The studies of oxysterol cytotoxicity, gene regulation and certain membrane effects can be carried *in vitro* with sophistic method in recent years. The problem is how to get enough number of oxysterol analogues. Our research interest is in the development of robust synthetic methodologies for synthesis of stereospecific oxysterol derivatives for studies of their anticancer activities and other bioactivities. (Zhao, 2002)

In addition, methods of analysis are also inadequate. For any sample, it is necessary to remove interfering materials to concentrate the analyte, optionally to hydrolyse oxysterol esters, to resolve oxysterols chromatographically, and to identify individual oxysterol by suitable independent means. For quantitation, it is also necessary to utilize appropriate means to measure sterols at the nanogram level. (Smith, 1996)

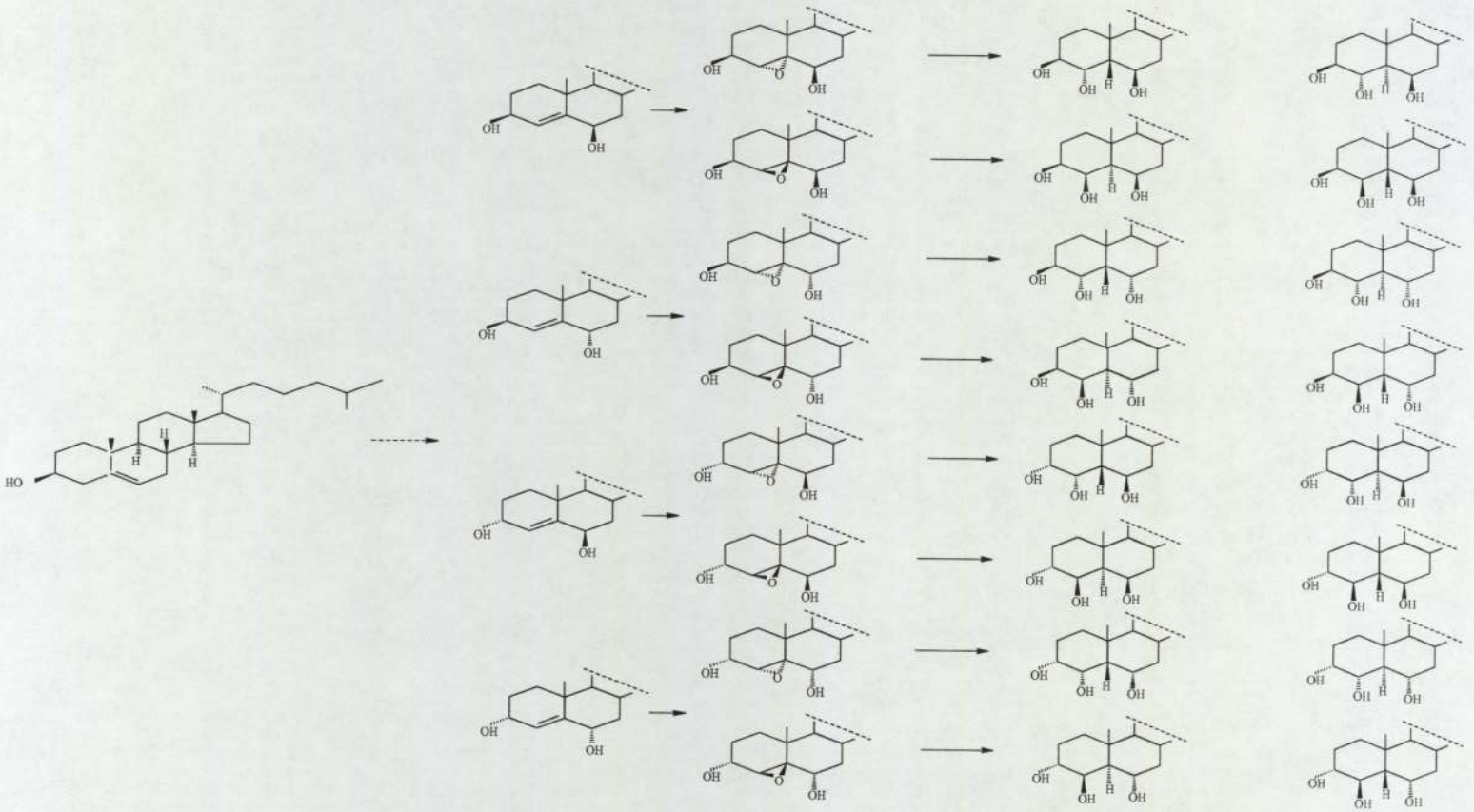
1.5 My one-year contribution to this field

1.5.1 Proposed goal of the project

On the whole, our research program was to develop efficient preparative methods for stereoselectively synthesizing polyhydroxyl sterol derivatives in quantities from commercially available bulk cheap materials for the studies of their structure-activity relationship against cancer.

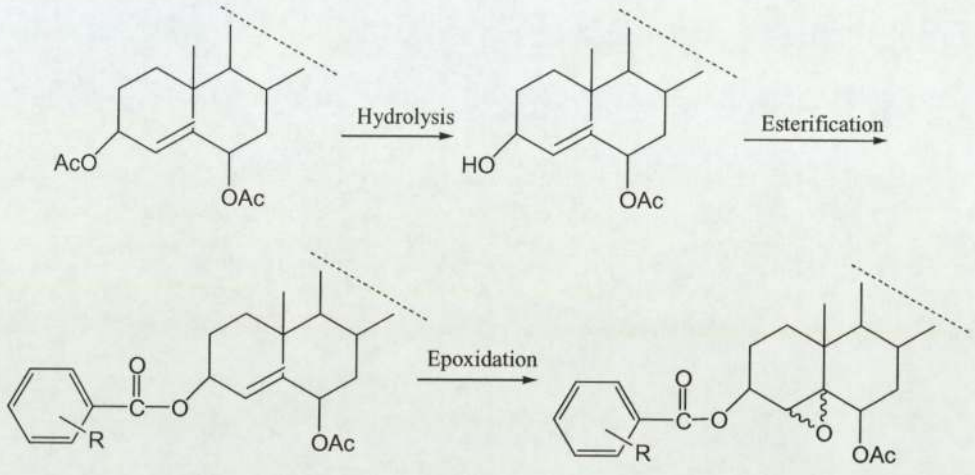
Now, we've been able to introduce hydroxyl groups to ring A at position C2 and C3 after the oxidation of relative position followed by reduction. (Xiong, 2001) At the same time, we've got four cholest-4-en-3β, 6β; 3β, 6α; 3α, 6β and 3α, 6α-diols and eight diastereomerically pure 4, 5-epoxycholestane with two hydroxyl groups attached at 3, 6 position. (Zhao, 2002)

My project is consisted of two parts. One is to explore an effective and stereoselective methodology to conduct a reductive cleavage of eight 4, 5-epoxycholestane-3, 6-diols to possibly obtain the sixteen cholestane-3, 4, 6-triols (Scheme 1-3).



Scheme 1-3 Outline of the first part of the project

The second is to investigate the effect of different 3β benzoyl ester on the epoxidation stereochemistry of cholest-4-en-3β, 6β-diol-6-acetate. (Scheme 1-4)



Scheme1-4 Outline of the second part of the project

In the first part of the project, to obtain the objective products, the eight isomers are supposed to be the best starting materials and it seems that epoxide ring should not be cleaved with difficulty. In the previous trial in our group, several methods are available to prepare 3, 4, 6-triols from different starting materials, but those methods have their limitations because they cannot be used for preparing all 16 isomers. What we need is an effective way to cleave the eight 4, 5-epoxycholestane-3, 6-diols in a manner that the 5-hydrogen is added with desired stereoselectivity with 5α or 5β control, therefore, the sixteen 3, 4, 6-triols can be synthesized at one time.

In the second part of the project, the starting material, cholest-4-en-3 β, 6β-diol-3, 6-diacetates, is subjected to a hydrolysis to become cholest-4-en-3 β, 6β-diol-6-acetate and then the free hydroxyl group at position 3 could be acelated with a number of benzoyl acids. All the benzoyl acids are bearing different electron donating and/or attracting groups at Para, Meta and Ortho positions. And then the intermediate is ready to be

epoxidated. In the project the reagent of epoxidation is m-CPBA. The final proportion of α and β oxidant rings in the product is determined by using the NMR technology.

1.5.2 Related chemical solutions

For the first part of the project, there are many related reactions that carried out in our group about the 4, 5-epoxycholestane ring cleavage. Most of them are splitting the ring using protic acid $HClO_4$ and Lewis acid $BF_3.OEt_2$ or $AlCl_3$. They are summarized as follows (The results in the tables only show the positions of the substituents, and basic structure remain the same). (Zhao, 2002)

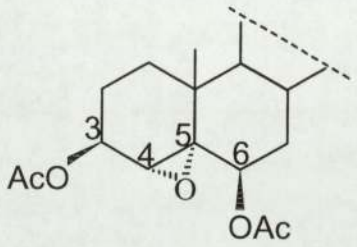


Figure 1-10 Starting Material 1-1

Reagents	Reaction Solvent		
	THF	CH_3CN	DCM
$HClO_4$	3β, 4β,5α,6β-tetrols, 3, 6-diacetate; 3β, 4β, 5α, 6β-tetrols, 4, 6-diacetate	3β, 4β,5α,6β-tetrols, 3, 6-diacetate; 3β, 4β, 5α, 6β-tetrols, 4, 6-diacetate	3β, 4β,5α,6β-tetrols, 3, 6-diacetate; 3β, 4α, 5β, 6β-tetrols, 3, 6-diacetate
$Al(ClO_4)_3$		3β, 4β, 5α, 6β-tetrols, 4, 6-diacetate	
$BF_3 \cdot Et_2O$	3β, 4β, 5α, 6β-tetrols, 4, 6-diacetate; 3β, 4α, 5β, 6β-tetrols, 3, 6-diacetate	3β, 4β, 5α, 6β-tetrols, 4, 6-diacetate (5% aq. CH_3CN)	
		3β, 4α, 5β, 6β-tetrols, 3, 6-diacetate	

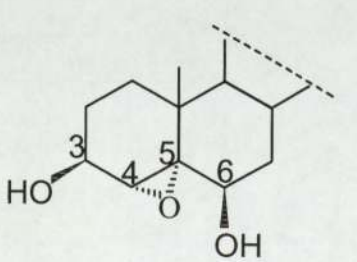
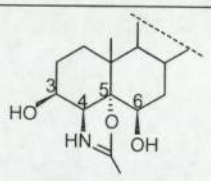
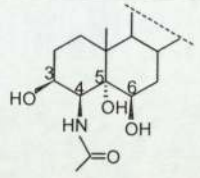
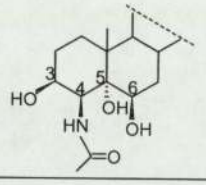


Figure 1-11 Starting Material 1-2

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$	3β, 4α, 5α, 6β-tetrols; 5α-F, 3β, 4α, 6β-triols	
$BF_3 \cdot Et_2O$	3β, 4α, 5α, 6β-tetrols; 5α-F, 3β, 4α, 6β-triols	
$AlCl_3$	6α-Cl, 3β, 4α, 5β-triols; 5α-Cl, 3β, 4α, 6β-triols; 4β-Cl, 3β, 5α, 6β-triols	6α-Cl, 3β, 4α, 5β-triols; 

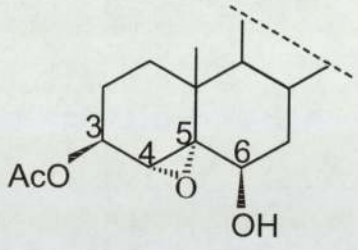
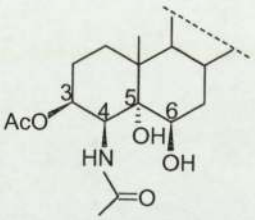


Figure 1-12 Starting Material 1-3

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	40 min	3β, 4β, 5α, 6β-tetrols, 3-acetate; 3β, 4β, 5α, 6β-tetrols, 4-acetate; 10:1	
	20 min		3β, 4β, 5α, 6β-tetrols, 3-acetate; 3β, 4β, 5α, 6β-tetrols, 4-acetate; 9:1

$BF_3 \cdot Et_2O$	1.5 hrs	3β, 4β, 5α, 6β-tetrols, 3-acetate; 3β, 4β, 5α, 6β-tetrols, 4-acetate; 1:20	
	1 hrs		3β, 4β, 5α, 6β-tetrols, 3-acetate; 3β, 4β, 5α, 6β-tetrols, 4-acetate; 1:5
$AlCl_3$		6α-Cl, 3β, 4α, 5β-triols, 3-acetate; 5α-Cl, 3β, 4α, 6β-triols, 3-acetate; 3β, 4β, 5α, 6β-tetrols, 3-acetate	

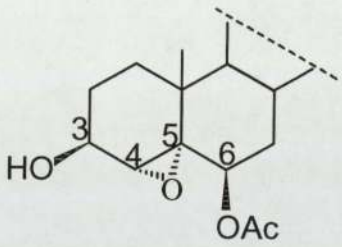
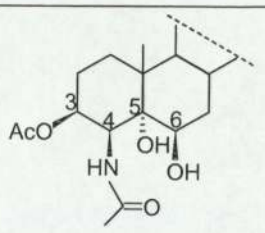


Figure 1-13 Starting Material 1-4

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	2 hrs	3β, 4α, 5β, 6β-tetrols, 6-acetate; 3β, 4α, 5β, 6β-tetrols, 5-acetate	
	30 min		3β, 4α, 5β, 6β-tetrols, 6-acetate
$BF_3 \cdot Et_2O$	5 hrs	3β, 4α, 5β, 6β-tetrols, 6-acetate	
	20 min		3β, 4α, 5β, 6β-tetrols, 6-acetate

$AlCl_3$		3β, 4β, 5α, 6β-tetrols, 6-acetate; 3β, 4α, 5β, 6β-tetrols, 6-acetate	

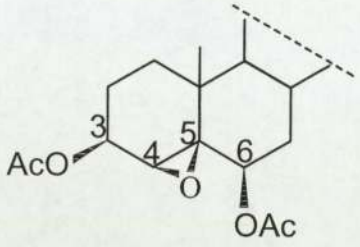
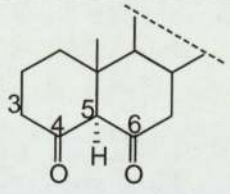
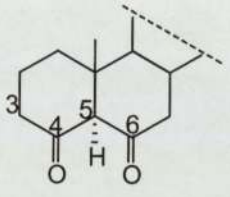
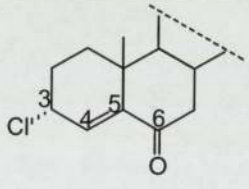


Figure 1-14 Starting Material 2-1

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$		
$BF_3 \cdot Et_2O$		
$AlCl_3$	4α-Cl, 3β, 5β, 6β-triols, 3, 6-diacetate	

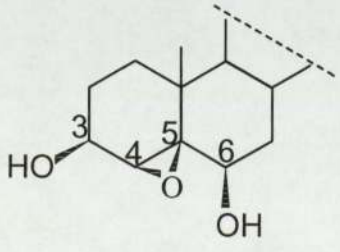
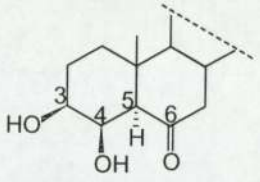
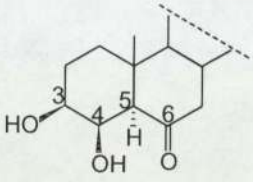
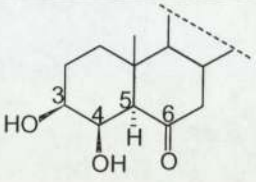


Figure 1-15 Starting Material 2-2

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$		
$BF_3 \cdot Et_2O$		
$AlCl_3$	4α-Cl, 3β, 5β, 6β-triols	

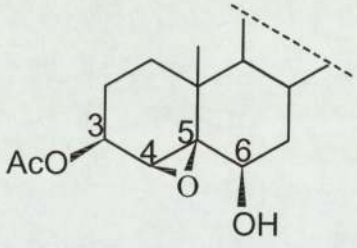
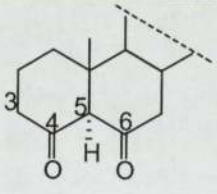
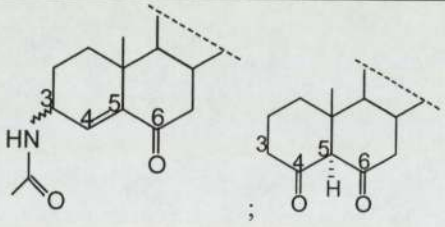
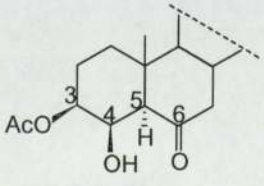
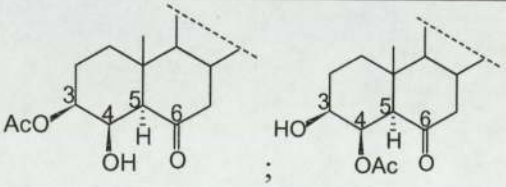
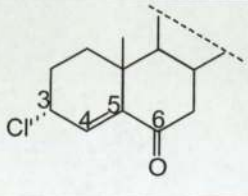


Figure 1-16 Starting Material 2-3

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$		
$BF_3 \cdot Et_2O$		
$AlCl_3$		

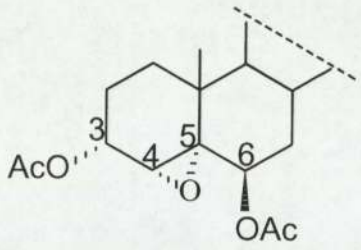


Figure 1-17 Starting Material 3-1

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	8 days	3α, 4α, 5β, 6β-tetrols	
	30 min		3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate >95%
$BF_3 \cdot Et_2O$	20 hrs	3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate >95%	
	30 min		3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate >83%
$AlCl_3$	16 hrs	3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate >71%	
	3 hrs		3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate >90%

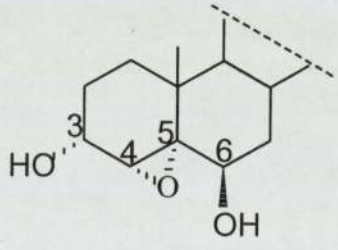


Figure 1-18 Starting Material 3-2

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$	3α, 4α, 5α, 6β-tetrols	
$AlCl_3$	5β-Cl, 3α, 4α, 6β-triols	5β-Cl, 3α, 4α, 6β-triols

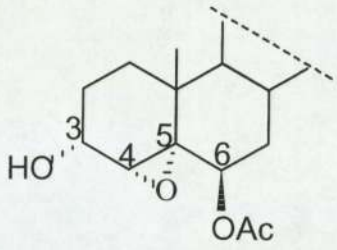


Figure 1-19 Starting Material 3-3

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$	3α, 4α, 5β, 6β-tetrols, 6-acetate	3α, 4α, 5β, 6β-tetrols, 6-acetate
$BF_3 \cdot Et_2O$	3α, 4α, 5β, 6β-tetrols, 6-acetate; 3α, 4α, 5β, 6β-tetrols, 5-acetate	3α, 4α, 5β, 6β-tetrols, 6-acetate
$AlCl_3$	3α, 4α, 5β, 6β-tetrols, 6-acetate	3α, 4α, 5β, 6β-tetrols, 6-acetate

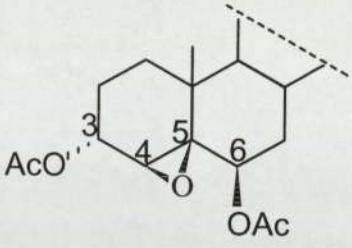


Figure 1-20 Starting Material 4-1

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	10 hrs	3α, 4α, 5β, 6β-tetrols, 6-acetate 90%	
	30 min		3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate 81%
$BF_3 \cdot Et_2O$	20 hrs	3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate >85%	
	30 min		3α, 4α, 5β, 6β-tetrols, 3, 6-diacetate 83% 3α, 4β, 5α, 6β-tetrols, 5, 6-diacetate 12%

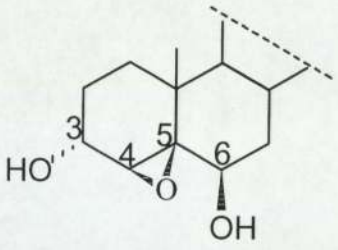
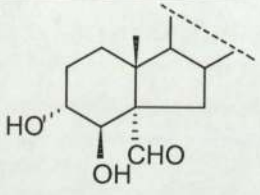
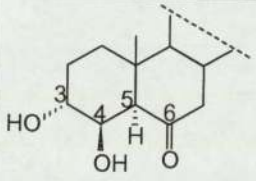
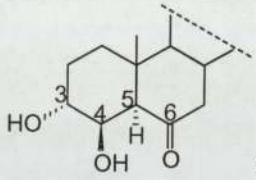
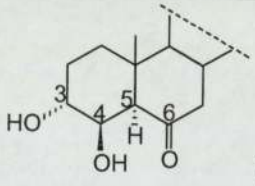


Figure 1-21 Starting Material 4-2

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	2 hrs		
	1 hrs		 75%
$BF_3 \cdot Et_2O$	6 hrs	 66%	
	30 min		 78%
$AlCl_3$		4α-Cl, 3α, 5β, 6β-triols	4α-Cl, 3α, 5β, 6β-triols

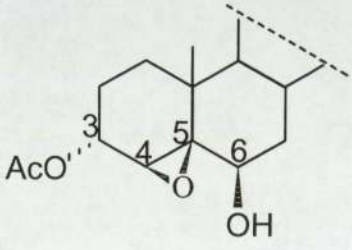
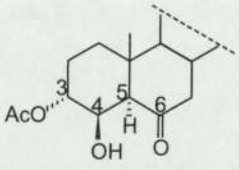


Figure 1-22 Starting Material 4-3

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	4 hrs	3α, 4α, 5β, 6β-tetrols, 3-acetate; 50% 3α, 4α, 5β, 6β-tetrols, 4-acetate 10%	
	40 min		3α, 4α, 5β, 6β-tetrols, 4-acetate 88%
$BF_3 \cdot Et_2O$	5 hrs	3α, 4α, 5β, 6β-tetrols, 3-acetate; mainly 3α, 4α, 5β, 6β-tetrols, 4-acetate; mainly 	
	6 hrs		3α, 4α, 5β, 6β-tetrols, 4-acetate 70%
$AlCl_3$	10 min	3α, 4α, 5β, 6β-tetrols, 4-acetate	
	5 min		3α, 4α, 5β, 6β-tetrols, 4-acetate 95%

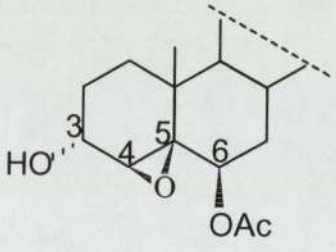


Figure 1-23 Starting Material 4-4

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$AlCl_3$	2 hrs	4α-Cl, 3α, 5β, 6β-triols, 6-acetate; 3, 4, 5, 6-tetrols 3:7; 90%	

	20 min		4α-Cl, 3α, 5β, 6β-triols, 6-acetate; 3, 4, 5, 6-tetrols 7:1; 90%

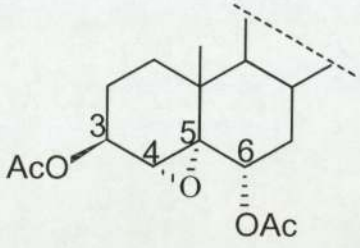


Figure 1-24 Starting Material 5-1

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	10 min	3β, 4β, 5α, 6α-tetrols, 4, 6-diacetate 77%	
	10 min		3β, 4β, 5α, 6α-tetrols, 4, 6-diacetate; 3β, 4β, 5α, 6α-tetrols, 3, 6-diacetate 5:4; 98%
$BF_3 \cdot Et_2O$	90 min	3β, 4β, 5α, 6α-tetrols, 4, 6-diacetate; 3β, 4β, 5α, 6α-tetrols, 3, 6-diacetate 1:2; 90%	
	10 min		3β, 4β, 5α, 6α-tetrols, 4, 6-diacetate; 3β, 4α, 5β, 6α-tetrols, 3, 6-diacetate 1:1; 95%
$AlCl_3$	30 min	3β, 4β, 5α, 6α-tetrols, 4, 6-diacetate; 51% 3β, 4β, 5α, 6α-tetrols, 3, 6-diacetate 5%	

	1 hrs		3β, 4β, 5α, 6α-tetrols, 4, 6-diacetate; 3β, 4α, 5β, 6α-tetrols, 3, 6-diacetate 20:7; 90%

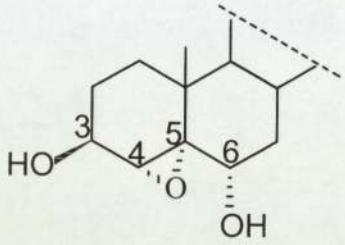
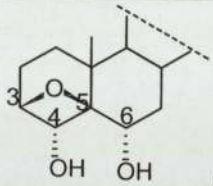
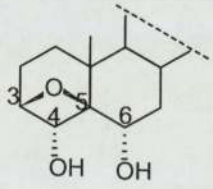
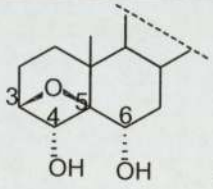
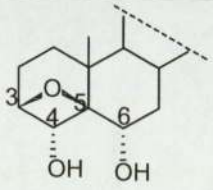


Figure 1-25 Starting Material 5-2

Reagents	Reaction Solvent		
	Time	THF	CH_3CN
$HClO_4$		3β, 4β, 5α, 6α-tetrols	Mess
$BF_3 \cdot Et_2O$	3 hrs	 48%	
	10 min		 trace
$AlCl_3$	30 min	 67%	
	30 min		 80%

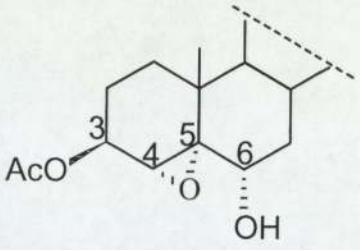


Figure 1-26 Starting Material 5-3

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	10 min	3β, 4β, 5α, 6α-tetrols, 4-acetate; 3β, 4β, 5α, 6α-tetrols, 3-acetate 4:6; 95%	
	10 min		3β, 4β, 5α, 6α-tetrols, 4-acetate; 3β, 4β, 5α, 6α-tetrols, 3-acetate 1:1; 98%
$BF_3 \cdot Et_2O$	30 min	3β, 4β, 5α, 6α-tetrols, 4-acetate 77%	
	10 min		3β, 4β, 5α, 6α-tetrols, 4-acetate 82%
$AlCl_3$	10 min	3β, 4β, 5α, 6α-tetrols, 4-acetate; 3β, 4β, 5α, 6α-tetrols, 3-acetate 3:1; 83%	

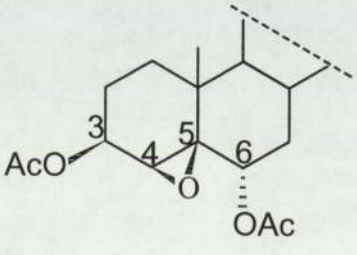


Figure 1-27 Starting Material 6-1

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$	3β, 4β, 5α, 6α-tetrols	

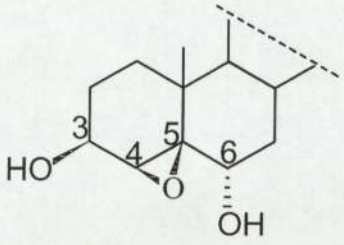


Figure 1-28 Starting Material 6-2

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$	3β, 4β, 5α, 6α-tetrols	

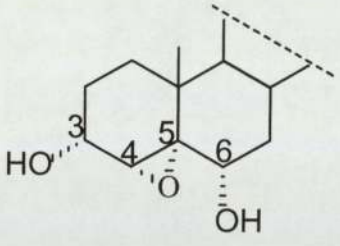


Figure 1-29 Starting Material 7-1

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$	3α, 4α, 5β, 6α-tetrols; 3α, 4β, 5α, 6α-tetrols	

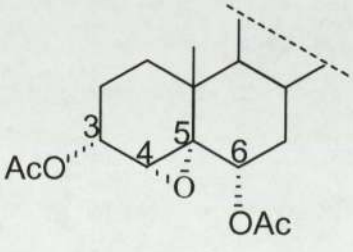
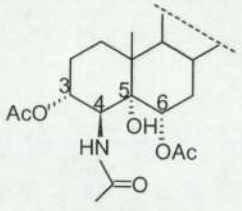


Figure 1-30 Starting Material 7-2

Reagents	Reaction Solvent	
	THF	CH_3CN
$HClO_4$	3α, 4α, 5β, 6α-tetrols; 3α, 4β, 5α, 6α-tetrols	

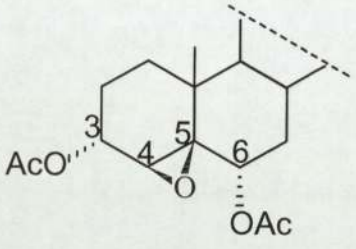


Figure 1-31 Starting Material 8-1

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	20 min	3α, 4α, 5β, 6α-tetrols, 3, 6-diacetate; 92% 3α, 4α, 5β, 6α-tetrols, 4, 6-diacetate 3%	
	30 min		3α, 4α, 5β, 6α-tetrols, 3, 6-diacetate; 56.5% 3α, 4α, 5β, 6α-tetrols, 4, 6-diacetate 33.5%
$BF_3 \cdot Et_2O$	9 hrs	3α, 4α, 5β, 6α-tetrols, 4, 6-diacetate	
	30 min		3α, 4β, 5α, 6α-tetrols, 3, 6-diacetate; 60% 3α, 4β, 5α, 6α-tetrols, 5, 6-diacetate 36%
$AlCl_3$	1hrs	3α, 4β, 5α, 6α-tetrols, 3, 6-diacetate >90%	

			3α, 4β, 5α, 6α-tetrols, 3, 6-diacetate; 3α, 4α, 5β, 6α-tetrols, 3, 6-diacetate; 3α, 4α, 5β, 6α-tetrols, 4, 6-diacetate

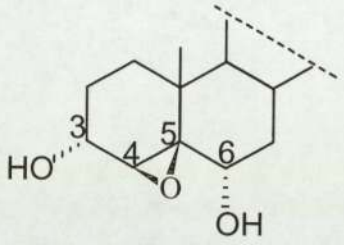
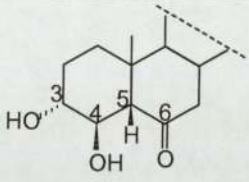
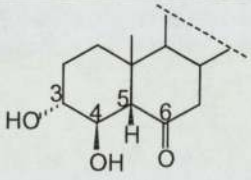


Figure 1-32 Starting Material 8-2

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	4 days	3α, 4β, 5α, 6α-tetrols	
	25 min		
$BF_3 \cdot Et_2O$	10 days		
$AlCl_3$	30 min	4α-Cl, 3α, 5β, 6α-triols 90%	
	30 min		4α-Cl, 3α, 5β, 6α-triols 80%

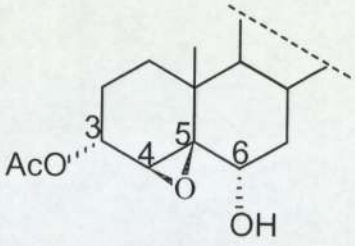
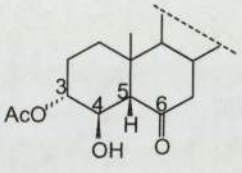
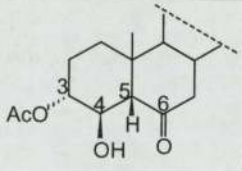
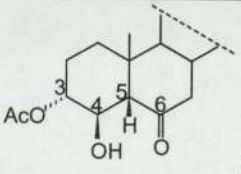
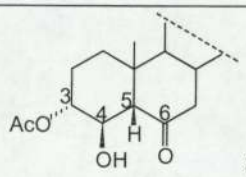
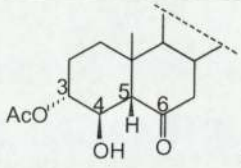


Figure 1-33 Starting Material 8-3

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	10 min	3α, 4α, 5β, 6α-tetrols, 3-acetate; 87% 3α, 4α, 5β, 6α-tetrols, 4-acetate 13%	
	10 min		3α, 4α, 5β, 6α-tetrols, 3-acetate; 24% 3α, 4α, 5β, 6α-tetrols, 4-acetate; 22% 3α, 4β, 5α, 6α-tetrols, 5-acetate; 21%  12%
$BF_3 \cdot Et_2O$	24 hrs	 ; 3α, 4α, 5β, 6α-tetrols, 4-acetate 89:11; 90%	
	10 min		 ; 34% 3α, 4α, 5β, 6α-tetrols, 4-acetate 8%

$AlCl_3$	10 min	 82%	
			 73%

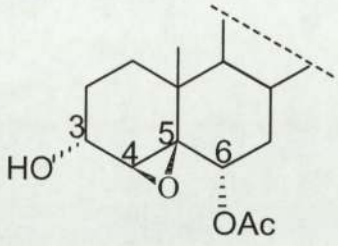
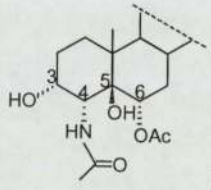
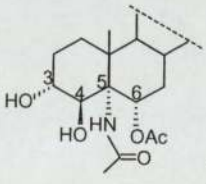
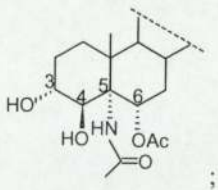
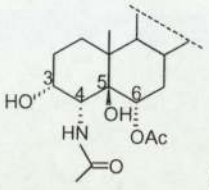
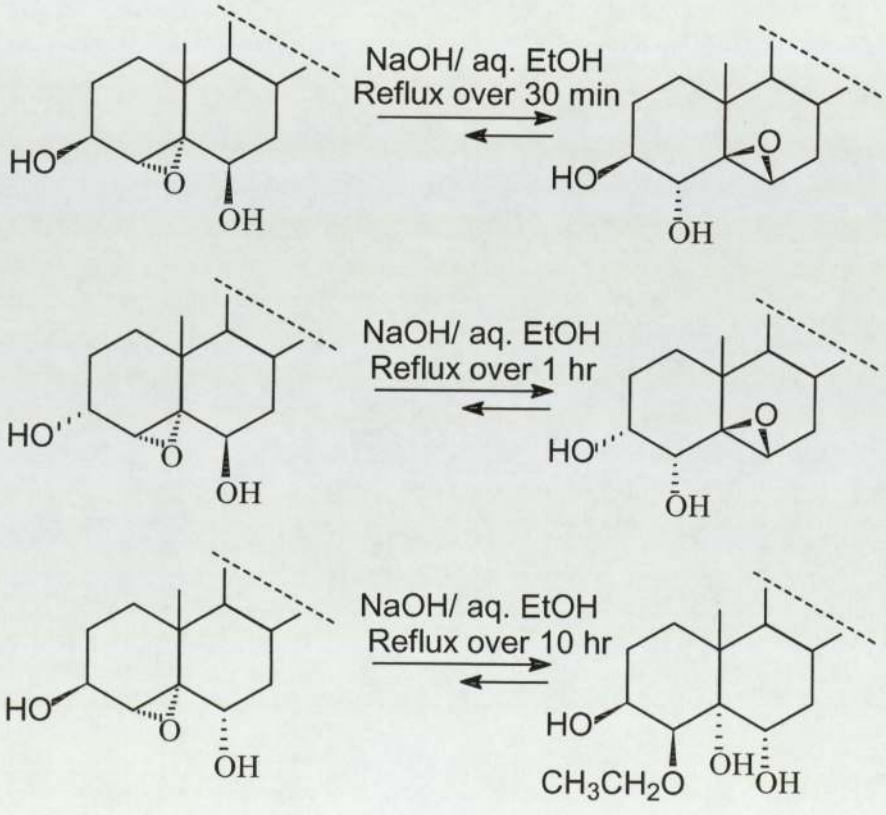


Figure 1-34 Starting Material 8-4

Reagents	Time	Reaction Solvent	
		THF	CH_3CN
$HClO_4$	10 min		3α, 4β, 5α, 6α-tetrols, 6-acetate; 40%  ; 17%  14%

$BF_3 \cdot Et_2O$	10 min		3α, 4β, 5α, 6α-tetrols, 6-acetate; 22%  ; 20%  18%
$AlCl_3$	90 min	4α-Cl, 3α, 5β, 6α-triols, 6-acetate 83%	
	10 min		4α-Cl, 3α, 5β, 6α-triols, 6-acetate 93%

The Payne rearrangements. (Scheme 1-5)



Scheme 1-5 The Payne rearrangements

1.5.3 The progress I made in one year

First part of my project was focused on the reductive cleavage of the epoxide ring of 4, 5-epoxycholestane-3, 6-diols. Sodium cyanoborohydride was tried first, combined with boron trifluoride diethyl ether complex. 45 times reactions were carried out in different combinations of reaction conditions such as temperature, the addition order of starting materials, reaction time, solvent and the ratio of the starting materials etc. But most reactions failed since the reaction did not happen at all, due to the weak reductive ability of sodium cyanoborohydride. Next, the method of borane combined with lithium borohydride was tried. This time, the reaction did happen. However, the epoxide ring was not cleaved in the direction expected. Most products were formed as the hydroxyl group went to the position 5 instead of position 4. Next, the mixed hydride was used, which are the different combination of lithium aluminium hydride and aluminium chloride. Part of the products formed with the hydroxyl group attached on the position 5 and part of the products did have the hydroxyl group going to the position 4. But unfortunately, the position 5 was not occupied by a hydrogen atom but a chloride. And then, the method of metal lithium in ethylenediamine was tested, which was reported as a much better way of reductively cleavage hindered epoxide ring. While the result also turned out that the hydroxyl group went to the position 5. Finally, several different Lewis acids in addition to lithium aluminium hydride were used, which could have different potency to polarize the carbon-oxygen bond in the epoxide ring. Therefore, with the proper strength of the polarization, it can benefit the hydride anion formed from lithium aluminium hydride to attack the carbon at position 5, and then obtained the desired product—3, 4, 6-triols. However, as far as about 20 Lewis acids that were being tried, all failed. When the Lewis acid was added four-fold of the starting material, the hydroxyl group always went to position 5, and the anion in the Lewis acid went to position 4. When the Lewis acid was added in large excess, the

hydroxyl group went to position 4 and the anion in the Lewis acid was attached at position 5.

