SYNTHESIS AND CHARACTERISATION OF BIOMATERIALS FOR USE AS MARKERS OF HEALTH AND DISEASE

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Rakesh Natverlal Desai

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ABSTRACT

The bicyclic[4.3.0]nonane ring system is commonly found in many complex bioactive natural products, such as spongians and several novel steroids. Previous examples of ester tethered cycloaddition reactions were limited to activated dienes and dienophiles. The synthesis of a range of precursors, in which an unactivated diene and dienophile were linked *via* an ester tether is described. Model studies into the synthesis of monocyclic and bicyclic lactones as possible pre requisites for the formation of the C/D rings of the spongian skeleton utilising intramolecular Diels-Alder reaction (IMDA) or alternative cyclisation methods such as free radical catalysed and Heck reaction were carried out with these substrates. However it was discovered that when the carbonyl group of the ester tether was in conjugation with the diene it caused a formidable challenge as none of the applied methods were found to be suitable for the cyclisation reactions.

Chapters two to five were focused on attempts to synthesise glucuronides of phytoestrogen metabolites (isoflavones and isoflavans) glucuronide due to their potential interest as anti cancer agents. Also the steroidal hormone estrone glucuronide (for fertility testing) and testosterone glucuronide (for use in clinical laboratories and for drug testing) for the purpose of developing multipurpose home monitor by adapting the platform technology previously developed and used in a point-of-care monitoring device known as the Ovarian Monitor.

The synthesis of phytoestrogen glucuronide is a relatively new concept and the literature revealed no successful chemical method to date. The desired phytoestrogen isoflavones required for stereoselective glucuronidation were successfully prepared from precursor deoxybenzoins using a new convenient and facile route. Reduction of the isoflavones to isoflavans was also carried out using standard literature procedures. Various activated and deactivated phenols (including a sterically hindered phenol) were successfully glucuronidated using various synthetic routes as model studies. The information garnered from the model studies was utilised for the glycosylation of isoflavones and isoflavan but numerous attempts to obtained the glucuronides by using direct methods failed. Even more reactive glycosyl donors such as the sulfoxide sugar and acetimidate sugar also failed to effect glycosylation of these phytoestrogen metabolites. The relative insolubility and instability of the chromene ring under acid-base reaction conditions were compounding problems for the isoflavones.

A new alternative route involving synthesis of the *O*-glucuronides by the prior synthesis of the glycosides, hydrolysis to the glucosides and then TEMPO mediated selective oxidation of the primary alcohol was successful for simple phenol, sterically hindered phenol and the steroids estrone and testosterone. However, this alternative route also failed to effect glucuronidation of the isoflavones. Attention was thus focused on the synthesis of isoflavone glucuronides using UDP-glucuronyl transferase. Towards this end (±) methoxy equol glucuronide has been synthesised enzymatically, and purified using chromatographic methods. The attempted enzymatic synthesis of formononetin gave instead the cleavage product 2-hydroxy, 4'-methoxy deoxybenzoin glucuronide. The glucuronides were fully characterised by ^{1}H-^{1}H 2D-COSY, ^{1}H-^{13}C 2D-HETCOR and DEPT spectra and the results unambiguously showed the β-linkage of the glucuronide ring with the aglycon moieties. The presence of the glucuronide ring at the C-4 position in 2-hydroxy, 4'-methoxy deoxybenzoin glucuronide was also confirmed by a long range coupling experiment (HMBC).

The stereochemical integrity of the estrone glucuronide (E1G) obtained using perester coupling, acetimidate coupling and TEMPO catalysed oxidation methods were clinically tested by comparison with a standard curve obtained with a sample produced by the Koenigs-Knorr method. Testosterone glucuronide was studied for use as a biomarker to validate the concept of a multi-purpose home monitor for a variety of analyte glucuronides of clinical interest. Testosterone glucuronide antibodies with high affinity were generated by immunisation of sheep and testosterone glucuronide-HEW lysozymes conjugates were prepared. The standard curve for TG clearly showed it can be used for measurement of urinary TG at physiological concentrations. This methodology can be extended for analyte glucuronides of interest and can be used for development of biomarkers for health and disease. Thus a multi-purpose home monitor is now a reality and exciting commercial and practical applications are expected in the future.