The second part of my work focused on effects of different substituted benzoic acids on the epoxidation of cholest-4-en-3β, 6β-diol-6-acetate-3-benzoate. First, cholest-4-en-3β, 6β-diol-3, 6-diacetate was hydrolysed at room temperature to obtain the mono-acetylated substrate with a free hydroxyl group at position 3. And then it went through the acylation with substituted benzoic acids or benzoyl chlorides to react with the free hydroxyl group in the substrate. The resulting product was then epoxidated by adding mCPBA and keeping cool in the fridge for one week. After the working-up, the product mixture was analysed by ^{1}H NMR. The ratio of the product bearing α and β epoxide ring was determined from the spectrum. 36 Different benzoic acids were tried and 30 of them worked to form esters properly. Those 30 results were enough to make a conclusion about the effect. Generally, no big differences were observed about the effects of different benzoyl substituent on the epoxidation judging from the proportion of α and β oxide ring in the products.

Chapter Two: Results and Discussion

2.1 Part I. Reductive cleavage of epoxide ring

2.1.1 Methodology and the reaction mechanisms

Ring opening reactions of the epoxides can be accomplished by reductive cleavage from a set of reagents and under either an acid or a basic condition to produce an alcohol product. Although the oxide ring is more active than open-chain ether and can be readily cleaved, hydrogenolysis of oxidants (epoxides) is complex since either of the C-O bonds may break, with or without inversion of configuration. This kind of compounds is prone to be attacked by a wide range of nucleophiles in both acidic and basic environments, resulting in ring opening. In the case of an unsymmetrical epoxide, a site attacked by nucleophiles depends largely on the reaction conditions.

Generally, there are two ways to open the ring. One is Markovnikov reaction, which is the hydroxyl group going to the more substituted carbon. The second is anti-Markovnikov reaction, which is the hydroxyl group going to the less substituted carbon.

In acidic aqueous solution, epoxides are opened to give diols by an anti- Markovnikov addition process. Particularly, in cyclic system, ring opening occurs to give the diaxial diol.

Base-catalyzed reactions, in which a nucleophile initiates a ring opening, usually involve breaking of the epoxide C-O bond at the less substituted carbon, since this is the position more open to nucleophilic attack. (Parker, 1959) And with the steroidal compounds, the reaction is only limited to a few highly reactive species. (Kirk, 1968)

The situation in acid-catalyzed reactions is more complex. However, the reaction occurs more readily. The protonation on the oxygen weakens the C-O bonds through greatly enhancing the normal polarisation of the C-O bond. Once the oxygen atom is protonated, there is partial positive charge on the oxygen atom, which makes the oxygen atom more likely to attract the electrons from the neighbouring carbon, therefore, the bond between the oxygen and the carbon in the ring is

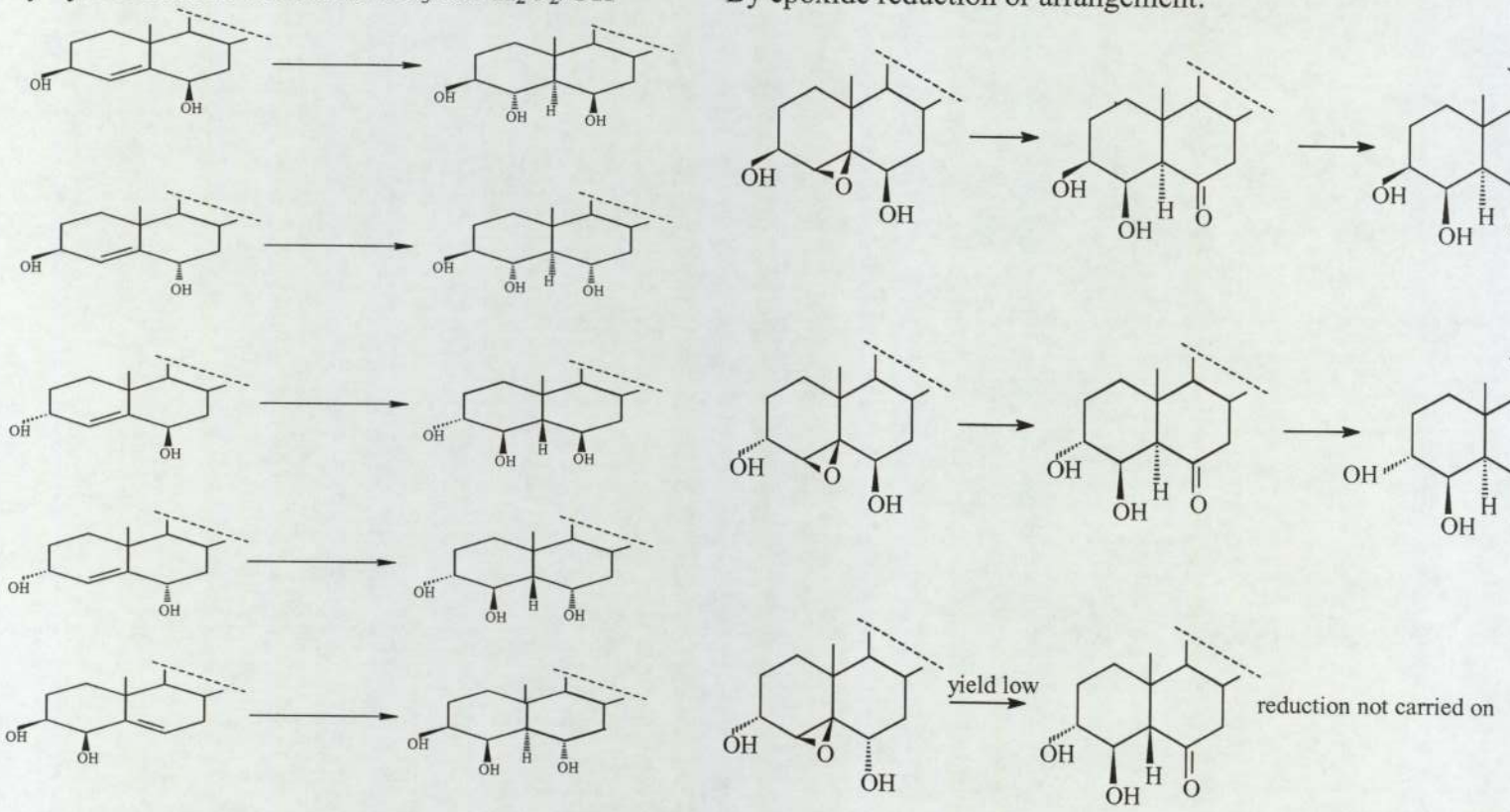
greatly weakened and makes the concerned carbon atom getting some positive charge and increases its ability to attract the nucleophiles. Thus, it facilitates rupture by weak nucleophiles and permits that it can be attacked by an anion or a solvent molecule on the opposite side of the ring in concern with breaking of a C-O bond from the opposite side. A S_N2 reaction occurs. If the C-O bonds are largely intact at the transition state, the nucleophile will become attached to the less substituted position for the same steric reasons that were cited for nucleophilic ring opening. If, on the other hand, C-O rupture is nearly complete when the transition state is reached, the opposite orientation is observed—the nucleophile will be attached to the more substituted position. This change in regiochemistry results from the ability of the more substituted carbon to stabilize the developing positive charge. In epoxy cyclohexane, including steroidal epoxides, the effect is to give a *trans* diaxially substituted product in almost every case. It follows from the principles of conformational analysis that every steroid epoxide should yield a unique product of ring opening, with the epoxide oxygen affording an axial hydroxyl group corresponding to the configuration of the epoxide, and the entering nucleophile becoming axially bonded at the other carbon atom. (Kirk, 1968)

Therefore, nucleophilic hydride transferring reagents, as considered for S_N2 process, attack epoxides at the less substituted position to afford the more substituted alcohol. While electrophilic reagents (i.e. BH_3, AlH_3, etc.) reverse opening is often observed to produce the less substituted alcohol, but mixture usually results. (Hutchins, 1981)

In this project, the anti-Markovnikov opening of the epoxide ring in the 4, 5-epoxycholestane-3, 6-diols were desired to afford the cholestane-3, 4, 6-triols. The structure determination is not difficult since the 3, 5, 6-triols and 3, 4, 5, 6-tetrols as well as many other compounds with related structures can be consulted for comparison. In the previous research in our group, several methods have been developed to prepare 3, 4, 6-triols (Scheme 2-1) (Zhao, 2002), but those methods have their

By hydroboration-oxidation: BH_3 and H_2O_2/OH^-

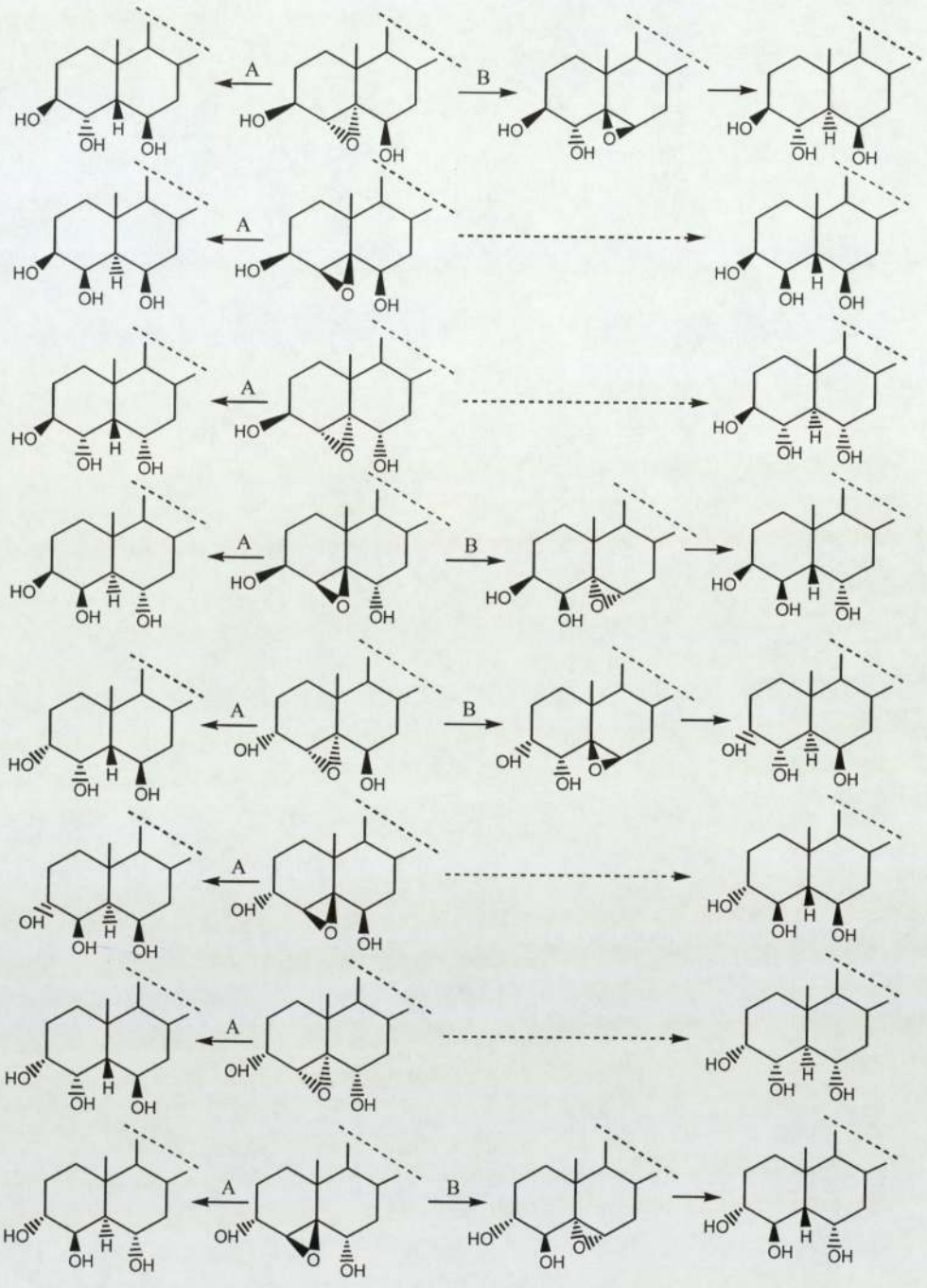
By epoxide reduction or arrangement:



Scheme 2-1 Other ways of 3, 4, 6-triols preparation

limitations and cannot be used for preparing all 16 isomers as a general method. What we need is some general ways to cleave the eight 4, 5-epoxide-3, 6-diols which can add the 5-hydrogen with stereoselectivity such as 5α or 5β control, therefore, the sixteen 3, 4, 6-triols could be synthesized easily with general methods.

According to the cleavage principle mentioned above, the nucleophile could only attack from the opposite side of the epoxide ring due to the great stereo barrier formed by the four-fused-ring system and the angle methyl group. There are at least two synthetic methods that can be employed to obtain the 16 desired isomers. Theoretically, among the 16 products desired, eight isomers could be obtained via one ideal method, that is, the epoxide ring could be opened directly with one reductive reagent. The other four desired isomers could be obtained by the rearrangement of corresponding starting materials to form the 5, 6-epoxy-cholestane-3, 4-diols first, and then going through the direct cleavage from the opposite side of the oxide ring. And the last four products could be obtained via a completely new oxide hydrogenation, which remains unknown. The prediction according to the above hypothesis is shown in Scheme 2-2.



Scheme 2-2 Envisaged synthetic sequences for producing the target compounds

A. Cleavage directly from the opposite side of the epoxide ring

B. Rearrangement first before the ring cleavage

A number of reagent combinations have been used to cleave epoxide at the more substituted position to get the less substituted alcohols. (Larock, 1999)

Usually, the methods of choices are $NaBH_3CN/BF_3{\cdot}Et_2O$, BH_3 in tetrahydrofuran, complex hydride ($LiAlH_4/AlCl_3$), zeolite support zinc borohydride, (Sreekumar, 1998) Me_3SiCl-Zn, dicycclopentadienyltitanium chloride and 1, 4-cyclohexadiene. (March, 1992)

Table 2-1 summaries literature reported most common hydride reagents, in an order of reductive ability toward epoxides from the strongest to the weakest. (Brown, 1979) Generally, there are two kinds of reducing agents: nucleophilic and electrophilic. Reagents of choice for reduction of epoxides to alcohols are hydrides and complex hydrides. A general rule of regioselectivity is that the reductions involving complex borohydrides and aluminohydrides, as well as their modified reagents appear to involve transfer of the hydride moiety from the complex anion to an electron deficient centre of the function group. They are nucleophilic. When used to cleave epoxide, they approach the oxide from the less hindered side, thus giving more substituted alcohols. (Hudlicky, 1984) As to the project, using this kind of reagent will form 3, 5, 6-triols instead of 3, 4, 6-triols since the bond between carbon 4 and oxygen is broken. In contrast, hydrides of electrophilic nature such as borane, especially in the presence of boron trifluoride, open the ring in the opposite direction and give predominantly less substituted alcohols. (Hudlicky, 1984) Therefore, acid reducing agents that act as electrophilic reductive agents can break the bond between carbon 5 and oxygen in the starting materials to obtain the desired 3, 4, 6-triols.

Table 2-1 Hydride Reagents (Brown, 1979)

Reactivity of hydride reagents from the strongest	**Reductive ability to epoxides**
$LiEt_3BH$ in THF (super hydride)	Rapid reaction (Markovnikov)

$LiAlH_4$ in THF	Rapid reaction (Markovnikov)
$LiAlH_4$: $AlCl_3$ 3:1 in ether	Rapid reaction (Markovnikov)
$LiAlH_4$: $AlCl_3$ 1:4 in ether	May give the less substitute alcohol as a major product
$Li(MeO)_3AlH$	Rapid reaction (Markovnikov)
AlH_3 in THF	Rapid reaction (Markovnikov)
9-BBN in THF	Borderline case
Sia_2BH in THF	Borderline case
BH_3 in THF	Very Slow (anti-Markovnikov)
$AlCl_3$ + $NaBH_4$ in Diglyme	Rapid reaction (Markovnikov)
LiCl + $NaBH_4$ in Diglyme	Rapid reaction (Markovnikov)
$Li(t\text{-}Bu)_3AlH$	Borderline case
$NaBH_4$ in Ethanol	Very slow or insignificant reaction
$NaBH_3CN$-BF_3OEt_2 in THF	Borderline case (anti-Markovnikov)

The results of the experiments carried out with these reagents and discussions will be presented in the next part 2.1.2.

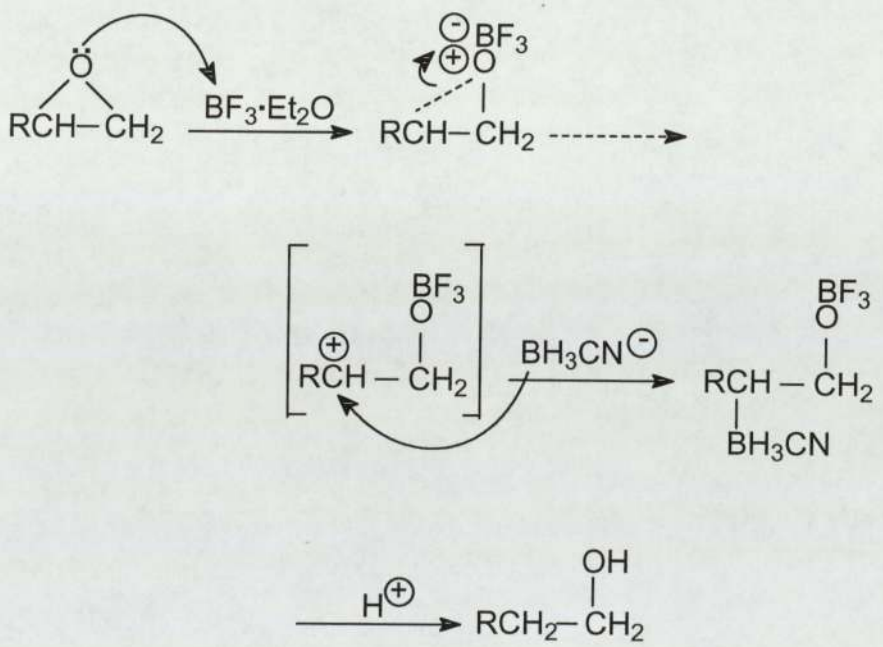
Method A. $NaBH_3CN/BF_3{\cdot}Et_2O$

Sodium cyanoborohydride and boron trifluoride diethyl ether complex was used in this method. Sodium cyanoborohydride with its strongly electron-withdrawing cyano group is a milder and more selective reducing agent than sodium borohydride. The characteristic of $NaBH_3CN$ is its stability in acid solution down to pH 3. Aldehydes and ketones are not affected by sodium cyanoborohydride in neutral solution, but they are readily reduced to the corresponding alcohol at pH 3. (Lane, 1975)

It was supposed that the unique acid stability of cyanoborohydride might be advantageous for regioselective opening of epoxides in which the less substituted alcohol would be preferentially produced by trapping of hydride at the site best able to accommodate a carbonium ion, which is the most stable. Activation of epoxides toward nucleophilic attack by

complexation with a Lewis acid is required since this moiety is essentially inert toward cyanoborohydride neutral or basic media. Boron trifluoride etherate in dry THF was considered to offer the most reliable and convenient reductive system. It was reported that $BH_3CN^-/BF_3{\cdot}Et_2O$ provides an effective combination for the regio and stereoselective cleavage of most epoxides to the less substituted alcohols, resulting from anti-Markovnikov ring opening. Further, the inertia of BH_3CN^- toward several other functional groups in acidic media (ester, acid, amide, cyano and nitro) recommends the reagent system when chemo selectivity is important. (Hutchins, 1981) This method was considered as the easiest and cleanest way for reduction of epoxides. (Taber, 1994)

The supposed mechanism is shown in Scheme 2-3.



Scheme 2-3 Mechanism of method A

The boron trifluoride etherate is a Lewis acid, which is electron deficient molecule. It readily accepts electron pair donated by the oxygen atom in the epoxide ring. And then the bond between the carbon and the oxygen is polarized. The BH_3CN^- then acts as a nucleophile tending to attack the more substituted carbon with the positive charge (carbonium

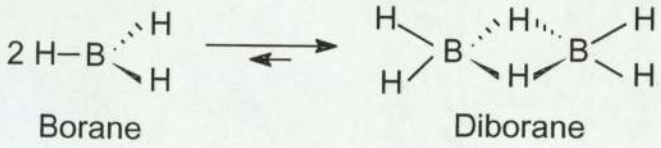
ion) from the back of the ring. The reason of this way of cleavage is that the more substitute carbonium ion is more stable due to the electron donating effects of the attached substituent (usually the alkane) that can distribute the positive charge. A general rule indicates that the order of decreasing stability or increasing energy content of similar carbonium ions is tertiary, secondary and primary. Therefore, according to the relative stability of them, the bond connected between the more substituted carbon and the oxygen was much more greatly weakened and prone to form the more stable carbonium ion. Thus, the hydroxyl group went to the less substituted carbon at last.

The bromocresol green used in the reaction functions as an acid-base indicator. It is yellow when pH range is from 3.8 to 5.4. Before the boron trifluoride was added, due the presence of the $NaBH_3CN$ and the indicator, the system appeared dark green or blue. With the addition of the Lewis acid boron trifluoride, the acidity of the system was becoming stronger. When the pH of the reaction system reached 5.4, falling into the yellow range of the indicator, the system turned to yellow. Usually, excessive boron trifluoride was added. Therefore, the other function of the boron trifluoride in addition to its being used as a Lewis acid is that it can maintain the acidity of the reaction system and make sure that the $NaBH_3CN$ can function well at its favourite acid condition.

Unfortunately, this method has never been used in sterol reaction system before. Therefore, according to its reported great regio and stereoselectivity combining with its availability and convenient operation, this method was worth being tried first.

Method B. $BH_3/LiBH_4$

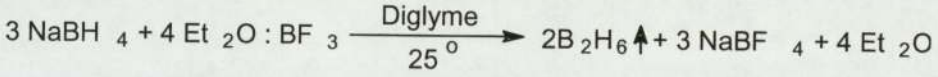
Borane (BH_3) is the simplest boron hydride, which could dimerize to diborane B_2H_6 in an equilibrium that lies overwhelmingly to the side of diborane under normal conditions of temperature and pressure.



Scheme 2-4 Equation of borane and diborane

Diborane is an inflammable toxic gas, which is available commercially or may be generated from $NaBH_4$ and BF_3 etherate complex in diglyme solution (diglyme is the dimethyl ether of diethylene glycol).

The commercially available diborane is in the form of a borane-Lewis base complex, such as BH_3-THF. It is relatively stable but still reactive and can be handled safely and easily. However, it usually has lower concentration. To make the reaction occur more rapidly and more completely, the self-generated diborane in our lab is preferred.



Scheme 2-5 Preparation of diborane

The gas may be generated externally and then introduced into the reaction flask or it may be produced in situ. (Pizey, 1977)

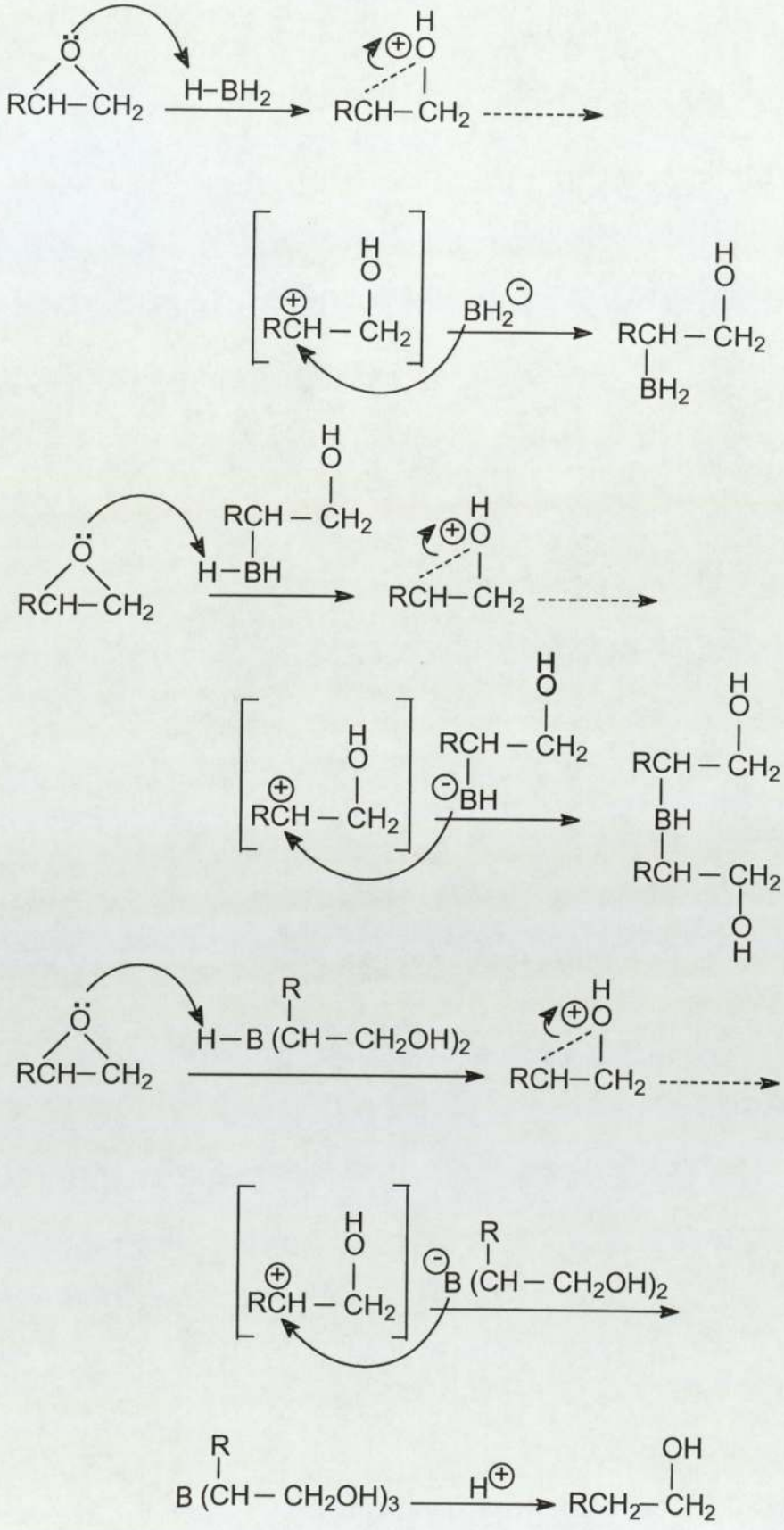
The reduction of epoxides with diborane is noteworthy since it has unusual characteristics and always gives rise to the less substituted carbinol in predominant amount, in contrast to reduction with complex hydrides. The best solvent for this reaction is tetrahydrofuran, but diglyme, ether, benzene, pentane, methylene dichloride, chloroform and carbon disulfide may also be used. (Brown, 1959) The borane and lithium borohydride can react rapidly with hydroxylic compounds. Consequently, this reagent must be used under anhydrous conditions with purified nonhydroxylic solvents.

In contrast to the simplicity of most other reactions, the reaction of diborane with epoxides is quite complex. Diborane itself in THF reacts slowly with epoxides to give only low yields of the expected alcohols, and

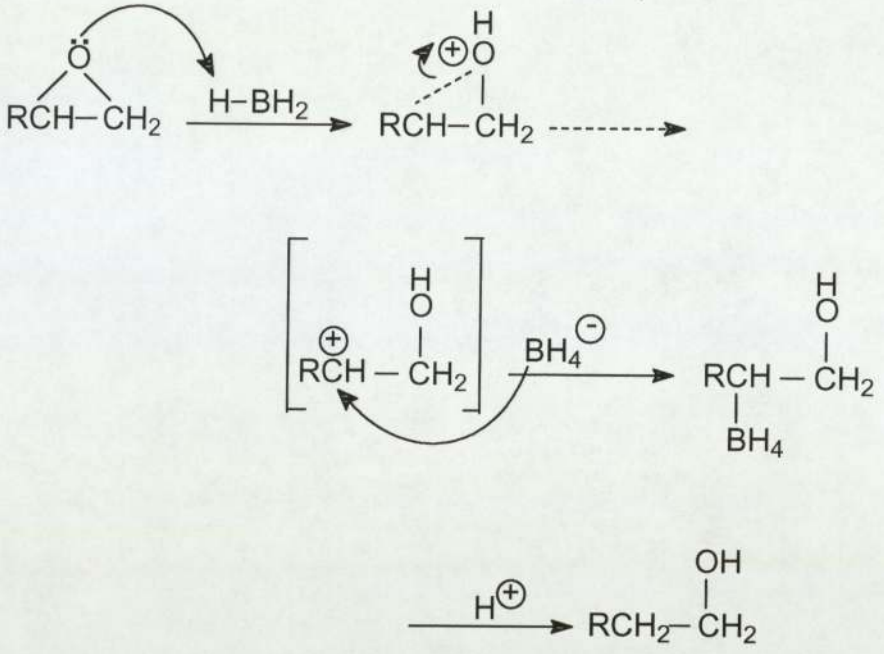
various side-reactions are complex and appear to be a consequence of the weak nucleophilic ability of diborane. (Buchanan, 1972) Better yields are obtained when BH_4^- is included in the reaction system, and a somewhat faster reaction, with similar products, could be realized by the use of higher concentration of the more soluble lithium borohydride. Therefore, the presence of even minor amounts of sodium or lithium borohydride has a major effect on both the reaction speed and its yield. (Brown, 1968)

Borane, as an electron-deficient molecule, functions through attack in an electron-rich centre in the functional group. (Brown, 1939) Thus diborane, borane and borane complex are acidic-type reducing agents that exhibit markedly different selectivity from the basic-type reducing agents, sodium and LAH. (Brown, 1957)

Therefore, the electrophilic nature of diborane determines the dominant product is that resulting from addition of the hydride at the more substituted carbon. The mechanism of the borane in the epoxide ring reductive cleavage is similar to the protonic acid in this kind of reaction.



Scheme 2-6 Mechanism of method B – 1 (Without BH_4^-)



Scheme 2-7 Mechanism of method B – 2 (With BH_4^-)

When the BH_4^- is involved in the reaction, the speed and the yield can be greatly increased since the nucleophilic ability of the BH_4^- is much stronger than the BH_2^- (because the Na^+ or Li^+ is much more tending to leave than H^+ does in borane). It is, to a certain extent, unnecessary to form the more steric hindered trimerized boron compound. While, due to the co-existence of BH_4^- and BH_2^- in the reaction system, the mechanism B-1 may also happen.

Borane is not found to be applied in the reductively cleavage of sterol epoxide ring system. Due to the failure of the method A, it was tried as the second method.

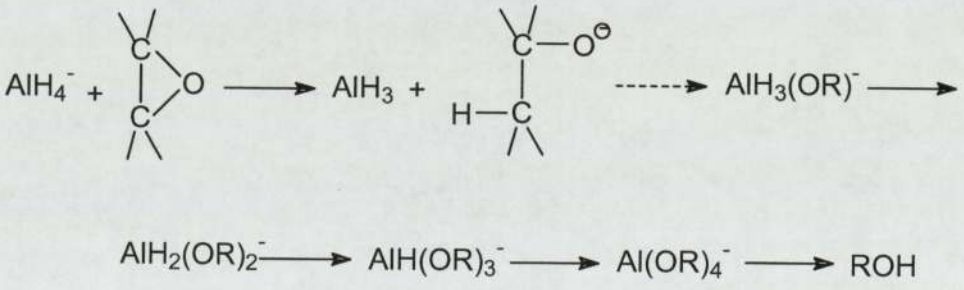
Method C. $LiAlH_4/AlCl_3$

The use of lithium aluminium hydride as a reducing agent in both organic and inorganic synthesis is well known. (Gaylor, 1956) Several years after the introduction of $LiAlH_4$ as reducing agent, a number of reports appeared using lithium aluminium hydride in combination with

aluminium chloride as a more selective reducing agent. (Nystrom, 1955; Nystrom, 1959; Eliel, 1956; Eliel, 1958)

Mixture of these two compounds has become known as the "mixed hydride" reagent. Although the mixture most commonly used has a $LiAlH_4/AlCl_3$ ratio of 1:4, other ratios have been used such as 3:1, 1:1 and 1:3. (Ashby, 1966) These reagents react with unsymmetrical epoxides in several ways, depending on which ratios of the two reagents are used. (Eliel, 1961; Rerick, 1962) The ratios used in my experiments were 1:4, 3:1 and 1:1.

The reduction course with lithium aluminium hydride only proceeds by an S_N2 pathway, which is a backside nucleophillic attack. It acts as a source of nucleophilic H^-. Although initial reaction involving $LiAlH_4$ proceeds by nucleophilic attack of $[AlH_4]^-$, there seems to be some question as to the nature of subsequent reducing species. Specifically there is evidence to indicate that in the reduction of epoxides by $LiAlH_4$, the space steric nature of the reducing species increases from $[AlH_4]^-$ to $[AlH_3OR]^-$ to $[AlH_2(OR)_2]^-$ and to $[AlH(OR)_3]^-$ etc., resulting in slower reaction and higher selectivity. (Fuchs, 1952; Pizey, 1974) (Scheme 2-8)

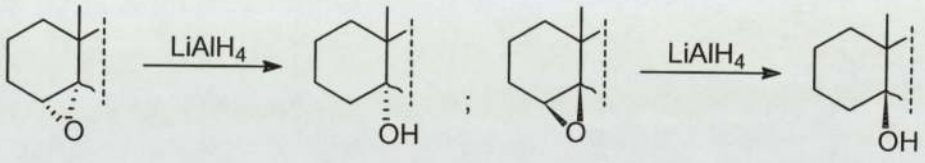


Scheme 2-8 Reductive cleavage of epoxide ring with lithium aluminium hydride

The solvent, generally diethyl ether or tetrahydrofuran, plays an essential part in the reaction since they also have an oxygen atom with an unshared pair of electron. Thus, in ether solution the lithium aluminium

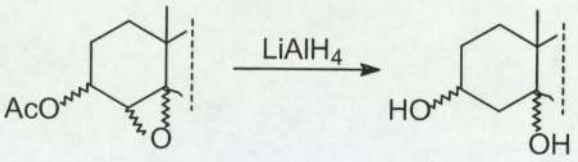
hydride exists largely as ionic aggregates of strongly solvated lithium ions and aluminohydride anions AlH_4^-. (Brown, 1951)

The steroid field is rich in examples of epoxide ring opening and, because of the existing conformational restraints, the contribution of various factors may be assessed. When the conformational effect in ring opening is complicated by steric or polar effects, lithium aluminium hydride gives a high yield of the axial alcohol. The behaviour of 4, 5-epoxides shows an interesting balance of steric and conformational factors. When the epoxide ring is α, attack by aluminium hydride ion occurs at the secondary carbon to yield the axial 5α-ol, due to a combination of steric and conformational factors. Reduction of the 4β, 5β-epoxides, Ring A, because of cis-fusion to Ring B, may adopt either of two half-chair forms of not widely different energies. Therefore, 4β, 5β-epoxycholestane also undergoes attack on the secondary carbon (C-4) yielding the 5-ol. (Scheme 2-9)



Scheme 2-9 Reductive cleavage of 4, 5-epoxycholestanes with lithium aluminium hydride

There are several instances of the reaction of hydroxyl-steroid epoxides with lithium aluminium hydride that might be expected to exhibit polar or neighbouring group effects. All of the 3-acetoxy-4, 5-epoxycholestanes and some related model compounds have been studied. The only products isolated have arisen from attack at C-4, in agreement with the unsubstituted epoxides. (Scheme 2-10) Polar effects should inhibit reduction at C-4, but apparently the steric hindrance to attack at C-5 is the overriding factor. (Buchanan, 1972)



Scheme 2-10 Reductive cleavage of 3-acetoxy-4, 5-epoxycholestanes with lithium aluminium hydride

In this method, a Lewis acid is added. The reductive cleavage of epoxides by a "mixed hydride" reagent has been examined and it was found that the direction of ring cleavage in the presence of a mixed hydride reagent differed fundamentally from that with lithium aluminium hydride alone. The mixed hydride with low aluminium chloride content presumably contained aluminium hydride, whereas in the presence of high aluminium chloride content, dichloroalane, $AlHCl_2$, is supposed to be predominant. Dichloroalane is a stronger Lewis acid and weaker hydride donor than aluminium hydride. (Pizey, 1974)

Although aluminium hydride polymerises in ether solution, stable solutions of this hydride can be obtained in tetrahydrofuran due to the formation of a covalent complex from aluminium hydride and the Lewis base, tetrahydrofuran. If molar ratios other than 3 moles of lithium aluminium hydride to 1 mole of aluminium chloride are used, a series of intermediate chloroaluminum compounds are produced. The ability of these aluminium compounds to serve as electrophilic reagents (i.e., Lewis acids) appears to decrease in the order: $AlCl_2H > AlClH_2 > AlH_3$. (Ashby, 1966; Ashby, 1968)

$$3LiAlH_4 + AlCl_3 \xrightarrow{THF} 3LiCl + 4AlH_3 \quad (1)$$

$$LiAlH_4 + AlCl_3 \xrightarrow{Et_2O} LiCl + 2AlClH_2 \quad (2)$$

$$LiAlH_4 + 3AlCl_3 \xrightarrow{Et_2O} LiCl + 4AlCl_2H \quad (3)$$

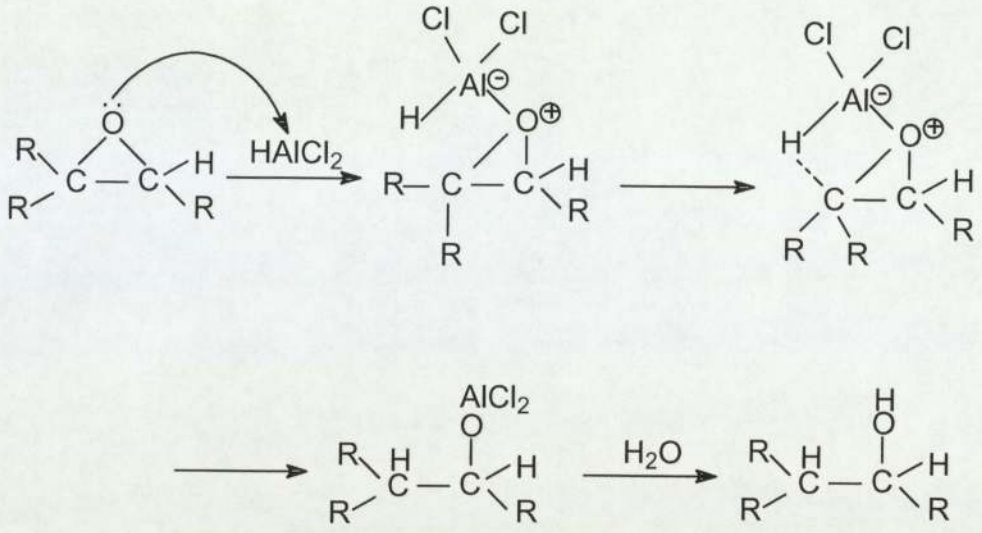
(Believed to be in solution as $LiAlCl_3H$)

$$LiAlH_4 + 4AlCl_3 \xrightarrow{Et_2O} LiAlCl_3H + 3AlCl_2H + AlCl_3 \quad (4)$$

Scheme 2-11 Different combinations of
lithium aluminium hydride and aluminium chloride

Although the final product of the reaction of lithium aluminium hydride and aluminium chloride in 1:3 ratio has been established as a mixture of 4 $AlCl_2H$ + LiCl, the complex of lithium chloride with 1 mole in 4 moles $AlCl_2H$ presents in order to explain the lithium chloride solubility. Thus in solution, the product of this reaction is probably 3 $AlCl_2H$ + $LiAlCl_3H$. Since $AlCl_2H$ is the product of least reduction of aluminium chloride, the reaction of lithium aluminium hydride and aluminium chloride in 1:4 ratio (most often used "mixed hydride" reagent) merely produces the same product as from the reaction in 1:3 ratio, but with one mole of aluminium chloride remaining in solution unreacted. Since the lithium chloride does not precipitate in this reaction either, it could be complexing with either the $AlCl_2H$ or $AlCl_3$. Thus the actual reaction product in solution could be represented by either eq. 3 or 4 or a mixture of both.

Obviously, the reaction of $LiAlH_4$ and $AlCl_3$ in 3:1, 1:1, 1:3 and 1:4 produces AlH_3, $AlClH_2$, $AlCl_2H$ and $AlCl_2H$ + $AlCl_3$, respectively. It was reported that the first step in the reduction of an organic functional group such as an epoxide involves complexation of the aluminium species at the oxygen atom. The resulting complex is relatively stable and thus reduction proceeds by a four-centre transition state to produce the less substituted alcohol. (Scheme 2-12)



Scheme 2-12 Mechanism of mixed hydride,
complex formation followed by reduction

As to those aluminium hydrides that contain more than one hydride, such as AlH_3 and $AlClH_2$, as long as the steric space permit, after the intermediates formation as $AlH_2(OR)$ and $AlClH(OR)$, they may go on to react with another epoxide molecule with their remaining hydride until all the hydrides are reacted.

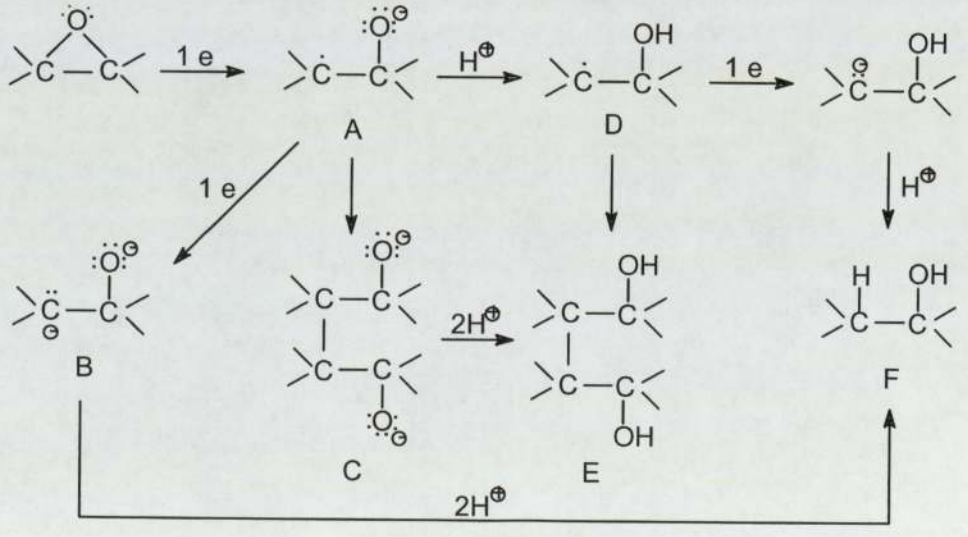
This method of mixed hydride was not found suitable to cleave the epoxide ring in steroid system. Hence, an alternative reagent was explored.

Method D. Li/ethylenediamine

The reductive ring opening of epoxides with dissolving metals such as lithium in ethylenediamine, a more reductive reagent combination than lithium aluminium hydride, gives rise to the more substituted alcohol and is a superior method for hindered epoxides and those epoxides that are resistant to reduction (Brown, 1970), because it can form the effective reagent, solvated electrons, which has a lower steric requirement. (Hallsworth, 1970)

Dissolving metal reductions were among the first reductions of organic compounds discovered some 150 years ago. Reduction is defined as acceptance of electrons. Electrons can be supplied by an electrode,

cathode or else by dissolving metals. If a metal goes into solution, it forms a cation and gives away electrons. A compound to be reduced accepts one electron and changes to a radical anion A. Such a radical anion may exist when stabilized by resonance, as in Li-ethylenediamine complexes with some ethers. In the absence of protons the radical anion may accept another electron and form a dianion B. Such a process is not easy since it requires an encounter of two negative species, an electron and a radical anion, and the two negative sites are close together. It takes place only with compounds that can stabilize the radical anion and the dianion by resonance. Rather than accepting another electron, the radical anion A may combine with another radical anion and form a dianion of a dimeric nature C. In the presence of protons, the initial radical anion A is protonated to a radical D which has two options, either to couple with another radical to form a 1, 4-diol E, or to accept another electron to form an alcohol F. The 1, 4-diol E and the alcohol F may also result from double protonation of the doubly charged intermediates C and B, respectively. (Scheme 2-13) (Hudlicky, 1984)



Scheme 2-13 Mechanism of method D

It was reported that some steroidal epoxides, which were quite unreactive to lithium aluminium hydride, were easily reduced with a large

excess of lithium in ethylamine. (Hallsworth, 1960; Hallsworth, 1957) The ethylenediamine (b.p. 116°C) is considered a more convenient medium to use than the much lower boiling ethylamine (b.p. 16°C) for routine laboratory reduction of hindered epoxides. And the reaction in ethylenediamine is more controllable (The lithium metal reduction in ethylamine is very vigorous, even at –20°C.) With either combination, similar results could be obtained. It was said that this method has a number of significant advantages over that of ethylamine and may be helpful in other instances where the reduction by complex hydrides fails. (Brown, 1970)

Therefore, it was chosen as the fourth method because all the aforementioned methods did not work.

Method E. $LiAlH_4$/different Lewis acids

With the failure of all the methods in literature, a new idea has to be generated to solve the problem. Overall, the solvent used is dried diethyl ether because the main reducing agent in the method is still $LiAlH_4$.

Reduction using lithium aluminium hydride only, it acts as a nucleophilic reducing agent, and the hydride is added at the less substituted carbon atom of the epoxide ring. As expected for an S_N2 process, cleavage usually results in the opening of the epoxide ring at the less substituted carbon (primary if possible) to give the more substituted saturated alcohol. Therefore, a tertiary alcohol is formed if possible. If not, a secondary alcohol is preferred.

To get the result converted, that is, to obtain the desired less substituted alcohol, the bond between the C-4 and the oxygen in the starting material 4, 5-epoxycholestane-3, 6-diols has to be polarized first. To polarize this bond, an electron-deficient molecule should get involved. The Lewis acids could serve as electron-deficient compounds, which can readily accept the free pair electron donated by the oxygen atom in the ring. Several different Lewis acids were being tried in order to find one

that could get the bond polarized to the maximal extent without ring rearrangement. Since the more substituted carbonium ion is more stable, the bond between C-5 and oxygen should be much more easily broken than the bond between C-4 and oxygen. At this point, provided by the lithium aluminium hydride, a hydride with its smallest volume among all other anions could easily attack the relative highly hindered C-5. The bond between the C-5 and the hydride then could be formed and at the same time the bond between C-5 and oxygen was cleaved. Therefore, the Lewis acid should be added first and allowed some time to have it form complex with the oxygen in the ring. And then the lithium aluminium hydride would be dropped into the system gradually. High temperatures should be avoided whenever possible because the complex formed may be destroyed and the reaction would go through the same reduction progress as that using lithium aluminium hydride alone. The course of the reaction was monitored by TLC. If no reaction seemed happening, then the reaction system was heated to reflux. The temperature was relatively low in this case because diethyl ether (b.p. 35°C) was used as the solvent.

2.1.2 Results and discussions

In most of reactions, the 4α,5–epoxy–5α–cholestane–3β, 6β–diol was used as the starting material (substrate), except those specified. The products shown in the following tables were only indicated the change at position C-3, 4, 5, 6 of the substrate. Other parts of the substrate structure remain the same.

Reductive cleavage of the sterol epoxide ring is not as easy and simple as cleavage of the epoxide ring in chain or cyclohexane structure. Especially for those specific target products in a selective cleavage, not only the characters of reductant had to be considered, but also needed to take care of the special configuration of the sterol that has a huge four hydrocarbon-fused-ring skeleton.

For this project, to obtain the desired product of cholestane-3, 4, 6-triols, the hydrogen atom had to be added to the C-5 only. Therefore, the bond between oxygen and the C-5 has to be broken, and the oxygen can only go to C-4. However, since the C-5 in the epoxide ring is also one of the joint carbons that shared by both ring A and ring B, combined with the shield effect from the 19β-methyl group connected with the neighbour C-10, there is not much space left for a big anion approaching it. This condition is even worse when the epoxide ring is α, which means the epoxide ring is under the nucleus ring-plane of the steroid molecule. Because the only attack direction of the reductant has to be from the opposite side of the epoxide ring, which is β—above the steroid plane and also at the same side as the C-19 methyl group.

Method A. $NaBH_3CN/BF_3{\cdot}Et_2O$

The results of the reactions by using the method A are listed in Table 2-2.

Table 2-2 Results of method A

Exp. No.	Solvent	Mole ratio of the reactants			Temp. (°C)	Time (hrs)	Result
		Substrate	$NaBH_3CN$	$BF_3{\cdot}Et_2O$			
1	THF(R)	1	2.5	20 (d)	r.t.	4	Mess
2	THF(R)	1	2.5	1.4 (d)	r.t. / Ref.	27.5	NR
3	THF(R)	1	2.5	1 (d)	Ref.	9	NR
4	THF(D)	1	2.5	2.3 (d)	Ref.	4	NR
5	THF(D)	1	2.5	4 (d)	Ref.	4	NR
6	THF(D)	1	2.5(d)	20	Ref.	4	Mess
7	THF(D)	1	2.5	1 (d)	Ref.	4	NR
8	THF(D)	1	1 (d)	2	Ref.	4	NR
9	THF(D)	1	2.5	20 (d)	Ref.	4	Mess
10	THF(D)	1	2.5	1 (d)	Ref.	4 days	NR
11	THF(D)	1	2.5	1 (d)	Ref.	15 days	NR
12	MeOH(R)	1	2.5	1 (d)	Ref.	21	NR
13	DMF(D)	1	2.5	1 (d)	Ref.	21	Mess
14	Dioxane (R)	1	2.5	1 (d)	Ref.	23	NR
15	Glyme(R)	1	2.5	1 (d)	Ref.	20	NR
16	THF(D)	1	2.5	1 (d)	Ref.	18	NR
17	THF(D)	1	1.23	Borane in excess	r.t.	36	NR
18	THF(D)	1	2.5	$(CH_3CO_2)_2Cu$	r.t.	4	NR
19	THF(D)	1	2.5	CsCl	r.t.	4	NR

20	THF(D)	1	2.5	$CoCl_2$	r.t.	4	NR
21	THF(D)	1	2.5	CdCl	r.t.	4	NR
22	THF(D)	1	2.5	CuI	r.t.	4	NR
23	THF(D)	1	2.5	$BaCl_2$	r.t.	4	NR
24	THF(D)	1	2.5	$FeCl_2$	r.t.	4	NR
25	THF(D)	1	2.5	4-NH_2-C_6H_4-SO_2H	r.t.	4	NR
26	THF(D)	1	2.5	(4-HO_2C-C_6H_4- CO_3) $_2$-Mg	r.t.	4	NR
27	THF(D)	1	2.5	2-Cl- C_6H_4-CH_2-CO-$NHNH_2$	r.t.	4	NR
28	THF(D)	1	2.5	H_2N-CO-$NHNH_2$	r.t.	4	NR
29	THF(D)	1	2.5	BrC-(CHO)=C(Br)-COOH	r.t.	4	NR
30	THF(D)	1	2.5	$(HCOOH)_2$	r.t.	4	NR
31	THF(D)	1	2.5	3, 4-di-OH-C_6H_3-CH=CH-COOH	r.t.	4	NR
32	THF(D)	1	2.5	C_6H_5-CH=CH-COOH	r.t.	4	NR
33	THF(D)	1	2.5	H_2N-CH_2 - CH_2-COOH	r.t.	4	NR
34	THF(D)	1	2.5	C_6H_5-COOH	r.t.	4	NR
35	THF(D)	1	2.5	LiCl	r.t.	4	NR
36	THF(D)	1	2.5	CuCl	r.t.	4	NR
37	THF (D)	1	2.5	$ZnCl_2$	r.t.	4	NR
38	CH_3CN (D)	1	2.5	1 (d)	r.t.	16	NR

39	CH_3CN (D)	1	2.5	1 (d)	r.t.	7 days	NR
40	DCM (D)	1	2.5	1 (d)	r.t.	7 days	NR
41	CH_3CN (D)	1	2.5	$AlCl_3$	r.t.	7 days	NR
42	THF(D)	1	2.5	$AlCl_3$	r.t.	7 days	NR
43	CH_3CN (D)	1	2.5	CuCN	r.t.	7 days	NR
44	THF(D)	1	2.5	CuCN	r.t.	7 days	NR

Note: Exp. No.=Experiment Number, Temp.=temperature, hrs=hours, R=Redistilled, D=Dried, d=dropwise, r.t.=Room temperature, Ref.=Reflux, NR=no reaction occurs

The Exp. No 2 repeated the method in literature. (Hutchins et al., 1981) When judging from the TLC that the reaction would not occur at room temperature, it was heated and kept refluxing for 24 hours. Still no reaction was detected. Then more $NaBH_3CN$ or $BF_3{\cdot}Et_2O$ was added respectively or at the same time, and fixed the reaction time as 4 hours refluxing. Again no reaction happened except Exp. No. 1, 6 and 9 that $BF_3{\cdot}Et_2O$ were added 20-fold of the substrate amount. Their ^1H NMR showed totally mess because the peak of 18-anglur methyl group (δ around 0.6-0.7) was split into several peaks, which should be a sharp single peak if there was only one product. It seemed that several compounds formed including one with aromatic structure since there were two multi-peaks between 7-8 ppm in the ^1H NMR. Too many products in these three reactions may be resulted from the addition of too many $BF_3{\cdot}Et_2O$ that could prompt the ring rearrangement or cleavage and other side-reactions. (Zhao, 2002)

The reversed addition of reactants was also tried in Exp. No. 8, no reaction was observed either.

The ratio of the reactants was changed back to 1:2.5:1 again, and tried to prolong the reaction time (Exp. No. 10, 11 and Exp. No. 38-44), the reaction solvent with the different boiling point (Exp. No. 7, THF, b.p. 66°C; Exp. No.12, MeOH, b.p. 64.7°C; Exp. No.13, DMF, b.p. 153°C; Exp. No.14, Dioxane, b.p. 100-102°C; Exp. No.15, Glyme, b.p. 83°C; Exp. No. 39, CH_3CN, b.p. 80-82°C; Exp. No. 40, DCM, b.p. 39.7°C), the different substrate with 3, 6-acetate attached (Exp. No.16). They all turned out where no reaction occurred and the final product was still the starting material except the one with DMF as solvent. Because DMF has much higher boiling point than any other solvent involved, when refluxing, the starting material might not bear such high temperature and was discomposed and showed mess in the ^1H NMR.

Next, different acids were used in order to polarize the carbon-oxygen bonds in epoxide ring system first before undergoing the reduction (Exp. No. 17-37, 41-44). A number of organic acids, inorganic Lewis acids and such compounds that may accept the electron pair were being tried. Again, no reaction occurred.

Over all, the reactions using $NaBH_3CN$ as reductant failed; most of them did not react at all. The reason could be the properties of the reactant itself. Due to the strongly electron-withdrawing cyano group presented in the molecule, as a nucleophile, its nucleophilic ability is too weak to approach the carbon with slight positive charge in the steroid nucleus, break the carbon oxygen bond and get the epoxide ring reduced.

Therefore, this method had to be abandoned and we moved on to the second method.

Method B. $BH_3/LiBH_4$

Although the complex of BH_3 in THF is commercially available, to obtain and guarantee the high concentration of the existence BH_3 in the reaction system, it was prepared whenever the reduction was carried out.

The results using this method are listed in Table 2-3.

Table 2-3 Results of method B

Exp. No.	Substrate	BH_3 generated	Mole ratio of reactants			Temp. (°C)	Time (days)	Result
			Substrate	$LiBH_4$	BH_3			
1		Ext.	1	1	Exc.	0/0	1	NR
2		Ext.	1	5	Exc.	0/r.t.	7	3β, 5α, 6β-triols
3		In situ	1	-	Exc.	r.t./r.t.	4	3β, 5α, 6β-triols
4		Ext.	1	5	Exc.	r.t./Δ	15	3β, 5α, 6β-triols
5		Ext.	1	5	Exc.	r.t./Δ	5	3α, 5α, 6β-triols
6		In situ	1	-	Exc.	r.t./Δ	7	Mess
7		Ext.	1	1	Exc.	r.t./r.t.	3	3α, 5α, 6α-triols
8		Ext.	1	1	Exc.	r.t./r.t.	3	3β, 5α, 6α-triols
9		Ext.	1	1	Exc.	0/r.t.	3	3α, 5β, 6β-triols
10		Ext.	1	1	Exc.	0/r.t.	3	3α, 5β, 6α-triols
11		Ext.	1	1	Exc.	0/r.t.	3	NR
12		In situ	1	-	Exc.	0/r.t.	3	5α-F, 3β, 4α, 6β-triols
13		Ext.	1	5	Exc.	0/r.t.	3	NR
14		In situ	1	-	Exc.	0/r.t.	3	5α-F, 3β, 4α, 6β-triols
15		Ext.	1	5	Exc.	0/r.t.	8	3β, 5α, 6β-triols
16		Ext.	1	$NaBH_3CN$ 5	Exc.	0/r.t.	5	NR
17		Ext.	1	$BF_3{\cdot}Et_2O$ 4	Exc.	0/r.t.	18	5α-F, 3β, 4α, 6β-triols

Note: Exp. No.=Experiment Number, Temp.= temperature, Ext.=externally, Exc.=excessively, r.t.=Room temperature, Ref.=Reflux, NR=no reaction occur, In the temperature column, the first one indicates the temperature during the borane going through or being generated in situ in the reaction system. The second one indicates the temperature during the reaction.

The borane gas was generated externally or in situ, no significant difference was noticed to the product formation (Exp. No.2 and 3). It tallies with the literature, which shows that "reductions with diborane give the same result regardless of whether diborane is generated externally or produced internally by the action of $NaBH_4$ and boron trifluoride." (Thakar, 1962)

The Exp. No. 1 repeated the condition mentioned in the literature. (Brown et al., 1968) There was no structure change found as to the starting material. Perhaps it was because the method in the literature was used to reductive cleavage the cyclohexane type compounds, which do not have such a great steric hindrance in the substrate molecule as that in the four-ring fused steroid system mentioned at the beginning of this part.

Many reactions involving borane complexes have unusually low activation energies. Consequently, most reactions occur readily at room temperature or below. These low temperatures tend to produce clean products with minimum side reactions. The solubility of diborane in ether solvents means that the reactions are usually homogeneous, proceed without induction periods, and easily controlled. (Wheeler, 1972) Since the Exp. No. 1 was carried all the way at 0^{o} and resulted in no reaction, room temperature, even being heated to reflux were applied. (Exp. No. 2-6, 15). Most of the products turned out to be the cholestane-3, 5, 6-triols. Obviously, it was the bond between C-4 and the oxygen that was broken, which resulted in the oxygen going to the C-5. When the reaction system was kept refluxing for longer time, there were many by-products formed, which led to the mess on the ^{1}H NMR.

The reductive cleavage using borane is one of the most generally applied methods to obtain a less substituted alcohol, which is widely introduced in the organic textbooks. The abnormal result for this specific isomer might not reappear for other isomers. Therefore, four other isomers of 4,5–epoxycholestane–3, 6–diol were used as the starting material to carry out the same borane reductive reaction respectively (Exp. No 7-10). Quite interestingly, all their products were turned out to be the

cholestane-3, 5, 6- triols. The ways of the cleavage were exactly the same as that in the reaction using the 4α,5–epoxy–5α–cholestane–3β, 6β–diol as starting material. Therefore, in this kind of epoxide ring reductive cleavage, the steric hindrance of the steroid compound is the key factor in determining the direction of the bond broken.

4α,5–epoxy–5α–cholestane–3β, 6β–diol-3, 6-diacetate was also served as starting material (Exp. No.11, 13). No reaction was detected. It was supposedly owing to the two acetates which could further increase the steric hindrance around the C-5, as well as C-4.

According to the borane reductive mechanism, the reaction might not stop at the point when $BH_2(OR)$ was formed. $BH_2(OR)$ might tend to get connected with the second OR to form $BH(OR)_2$ or even $B(OR)_3$. However, this could not happen in our compound's reduction. There were two reasons. First, the borane was highly excessive in the reaction system. Second, the greatly steric hindered steroid structure made it impossible to get two or three such a big molecule OR connected with the boron atom. Therefore, the reaction had to stop at the first stage.

As to the Exp. No. 12, 14 and17, although the oxygen did go to the C-4 as desired, the fluoride was attached at C-5 at the same time. This might be due to the fluoride ion present in the reaction system. Many commercially available $BF_3{\cdot}Et_2O$, as well as the one with long-time storage, appears to contain fluoroboric acid (HBF_4) in equilibrium with BF_3 + HF. The later can apparently act as a source of fluoride ions and lead to epoxide-opening to form fluorohydrins. (Kirk, 1968) A general rule mentioned in 2.1.1 indicates that the order of decreasing stability or increasing energy content of similar carbonium ions is tertiary, secondary and primary. Therefore, according to the relative stability of them, the bond connected the more substitute carbon and the oxygen was much more greatly weakened and more easily being attacked by the strong electronegative atom—fluoride. The hydroxyl group was remained with C-4.

For the Exp. No. 16, no reaction occurred using the $NaBH_3CN$ instead of $LiBH_4$. Apparently, it was due to the same reason as indicated in method A.

Method C. $LiAlH_4/AlCl_3$

This method mainly lies in the ratio of the mixed hydride—lithium aluminium hydride and the aluminium chloride. The results are shown in the Table 2-4.

Table 2-4 Results of method C

Exp. No.	solvent	Mole ratio of the reactants			Ratio of mixed hydride	Temp. (°C)	Time (hrs)	Result
		Subs-trate	$LiAlH_4$	$AlCl_3$				
1	Et_2O(D)	1	4	1.3	3:1	0/Ref.	2	3β, 5α, 6β-triols
2	Et_2O(D)	1	2	8	1:4	0/Ref.	2	5α-Cl-3β, 4α, 6β-triols
3	THF(D)	1	4	1.3	3:1	0/Ref.	2	3β, 5α, 6β-triols
4	THF(D)	1	2	6	1:3	0/Ref.	2	3β, 5α, 6β-triols
5	THF(D)	1	2	8	1:4	0/Ref.	2	5α-Cl-3β, 4α, 6β-triols
6	Et_2O(D)	1	4	4	1:1	0/Ref.	2	3β, 5α, 6β-triols
7	Et_2O(D)	1	2	6	1:3	0/Ref.	2	5α-Cl-3β, 4α, 6β-triols
8	THF(D)	1	4	4	1:1	0/Ref.	2	5α-Cl-3β, 4α, 6β-triols

Note: Exp. No.=Experiment Number, Temp.=temperature, hrs=hours, D=dried, Ref.=reflux, In the temperature column, the first one indicates the temperature when the substrate was dropped into the reaction system. The second one indicates the temperature during the reaction.

As indicated by the results in the Table 2-4, no matter what solvent used, the mixed hydride with ratio of $LiAlH_4$:$AlCl_3$ 3:1 always gave the alcohol with the hydroxyl group attached at the C-5 (Exp.No.1, 3). The functional reductant in this condition was alane AlH_3. (As discussed in 2.1.1) It might go through the same reductive course as BH_3. As explained in method B, the products with 5-hydroxyl group were as expected.

While with the ratio of $LiAlH_4$:$AlCl_3$ 1:4, it always gave the alcohol with the hydroxyl group attached at the C-4 and with the chloride atom attached at C-5 at the same time in spite of the solvent. (Exp.No.2, 5). It might be due to one spare mole of the $AlCl_3$ presence in the system as a source of chloride ion. Same as the reason of fluoride ion in the BF_3 might result the fluorohydrins, the chloride ion might be responsible for the chlorohydrins.

An interesting case was found. When the mixed hydride ratio was $LiAlH_4$:$AlCl_3$ 1:3 and the reaction occurred in the THF (Exp. No 4), the product turned out to be cholestane-3β, 5α, 6β-triol. While the reaction carried out in diethyl ether (Exp. No 7), 5α-Cl-cholest-3β, 4α, 6β-triol was afforded. However, when the mixed hydride of the ratio $LiAlH_4$: $AlCl_3$ 1:1, the result was totally reversed. When the reaction occurred in diethyl ether (Exp. No 6), the product turned out to be cholestane-3β, 5α, 6β-triol. While the reaction carried out in the THF (Exp. No 8), 5α-Cl-cholest-3β, 4α, 6β-triol was afforded. The reason for this phenomenon remains unclear.

Method D. Li/ethylenediamine

Only one experiment was carried out by using this method. This method was reported to be a stronger reductive-reagent combination than

lithium aluminium hydride, which gave rise to the more substituted alcohol and was a superior method for cleavage hindered epoxides that were resistant to reduction (Brown, 1970). The result product cholestane-3β, 5α, 6β-triol was obtained as expected.

Method E. $LiAlH_4$/different Lewis acids

Nearly 20 different Lewis acids were tried in this method. The results are shown in the following Table 2-5.

Table 2-5 Results of method E

Exp. No.	Lewis Acids	Result
1	CsCl	NR
2	CuI	NR
3	$CuBr_2$	NR
4	$CuCl_2$	4β-Cl, 3β, 5α, 6β-triols
5	CuCl	4β-Cl, 3β, 5α, 6β-triols
6	$SnCl_4$	5α-Cl, 3β, 4α, 6β-triols
7	$ZnCl_2$	5α-Cl, 3β, 4α, 6β-triols
8	NH_4Cl	5α-Cl, 3β, 4α, 6β-triols
9	$CoCl_2$	4β-Cl, 3β, 5α, 6β-triols
10	CdI	NR
11	$FeCl_2$	4β-Cl, 3β, 5α, 6β-triols
12	$MnCl_2$	4β-Cl, 3β, 5α, 6β-triols
13	$NiCl_2$	4β-Cl, 3β, 5α, 6β-triols
14	$HgCl_2$	4β-Cl, 3β, 5α, 6β-triols; 3β, 5α, 6β-triols
15	$BaCl_2$	3β, 5α, 6β-triols; 4β-Cl, 3β, 5α, 6β-triols;
16	$CeSO_4$	5α-Cl, 3β, 4α, 6β-triols
17	$CrCl_3$	4β-Cl, 3β, 5α, 6β-triols

18	$BF_3 \cdot Et_2O$	5α-F, 3β, 4α, 6β-triols

Note: Exp. No.=experiment number, NR=no reaction

Lewis acids are defined as molecules which act as electron pair acceptors, featured with a vacant orbital. The proton is an important special case, but many other species can play an important role in the catalysis of organic reactions. The most important in organic reactions are metal cations and covalent compounds of metals. Metal cations that play prominent roles as catalysts include the alkali metal monocations Li^+, Na^+, K^+, Cs^+ and Rb^+, the divalent ions Mg^{2+}, Ca^{2+} and many of the transition metal cations. The most commonly employed of the covalent compounds include boron trifluoride, aluminium trichloride, titanium tetrachloride, and stannic tetrachloride. Various other derivatives of boron, aluminium and titanium are also employed as Lewis acid catalysts.

The catalytic activity of metal ions originated in the formation of a donor acceptor complex between the cation and the reactant, which must act as a Lewis base. The result of the complexation is that the donor atom becomes effectively more electropositive. All functional groups that have unshared electron pairs are potential electron donors. The oxygen in the epoxide ring is one of the specially prominent electron donors in reaction chemistry.

Compounds such as boron trifluoride and aluminium chloride form complexes by accepting an electron from the donor molecule. The same functional groups that act as lone pair donors to metal cations form complexes with boron trifluoride, aluminium trichloride and related compounds. Since in this case the complex is formed between two neutral species, it, too, is neutral, but a formal positive charge develops on the donor atom, and a formal negative charge on the acceptor atom. The result is to increase the effective electronegativity of the donor atom and increase the electrophilic ability of the complex functional group.

A simple table of Lewis acidities based on some quantitative measurement, in terms of soft and hard acids and bases, is shown as below.

Table 2-6 Hard and soft acids and bases

Hard bases	Soft bases	Borderline bases
H_2O OH^- F^- AcO^- SO_4^{2-} Cl^- CO_3^{2-} NO_3^- ROH RO^- R_2O NH_3 RNH_2	R_2S RSH RS^- I^- R_3P $(RO)_3P$ CN^- RCN CO C_2H_4 C_6H_6 H^- R^-	$ArNH_2$ C_5H_5N N_3^- Br^- NO_2^-
Hard acids	Soft acids	Borderline acids
H^+ Li^+ Na^+ K^+ Mg^{2+} Ca^{2+} Al^{3+} Cr^{2+} Fe^{3+} BF_3 $B(OR)_3$ $AlMe_3$ $AlCl_3$ AlH_3 SO_3 RCO^+ CO_2 HX (hydrogen-bonding molecules)	Cu^+ Ag^+ Pd^{2+} Pt^{2+} Hg^{2+} BH_3 $GaCl_3$ I_2 Br_2 CH_2 carbenes	Fe^{2+} Co^{2+} Cu^{2+} Zn^{2+} Sn^{2+} Sb^{3+} Bi^{3+} BMe_3 SO_2 R_3C^+ NO^+ GaH_3 $C_6H_5^+$

Soft bases: The donor atoms are of low electronegativity and high polarizability and are easy to oxidize. They hold their valence electrons loosely.

Hard bases: The donor atoms are of high electronegativity and low polarizability and are hard to oxidize. They hold their valence electrons tightly.

Soft acids: The acceptor atoms are large, have low positive charge, and contain unshared pairs of electrons (p or d) in their valence shells. They have high polarizability and low electronegativity.

Hard acids: The acceptor atoms are small, have high positive charge, and do not contain unshared pairs of electrons in their valence shells. They have low polarizability and high electronegativity.

While it just can be a through reference since Lewis acidity depends on the nature of the base. Quantitatively, the following approximate sequence of acidity of Lewis acids of the type MXn has been suggested, where X is a halogen atom or an inorganic radical: $BX_3 > AlX_3 > FeX_3 > GaX_3 > SbX_5 > SnX_4 > AsX_5 > ZnX_2 > HgX_2$.

Therefore, for the reductive cleavage of 4α,5–epoxy–5α–cholestane–3β, 6β–diol, theoretically, the different acidity of the Lewis acid should have different ability and strength to complex with the oxygen atom in the epoxide ring. Once a proper acid was chosen, the bond between the C-5 and the oxygen could be polarized to the greatest extent. At this point, a hydride donated by lithium aluminium hydride can easily attack the C-5, and then along with the rupture of the bond between C-5 and the oxygen, the expected product cholestane-3β, 4α, 6β–triols could be formed.

However, the experiment result turns out in another way. The anion presented in the Lewis acid molecule got involved in the reaction unavoidable, which results in the products were shown in the table. With the bond cleavage, the hydroxyl group went to one carbon in the epoxide ring and at the same time the halogen atom was attached to the other one. Only chloride and fluoride were found to undergo such an addition. Bromide and iodine (Exp. No. 2, 3, 10) were not observed to connect with any carbon in the ring, possibly because they were too big to approach the related carbon. In the Exp. No. 1, CsCl was seemed that it had no effect on the epoxide ring too. It was strange that, in those no reaction cases, the hydride in the reaction system could not function either. The starting material remained unchanged.

For those other cases, the position of the anion (chloride or fluoride) attached seemed to be determined by the amount of Lewis acid added. During the course of the experiments, it was found that when the mole ratio of the Lewis acid : starting material 4:1, the hydroxyl group goes to C-5; when the amount of the Lewis acid is greatly excessive over the starting material, the hydroxyl group goes to C-4. The results also showed that when the anion attacked the C-4, it tended to approach from the other side of the epoxide ring. According to the starting material 4α,5–epoxy–

5α–cholestane–3β, 6β–diol used, it approached from the upper side of the molecule to afford the β chloride substitute. When the anion attacked the

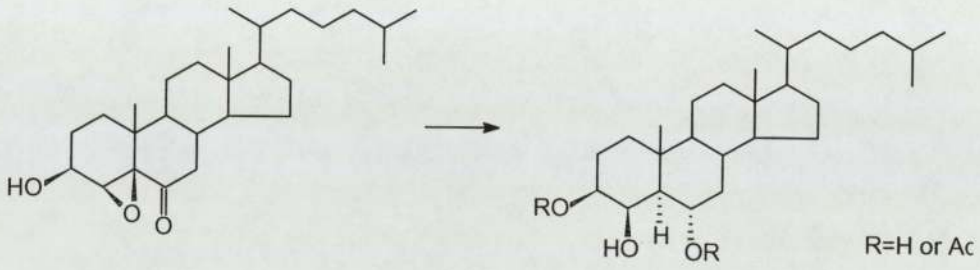
C-5, it tended to approach from the same side of the epoxide ring. As to the starting material 4α,5–epoxy–5α–cholestane–3β, 6β–diol used, it approached from the down side of the molecule to afford the α chloride substitute. It was easy to understand the backside attack in the former case, but the reason that it preferred to attack from more hindered side of the epoxide ring in the latter case was not clear. If the bond between the C-5 and oxygen was broken first, due to the higher hindrance of the β side of the molecule (discussed in the beginning 2.1.2), it might approach the more stable tertiary carbonium ion from the less hindered side, which was easily understood. However, in this condition, it should have the same chances for the hydride from lithium aluminium hydride to be attached at position 5, which could turn out to be the needed product—3, 4, 6-cholestane-triols.

Over all, five methods were studied although none worked perfectly for producing the target compounds. Further investigation is needed.

2.1.3 Prospective working-out ways to the problem

Since all five methods employed in the project had miscellaneous results, and for preparing our expected products, obviously we need to look for new methodologies for this purpose. Possibly, two directions may help the project out: 1) consult the literature again and more widely to see if there are any other better reductive ring-cleavage methods available that could be used in the epoxide sterol system; 2) design new reagents and choose other convenient starting materials to obtain those 16 isomers of 3, 4, 6-cholestane-triols.

For the first working-out direction, during writing-up period, an article was found which was related to the reduction of some α, β-epoxyketones of cholestane series with sodium in liquid ammonia. (Jablonski, 1970) One thing should be pointed out that in this literature, 5α-cholestane-3β, 4β, 6α-triol and its 3, 6-diacetate were prepared from the 4β, 5-epoxycoprostan-3β-ol-6-one. (Scheme 2-14)



Scheme 2-14 A way of cholestane-3, 4, 6-triols preparation in literature

Because the reduction of sodium in liquid ammonia is not easy to be handled and controlled, the method is still not being tested yet.

For the second working-out method, some other more convenient starting materials have to be prepared first, which may be another even more difficult project, such as 3, 4-epoxycholestane-6-ol. This will need more efforts and cooperation within the group.

2.2 Part II. Effects of benzoyl esters on epoxidation

2.2.1 The mechanisms of the epoxidation

After the hydrolysis of the acetate at the C-3, the Cholest-4-en-3β, 6β-dial-6-acetate was ready to be acylated by different substituted benzoic acids or benzoyl chlorides, followed by the key reaction of epoxidation.

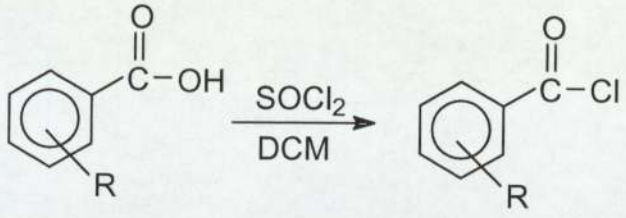
The product of hydrolysis was reported in the literature. (Ishigure, 1980) However, its data of ^{13}C NMR chemical shift values and IR absorption values did not match well with the results of experiment. (Table 2-7)

Table 2-7 The comparison of the data of the intermediate (Cholest-4-en-3β, 6β-dial-6-acetate) in literature and those from the experiments

In literature		From the experiments
a. ^{13}C NMR chemical shift values (ppm)		
C-3	65.254	67.7131
C-4	65.133	131.7499
C-5	64.083	142.1754
C-6	74.984	75.6357
C-19	17.362	20.7249
Carbonyl of acetyl group	170.280	170.2378
b. IR absorption values (cm^{-1})		
1738, 1234		3523, 2930, 2862, 2843, 1718, 1470, 1374, 1259, 1016

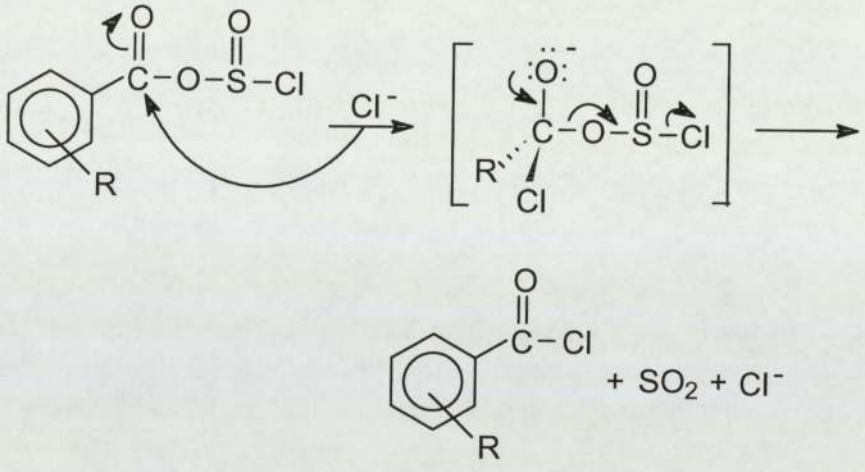
The data generated from the experiment were correct because the chemical shift of the double bond carbon should be positioned between 100 and 150 ppm.

During the experiments, it was found that some benzoic acids did not react with the substrate readily. They had to be converted into benzoyl chloride first by treatment with thionyl chloride ($SOCl_2$).



Scheme 2-15 Conversion of benzoic acid into benzoyl chloride

The reaction occurs by a nucleophilic acyl substitute pathway in which the benzoic acid is first converted into a reactive chlorosulfite intermediate, which is then attacked by a nucleophilic chloride. (Scheme 2-16) (McMurray, 1996)



Scheme 2-16 Mechanism of Anyl Chlororization

Acid halides are among the most reactive of carboxylic acid derivatives and can be converted into ester much easier than carbonic acids.

The next step was esterification, involving the substrate and the benzoic acid or benzoyl chloride together.

Usually, esterification is catalyzed by acids. Another way to esterify a carboxylic acid is to treat it with an alcohol in the presence of a dehydrating agent. One of the agents is dicyclohexylcarbodiimide (DCC).

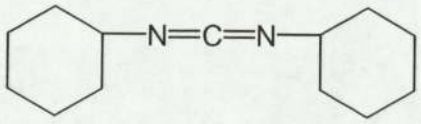
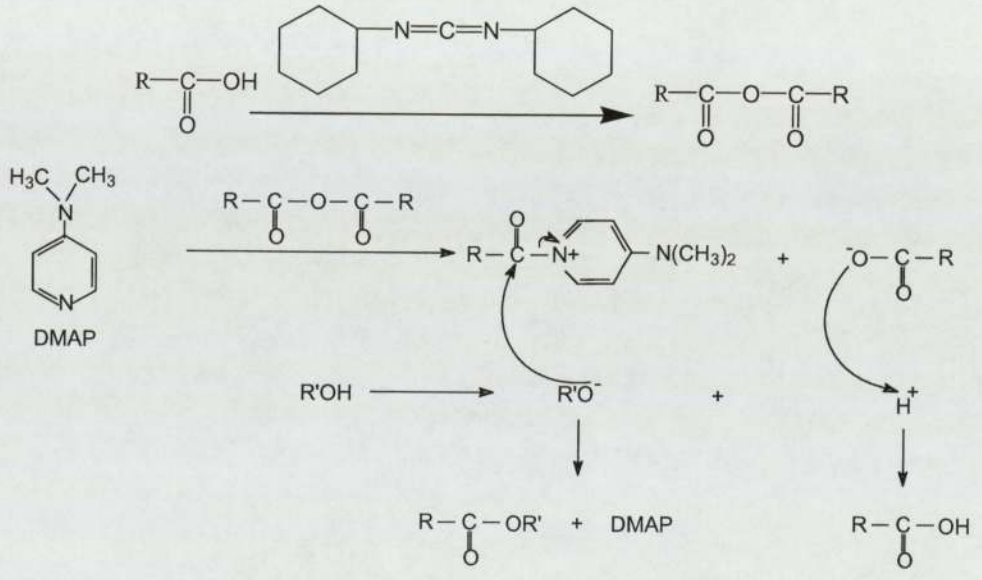


Figure 2-1 DCC

However, there are limitations to the usage of DCC; yields are variable and N-acylureas are side products. While with the presence of catalytic amount of the 4-dimethylaminopyridine (DMAP), it accelerates the DCC-activated esterification of carboxylic acids with alcohols or thiols to such an extent that formation of side products is suppressed and even sterically demanding esters are formed in good yields at room temperature. (Neises, 1978)

The reaction is based on a requirement for both DCC and aminopyridine catalyst. The mechanism is shown as in the Scheme 2-17. (Hassner, 1978)



Scheme 2-17 Mechanism of esterification using DCC and DMAP

As for the epoxidation, there are several methods for oxidation of different olefins. They are listed in the Table 2-8.

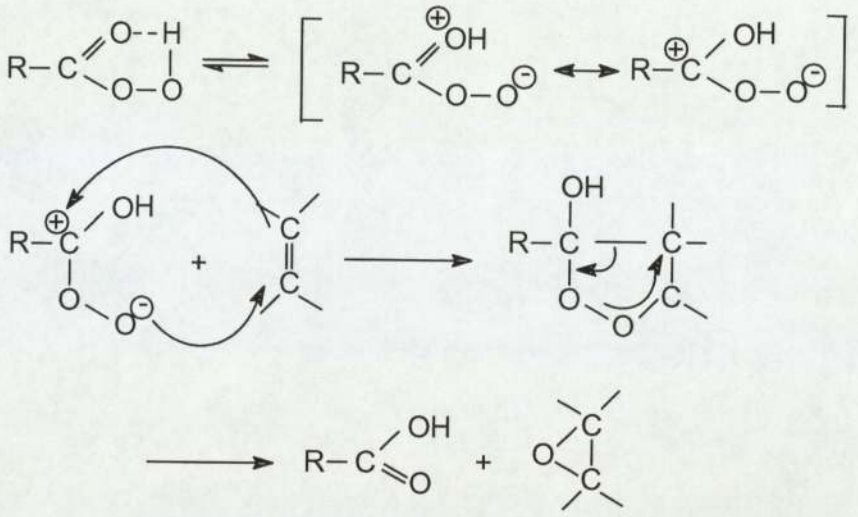
Table 2-8 Olefin Epoxidation Methods

Epoxidation Method		Target Olefin
Electrophilic reagents	*m*CPBA	Electron rich olefins, allylic or homoallylic alcohols
	DMDO	Electron rich olefins epoxidated preferentially, but will be effective to most olefins
Reagents requiring a directing group	$VO(acac)_2$ / TBHP	Good for allylic and homoallylic alcohols
	$Ti(Oi\text{-}Pr)_4$ / TBHP / DET	Sharpless ASYMMETRIC epoxidation of allylic and homoallylic alcohols
Nucleophilic reagents	TBHP / NaOH	Dr-unsaturated carbonyl systems

In a laboratory, the most often used reagent for oxidation of alkenes is the organic peroxyacids, such as peroxyacetic acid, peroxybenzoic acid, 3-chloroperoxybenzoic acid (mCPBA) etc.

To this particular substrate, mCPBA showed superior reactivity and induced satisfactory stereoselectivity and gave well to excellent yield. Therefore, it was chosen to be the oxidizing reagent for this reaction although the reaction rates were lower due to the increased steric hindrance and lack of coordination between mCPBA and the free hydroxyl group. (Zhao, 2001)

As a peroxyacid using in the preparation of epoxide from alkene, mCPBA transfers oxygen to an alkene with syn-stereochemistry, that is, adds it to the same side of the molecule, without the conversion of the original configuration of the olefin. Usually, it is the oxygen atom farthest from the carbonyl group that is transferred. (Scheme 2-18) (McMurray, 1996)



Scheme 2-18 Mechanism of epoxidation using peroxyacid

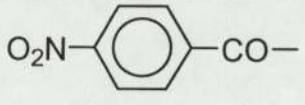
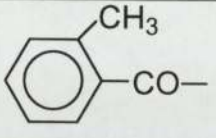
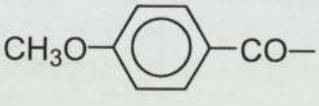
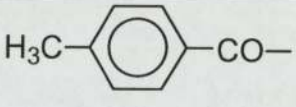
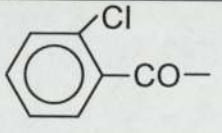
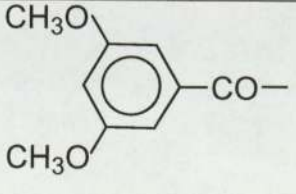
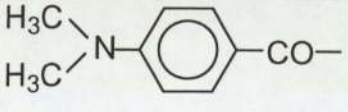
It is a kind of stereospecific reaction, which means that if the starting material has different stereochemistry, the product will also have the corresponding different stereochemistry.

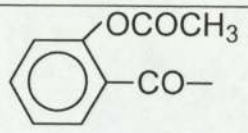
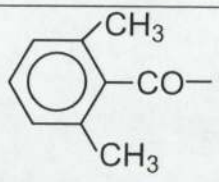
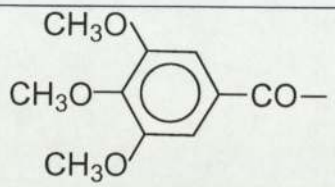
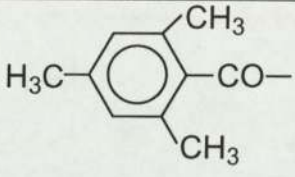
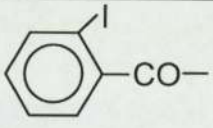
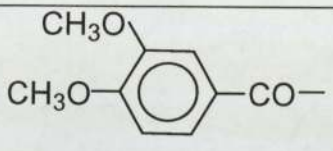
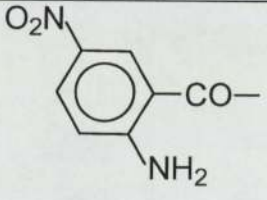
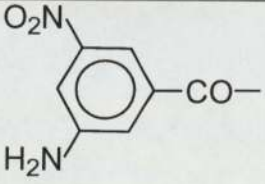
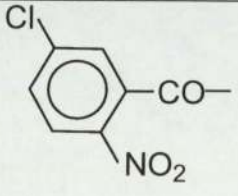
The benzoyl esters attach at the position 3 are differently substituted, not only the positions such as ortho-, meta- and/or para-oriented, but also the groups with different electronic withdrawing or donating characters. Due to the allylic position of benzoyl ester to the double bond, the substituted groups attached on the benzene ring will affect the course of the epoxidation via the combination effects of electron-inducing effect and conjugative effect. This will be discussed in the next part.

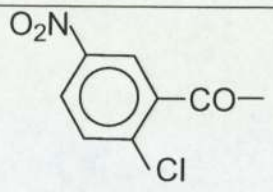
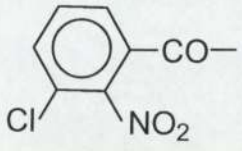
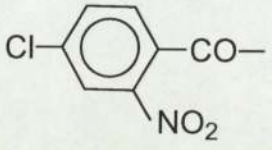
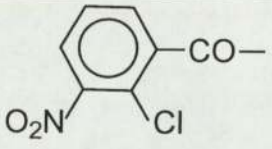
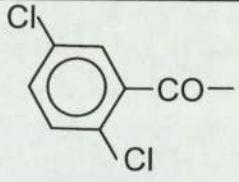
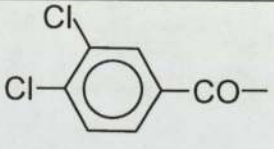
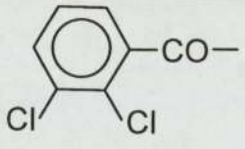
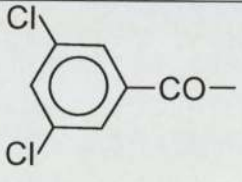
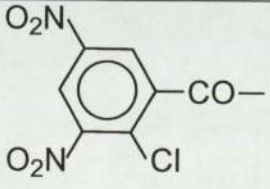
2.2.2 The results, discussion and conclusion

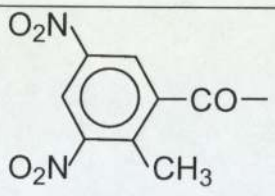
Thirty different benzoic acids or benzoyl chlorides with different substituent were attached on the substrate to afford thirty different cholest-4-en-3β, 6β-diol-6-acetate-3-substituted esters. And then they went through the epoxidation to tell what the stereochemistry effects of the substituted benzoyl esters are on the epoxidation, that is, what the proportion of the α, β ring product is formed respectively. The result is listed in the Table 2-9.

Table 2-9 Results of epoxidation of cholest-4-en-3β, 6β-diol-6-acetate-3-substituted esters

Product No.	The benzoyl at the position 3 of the substrate	α	β
1		61	39
2		75	25
3		69	31
4		72	28
5		76	24
6		60	40
7		71	29

8		80	20
9		87	13
10		65	35
11		90	10
12		46	54
13		75	25
14		74	26
15		68	32
16		67	33

17		64	36
18		72	28
19		72	28
20		74	26
21		67	33
22		68	32
23		76	24
24		70	30
25		68	32

26		60	40
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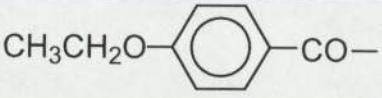
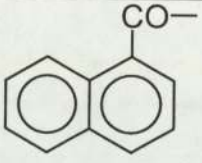
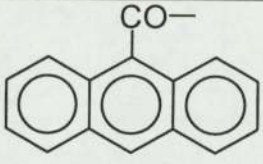
28		73.5	26.5
29		73	27
30		81	19

Note: Exp. No.=experiment number, the data in α or β column represents the percentage of the product with α or β ring in the final mixture respectively.

Because of the conjugated (resonance) and inductive effect, the substituent on the 3-benzoyl ester ring may affect the epoxidation course of the double bond in the substrate 4-en-3β, 6β-dial-6-acetate-3-benzoyl esters.

Multiple bonds that alternate with single bonds are said to be conjugated. Resonance effects are due to the overlap of a *p* orbital on the substituent with a *p* orbital on the aromatic ring and result in withdraw or donation of electrons through π bonds. Carbonyl, cyano and nitro substituent, for example, are electron withdrawing from the aromatic ring by resonance. π electrons flow from the rings to the substituent, placing a positive charge in the ring. An inductive effect is simply the shifting of electrons in a bond in response to the electronegativity of nearby atoms.

The common feature of all activating groups is that they donate electrons to the ring, thereby stabilizing the carbocation intermediate from electrophilic addition and causing it to form faster. Hydroxyl, alkoxyl and amino groups are activating because their stronger electron-donating resonance effect outweighs their weaker electron-withdrawing inductive effect. Alkyl group are activating because of their electron-donating inductive effect. The common feature of all deactivating groups is that they withdraw electrons form the ring, thereby destabilizing the carbocation intermediate and causing it to form more slowly. Carbonyl, cyano and nitro groups are deactivating because of both electron-withdrawing resonance and inductive effects. Halogens are deactivating because their weaker electron-donating resonance effect.

A summary of the activating and directing effects of substituent in electrophilic aromatic substitution is shown in Table 2-10.

Table 2-10 Substituent effects in electrophilic aromatic substitution

Substituent	Reactivity	Orientation	Inductive effect	Resonance effect
$-CH_3$	Activating	Ortho, Para	Weak, Electron-donating	None
-OH, $-NH_2$	Activating	Ortho, Para	Weak, Electron-withdrawing	Strong, Electron-donating
-F, -Cl, -Br, -I	Deactivating	Ortho, Para	Strong Electron-withdrawing	Weak, Electron-donating
$-N^+(CH_3)_3$	Deactivating	Meta	Strong Electron-withdrawing	None
$-NO_2$, -CN,	Deactivating	Meta	Strong	Strong

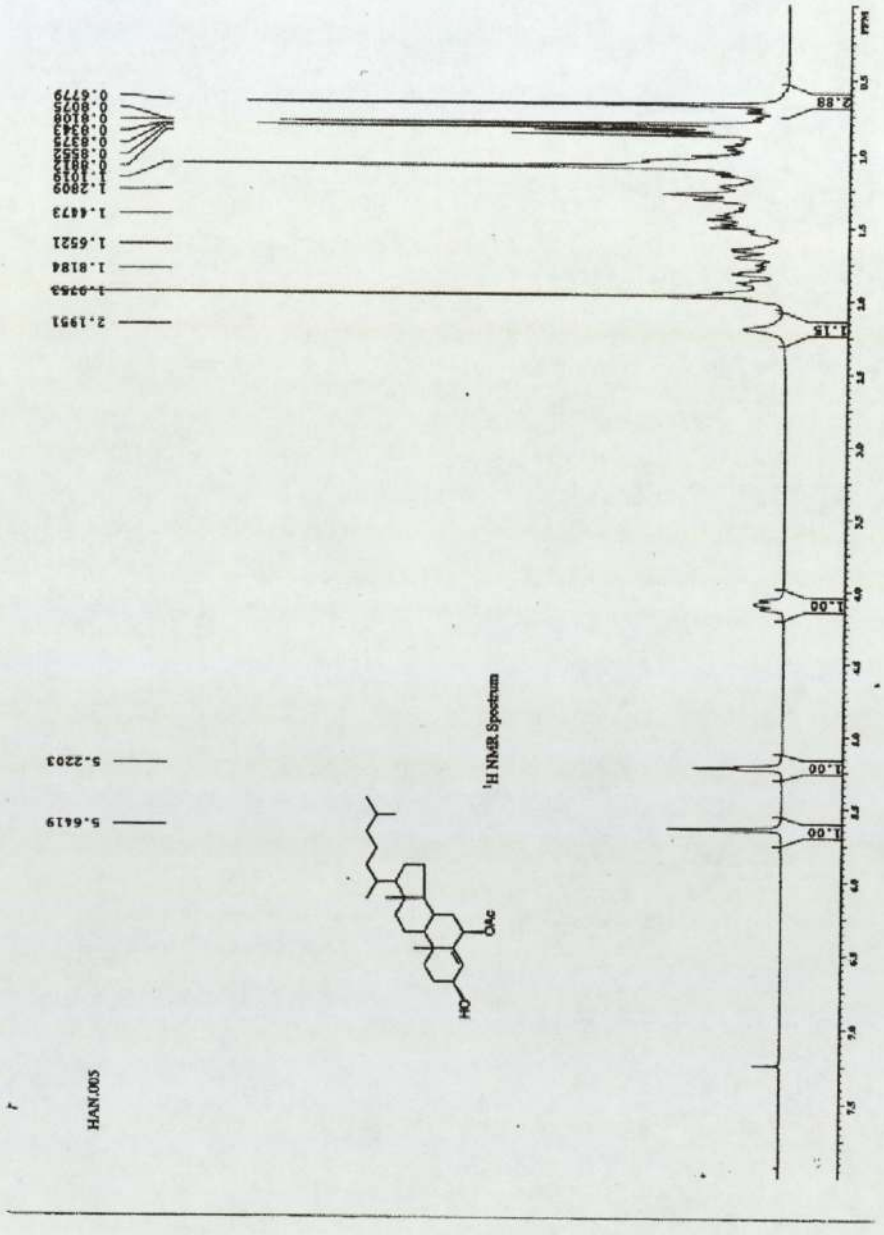
-CHO, $-CO_2CH_3$, $-COCH_3$			Electron-withdrawing	Electron-withdrawing

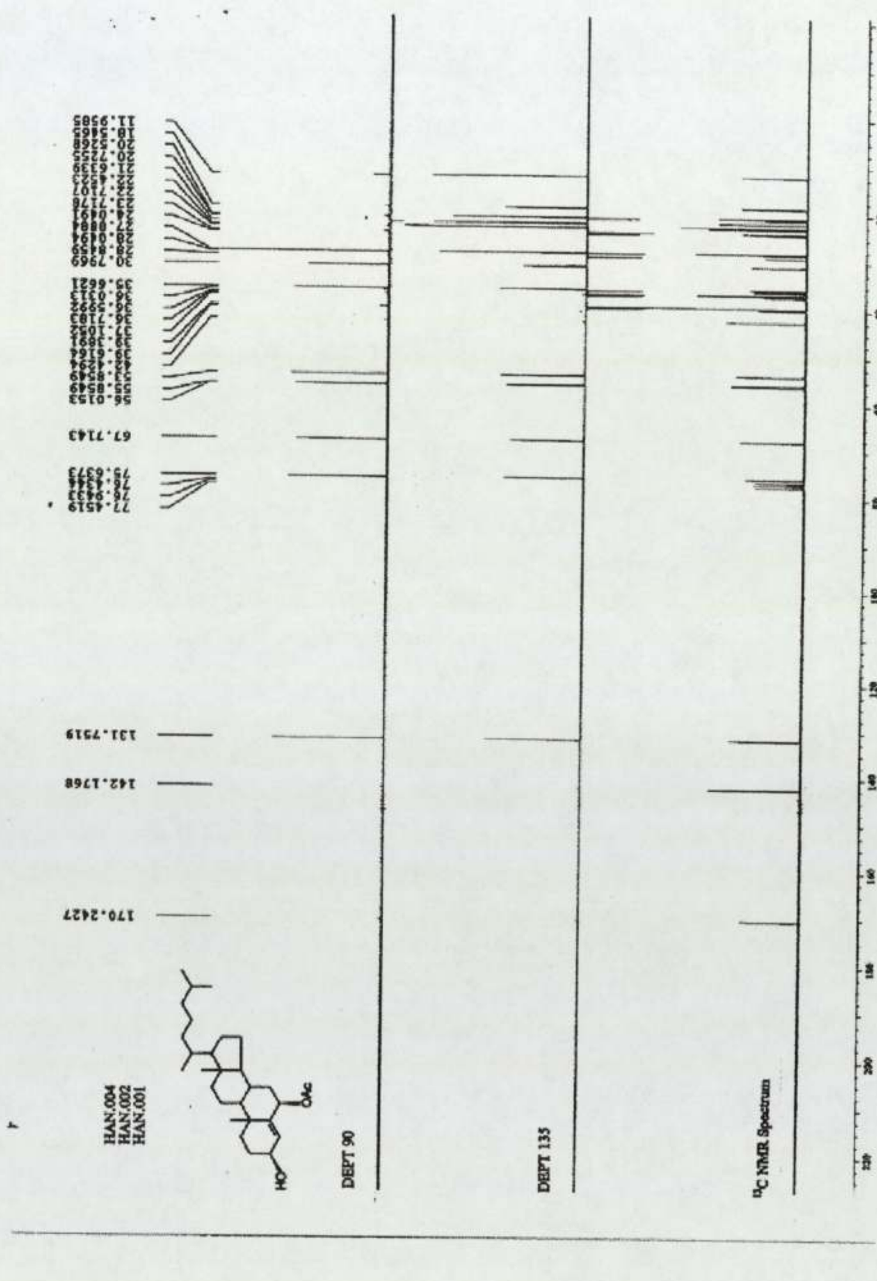
Generally, what we are trying to identify those substitutions effects on the epoxidation lies on the epoxide ring's configuration of the products we obtained. After the epoxidation, the double bond between C4 and C5 formed an epoxide ring and this ring could be α or β configuration. With the different substituent on the benzene ring, the electronic characteristic of the substituent would affect the formation of α or β configuration in the product. As indicated in the published paper, (Zhao, 2001) the ratio of product of α or β ring formed from the staring material with 3-OBz and 6-OAc is 77:23, and from the substrate 3, 6-diacetate, the ratio is 82:18. The results above indicated that:

1. Generally, both electron-withdrawing groups (as $-NO_2$ and –Cl) and electron-donating groups (as $-CH_3$ and $-OCH_3$) could cause an increase in ratio of the β configuration, except Exp. No.8, 9, 11 and 30 compared with the result 77:23 from the substrate with 3-OBz.
2. From the Exp. No 2 and 4, the substituent at 4-position might result in higher ratio of β ring configuration product than it at 2-position did.
3. From the Exp. No 2, 4, 9 and 11, the more the methyl groups attached on the benzene ring, the less the β ring configuration ratio might result.
4. As to Exp. No. 12 that the result controverts to above —the product with β configuration was dominated in the mixture. The reason for this was not clear.

Therefore, it seemed that the substituents attached on the benzene ring at the C-3 of cholest-4-en-3β, 6β-diol-6-acetate-3-substituent esters have little effect on the stereochemistry outcome of their epoxidations.

2.2.3 The related spectra


HAN.005
^{1}H NMR Spectrum
OAc
HO
5.6619
5.2203
2.1951
1.9763
1.8184
1.6521
1.6473
1.2809
1.1019
0.8952
0.8375
0.8343
0.8108
0.8075
0.6779
2.88
1.15
1.00
1.00
1.00


HAN.004
HAN.002
HAN.001
DEPT 90
DEPT 135
13C NMR Spectrum
170.2427
142.1768
131.7519
67.7143

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Acquired : 17 Jun 102 9:09 am using AcqMethod 3ESPOSSM G0.3G0.050
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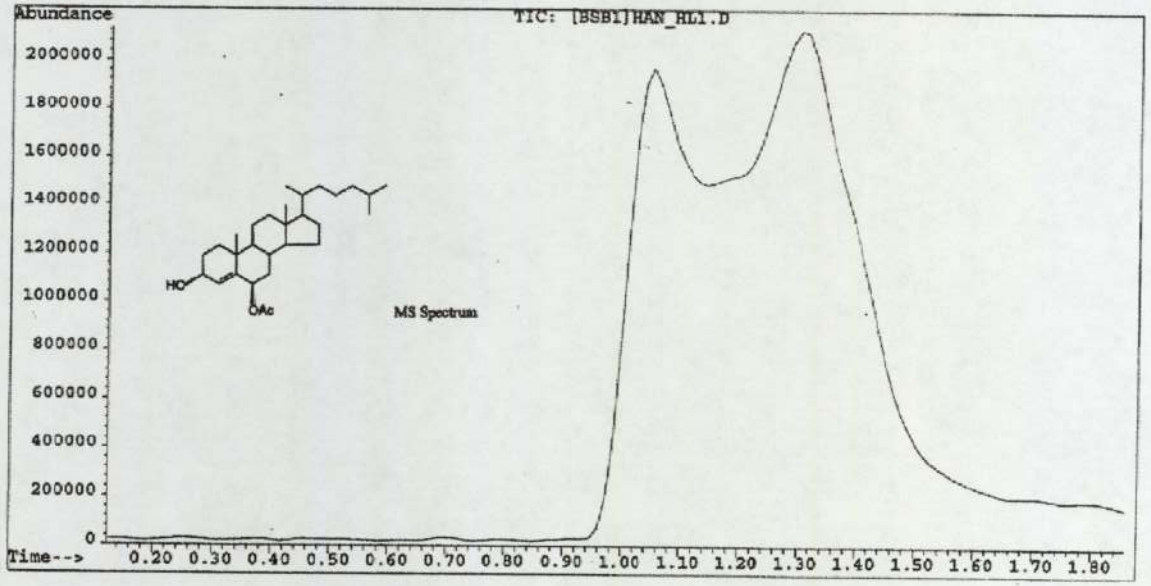
Sample Name: HL-1
Misc Info : 17TH JUNE 2002, ELECTROSPRAY +
Vial Number: 1



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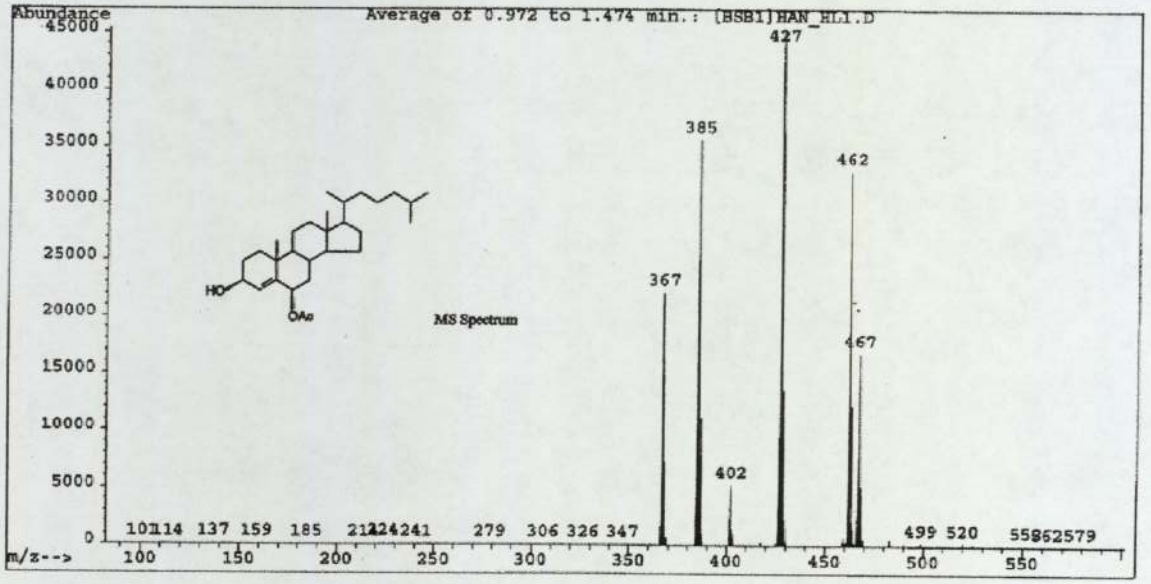

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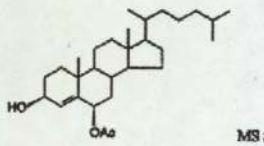
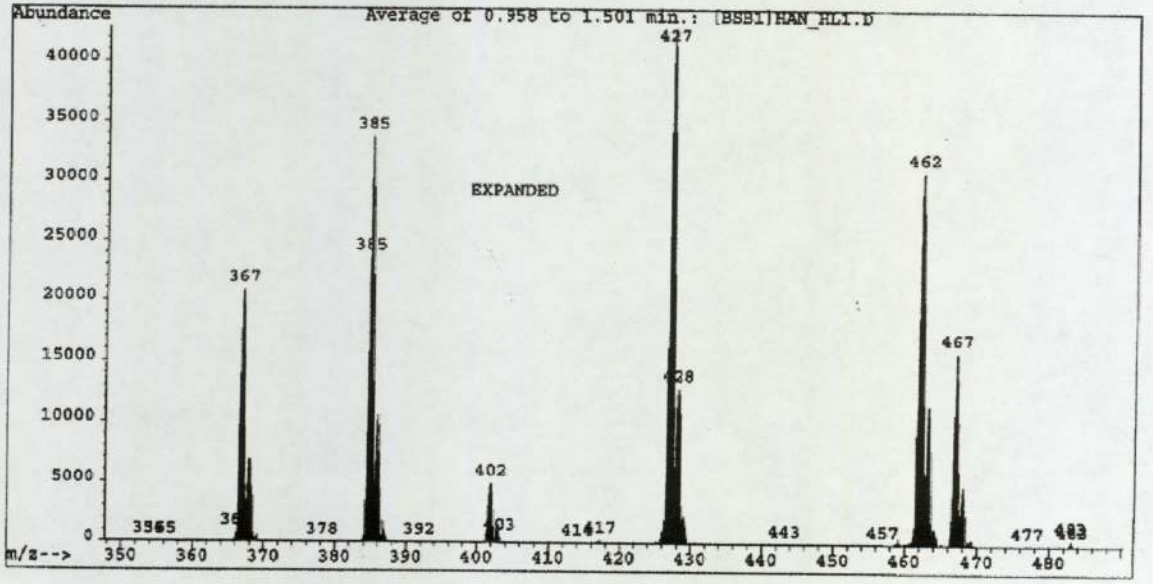
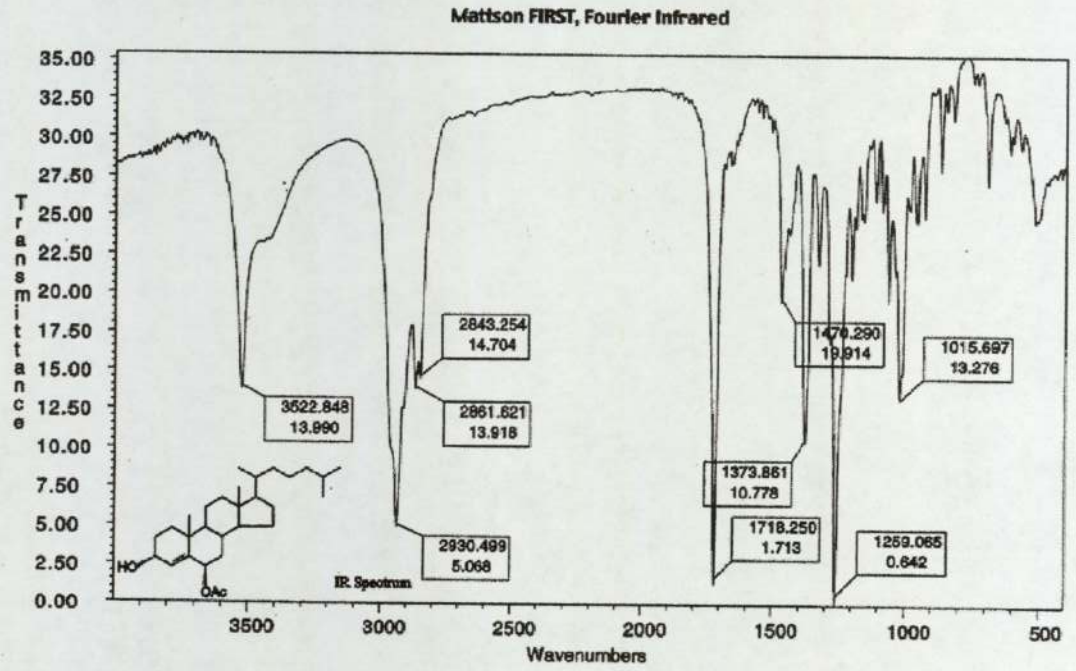
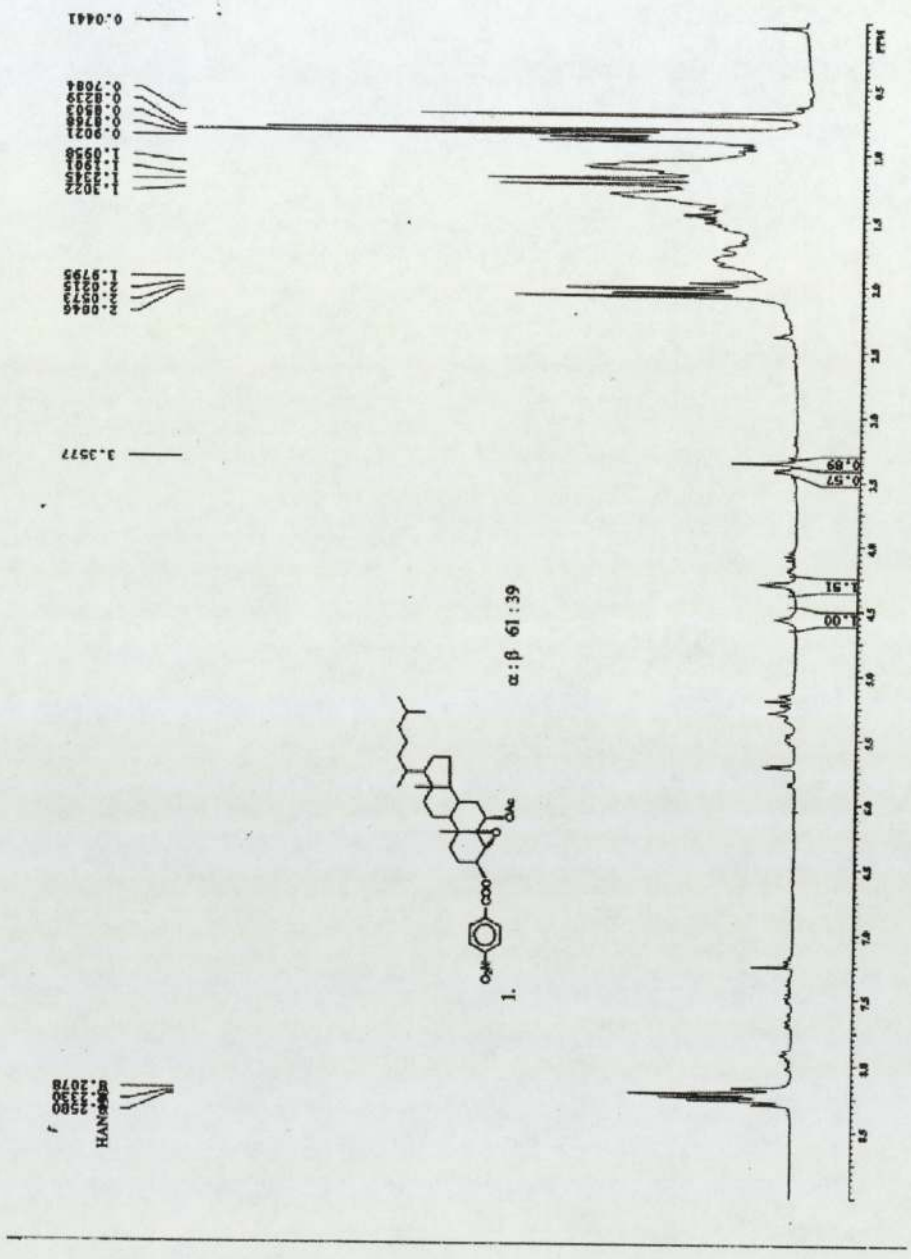


MS Spectrum




Mattson FIRST, Fourier Infrared
Transmittance
35.00
32.50
30.00
27.50
25.00
22.50
20.00
17.50
15.00
12.50
10.00
7.50
5.00
2.50
0.00
3500
3000
2500
2000
1500
1000
500
Wavenumbers
3522.848
13.990
2843.254
14.704
2861.621
13.918
2930.499
5.068
1373.861
10.778
1718.250
1.713
1259.065
0.642
1015.697
13.276
HO
OAc
IR Spectrum


α : β 61 : 39
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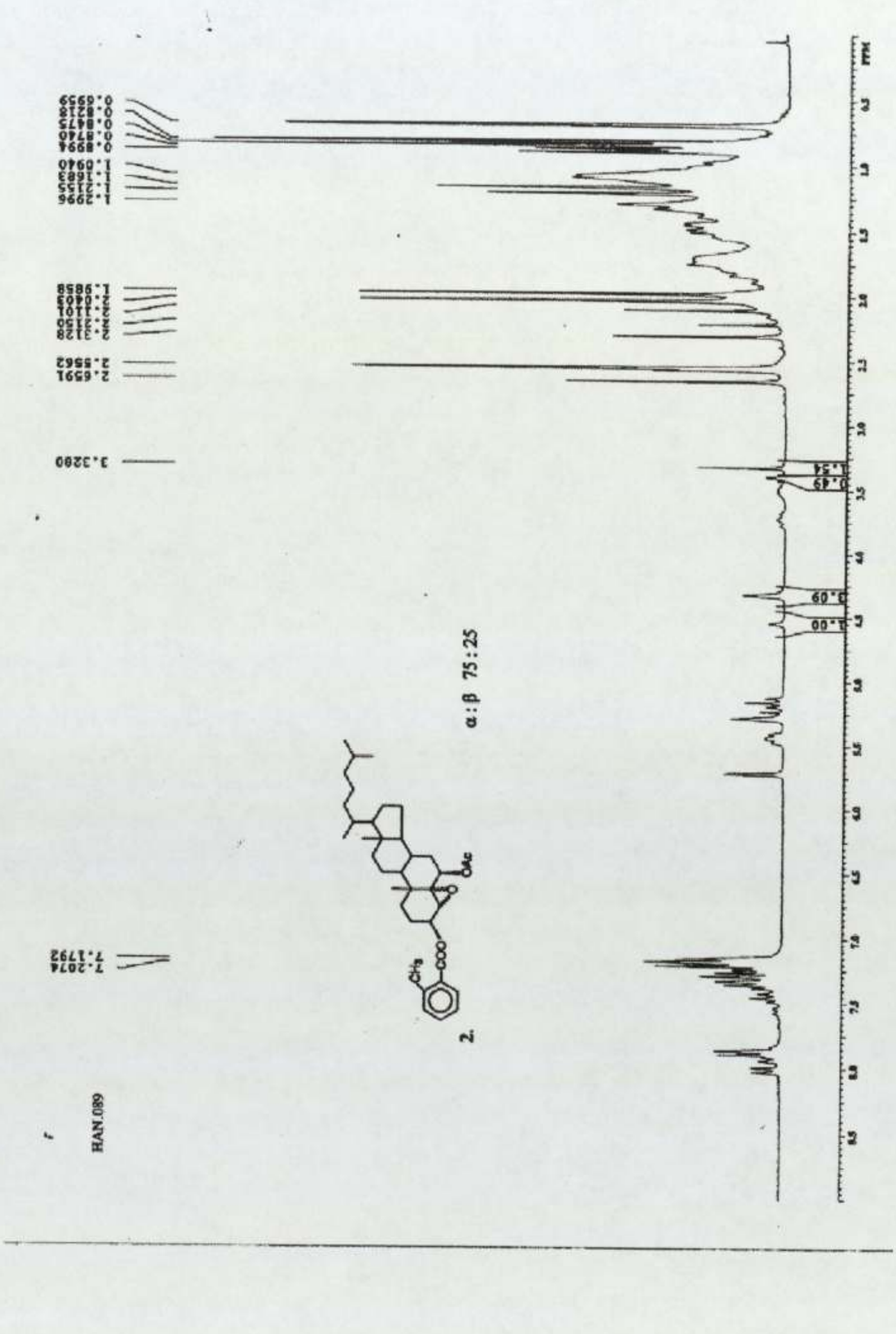
0.89
0.57
1.91
1.00



HAN.089
α:β 75:25
2
OAc
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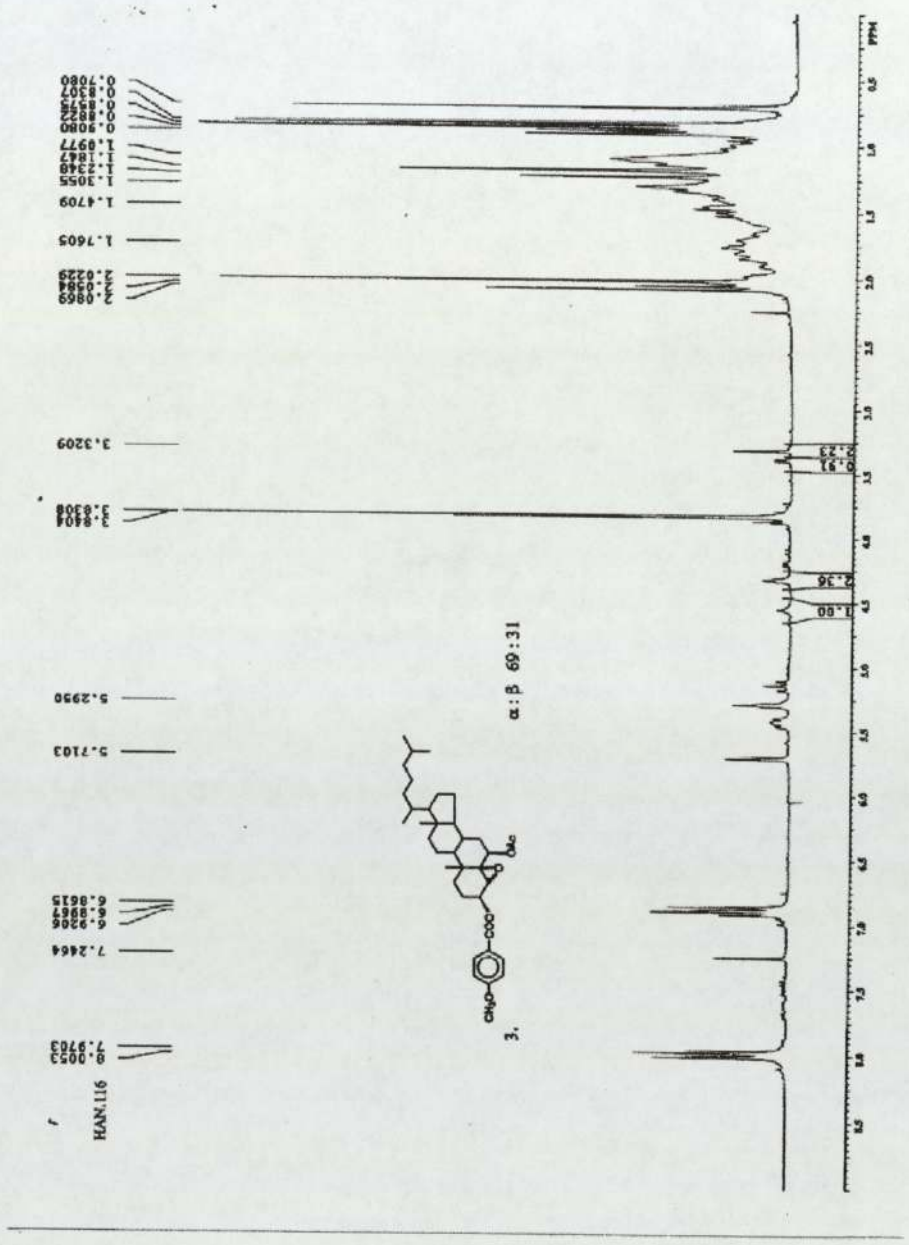
0.8475
0.8740
0.8894
1.0960
1.1683
1.2155
1.2996
1.9858
2.0405
2.1101
2.2150
2.3128
2.5562
2.6591
3.3280
7.1792
7.2074
ppm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3.8308
3.3209
2.0869
2.0584
2.0229
1.7605
1.4709
1.3055
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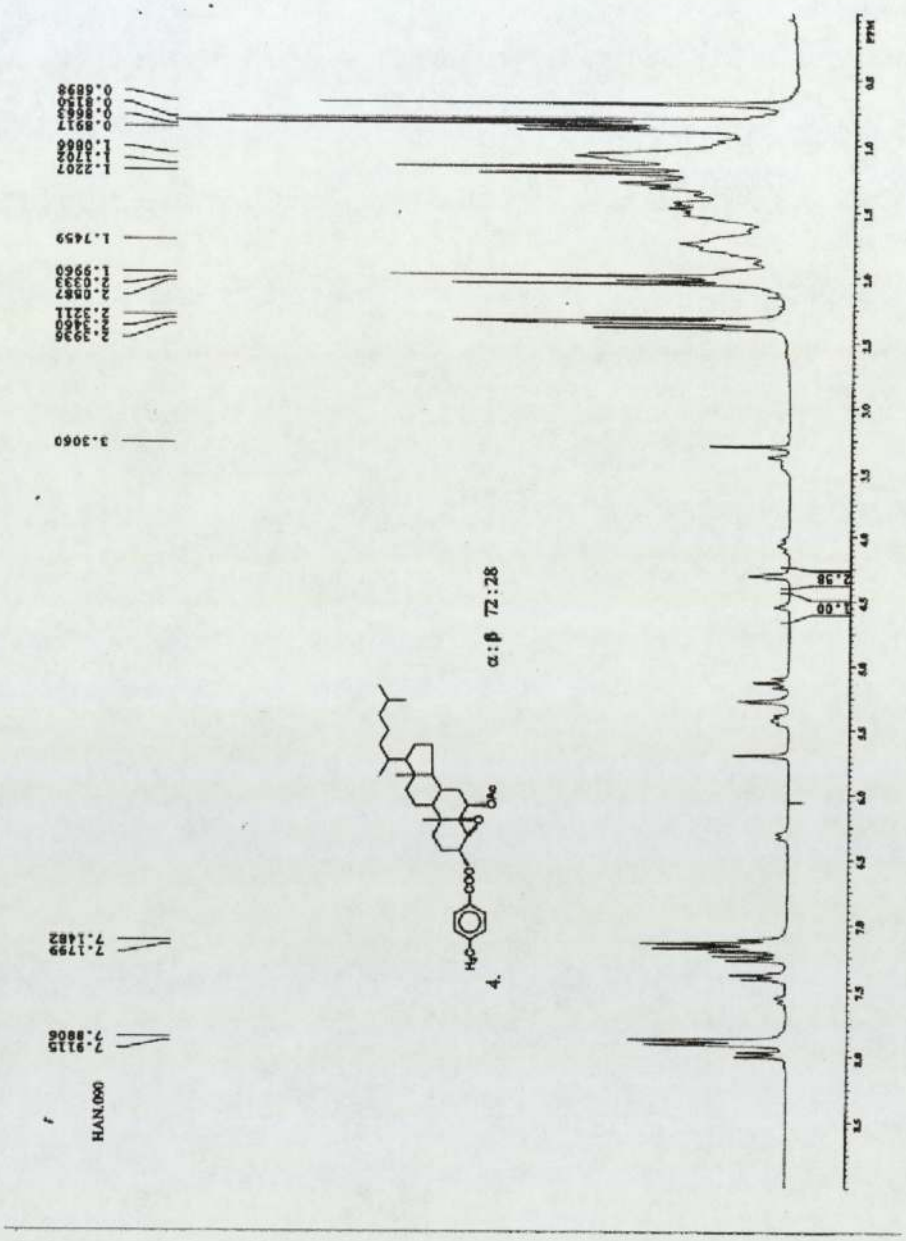
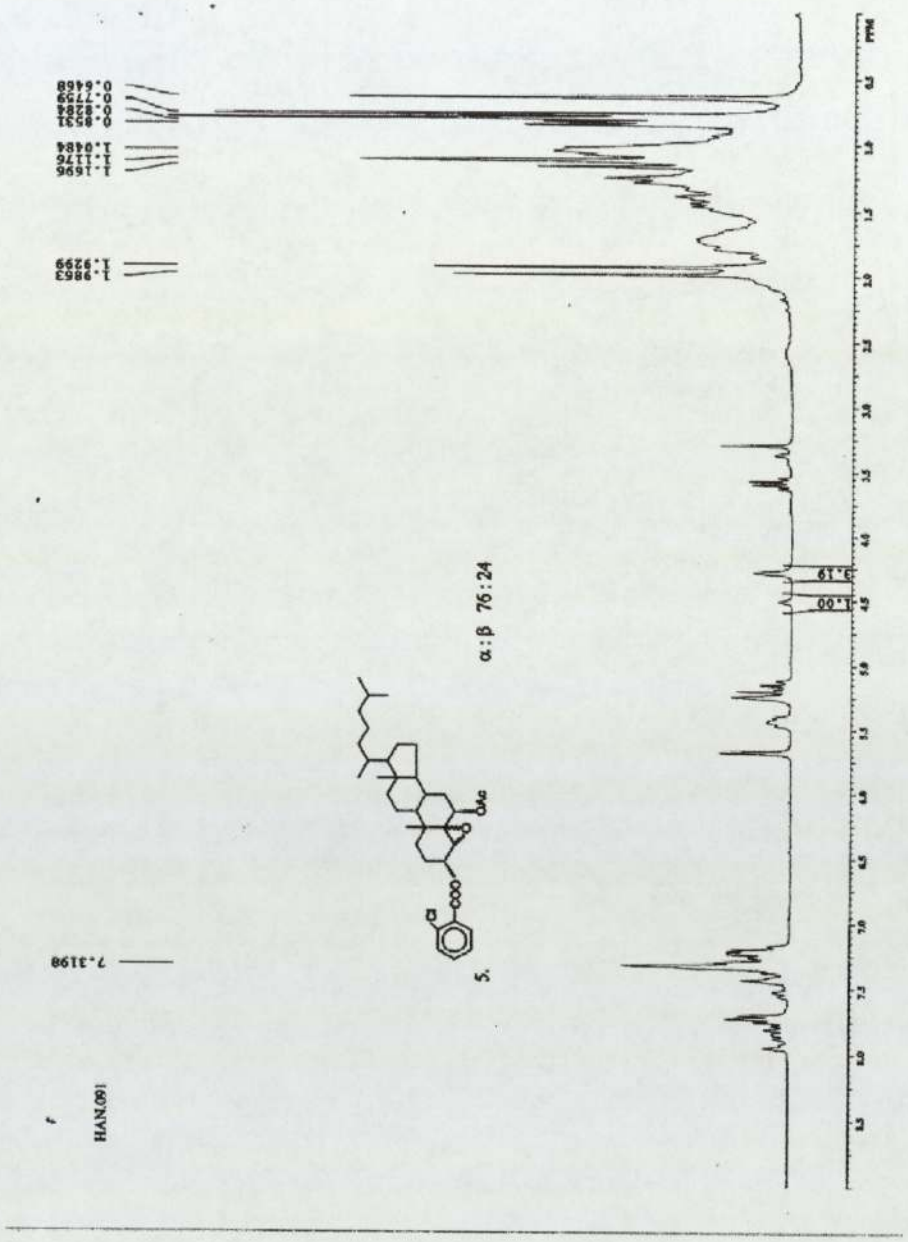
0.7080
α:β 69:31
PPM


α:β 72:28
4.
HAN.090
PPM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1.1696
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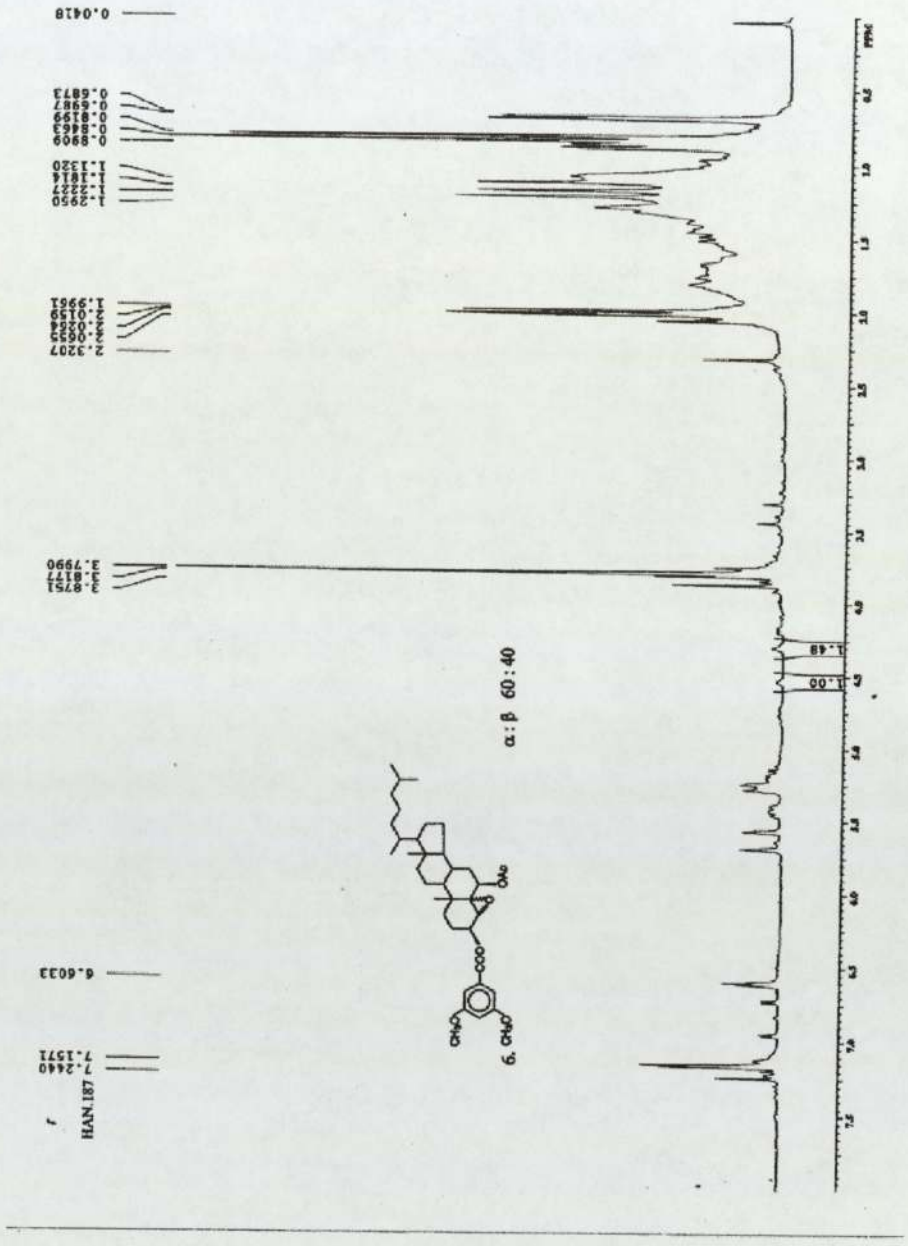
0.6468
α:β 76:24
3.19
1.00
PPM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1.9961
1.2950
1.2277
1.1816
1.1320
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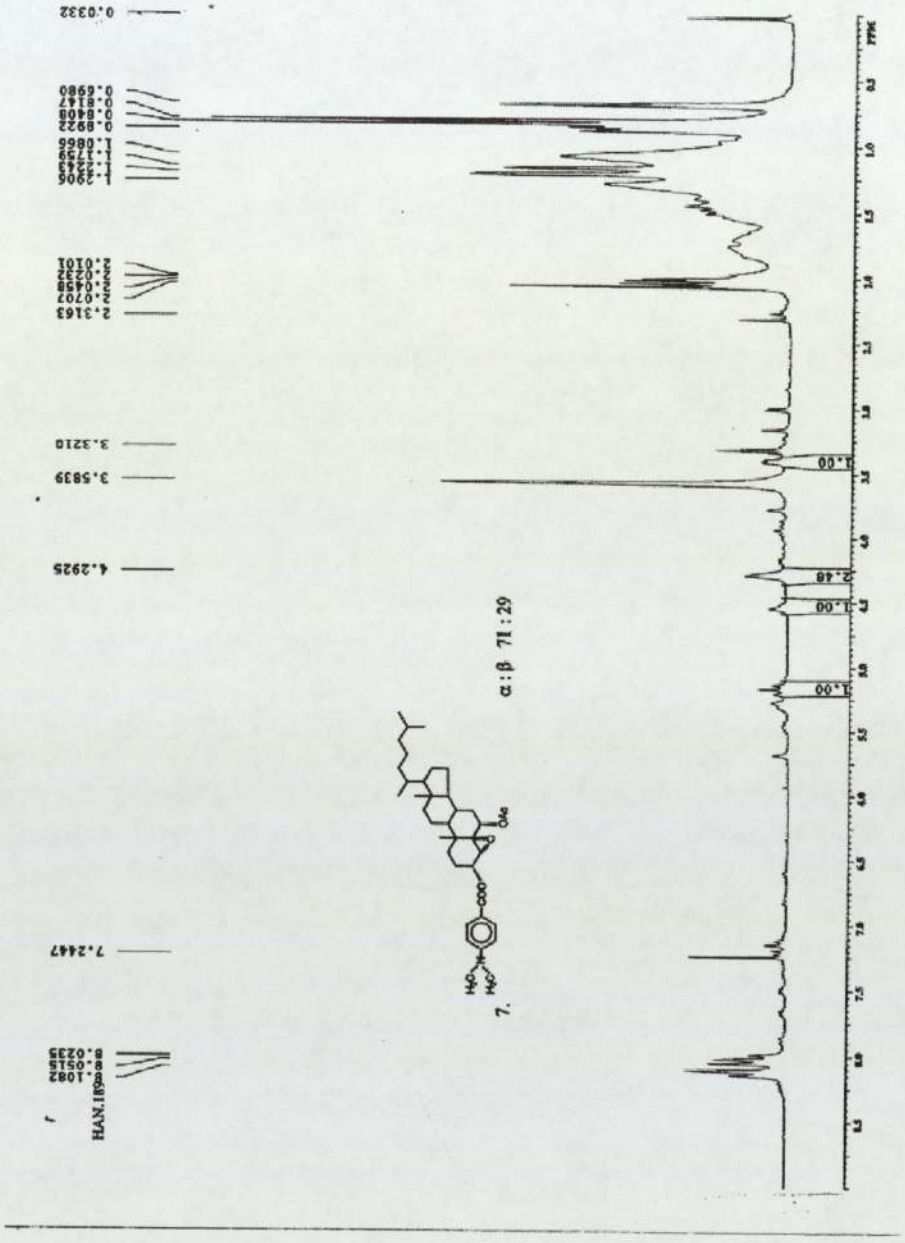
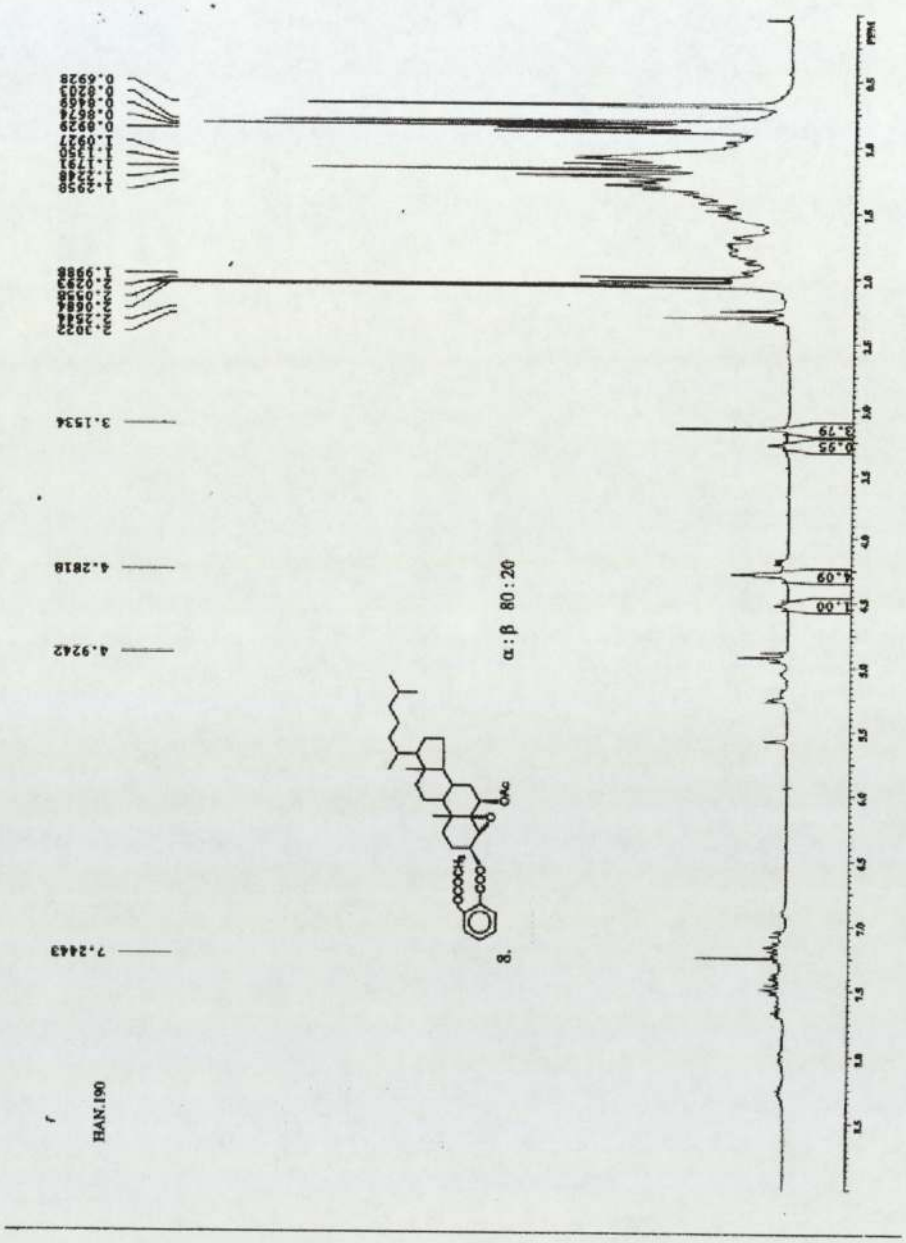
PPM


α:β 71:29


HAN.190
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3.1534
α:β 80:20
8.
1.00
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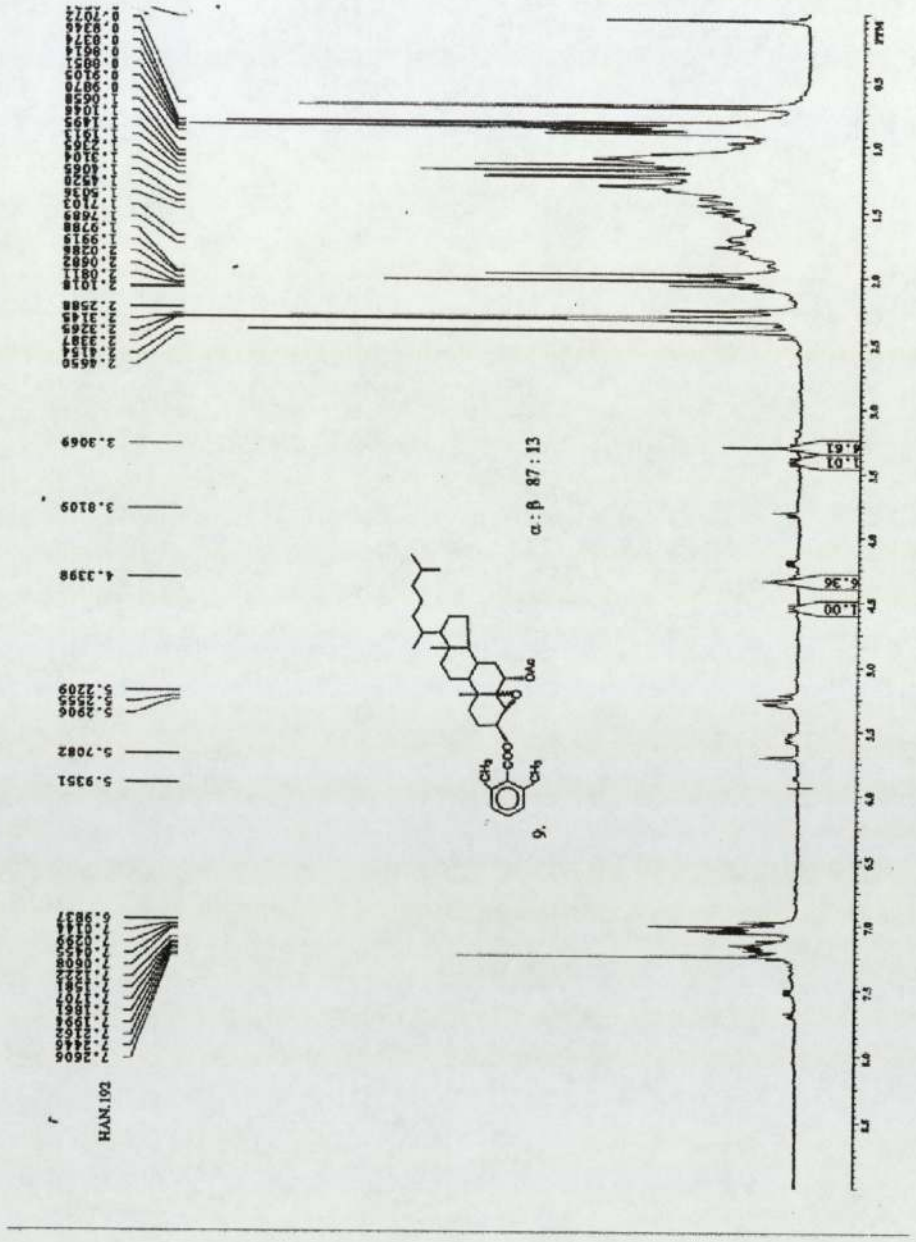
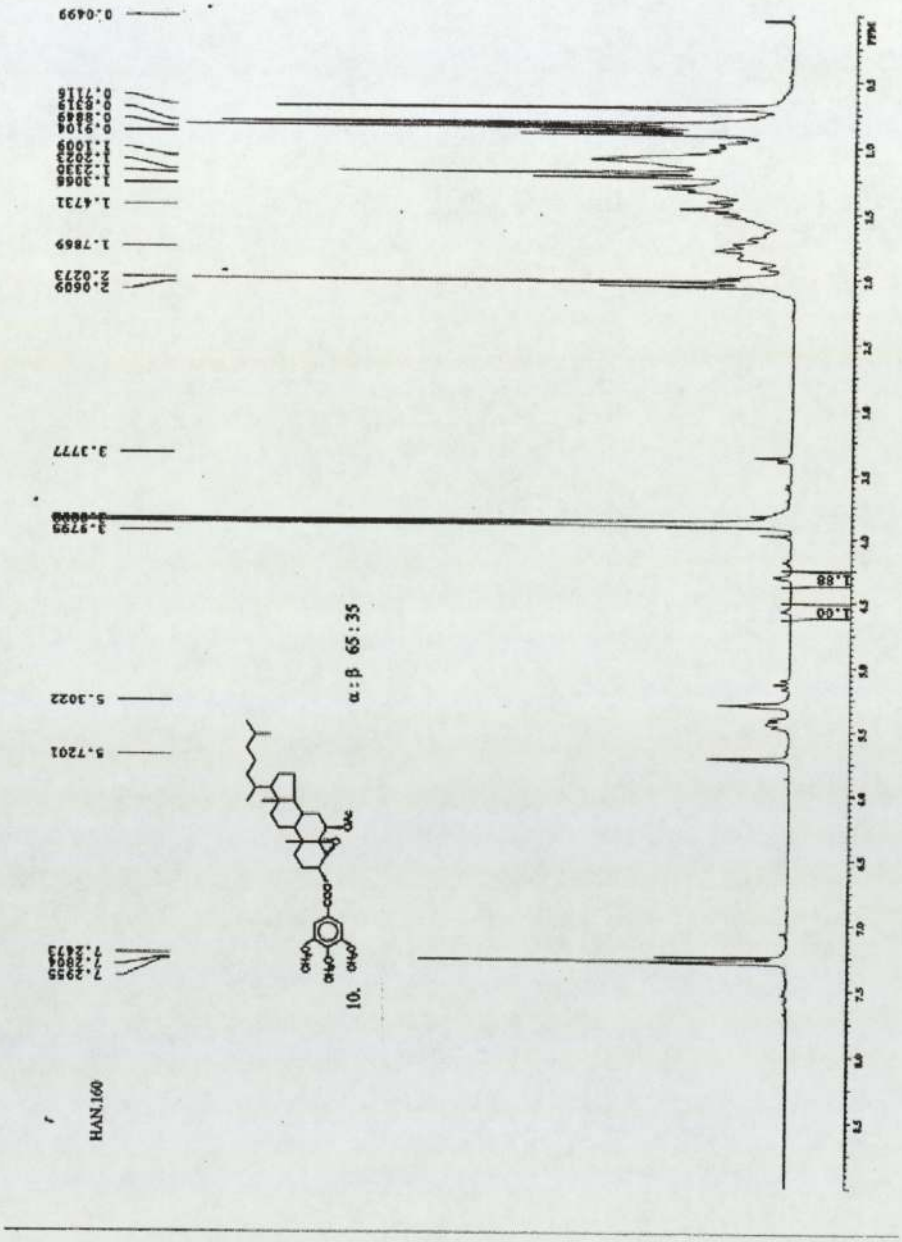
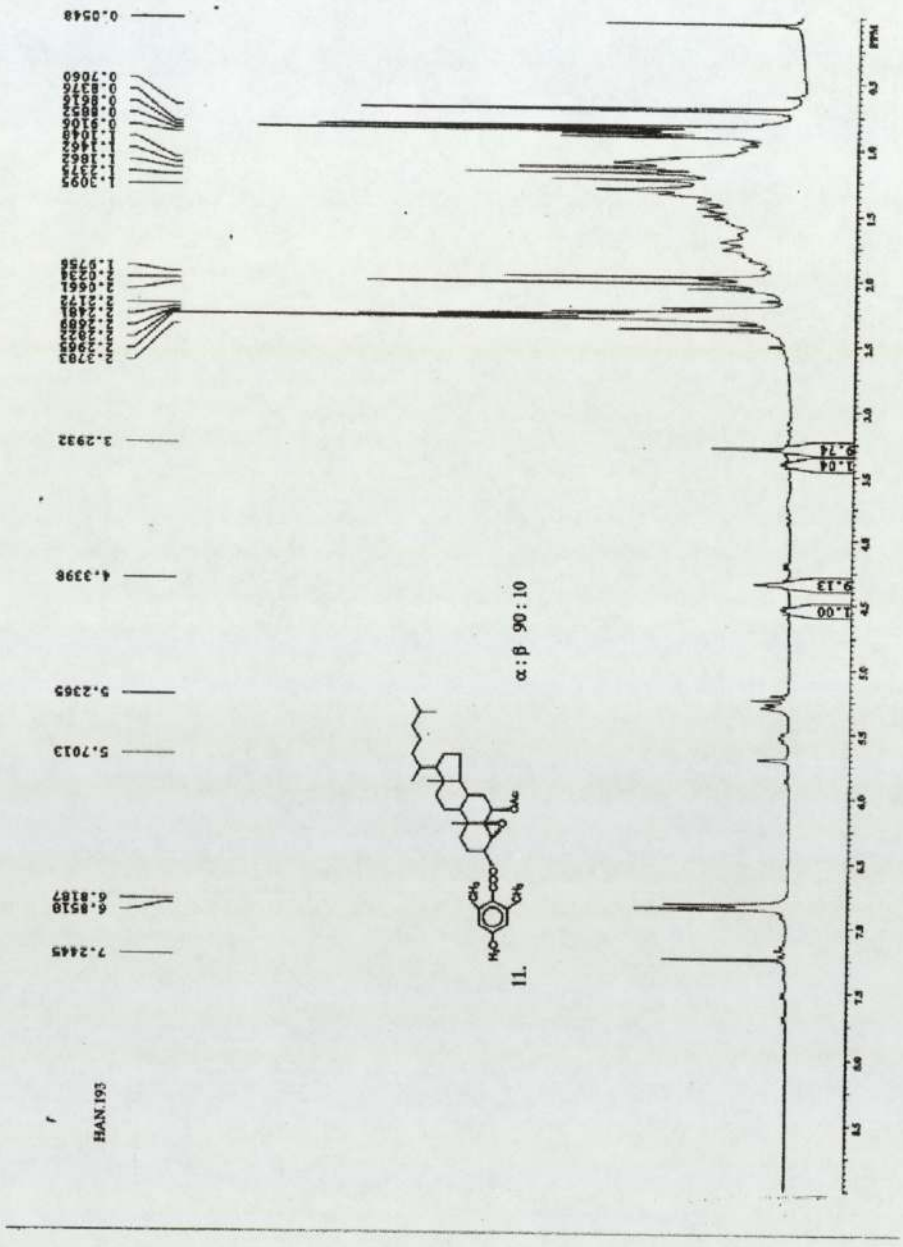
0.79
PPM


α : β 87 : 13
9.


HAN.160
10.
α : β 65 : 35
1.00
1.88
PPM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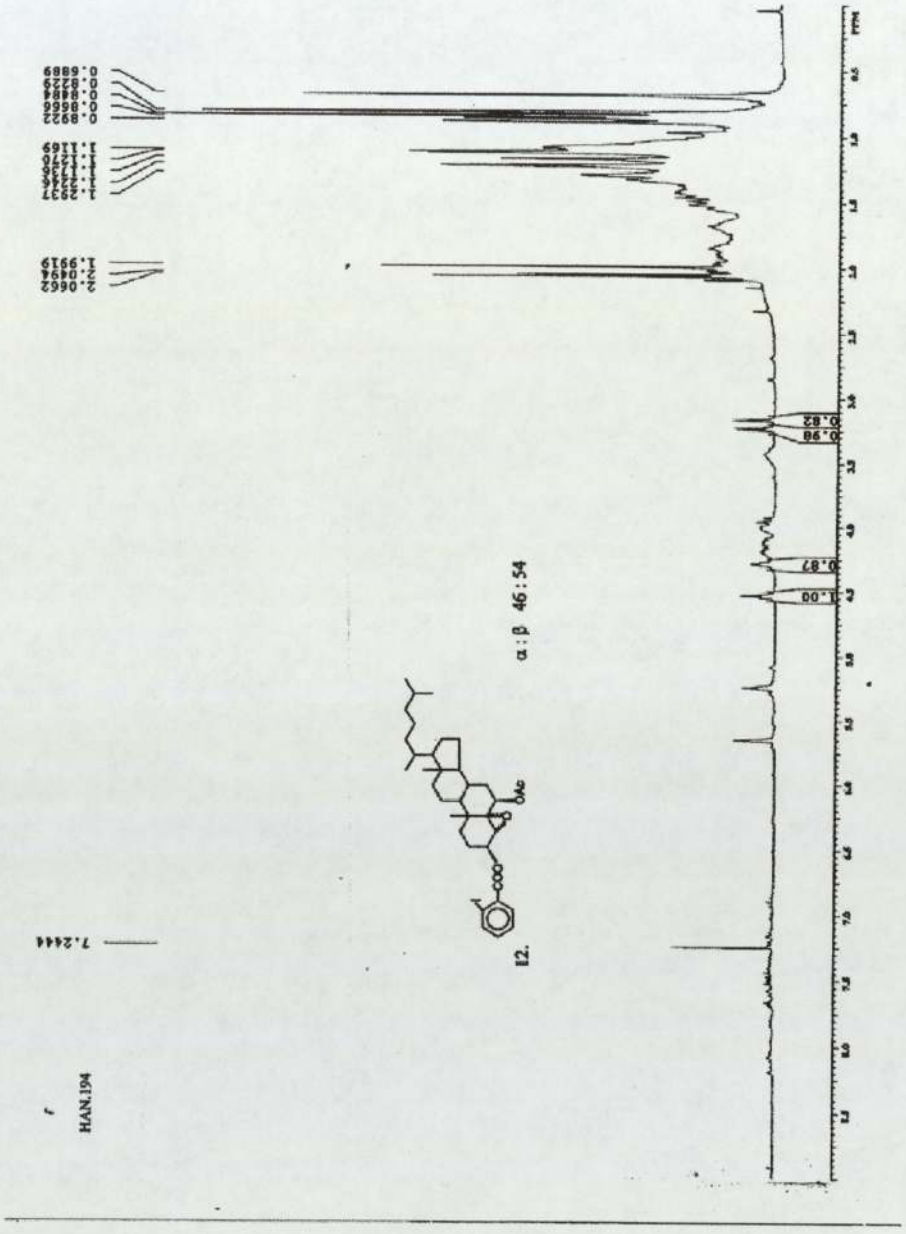
5.2363
4.3398
3.2932
α:β 90:10
11.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12.
α:β 46:54
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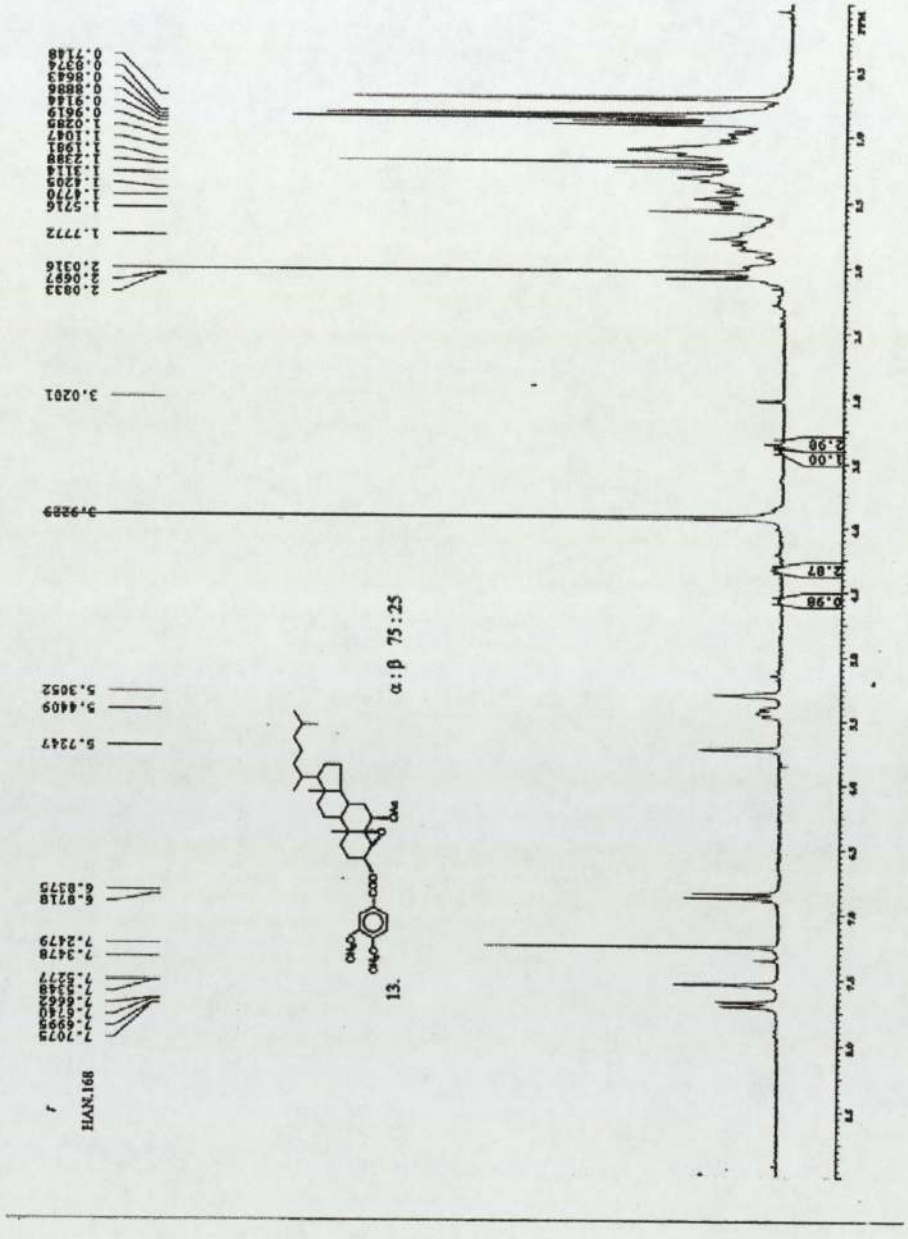
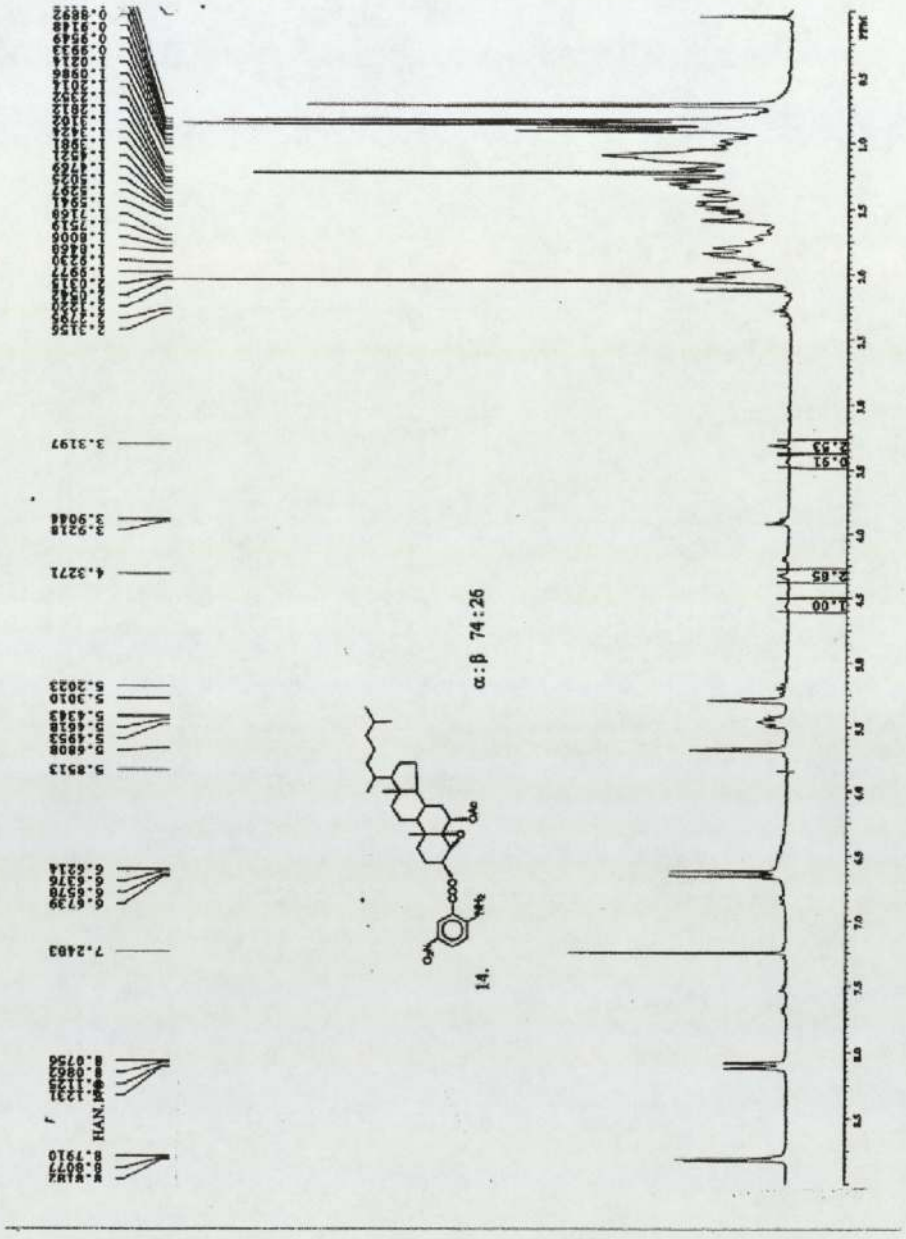
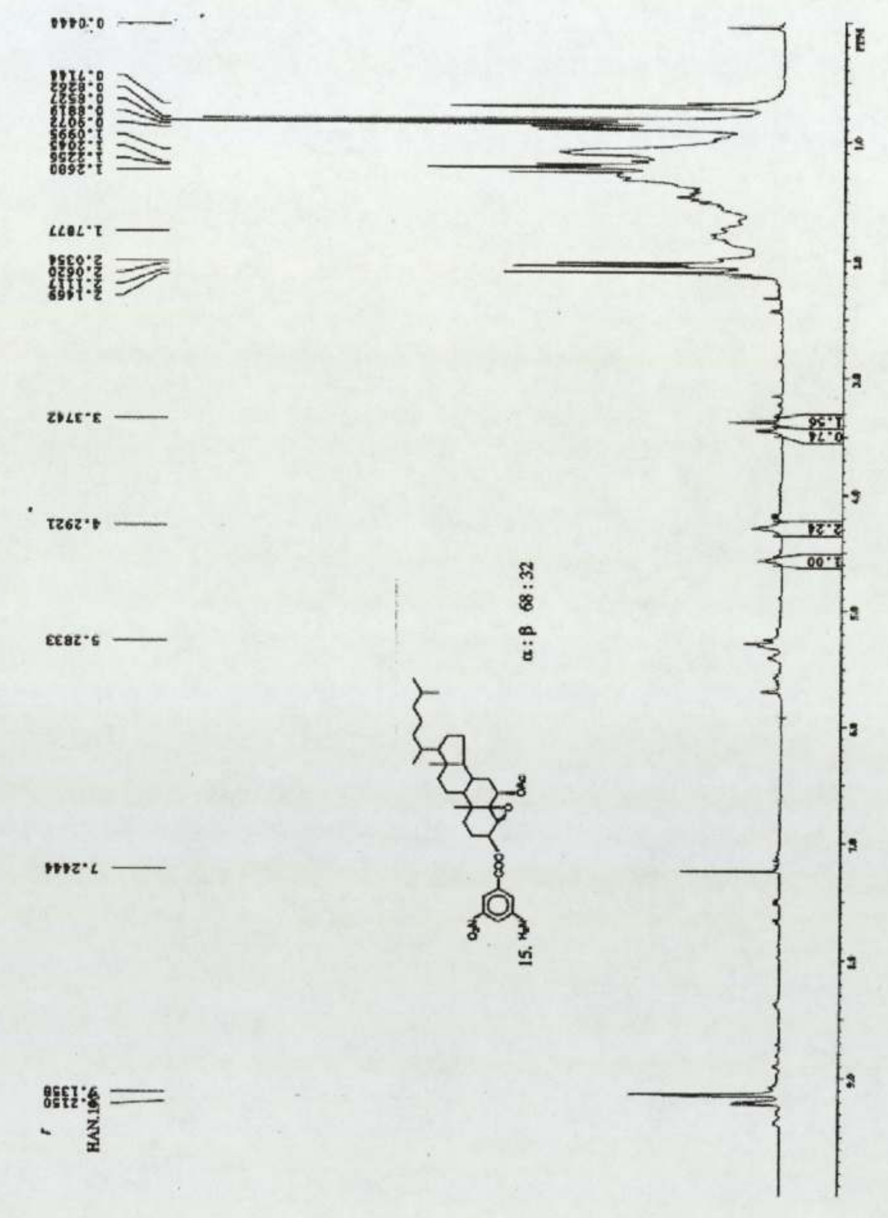
0.87
1.00


HAN.168
13.
α:β 75:25


14.
α:β 74:26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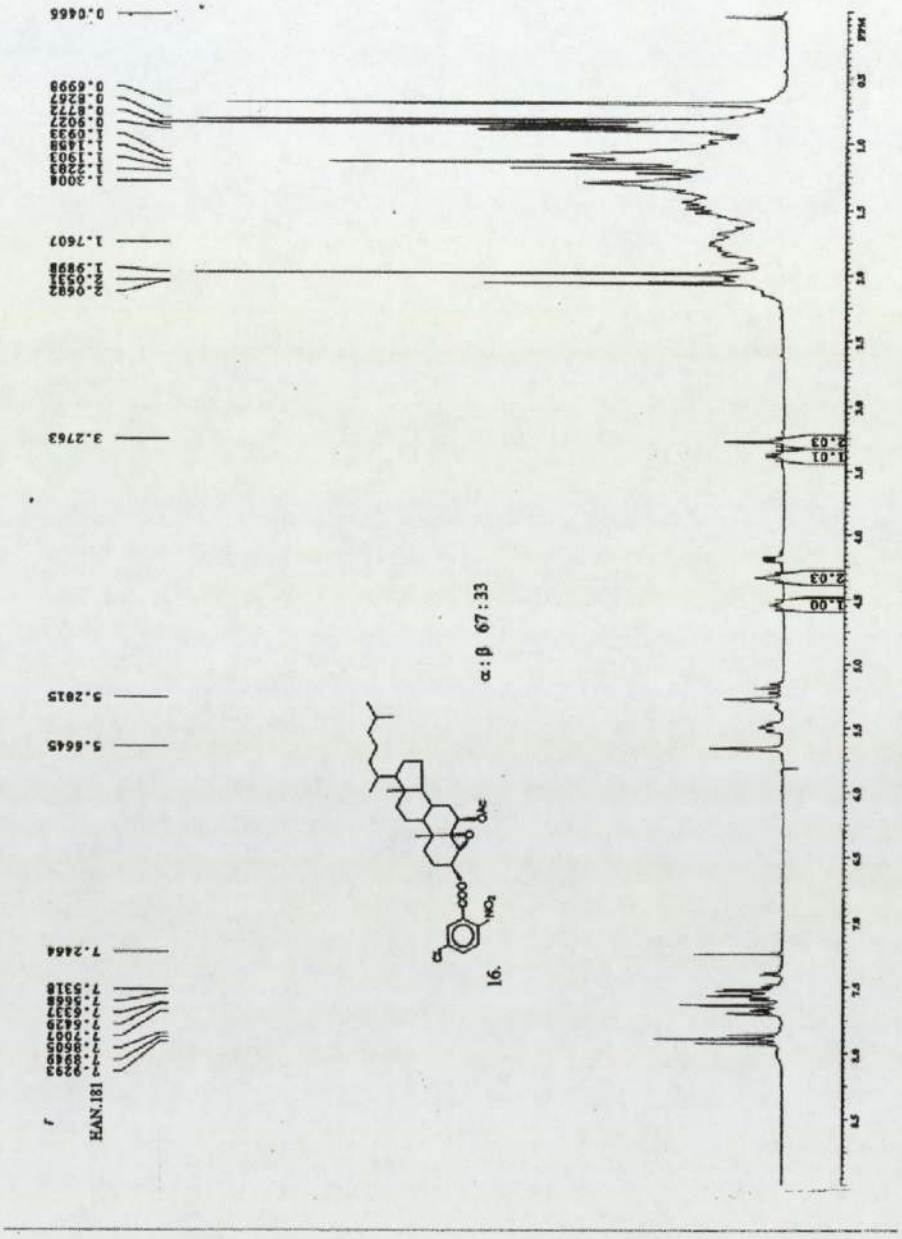
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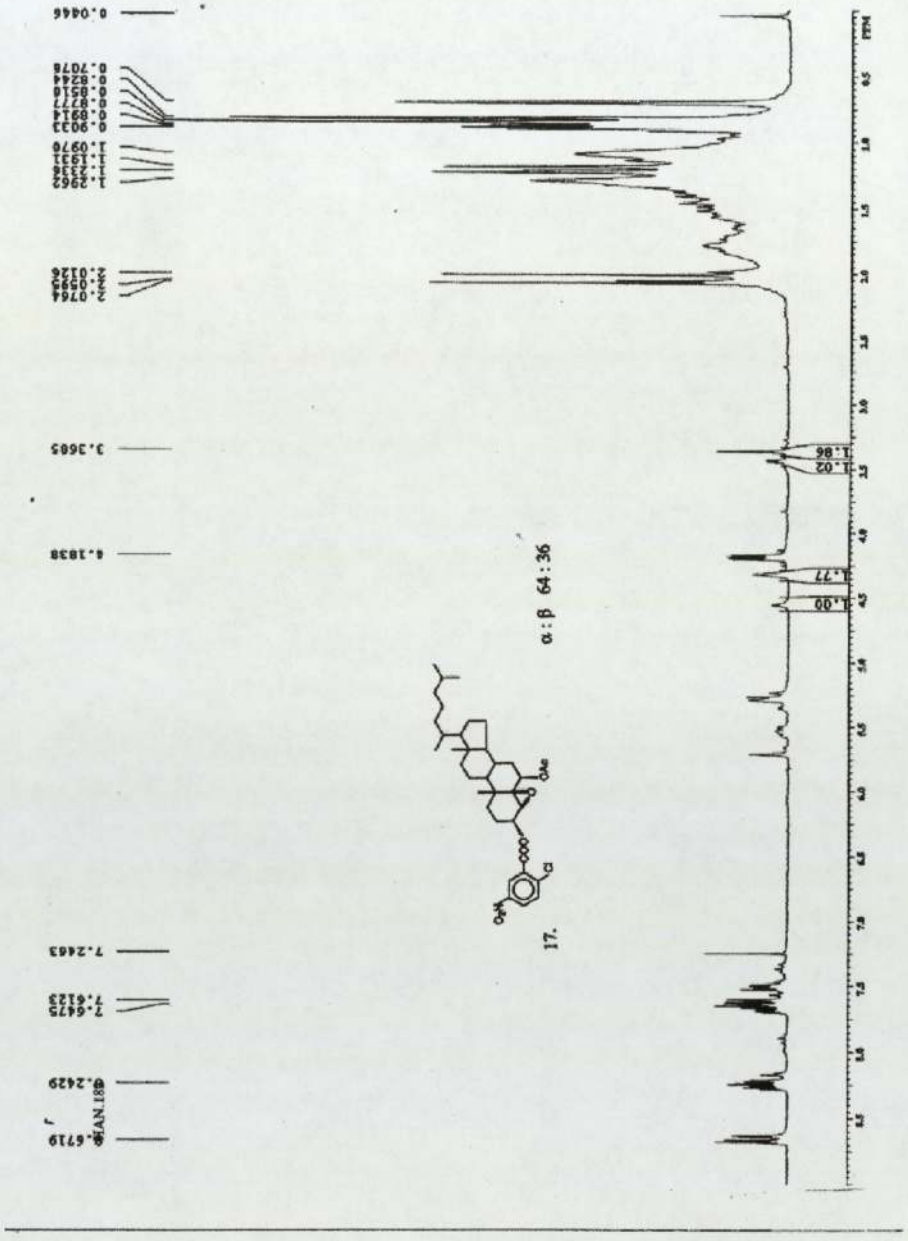
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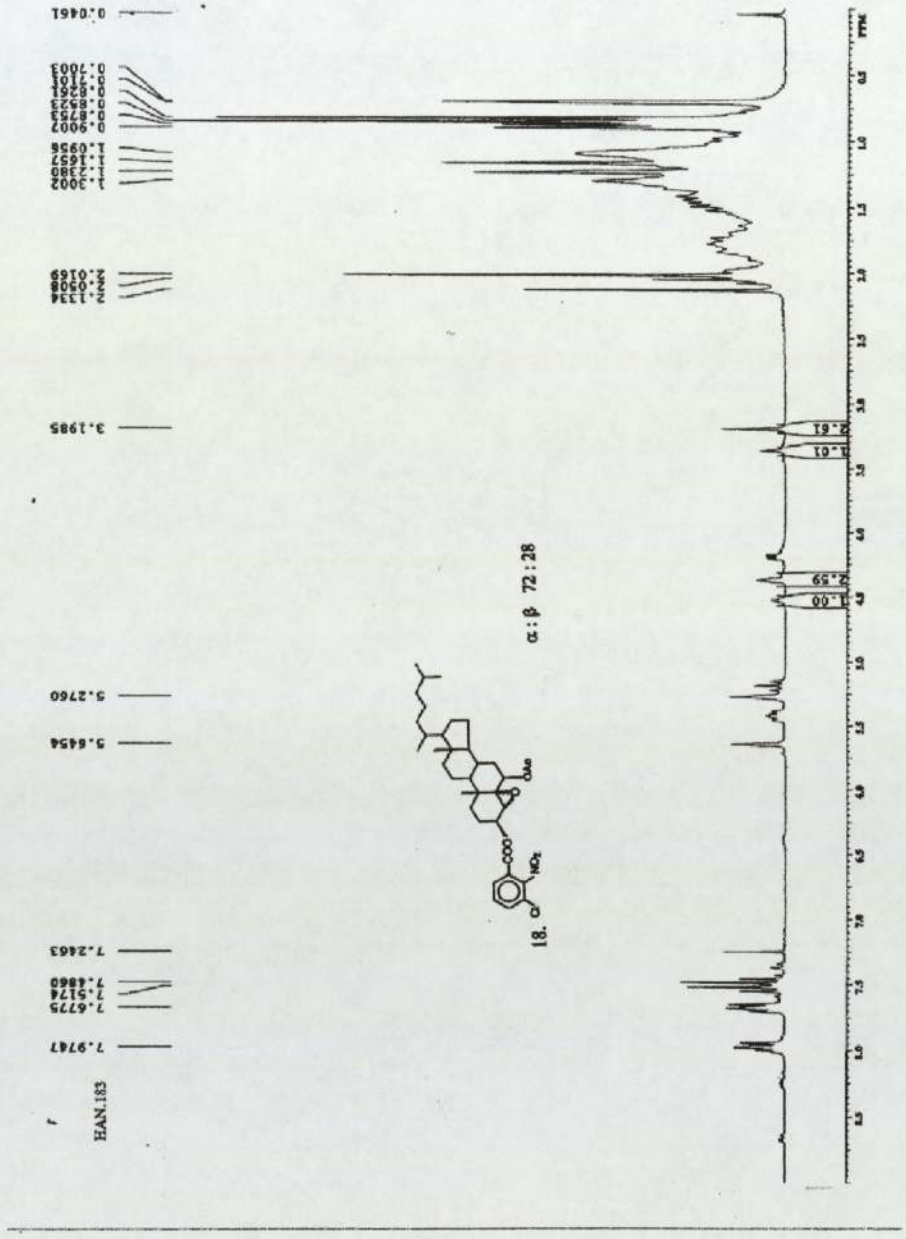
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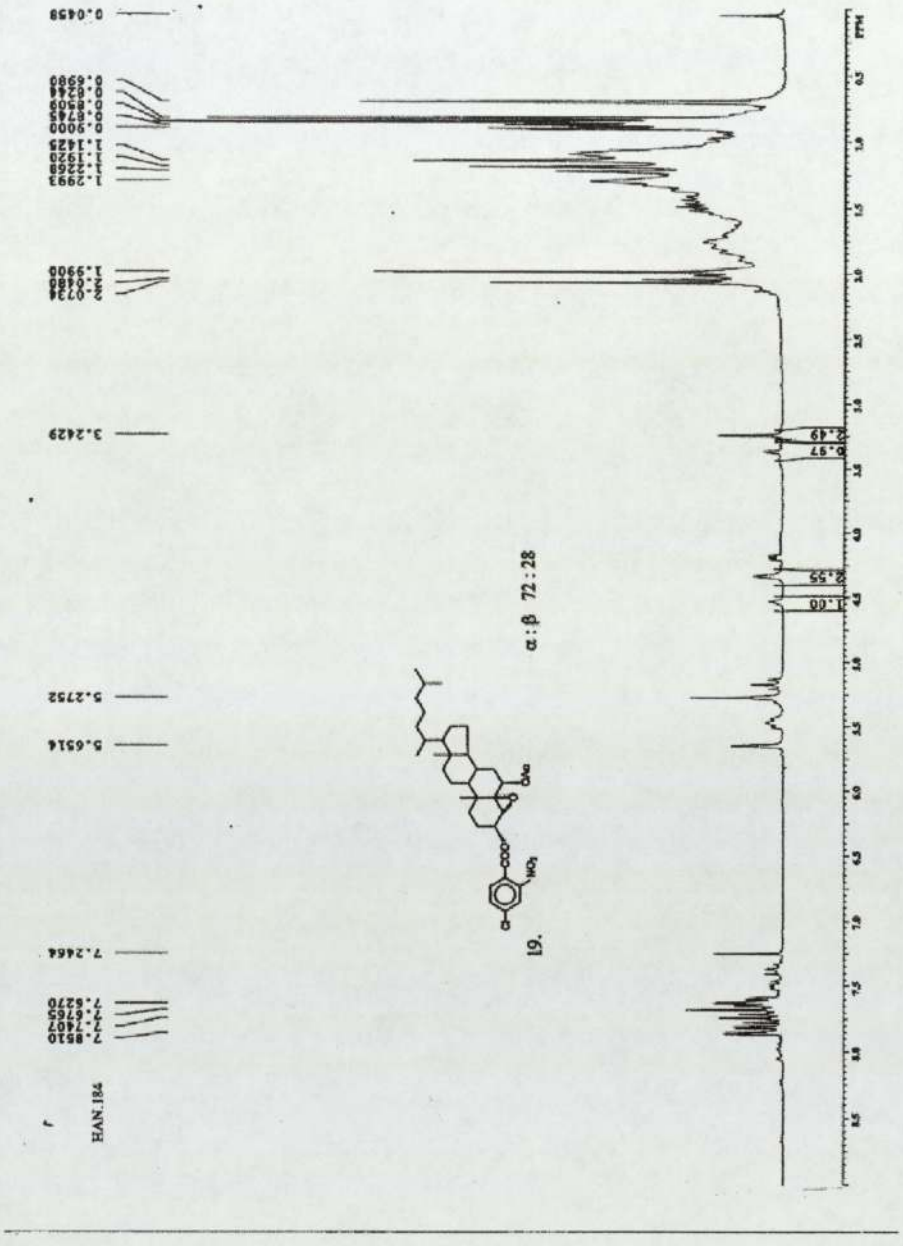
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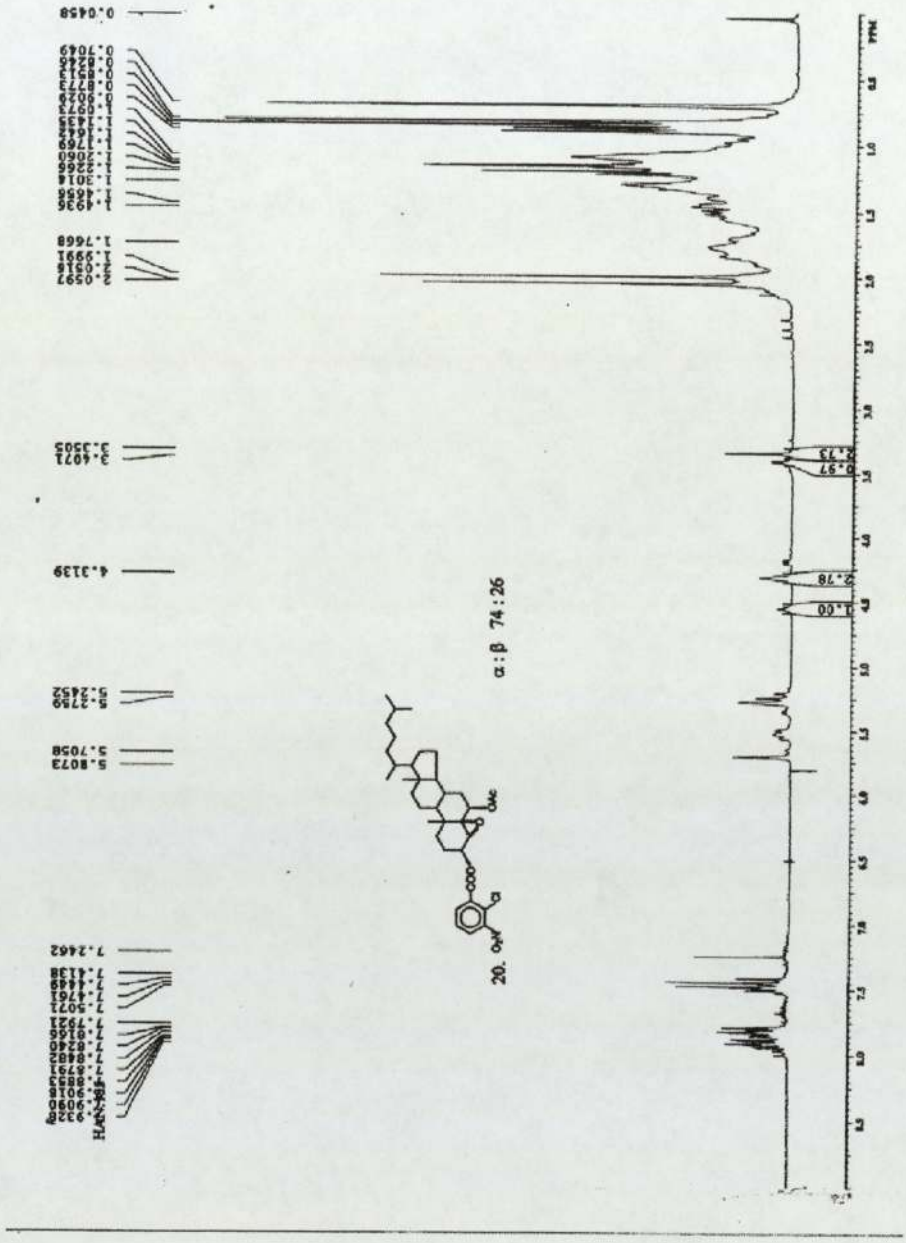
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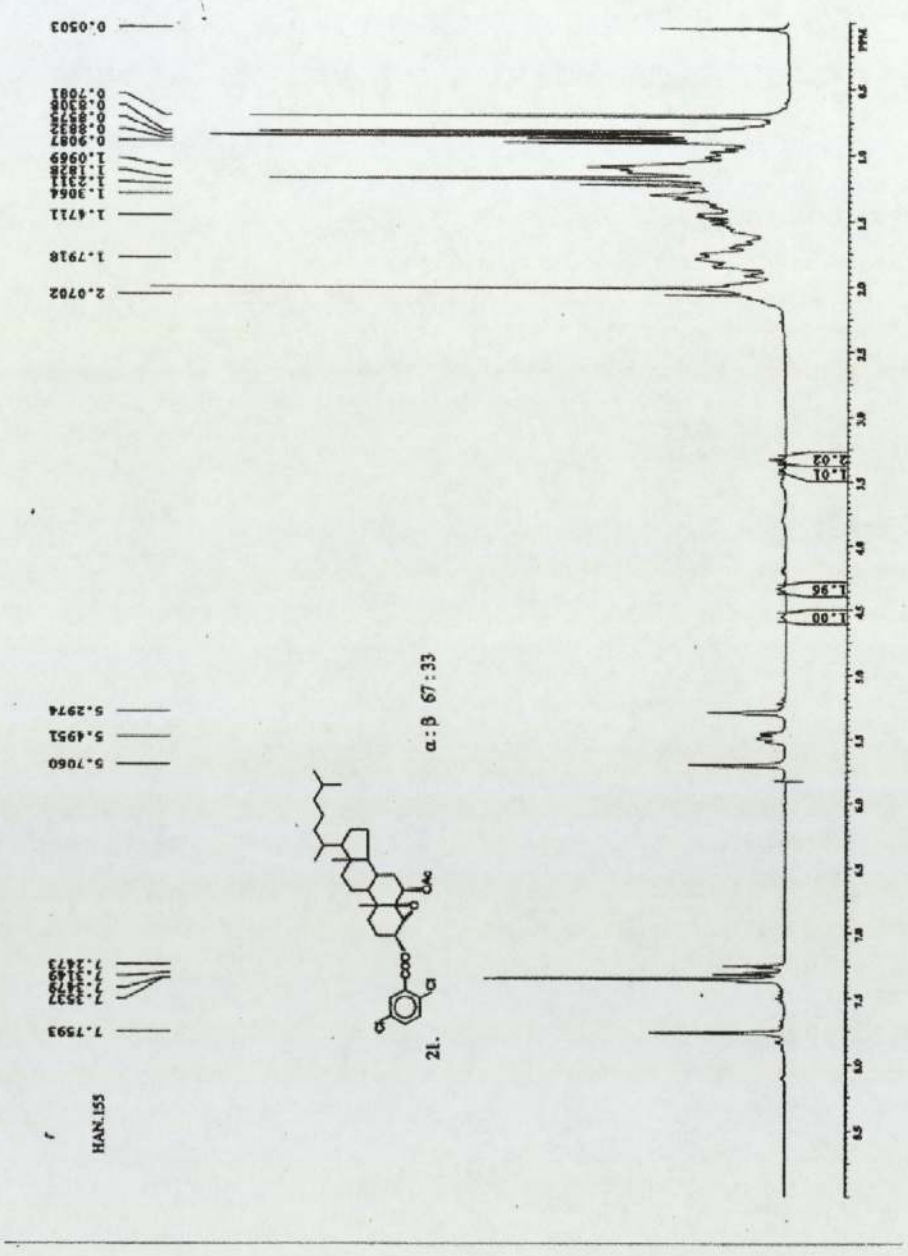
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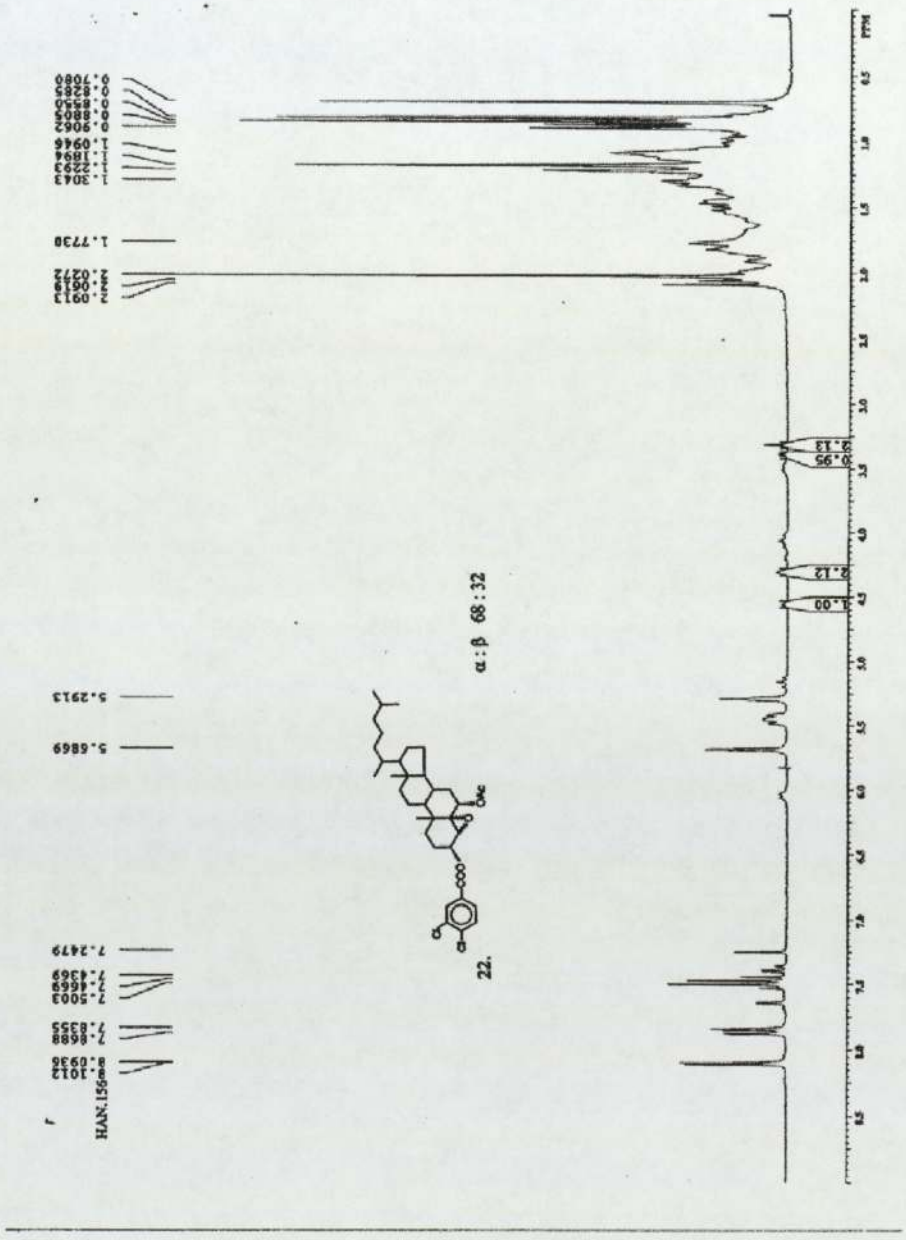
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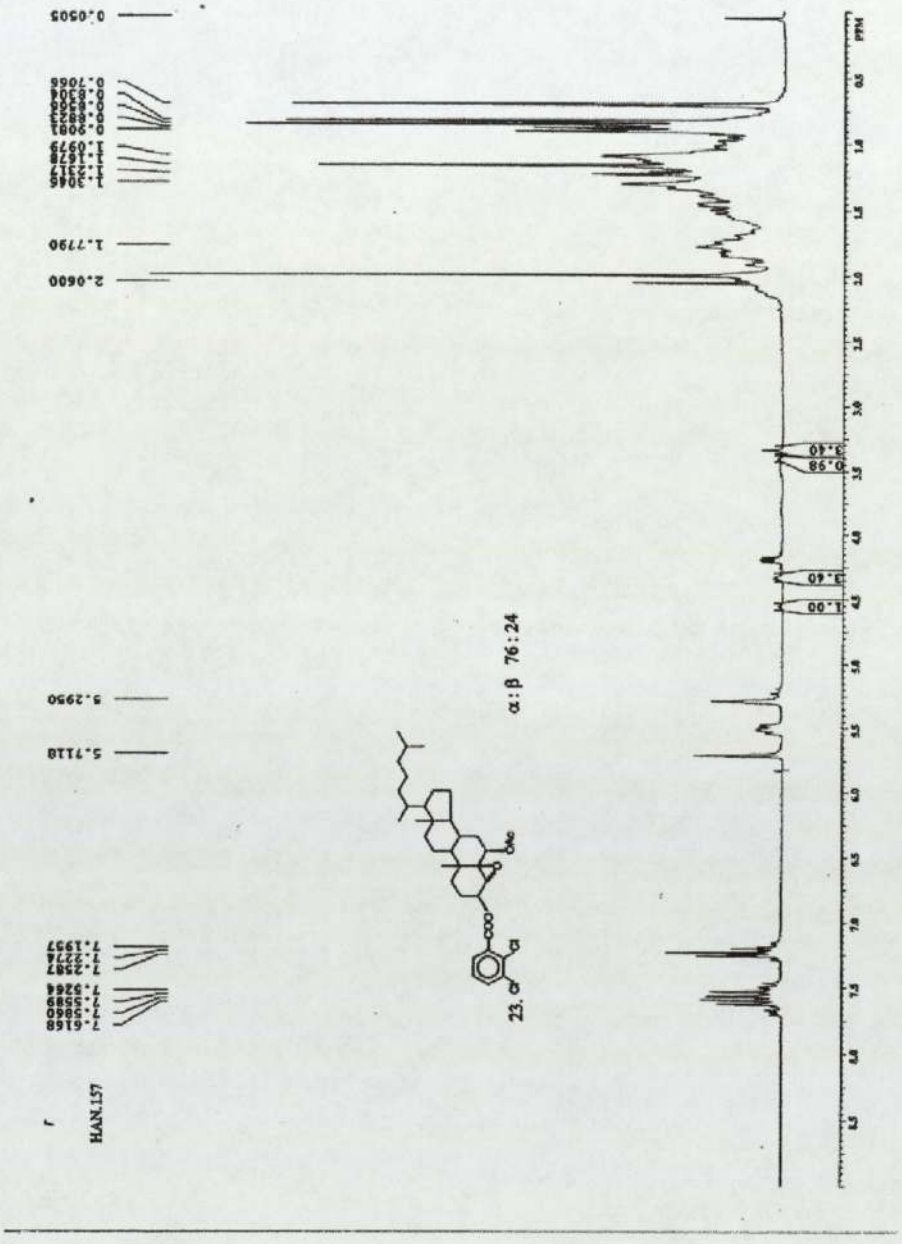
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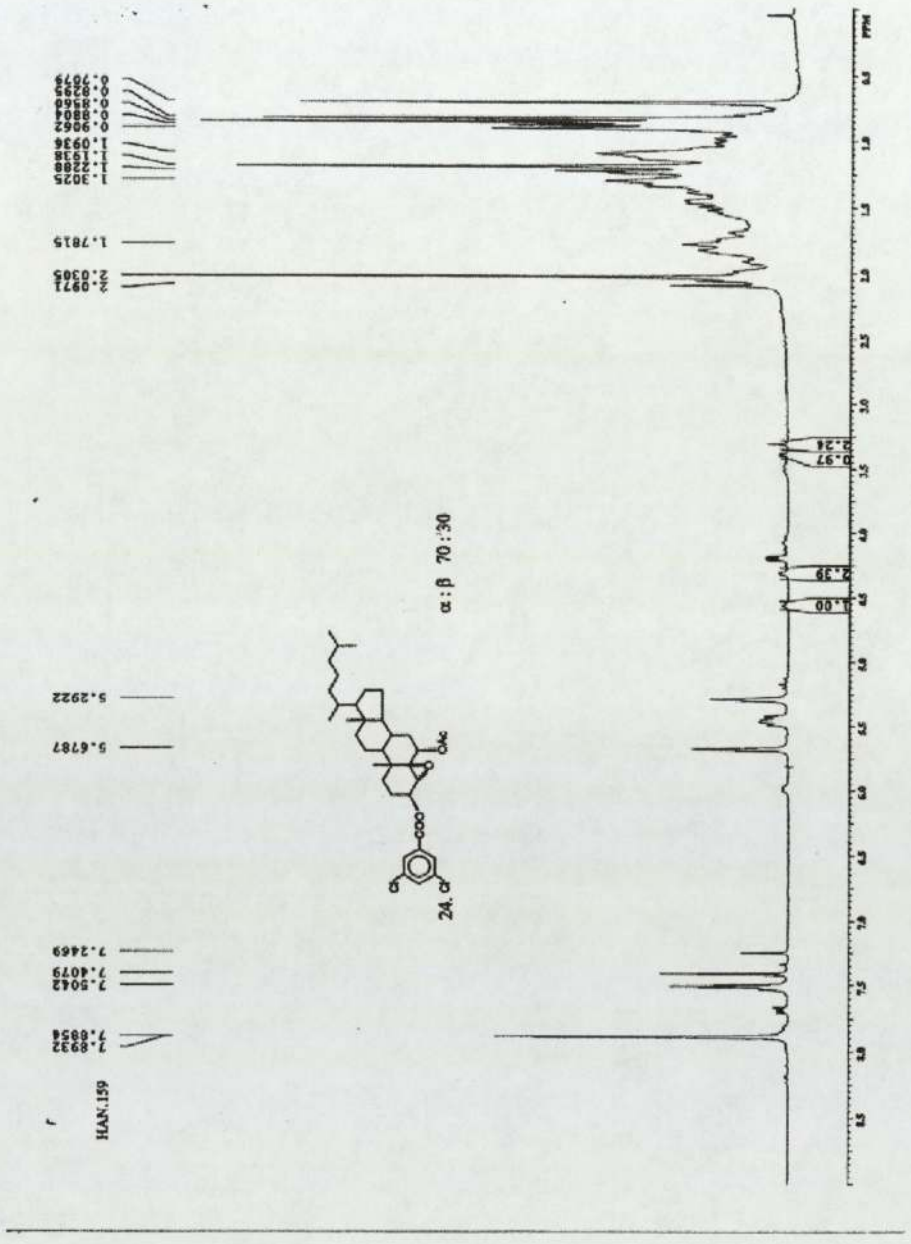
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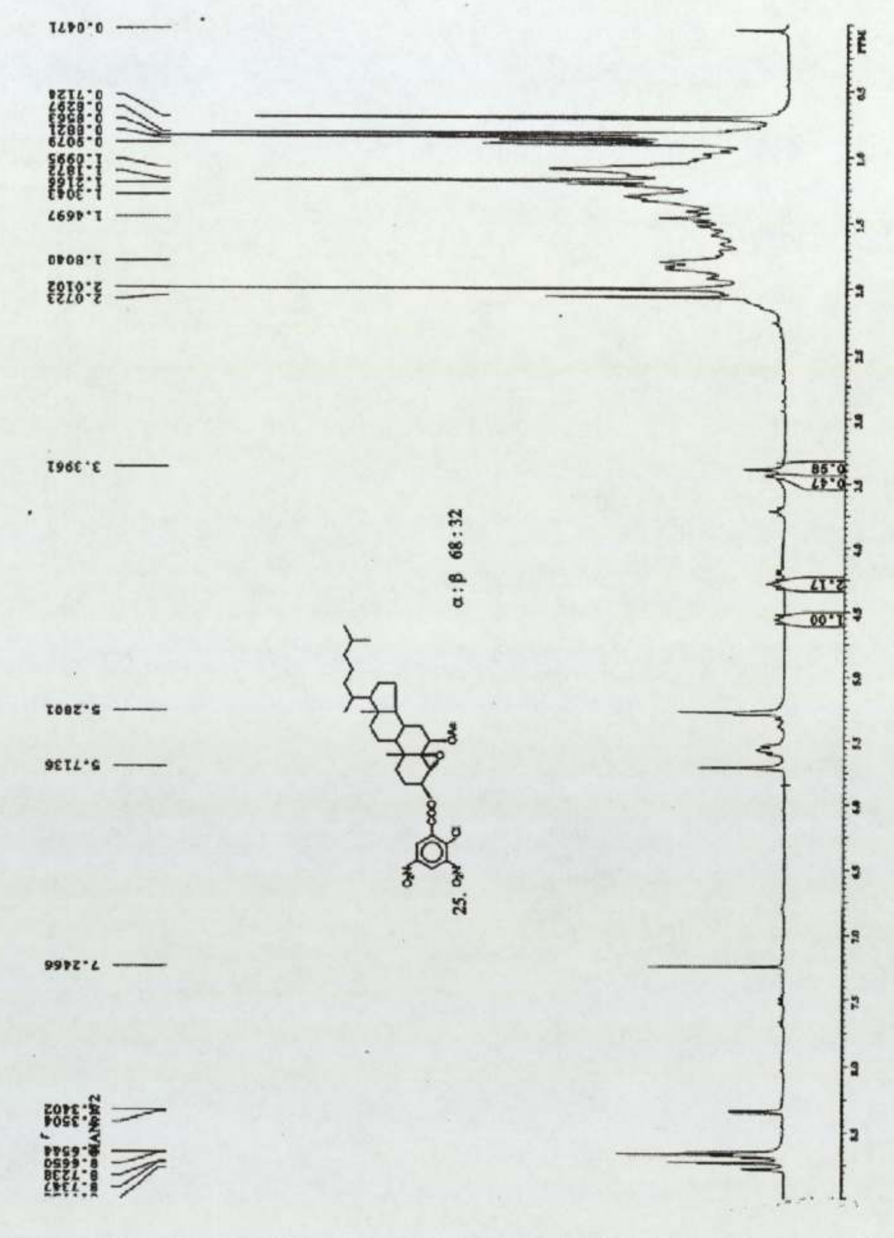
PPM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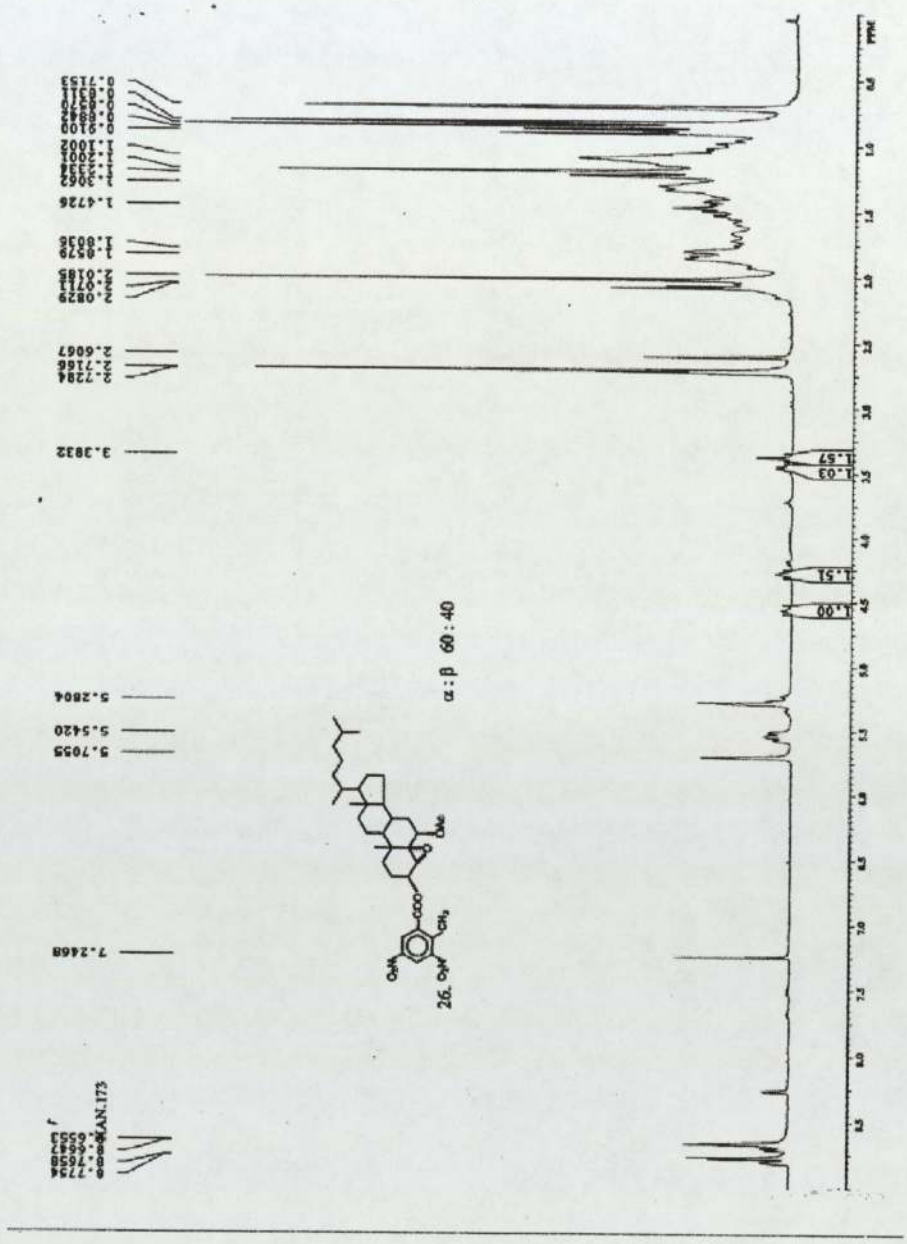
α:β 68:32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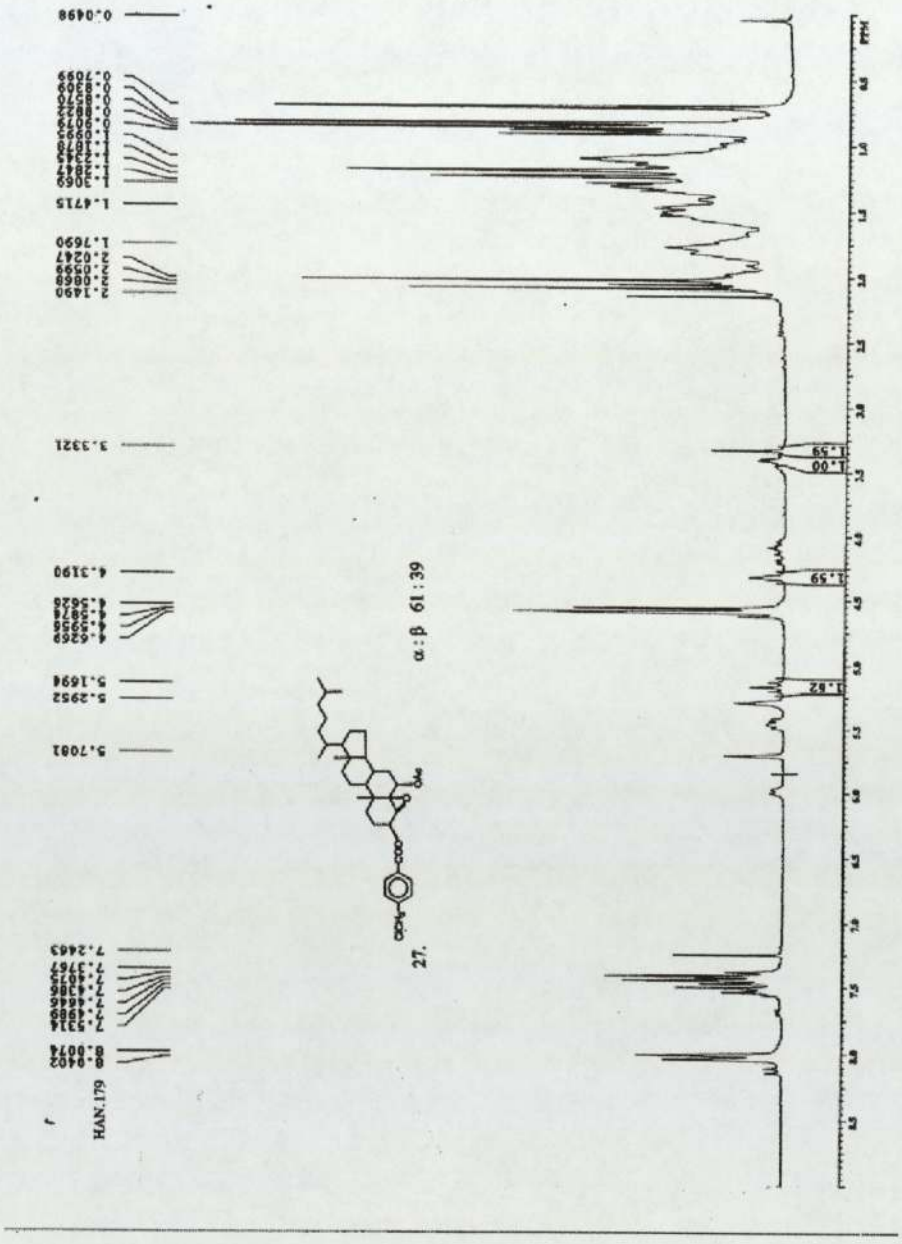
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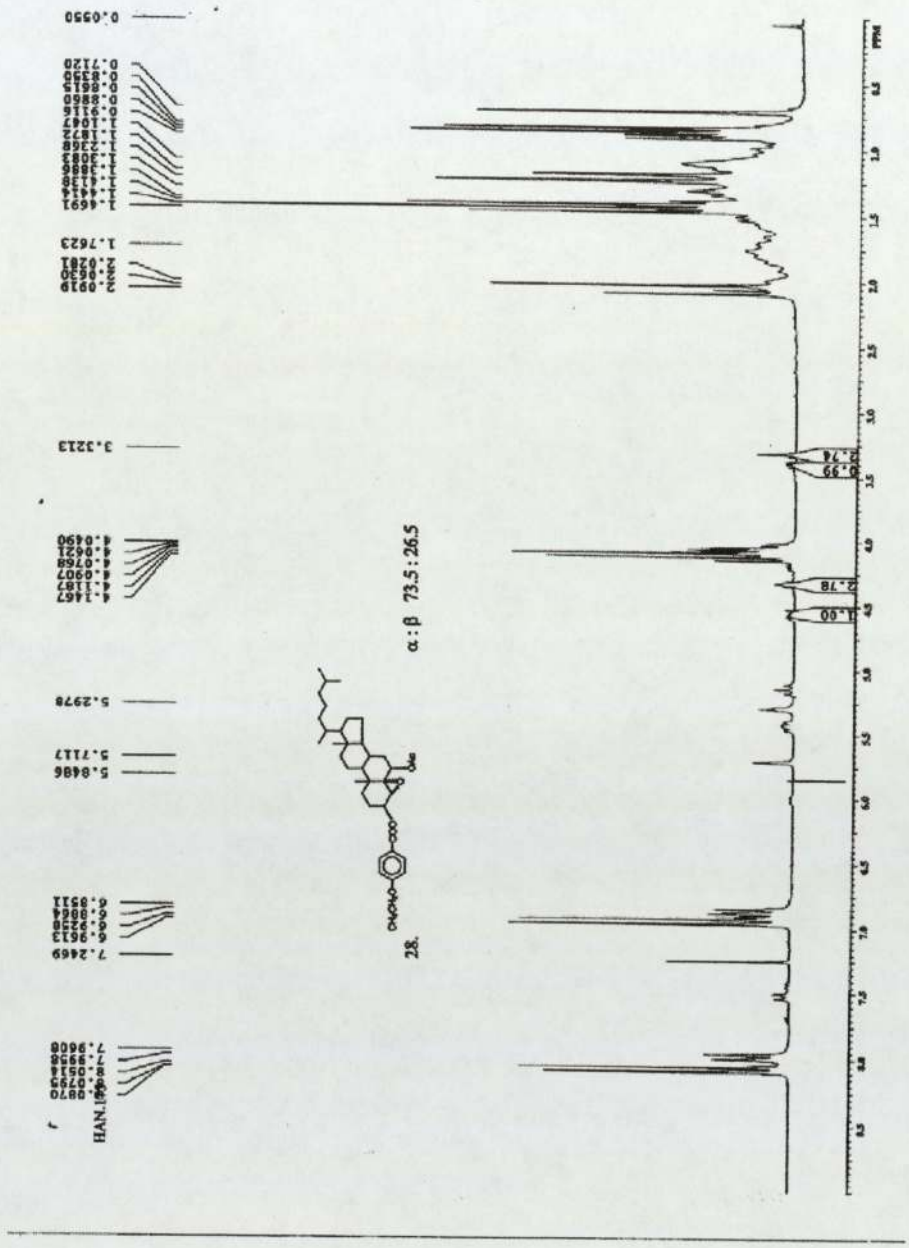
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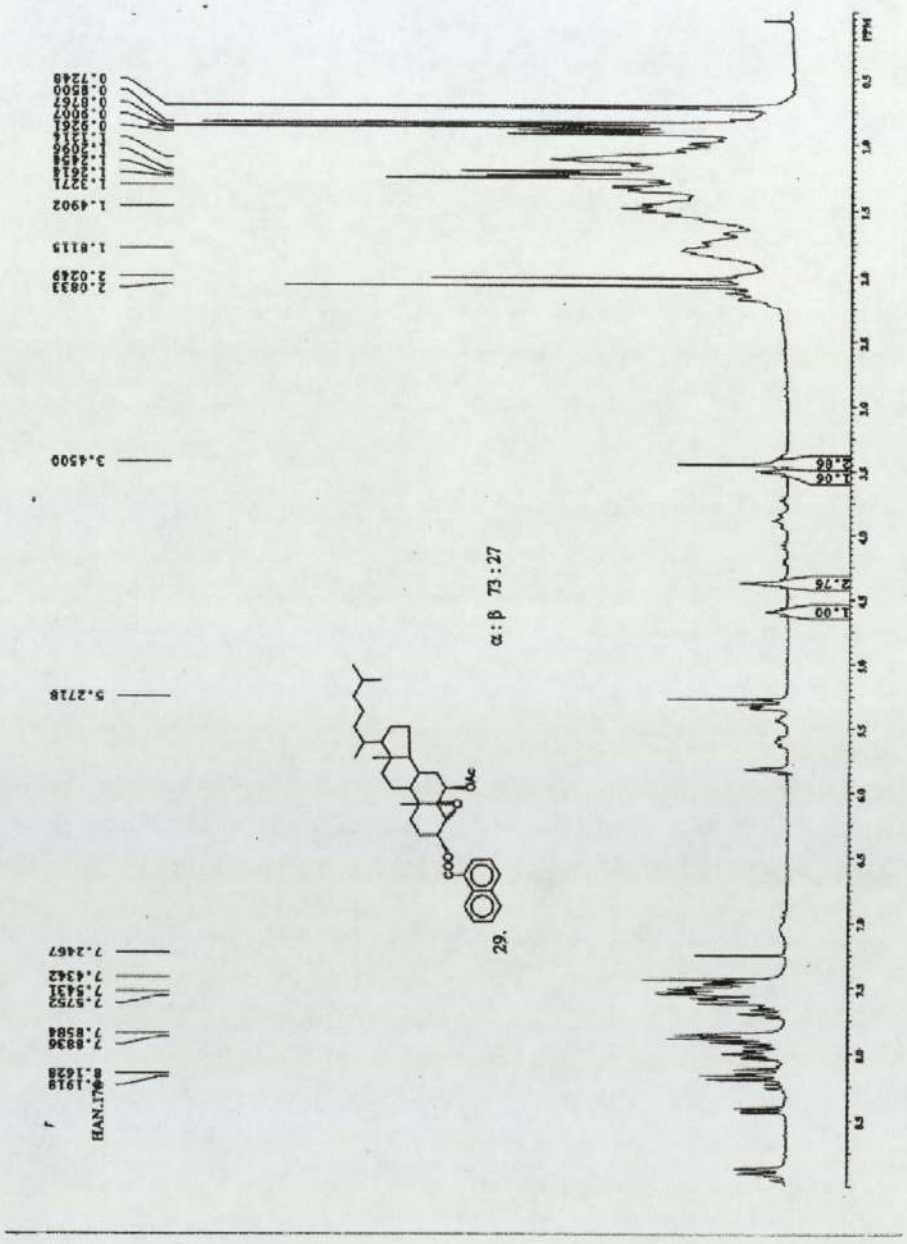
α : β 73.5 : 26.5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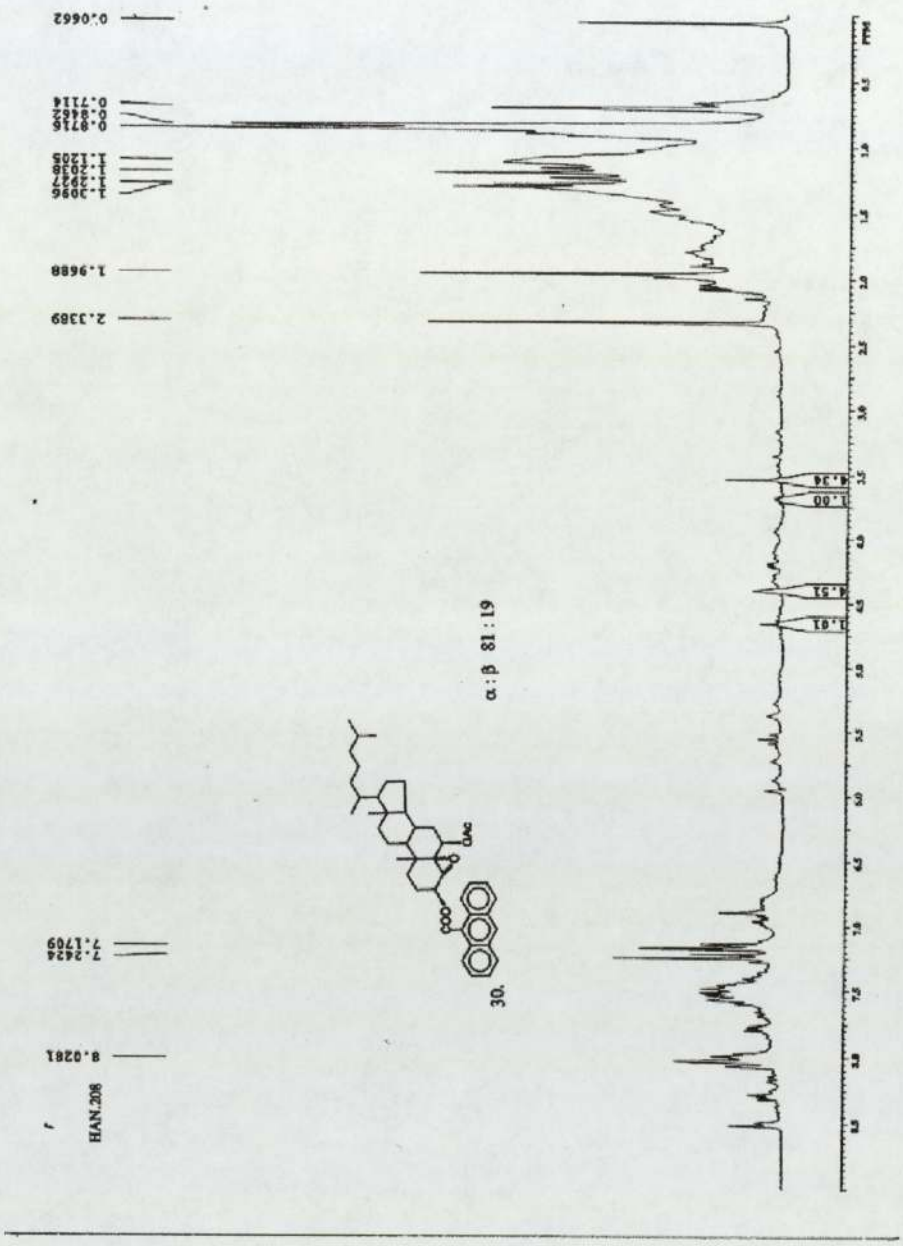
α:β 73:27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1.2977
1.2038
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0.8716
0.8662
0.7114
0.0662
PPM

Chapter Three: Experimental

Unless otherwise stated the following procedures were adopted:

Melting points were taken on Reichert-Jung Microthermal, uncorrected. I.R. spectra were recorded on a Mattson 3000 instrument and N.M.R. spectra at 250 MHz on a Bruker AC-250. Mass spectra were obtained on a HP G1034C GC/LC-MS Chemstation using atmospheric chemical ionisation (ACPI) and electrospray (ES) 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.

Thin-layer-chromatography (TLC) was carried out using aluminium backed Merck Silica Gel 60 F_{254} plates and 50% sulphuric acid water solution was used to make the points visible.

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

Part I. Reductive cleavage of 4, 5-epoxycholestane-3, 6-diols

3.1.1 Chemical Synthesis Methods

Method A, Sodium cyanoborohydride and boron trifluoride diethyl ether complex (Hutchins et al., 1981)

A solution of the epoxide starting material (10 mmol), $NaBH_3CN$ (14-30 mmol), and a small quantity of bromocresol green indicator in 40 ml of dry THF was stirred, while BF_3OEt_2 in a few millilitres of THF was added dropwise until a colour change to yellow was noted and stirring was continued at the room temperature or with refluxing. For some examples, additional BF_3OEt_2 was required periodically to maintain the acidity. Upon completion the reaction were diluted with brine and exhaustedly

extracted with dichloromethane (DCM). After the solution was dried (Na_2SO_4), solvent was removed on a rotary evaporator and got the residue.

Method B, Borane and lithium boron hydride (Brown et al., 1968)

A dry 100ml flask, fitted with a rubber syringe cap and a magnetic stirring bar, was placed in an ice bath and flushed with dry nitrogen and then maintained under a static atmosphere of the gas. Then 10 mmol of borane (4.4 ml of a 2.28 M solution in THF), 10 mmol of Lithium boron hydride (5.6 ml of a 1.79 M solution in THF), and 20 ml of THF were introduced into the flask. At 0 °C, 10 mmol epoxide in 10 ml of THF was added dropwise. After 24 hours at 0 °C, 10 ml of a 1:1 mixture of 1.0 M sulphuric acid and THF was added to the reaction mixture and stirred for 5 minutes. Potassium carbonate was added to saturate the aqueous phase, and the THF phase was separated, dried over anhydrous magnesium sulfate. Solvent was evaporated and the solid was obtained.

a. Borane Generation Externally (Wheeler et al., 1972)

Diborane is generated by adding slowly a solution of 0.51 g (13.5 mmol) of sodium borohydride in 10 ml of diglyme to a stirred solution of 2.85 ml (22.7 mmol) of boron trifluoride-etherate (about 45% boron trifluoride) in 5 ml of diglyme. The resulted diborane was carried by a current of dry nitrogen into the stirred solution of reaction flask, which was fixed with outlet tube to release extra unreacted diborane is eliminated by passing the emerging gas through a solution of acetone. All joints in the apparatus are polyvinyl chloride or rubber covered with silicon grease. The slow current of diborane is maintained for 1 hour. After rapid sweeping by dry nitrogen the excess of reagent is destroyed by addition of ethylene glycol to the generator.

b. Borane Generation in situ (Hudlicky, 1984)

In a three-necked flask equipped with a magnetic stirrer, a dropping funnel, and thermometer and reflux condenser, 0.51 g (13.5 mmol) of sodium borohydride is dissolved in 10 ml of diglyme with stirring. After addition of 0.125 g (0.4 mmol) of epoxide compound, a solution of 2.85 ml (22.7 mmol) of boron trifluoride-etherate (about 45% boron trifluoride) in 5 ml of diglyme is added through the dropping funnel while the mixture is stirred vigorously. The rate of addition is adjusted so that the temperature inside the flask does not rise above 50 °C. Ice bath was needed if it was necessary. After all of the boron trifluoride-etherate has been added, the reaction mixture is stirred at room temperature until the products appeared judging from the TLC. After the reaction, the mixture was poured into ice water and extracted with DCM followed by drying with anhydrous magnesium sulfate. Solvent was evaporated and the solid was obtained.

Method C, Mixed hydride—lithium aluminium hydride and aluminium chloride

a. The ratio of lithium aluminium hydride and aluminium chloride is 1:4 (Ashby, 1966)

To 5.3 g (0.04 mol) of anhydrous aluminium chloride was added 50 ml of anhydrous ether at ice-bath temperature, followed by 11.1 ml (0.01 mol) of 0.91 M ethereal lithium Aluminium hydride. After stirring the clear ethereal solution for 0.5 hour, 1.35 g (0.005 mol) of epoxide starting material in 50 ml ether was added. The mixture was refluxed for 2 hours. Water was added to destroy the complex hydride, and extracted with DCM. The organic layer was dried with sodium sulfate, and evaporated to obtained the solid.

b. The ratio of lithium aluminium hydride and aluminium chloride is 3:1 (To form alane AlH_3) (Lansbury et al., 1967)

To a solution of 0.71 g (5.3 mmol) of anhydrous aluminium chloride in 50 ml of ether was added 0.60 g (15.8 mmol) of lithium aluminium hydride at 0 °C. After stirring for 45 min at room temperature, a solution of 10.5 mmol of epoxide compound in 50 ml of ether was added during 45 min and the solution then allowed to reflux for 2 hours. The reaction mixture was hydrolysed with 50 ml of 10% hydrochloric acid, extracted with DCM. The organic layer was dried with sodium sulfate, and evaporated to obtain the solid.

c. The ratio of lithium aluminium hydride and aluminium chloride is 1:1 (Hudlicky, 1984)

A three-necked flask equipped with a magnetic stirrer, a dropping funnel and an efficient reflux condenser connected to a dry-ice trap is charged with 3.8 g (0.1 mol) of lithium aluminium hydride and 100 ml of anhydrous ether. Through the dropping funnel a solution of 13.3 g (0.1 mol) of anhydrous aluminium chloride was added rapidly. Five minutes after the last addition of halide, a solution of 0.1 mol epoxide compound in 200 ml of ether was added dropwise to the well-stirred solution. One hour after the last addition of the epoxide compound, decomposition of the reaction mixture and excess halide was carried out by addition of water dropwise followed by 140 ml of 6 N H_2SO_4 and then was diluted with 100 ml water, extracted with DCM. The organic layer was dried with sodium sulfate, and evaporated to obtained the solid.

Method D, Lithium-Ethylenediamine (Brown et al., 1970)

To a three-necked flask, fitted with a septum outlet, thermometer, and magnetic stirring bar and protected under nitrogen, was added 10 mmol of epoxide compound and 10 ml of anhydrous ethylenediamine, then 0.21g (30 mmol) of lithium wire, cut into 2 mm pieces and washed with pentane, was added at room temperature with vigorous stirring. The reduction was exothermic above room temperature, but a water bath was necessary to keep the temperature at 50°C for 1 hour. Colour changing was observed

during the reduction, but the reduction was completed when a blue-purple colour persists. The reaction mixture was cooled and 10 ml of water was added to destroy excess reagent. Extracted with 20 ml of DCM and dried with sodium sulfate. Evaporated to obtain the solid.

Method E, Lithium aluminium hydride and different Lewis acids

To a diethyl ether solution of epoxide starting material (0.1 mmol), added dropwise the etherate solution of Lewis acid (0.6 mmol) while under the ice-bath cooled. The mixture was kept stirring for 2 hours and then the etherate solution of lithium aluminium hydride (0.2 mmol) was added also as being cooled with ice-bath. If no reaction occurred judging from TLC, the reaction mixture was then heated and kept refluxing for another 2 hours and then the following-up was performed as described before.

General procedure for the acetylation of resulting products with free hydroxyl group (Zhao, 2002)

a. A basic as a catalyst:

Sterol (2.00 mmol), acetic anhydride (1.0 ml for every hydroxyl group) and pyridine (0.05 ml) were heated at reflux in toluene (10.0 ml) for the given period. For more stereo hindered sterols, DMAP (0.10 mmol) was used additionally. After cooling down, the toluene was evaporated and the residue was taken up with DCM, washed with 5% HCl and dried over sodium sulfate. Removal of the solvent gives the product.

b. An acid as a catalyst:

Sterol (2.00 mmol) and acetic anhydride (5 ml) were stirred in room temperature, following with the addition of boron trifluoride etherate (0.40 mmol). After a given period, the reaction was quenched by pouring the resulting mixture into ice water (40 ml), extracted with DCM, and dried over sodium sulfate. Removal of the solvent gives the product

3.1.2 The physiochemical data of the starting materials and the products (Zhao, 2002)

4α, 5–Epoxy–5α–cholestane–3β, 6β–diol (Starting Material 1-2)

Mp 166 – 167°C (Ref.); $[\alpha]_D^{15}$= +23° (c 10.0, $CHCl_3$); IR: ν_{max} 3500-3300, 2949, 2865, 1469,1385 and 1051 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.02 (dd, J=8.0 and 0.5Hz, 1H, H-3), 3.22 (s, 1H, H-6), 2.93 (s, 1H, H-4), 1.26 (s, 3H, CH_3-19), 0.67 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5X CH_3: 12.0, 17.7, 18.6, 22.5, 22.7; 10 X CH_2: 20.6, 23.8, 24.1, 26.5, 28.1, 29.4, 35.9, 36.1, 39.4, 39.5; 9 X CH: 27.9, 30.4, 35.7, 49.7, 55.4, 56.1, 63.6, 64.6, 73.5; 3 X C: 34.7, 42.5, 65.9; MS(ES^+): m/z 441($M+Na^+$,100).

4α, 5–Epoxy–5α–cholestane–3β, 6β–diol, 3,6-diacetate (Starting Material 1-1)

Obtained via recrystallization from methanol as white needles. $[\alpha]_D^{15}$= +18° (c 10.0, $CHCl_3$); Mp 157-158°C; IR: ν_{max} 2954, 2850, 1743, 1465,1367, 1240 and 1027 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.93 (dd, J = 8.2 and 0.6Hz, 1H, H-3), 4.28 (m, 1H, H-6), 3.16 (s, 1H, H-4), 1.19 (s, 3H, CH_3-19), 0.70 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 7 X CH_3: 11.9, 17.0, 18.5, 20.4, 20.9, 22.4, 22.6; 10 X CH_2: 20.4, 22.8, 23.7, 23.9, 28.0, 29.3, 33.6, 36.0, 39.3, 39.4; 9 X CH: 27.8, 30.8, 35.6, 49.4, 55.2, 56.0, 62.2, 66.9, 74.2; 5 X C: 34.5, 42.4, 63.2, 169.5, 169.7; MS (ES^+): m/z 503($M+H^+$, 40), 525 ($M+Na^+$,100).

4β, 5–Epoxy–5β–cholestane–3β, 6β–diol (Starting Material 2-2)

Mp 162–163°C (Lit. 164-165°C, Rosenheim et al. 1937); $[\alpha]_D^{15}$ = -7° (c 10.0, $CHCl_3$); $[\alpha]_D^{20}$ = -7.5° (c 0.6, $CHCl_3$); IR: ν_{max} 3500-3300, 2960, 2848, 1463,1380 and 1059 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 3.96 (m,1H, H-3), 3.32 (m, 1H, H-6), 3.22 (d, J=3.2Hz, 1H, H-4), 1.16 (s, 3H, CH_3-19), 0.70 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5 X CH_3: 11.9, 18.5, 19.6, 22.5, 22.7; 10 X CH_2: 21.3, 23.7, 24.1, 25.5, 28.7, 32.5, 36.0, 36.8, 39.4, 39.7; 9

X CH: 27.9, 29.2, 35.6, 50.6, 55.9, 56.1, 65.1, 66.1, 74.0; 3 X C: 35.1, 42.4, 69.4; MS (ES^+): m/z 441($M+Na^+$,100).

4α, 5–Epoxy–5α–cholestane–3β, 6α–diol (Starting Material 5-2)

White solid. Mp 180 – 181 °C; $[\alpha]_D^{20}$= +49° (c 1.0, $CHCl_3$); HRMS(ES^+): m/z 436.3788 ($M+NH_4^+$, $C_{24}H_{50}NO_3$ requires 436.3791); IR: ν_{max} 3500-3300, 2937, 1469,1382 and 1074 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 3.95 (m, 2H, H-3 & H-6), 3.39 (s, 1H, H-4), 1.07 (s, 3H, CH_3-19), 0.65 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5 X CH_3: 11.9, 17.1, 18.5, 22.5, 22.7; 10 X CH_2: 20.7, 23.9, 24.1, 25.7, 28.1, 29.0, 36.0, 38.1, 39.4, 39.5; 9 X CH: 27.9, 34.3, 35.7, 49.3, 55.4, 56.2, 60.1, 64.9, 65.4; 3 X C: 36.2, 42.6, 69.3; MS (ES^+): m/z 441($M+Na^+$, 100).

4β, 5–Epoxy–5β–cholestane–3β, 6α–diol (Starting Material 6-2)

Obtained via flash chromatography (ether as elutent) as white solids. Mp 66-67 °C; $[\alpha]_D^{20}$ = +30° (c 1.0, $CHCl_3$); HRMS (ES^+): m/z 436.3787 ($M+NH_4^+$, $C_{24}H_{50}NO_3$ requires 436.3791); IR: ν_{max} 3500-3300, 2938, 2865, 1463,1378 and 1066 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 3.95(m, 2H, H-3 & H-6), 3.65 (s, 1H, H-4), 0.95(s, 3H, CH_3-19), 0.62(s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5 X CH_3: 11.8, 18.5, 19.0, 22.5, 22.7; 10 X CH_2: 21.3, 23.7, 24.1, 25.3, 27.9, 28.0, 36.0, 37.8, 39.3, 39.5; 9 X CH: 27.9, 29.4, 35.7, 46.4, 55.9, 56.1, 65.8, 66.1, 74.3; 3 X C: 36.4, 42.4, 71.0; MS (ES^+): m/z 441($M+Na^+$, 100).

4α, 5–Epoxy–5α–cholestane–3α, 6β–diol (Starting Material 3-2)

Obtained via hydrolysis of the 6-acetate and recrystallised from chloroform/hexane as granular crystals. Mp 152 – 154 °C; $[\alpha]_D^{20}$= +57° (c 10.0, $CHCl_3$); HRMS(ES^+): m/z 419.3528 ($M+H^+$, $C_{24}H_{47}O_3$ requires 419.3525); IR: ν_{max} 3500-3300, 2939, 2865, 1469,1380 and 1034 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.06 (m, 1H, H-3), 3.26 (m, 2H, H-6 & H-4), 1.18 (s, 3H, CH_3-19), 0.68 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5 X CH_3:

12.0, 17.3, 18.5, 22.5, 22.7; 10 X CH_2: 20.6, 23.8, 24.1, 26.8, 28.1, 29.5, 35.7, 36.0, 39.4, 39.6; 9 X CH: 27.9, 30.3, 35.7, 50.2, 55.5, 56.1, 62.8, 63.3, 73.8; 3 X C: 34.6, 42.5, 67.7; MS (ES^+): m/z 436 ($M+NH_4^+$, 55), 236(50), 214(100).

4β, 5–Epoxy–5β–cholestane–3α, 6β–diol (Starting Material 4-2)

Obtained via hydrolysis of its 3,6-diacetate and recrystallised from DCM/hexane as white solids. Mp 137 – 138 °C; $[\alpha]_D^{20} = +10°$ (c 1.0, $CHCl_3$); HRMS (ES^+): m/z 419.3517 ($M+H^+$, $C_{24}H_{47}O_3$ requires 419.3525); IR: ν_{max} 3500-3300, 2942, 2865, 1463,1378 and 1058 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 3.94 (dd, J = 9.1, 2.6 Hz, 1H, H-3), 3.35 (m, 1H, H-6), 2.93 (s, 1H, H-4), 1.14 (s, 3H, CH_3-19), 0.69 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5 X CH_3: 11.9, 18.5, 20.6, 22.5, 22.7; 10 X CH_2: 21.0, 23.8, 24.2, 25.3, 27.3, 28.1, 35.8, 36.0, 39.4, 39.6; 9 X CH_2: 7.9, 29.4, 35.7, 46.4, 55.9, 56.1, 65.8, 66.1, 74.3: 3 X C: 35.8, 42.5, 67.6; MS (ES^+): m/z 441 ($M+Na^+$, 100), 419 ($M+H^+$, 39), 236 (61), 214 (37).

4α, 5–Epoxy–5α–cholestane–3α, 6α–diol (Starting Material 7-1)

Obtained via recrystallistion from chloroform/hexane as white solid. Mp 173-174 °C; $[\alpha]_D^{20} = +64°$ (c 4.0, $CHCl_3$); HRMS(ES^+): m/z 436.3790 ($M+NH_4^+$, $C_{24}H_{50}NO_3$ requires 436.3791); IR: ν_{max} 3500-3300, 2943, 1469,1376 and 1042 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.04 (m, 1H, H-3), 3.87 (m, 1H, H-6), 3.71(d, J = 4.4 Hz, 1H, H-4), 0.98 (s, 3H, CH_3-19), 0.65 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5 X CH_3: 11.9, 16.6, 18.5, 22.5, 22.7; 10 X CH_2: 20.6, 23.8, 24.1, 26.6, 28.0, 28.1, 36.0, 38.6, 39.4, 39.5; 9 X CH: 27.9, 34.3, 35.7, 49.8, 55.4, 56.1, 57.7, 62.5, 64.8; 3 X C: 36.2, 42.5, 70.5; MS (ES^+): m/z 441 ($M+Na^+$, 100), 401 (55), 214 (71), 114 (43).

4β, 5–Epoxy–5β–cholestane–3α, 6α–diol (Starting Material 8-2)

Obtained via hydrolysis of the 3-acetate and flash chromatography as white solid. Mp 99-100 °C; $[\alpha]_D^{20}$= +25° (c 3.0, $CHCl_3$); HRMS(ES^+): m/z 436.3793 ($M+NH_4^+$, $C_{24}H_{50}NO_3$ requires 436.3791); IR: ν_{max} 3500-3300, 2937, 1467,1365 and 1078 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.01 (m, 2H, H-3 and H-6), 3.36 (s, 1H, H-4), 0.96 (s, 3H, CH_3-19), 0.65 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 5 X CH_3: 11.8, 18.5, 18.9, 22.4, 22.7; 10 X CH_2: 21.1, 23.8, 24.2, 24.6, 26.4, 28.1, 36.0, 37.7, 39.4, 39.5; 9 X CH: 27.9, 34.0, 35.7, 45.5, 55.6, 56.2, 59.8, 65.9(two carbon); 3 X C: 36.0, 42.5, 69.8; MS (ES^+): m/z 436 ($M+NH_4^+$, 62), 401 (100), 236 (15), 214 (18).

5α-Cholestan-3β, 5, 6β-triol (Product 1)

M.p.239-241°C (Lit. 237-239°C, Kimura et al., 1976) IR: ν_{max} 3500-3300, 2937, 2865, 1465,1375 and 1039 cm^{-1}; ^1H NMR ($CDCl_3$): 4.08(m, 1H, H-3) 3.52(s, 1H, H-6), 1.16 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); ^1H NMR (DMSO-d_6): 4.39(d, J=4.1Hz, 1H, OH-6), 4.16 (d, J=5.7Hz, 1H, OH-3), 3.79(m,1H, H-3) 3.63(s, 1H, OH-5), 3.28(m, 1H, H-6), 1.00 (s, 3H, CH_3-19), 0.61 (s, 3H, CH_3-18); MS (ES^-): m/z 443 ($M+Na^+$, 63), 385 (100), 438 (46).

5α-Cholestan-3β, 5, 6β-triol-3, 6-diacetate (Product 1a)

M.p.167-169°C (Lit. 169-170°C, Kimura et al., 1972) IR: ν_{max} 3485, 2941, 2871, 1736, 1465, 1378, 1267 and 1027 cm^{-1}; ^1H NMR ($CDCl_3$): 5.11(m, 1H, H-3), 4.67 (s, 1H, H-6), 2.04, 1.99 (both s, 6H, the acetyl CH_3); 1.12 (s, 3H, CH_3-19), 0.65(s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 12.0, 16.1, 18.5, 21.3, 21.4, 22.4, 22.7; (CH_2): 20.9, 23.9, 24.0, 26.6, 28.1, 31.2, 31.7, 36.1, 36.4, 39.4, 39.8; (CH): 27.9, 30.6, 35.8, 44.6, 55.6, 56.2, 70.9, 76.2; (C): 38.2, 42.6, 74.7, 170.2, 170.7; MS (ES^+): m/z 522 ($M+NH_4^+$, 100).

5β-Cholestan-3α, 5, 6β-triol (Product 2)

M.p. 172-174°C IR: ν_{max} 3500-3300, 2942, 2865, 1457,1370, 1054 and 1029 cm^{-1}; ^1H NMR ($CDCl_3$): 3.92(m, 1H, H-3) 3.55(s, 1H, H-6), 0.99 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); MS (ES^+): m/z 443 ($M+Na^+$, 100); 385(66).

5β-Cholestan-3α, 5, 6β-triol-3, 6-diacetate (Product 2a)

M.p. 61-63 °C; IR: ν_{max} 3500-3300, 2943, 2867, 1737, 1463,1365, 1240, 1162, 1059 and 1032 cm^{-1}; ^1H NMR ($CDCl_3$): 4.95-5.11 (m, 1H, H-3), 4.70 (s, 1H, H-6), 2.07, 2.00 (both s, 6H, the acetyl CH_3); 0.97 (s, 3H, CH_3-19), 0.65(s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 11.9, 17.5, 18.5, 21.2, 21.3, 22.4, 22.7; (CH_2): 20.8, 23.7, 24.0, 25.8, 28.0, 30.0, 33.0, 36.0, 37.7, 39.3, 39.6; (CH): 27.8, 30.5, 35.6, 42.9, 56.0, 56.1, 70.6, 77.2; (C): 39.6, 42.4, 74.8, 170.2, 170.9; MS (ES^+): m/z 522 ($M+NH_4^+$, 100).

5β-Cholestan-3α, 5, 6α-triol (Product 3)

M.p. 104-106°C; HRMS (ES^+): m/z 438.3949 ($M+NH_4^+$, $C_{27}H_{52}NO_3$ requires 438.3947); IR: ν_{max} 3500-3300, 2934, 2865, 1463, 1377, 1050 and 970 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 3.96 (m, 1H, H-3), 3.72 (dd, 1H, H-6), 0.85 (s, 3H, CH_3-19), 0.62 (s, 3H, CH_3-18); MS (ES^-): m/z 419 ($(M-H)^-$, 100), 435 (56); MS (ES^+): m/z 443 ($M+Na^+$, 57), 385 (66), 864 (100).

5β-Cholestan-3α, 5, 6α-triol-3, 6-diacetate (Product 3a)

Afford as colorless gum. IR: ν_{max}2951, 2867, 1737, 1462, 1381, 1369, 1239 and 1026 cm^{-1}; ^1H NMR ($CDCl_3$): 4.99-5.10 (m, 1H, H-3), 4.86-4.99 (dd, 1H, H-6), 2.04, 2.00 (both s, 6H, the acetyl CH_3); 0.90 (s, 3H, CH_3-19), 0.61 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 11.8, 16.2, 18.5, 21.2, 21.3, 22.4, 22.7; (CH_2): 21.0, 23.7, 24.0, 25.9, 28.0, 29.4, 32.3, 33.3, 36.0, 39.3, 39.6; (CH): 27.9, 33.7, 35.6, 42.7, 56.0, 56.1, 70.9, 76.2; (C): 40.6, 42.5, 76.5, 170.4, 171.4; MS (ES^+): m/z 522 ($M+NH_4^+$, 100), 487 (52).

5-Fluoro-5α-cholestane-3β, 4α, 6β–triol (Product 4)

M.p. 239-242°C; IR: ν_{max} 3500-3300, 2941, 2865, 1468, 1385 and 1070 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.26 (s, 1H, H-6), 3.75-3.97 (m, 2H, H-3 & H-4), 1.14 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); MS (ES^+): m/z 461 ($M+Na^+$, 100), 236 (60), 214 (70).

5-Fluoro-5α-cholestane-3β, 4α, 6β–triol-3, 4, 6-triacetate (Product 4a)

Foam. IR: ν_{max} 3470, 2954, 2871, 1753, 1473, 1371, 1245, 1225 and 1050 cm^{-1}; ^1H NMR ($CDCl_3$): 5.25-5.45 (dd, 1H, H-4), 5.08 (br s, 2H, H-3 & H-6), 1.18 (s, 3H, CH_3-19), 0.64 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 11.9, 15.4/15.5, 18.5, 20.9, 21.1, 21.4, 22.4, 22.7; (CH_2): 20.5, 23.7, 23.9, 25.0, 28.0, 31.4, 36.0, 39.3, 39.4; (CH): 27.8, 29.5, 35.6, 45.4, 55.3, 55.9, 66.1/66.7, 69.9/70.1, 72.2/72.3; (C): 40.2/40.4, 42.3, 96.3/99.1, 169.7, 170.0, 170.3; ^{19}F NMR ($CDCl_3$): 1.99, 2.11.

5-Chloro-5α-cholestane-3β, 4α, 6β–triol (Product 5)

M.p. 190-193°C; IR: ν_{max} 3500-3300, 2942, 2865, 1458, 1380 and 1068 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.28 (s, 1H, H-6), 4.07 (d, J=8.6 Hz, 1H, H-4), 3.95 (m, 1H, H-3), 1.30 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); MS (ES^+): m/z 472 ($M+NH_4^+$, 20), 214 (100).

5-Chloro-5α-cholestane-3β, 4α, 6β–triol-3-acetate (Product 5a)

M.p. 147-150°C; ^1H NMR ($CDCl_3$): 5.21 (m, 1H, H-3), 4.28 (s, 1H, H-6), 4.23 (d, 1H, H-4), 2.07 (s, 3H, acetyl CH_3), 1.29 (s, 3H, CH_3-19), 0.65 (s, 3H, CH_3-18).

4β-Chloro-5α-cholestane-3β, 5α, 6β–triol (Product 6)

M.p. 155-158°C; IR: ν_{max} 3500-3300, 2937, 2861, 1466, 1380 and 1012 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 4.33 (m, 1H, H-3), 4.18 (d, J=4.0 Hz, 1H, H-4), 3.81 (s, 1H, H-6), 1.44 (s, 3H, CH_3-19), 0.66 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 12.1, 15.9, 18.6, 22.5, 22.7; (CH_2): 20.3, 23.8,

24.0, 26.2, 28.1, 32.8, 34.4, 36.0, 39.4, 39.7; (CH): 27.9, 29.9, 35.7, 47.5, 55.9, 56.1, 67.7, 70.8, 76.9; (C): 38.8, 42.6, 75.8; MS (ES^+): m/z 214 (100).

3.1.3 Working-up procedures & Analytical methods

The solid obtained from the chemical synthetic methods was analysed by TLC first. For those compounds without acetate group attached, the eluent is 1:1, 2:1, 3:1 or 5:1 of DCM : Acetone, or 1:1 of Ethyl acetate : Hexane. For those compounds with acetate, the eluent is 20:1 of DCM : Acetone, or 1:3 of Ethyl acetate : Hexane. Take the eluent 3:1 of DCM : Acetone, or 1:1 of Ethyl acetate : Hexane for example. The spot of the starting material without acetate should appear at the position that retention factor (Rf, the ratio of the distance of the sample spot to the distance of the solvent front) equals to 0.5. With the opening of the epoxide ring, another one or two free hydroxyl groups would form. Therefore, the polarity of the product should be greater than the original starting material. Thus the spot position of the desired product should lower than the spot position of the starting material. That is, the product should have smaller retention factor.

If the spots on the TLC for the product were more than one, then the mixture was needed to do the column chromatography to separate the compounds one from another. Usually the eluent used in the column separation was different according to the different compound. For those compounds without acetate group, the eluent is 3:1 of DCM : Acetone, or 1:1 of Ethyl acetate : Hexane. For those compounds with acetate group, the eluent is 20:1 of DCM : Acetone, or 1:3 of Ethyl acetate : Hexane. The quantity of the silicon gel used was determined by the amount of the mixture. Generally, for the absorption of the sample, 2 g of the silicon gel was used for 1 g sample. For the column furnishing, 30 g silicon gel was used for 1 g sample. Approximately 2.2 ml silicon gel weighs 1 g. Wet column separation was often used, which is the silicon gel was soaked in the eluent first before it was furnished into the column. The column

separation might be repeated under the same condition until the compounds in the mixture was completely separated from each other and judging from the TLC, there was only one spot for each sample.

If only one spot on the TLC appears for one sample, this compound could be used for the NMR analysis.

The compound was analysed by the ^{1}H NMR first. The spectrum obtained was compared with the available standard spectrums to determine what it is and if it is the desired compound. To confirm the structure of the compound obtained with free hydroxyl groups, it usually went on a further step of acetylation, and then did the separation if needed, and also did the ^{1}H NMR testing.

For the determination of the product, ^{13}C NMR, IR, MS and optical rotation $[\alpha]_D$ were further recorded, as well as the melting point. For the product 4 with the fluoride atom attached at the position 5, the ^{19}F NMR was also tested. All the compounds were dissolved in $CDCl_3$ when performing NMR test.

3.2 Part II. Effects of benzoyl esters on epoxidation of cholest-4-en-3β, 6β-diols-6-acetate.

3.2.1 Chemical Synthesis Methods

General procedure for the sterol acetates hydrolysis (Zhao, 2002)

a. Hydrolysis by heating

The oxysterol acetate (2.0 mmol) was dissolved in ethanol (20 ml) (if it was not dissolved at room temperature heating the mixture to 40-50 °C), a solution of sodium hydroxide (0.4 g, 10 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 cooling down the mixture was poured into water and the solid was collected by filtration, if the product is oil then extracted with suitable solvent, dried with sodium sulfate and then the solvent was evaporated in vacuum to get the product.

b. Hydrolysis in room temperature

The oxysterol acetate (2.0 mmol) was dissolved in DCM and ethanol (make a small volume as possible), a solution of sodium hydroxide (0.4 g, 10 mmol) in water (2 ml) was added and the mixture was kept at room temperature for 1 hr. After cooling down the mixture was poured into water and the solid was collected by filtration. If the product is oil then extracted with suitable solvent, dried with sodium sulfate and then the solvent was evaporated in vacuum to get the product.

General procedure for acyl chlorides

To the solution of 0.1 mol substitute benzoic acid in 10 ml DCM, 0.5 mol thionyl chloride ($SOCl_2$) was added. The reaction system was heated and

kept refluxing for 4 hours, and then the result mixture was evaporated in vacuum and dried. It was used directly in the next step without further purification.

General procedure of esterification using DCC and DMAP (Hassner, 1978)

A solution of benzoic acid or benzoyl chloride (0.2 mol), N, N'-dicyclohexylcarbodiimide (DCC, 0.2 mol), the alcohol (0.011 mol) and 4-dimethylaminopyridine (DMAP, 0.002 mol) in diethyl ether or DCM (25-50 ml) was allowed to stand a room temperature until esterification was complete (3-16 hours) until the reaction finished judging from the TLC. The precipitated urea was filtered and the filtrate washed with water (3 X 50 ml), dried with sodium sulfate and the solvent was evaporated in vacuum in order that the product ester crystallized.

General procedure of mCPBA epoxidation (Zhao, 2002)

The cholest-4-en-3, 6-diol or its derivative (1.00 mmol) and mCPBA (207 mg, 1.20 mmol) were dissolved in DCM (10 ml) and the mixture was stirred at room temperature for a given period. The resulting mixture was washed with 10% aqueous sodium hydroxide and water, and then dried over sodium sulphate. Removal of the solvent gave the crude product for the ratio of the α and β epoxide ring determination.

3.2.2 The data of the starting materials and the products

Cholest-4-en-3β, 6β-diol-3, 6-diacetate (Starting material) (Zhao, 2002)

White solid. M.p. 133-135 °C; IR: ν_{max} 3500-3300, 2952, 2871, 1753, 1471,1383, 1240, 1008, 705 and 609 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 5.58 (s, 1H, H-4), 5.26 (s, 1H, H-6), 5.19 (m, 1H, H-3), 1.17 (s, 3H, CH_3-19), 0.69 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C (CH_3): 11.8, 18.4, 20.2, 20.9, 21.2,

22.3, 22.6; (CH_2): 20.6, 23.6, 23.9, 24.5, 27.9, 35.9, 36.3, 36.9, 39.3, 39.5; (CH): 27.7, 30.5, 35.6, 35.6, 53.6, 55.7, 55.7, 55.9, 69.9, 74.6, 127.4; (C): 36.3, 42.2, 143.5, 169.1, 169.9; MS (ES^+): m/z 509 ($M+Na^+$, 100).

Cholest-4-en-3β, 6β-diol- 6-acetate (Intermediate)

M.p. 136.4-137.6°C; $[\alpha]_D^{15}$= +10.5° (c 10.0, $CHCl_3$); IR: ν_{max} 3523, 2930, 2862, 2843, 1718, 1470, 1374, 1259, 1016 cm^{-1}, ^1H NMR ($CDCl_3$) δ_H 5.64 (s, 1H, H-4), 5.22 (m, 1H, H-6), 4.08 (m, 1H, H-3), 1.10 (s, 3H, CH_3-19), 0.67 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 6 X CH_3: 12.0, 18.5, 20.7, 21.6, 23.7, 24.0; 10 X CH_2: 20.7, 23.7, 24.0, 28.1, 28.8, 36.0, 36.6, 37.1, 39.4, 39.6; 9 X CH: 28.0, 30.8, 35.7, 53.9, 55.9, 56.0, 67.7, 75.6, 131.8; 4 X C: 36.7, 42.4, 142.2, 170.2; MS (ES^+): m/z 467 ($M+Na^+$, 37), 427 (100)

4β,5–epoxy–5β–cholestane–3β, 6β–diol, 3, 6-diacetate (Compare compound 1) (Zhao, 2002)

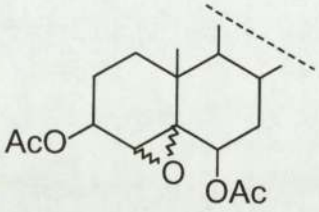
Obtained via recrystallization from methanol as off-white needles. Mp 160-161 °C (Lit.[18] 154-155°C); $[\alpha]_D^{15}$= -60° (c 10.0, $CHCl_3$; Lit.[18] $[\alpha]_D^{19}$ = -58.5°, c 1.0, $CHCl_3$); IR: ν_{max} 2948, 2871, 1743, 1463,1369, 1239 and 1027 cm^{-1}; ^1H NMR ($CDCl_3$): δ_H 5.01 (m, 1H, H-3), 4.51(m, 1H, H-6), 3.27 (d, J = 3.0 Hz, 1H, H-4), 1.14 (s, 3H, CH_3-19), 0.68 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 7 X CH_3: 11.8, 18.4, 18.9, 20.7, 21.0, 22.4, 22.6; 10 X CH_2: 21.9, 22.3, 23.6, 24.0, 27.9, 32.2, 35.2, 35.9, 39.2, 39.4; 9 X CH: 27.7, 29.9, 35.5, 50.0, 55.4, 55.9, 60.8, 68.6, 75.4; 5 X C: 35.6, 42.3, 64.8, 169.3, 170.1; MS(ES^+): m/z 525 ($M+Na^+$, 100).

4α, 5–Epoxy–5α–cholestane–3β, 6β–diol, 3, 6-diacetate (Compare compound 2) (Zhao, 2002)

Obtained via recrystallization from methanol as white needles. $[\alpha]_D^{15}$= +18° (c 10.0, $CHCl_3$); Mp 157-158°C; IR: ν_{max} 2954, 2850, 1743, 1465,1367, 1240 and 1027; ^1H NMR ($CDCl_3$): δ_H 4.93 (dd, J = 8.2 and 0.6Hz, 1H, H-3), 4.28 (m, 1H, H-6), 3.16 (s, 1H, H-4), 1.19 (s, 3H, CH_3-

19), 0.70 (s, 3H, CH_3-18); ^{13}C NMR ($CDCl_3$): δ_C: 7 X CH_3: 11.9, 17.0, 18.5, 20.4, 20.9, 22.4, 22.6; 10 X CH_2: 20.4, 22.8, 23.7, 23.9, 28.0, 29.3, 33.6, 36.0, 39.3, 39.4; 9 X CH: 27.8, 30.8, 35.6, 49.4, 55.2, 56.0, 62.2, 66.9, 74.2; 5 X C: 34.5, 42.4, 63.2, 169.5, 169.7; MS (ES^+): m/z 503 ($M+H^+$, 40), 525 ($M+Na^+$,100).

Data for comparing and for referring the positions of key peaks

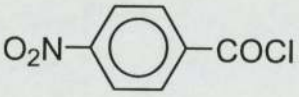


4, 5-α epoxide ring: ^1H NMR ($CDCl_3$): δ_H 4.93 (m, 1H, H-3), 3.16 (m, 1H, H-4);

4, 5-β epoxide ring: ^1H NMR ($CDCl_3$): δ_H 5.01 (m, 1H, H-3), 3.27 (m, 1H, H-4)

4, 5–Epoxycholestane–3β, 6β–diol - 6-acetate-3-substitute benzoyl ester (Products) with key peaks for judging

Different substitute benzoyl chlorides or benzoic acids at position 3:

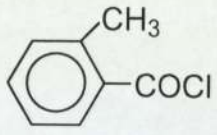


1.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(4-nitro benzoate), 6-acetate

4, 5-α epoxide ring: ^1H NMR ($CDCl_3$): δ_H 4.30 (m, 1H, H-3), 3.30 (m, 1H, H-4);

4, 5-β epoxide ring: ^1H NMR ($CDCl_3$): δ_H 4.53 (m, 1H, H-3), 3.37 (m, 1H, H-4)



2.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-methyl benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.30 (m, 1H, H-3), 3.35 (m, 1H, H-4);

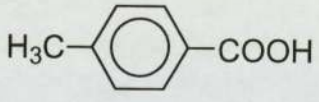
4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.53 (m, 1H, H-3), 3.40 (m, 1H, H-4)

3.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(4-methoxy benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.30 (m, 1H, H-3), 3.32 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.53 (m, 1H, H-3), 3.40 (m, 1H, H-4)

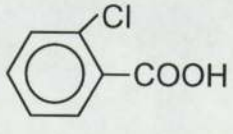


4.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(4-methyl benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.30 (m, 1H, H-3), 3.30 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.53 (m, 1H, H-3), 3.37 (m, 1H, H-4)

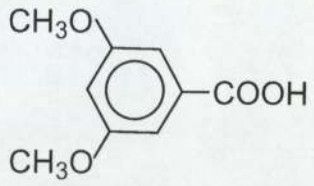


5.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-chloro benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.30 (m, 1H, H-3), 3.30 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.53 (m, 1H, H-3), 3.37 (m, 1H, H-4)

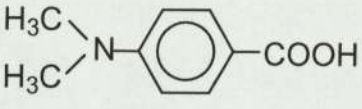


6.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3, 5-dimethoxy benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.31 (m, 1H, H-3);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.54 (m, 1H, H-3)

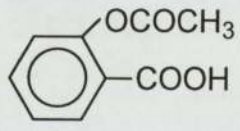


7.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(4-dimethylamino benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.29 (m, 1H, H-3);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.55 (m, 1H, H-3)

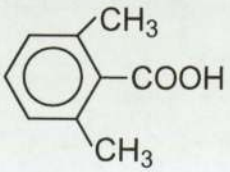


8.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-acetate benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.28 (m, 1H, H-3), 3.15 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.52 (m, 1H, H-3), 3.28 (m, 1H, H-4)

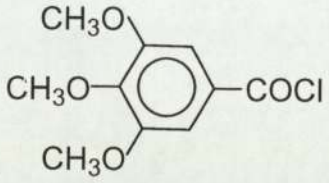


9.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2, 6-dimethyl benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.34 (m, 1H, H-3), 3.07 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.55 (m, 1H, H-3), 3.41 (m, 1H, H-4)

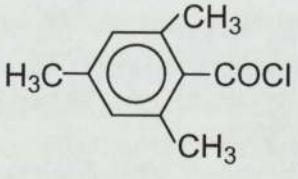


10.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3, 4, 5-trimethoxy benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.31 (m, 1H, H-3), 3.38 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.41 (m, 1H, H-4)

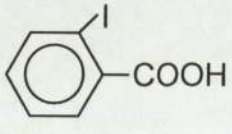


11.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2, 4, 6-trimethyl benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.34 (m, 1H, H-3), 3.29 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.54 (m, 1H, H-3), 3.41 (m, 1H, H-4)

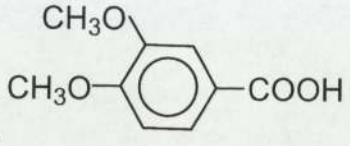


12.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-iodo benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.29 (m, 1H, H-3), 3.17 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.53 (m, 1H, H-3), 3.24 (m, 1H, H-4)

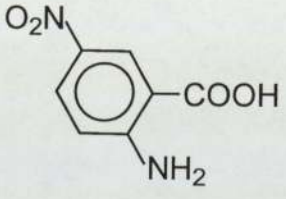


13.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3, 4-dimethoxy benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.33 (m, 1H, H-3), 3.35 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.57 (m, 1H, H-3), 3.41 (m, 1H, H-4)

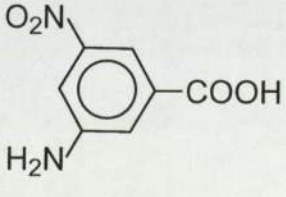


14.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3, 6-diamino benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.33 (m, 1H, H-3), 3.32 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.44 (m, 1H, H-4)

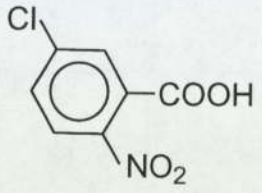


15.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3, 5-dinitro benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.29 (m, 1H, H-3), 3.37 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.57 (m, 1H, H-3), 3.46 (m, 1H, H-4)

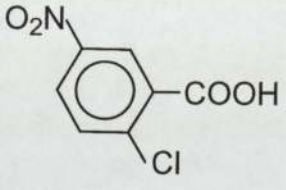


16.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(5-chloro-2-nitrobenzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.34 (m, 1H, H-3), 3.27 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.55 (m, 1H, H-3), 3.39 (m, 1H, H-4)

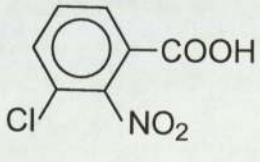


17.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-chloro-5-nitrobenzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.34 (m, 1H, H-3), 3.37 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.55 (m, 1H, H-3), 3.45 (m, 1H, H-4)

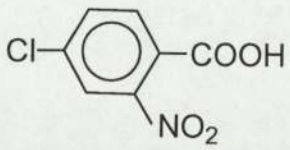


18.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3-chloro-2-nitrobenzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.37 (m, 1H, H-3), 3.27 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.55 (m, 1H, H-3), 3.37 (m, 1H, H-4)

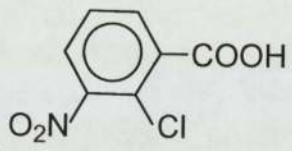


19.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(4-chloro-2-nitrobenzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.34 (m, 1H, H-3), 3.27 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.55 (m, 1H, H-3), 3.39 (m, 1H, H-4)

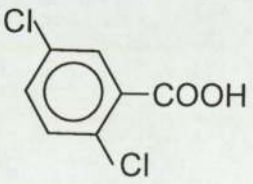


20.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-chloro-3-nitrobenzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.34 (m, 1H, H-3), 3.35 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.55 (m, 1H, H-3), 3.42 (m, 1H, H-4)

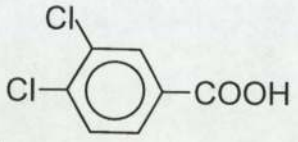


21.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2, 5-dichloro benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.34 (m, 1H, H-3), 3.34 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.41 (m, 1H, H-4)

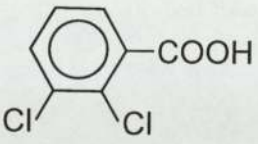


22.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3, 4-dichloro benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.30 (m, 1H, H-3), 3.32 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.40 (m, 1H, H-4)

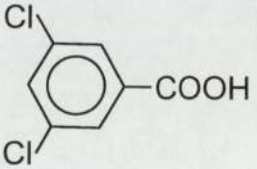


23.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2, 3-dichloro benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.33 (m, 1H, H-3), 3.34 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.41 (m, 1H, H-4)

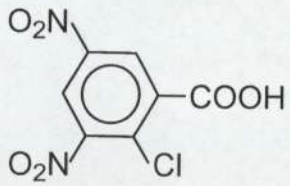


24.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(3, 5-dichloro benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.31 (m, 1H, H-3), 3.31 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.39 (m, 1H, H-4)

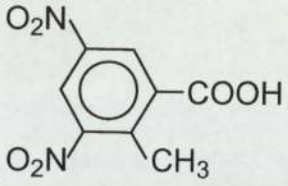


25.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-chloro, 3, 5-dinitro benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.29 (m, 1H, H-3), 3.40 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.45 (m, 1H, H-4)

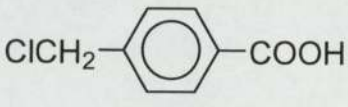


26.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(2-methyl, 3, 5-nitrobenzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.28 (m, 1H, H-3), 3.38 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.46 (m, 1H, H-4)

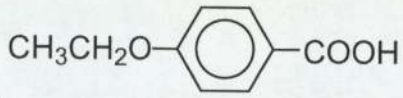


27.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(4-chloromethyl benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.32 (m, 1H, H-3), 3.33 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 3.41 (m, 1H, H-4)

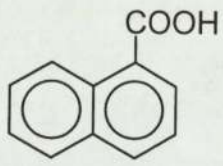


28.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(4-ethoxy benzoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.33 (m, 1H, H-3), 3.32 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.56 (m, 1H, H-3), 3.40 (m, 1H, H-4)

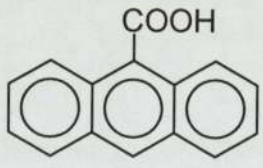


29.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(1-naphthoate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.38 (m, 1H, H-3), 3.45 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.60 (m, 1H, H-3), 3.51 (m, 1H, H-4)



30.

Cholestane-3β, 6β-diol, 4,5-epoxy, 3-(9-anthroate), 6-acetate

4, 5-α epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.41 (m, 1H, H-3), 3.54 (m, 1H, H-4);

4, 5-β epoxide ring: ^{1}H NMR ($CDCl_3$): δ_H 4.67 (m, 1H, H-3), 3.69 (m, 1H, H-4)

3.2.3 Working-up procedure & Analytical Methods

The solid obtained from the chemical synthetic methods was analysed by TLC first. The eluent is 20:1 of DCM : Acetone, or 1:3 of Ethyl acetate : Hexane. The spot of the starting material was at position with Rf value 0.8. After the acetate at position 3 was hydrolysed, the spot of the intermediate was at the position with Rf value 0.2. Thus, the polarity of the product with a free hydroxyl group was greater than the starting material with both hydroxyl groups acetated.

Generally, the products of hydrolysis were not pure with some starting material remaining. Therefore, there were two spots on the TLC. In order to ensure the quality of the following two steps, the mixture was needed to do the column chromatography to separate the compounds one from another. The eluent used was 20:1 of DCM : Acetone, or 1:3 of Ethyl acetate : Hexane. The quantity of the silicon gel used and the method of column separation were the same as described in the Working-up procedure and Analytical methods in Part I. The eluent was collected and evaporated to get the solid. And then this compound could be used for the NMR testing.

The compound was analysed by the ^{1}H NMR first. The spectrum obtained was compared with the available standard spectrums to determine what it is and if it is the compound desired. For the determination of the product, ^{13}C NMR, IR, MS and specific rotation $[\alpha]_D$ were further tested, as well as the melting point.

After the esterification, the intermediate obtained was also needed to do the column separation as described previously. When the relative pure esters were obtained, they were moved on to the final step and also the key step—epoxidation.

Principally, the crude product would not give furthering purification and did the ^{1}H NMR test directly, in case furthering purification would disturb the ratio of the product. Occasionally, some samples gave a mass on the NMR spectrum. And they had to be separated by the column chromatography as described before and did the NMR test

later one more time. While these two ways were compared later and it was found that the difference between with and without column purification to the ratio of product with α or β epoxide ring was not significant.

For the method of judging the ratio of the product with α or β epoxide ring, it was completely determined from the ^{1}H NMR spectrum.

First, the characteristic peaks that belong to the product with α or β epoxide ring respectively were recognized. Usually the peaks of H-3 and H-4 could be noticed more easily. The peaks that represent H-3 and H-4 belong to the product with α epoxide ring were often situated to the right of those belong to the product with β epoxide ring. That is, they were situated at higher field than the other two respectively. Usually, the peak of H-3 positioned around 4.28 – 4.60 ppm, and the peak of H-4 positioned around 3.15 -3.55 ppm.

Secondly, the four characteristic peaks were integrated respectively on the computer. Thereafter, the four peaks were enlarged in two pairs—the two peaks belong to H-3 on one paper and the other two represent H-4 on one paper. Cut off the areas of the four peaks from the spectra and weigh them using an accurate balance. The mass of those peaks was recorded for the calculation described blow.

The peak mass of H-3 of the product with β epoxide ring was divided by the peak mass of H-3 of the product with α epoxide ring. This value was called A.

The peak mess of H-4 of the product with β epoxide ring was divided by the peak mess of H-4 of the product with α epoxide ring. This value was called B.

The integration value of H-3 of the product with β epoxide ring by computer was divided by the integration value of H-3 of the product with α epoxide ring by computer. This value was called A'.

And the integration value of H-4 of the product with β epoxide ring by computer was divided by the integration value of H-4 of the product with α epoxide ring by computer. This value was called B'.

If A and A' or B', and/or B and A' or B' (at least one pair of them), had no significant difference (their difference was smaller than 4%), the ratio of product with α or β epoxide ring was the mean of the integration value by computer. Therefore the method of peak cut and weighed was just to confirm the integration value by computer.

Take the product mixture 1 for example. (From the spectrum HAN.130)

The weight of H-3 (4.3 ppm) of the product with α epoxide ring was 0.2141 g.

The weight of H-3 (4.53 ppm) of the product with β epoxide ring was 0.1346 g.

The weight of H-4 (3.3 ppm) of the product with α epoxide ring was 0.2897 g.

The weight of H-4 (3.37 ppm) of the product with β epoxide ring was 0.1545 g.

The ratio of the peak weight:

For H-3: 0.1346/0.2141=0.6287=A

For H-4: 0.1545/0.2897=0.5333=B

The ratio of the integration value by computer:

For H-3: 1/1.51=0.6623=A'

For H-4: 0.57/0.89=0.6404=B'

Since B was greatly different from A, A' and B'. It had to be ignored.

Because the difference between A and A' and B' was smaller than 4% (only 1%-3%), the ratio of product with α or β epoxide ring was the mean of the integration value by computer. That is,

(0.6623+0.6404)/2=0.65135

Therefore the product with β epoxide ring in the mixture occupied:

0.65135/(1+0.65135)=0.3944=39.44%

And the product with β epoxide ring in the mixture occupied: 1/(1+0.65135)=0.6056 =60.56%

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