Errata

Page	Line	Amendment
ii	18	an attempts should read attempts
iii	2	obtain the glucuronides by
vi	21	tetraene
viii	28	isoflavone
ix	1	donors
xiv	15	spectroscopy
xv	5	fourier
23	Scheme 1.16	hydrogenation
82	-	Figure 1.18
95	11	thyroglobulin
96	-	Ag-Ab complex
108	9	all
114	-	**2.15, 2.16, 2.18, 2.19** should read R_2=H
116	17	position
120	29	Blackwell
123	5	led
126	6	stereochemistry
129	5	Cyclopentadienyl
130	7	separation
131	14	boron
141	7	pyrrolidine
142	1	re-esterification
142	4	phosphorus
143	7	selectivity
145	8	intermediate
145	11	phosphorus
145	16	benzyl
152	13	recrystallised
156	4	Trihydroxy
171	13	Friedel-Crafts
178	14	acetic
181	8	recrystallisation
182	14	m-prepthalic

Page	Line	Amendment
183	8	$KMnO_4$
184	15	conveniently
186	30	(should be σ
187	4	Yield
189	-	OTs should be OH **(3.124)**
190	2	more deactivated
193	7	cleavage
193	5	bromosugar
194	3	glucuronidation
202	7, 9	acetyl
205	5	sulfoxide
209	24	*Lett.*
211	12	1988, *42B*
212	1	United states
219	19	4.2
247	4	Wellington
251	4	acetyl
264	11	2,6-dimethyl phenol
266	2	Stevenson
267	7	chromene
271	3	glucuronide
282	21	regioselectivity
288	21	desiccation
291	26	testosterone
292	2	dichlorophosphate
306	13	recrystallisation
318	5	Bowers[15]
318	23	co-workers[18]
337	31	*Lett.*
338	5	steroid.

All chemical names with propyonate should read propynyl ester in Chapter 1.

Scheme numbers should move up by text 1 in pages 61-77. 1.30 should read 1.31, 1.31 should read1.32 and so on.

This thesis is dedicated to my parents :

Natverlal K. Desai and

Manjula N. Desai

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ABBREVIATIONS

Δ	reflux
A_{280}	absorbance at 280 nm
AB	antibody
Ac	acetyl
AcOH	acetic acid
AIBN	2, 2'-azo-*bis*-isobutyronitrile
Aq.	Aqueous
Ar	aryl
BHT	2,6-di-*tert*-butyl-4-methylphenol
Bn	benzyl
BSA	bovine serum albumin
BTEAB	benzyl triethyl ammonium bromide
Bz	benzoyl
COSY	correlated specroscopy
CH_2Cl_2	dichloromethane
d	day(s) or doublet
DA	Diels-Alder
DCC	dicyclohexylcarbodiimide
DEPT	distortionless enhancement by polarization transfer
DIBAL-H	diisobutylaluminium hydride
DMAP	*N,N*-dimethylaminopyridine
DMF	*N,N*-dimethylformamide
DMSO	dimethylsulfoxide
E1G	estrone glucuronide
E1G[H]	estrone glucuronide (acid form)
EI	electron impact
EIA	enzyme immunoassay
eq	molar equivalents
Et_2O	diethyl ether
EtOAc	ethylacetate
EtOH	ethanol

Et_3N	triethylamine
eV	electron Volts
FMO	frontier molecular orbital
FPLC	Fast protein liquid chromatography
FT	Frourier transform
h	hour(s)
H_2O	water
Hex	hexane
HETCOR	heteronuclear chemical-shift correlation spectroscopy
HEWL	hen egg white lysozyme
HMBC	heteronuclear multiple bond correlation spectroscopy
HOMO	highest occupied molecular orbital
HPLC	high performance liquid chromatography
Hz	hertz
I.D.	internal diameter
IR	infra-red
IMDA	intramolecular Diels-Alder reaction
LUMO	lowest unoccupied molecular orbital
M	mol L-1
MA	maleic anhydride
Me	methyl
MeOH	methanol
Min	minute
MP	melting point
NHS	*N*-hydroxysuccinimide
NK	natural killer
NMR	nuclear magnetic resonance
Ph	phenyl
PhMe	toluene
PhH	benzene
ppm	parts per million
Py	pyridine
RIA	radio imminoassay
R_f	retention factor

RT	room temperature
s	singlet
S.M.	starting material
SOMO	singly occupied molecular orbital
t	time or triplet
ΔT	change in transmission
TBHS	tetrabutyl ammonium hydrogen sulfate
TBAB	tetrabutyl ammonium bromide
TG	testosterone glucuronide
TIMC	tandem intramolecular cyclisation
TIMDA	tandem intramolecular Diels-Alder
TLC	thin layer chromatography
Tris	tris(hydroxymethyl)aminomethane
WHO	world health organisation

CHAPTER 1 NEW SYNTHETIC STRATEGIES FOR SPONGIAN DITERPENOIDS

1.1 INTRODUCTION

1.1.1 Spongian Diterpenoids: Isolation, Structure And Biological Activity

A wide range of biologically important natural products are terpenoid in origin. From as early as 1800 considerable attention has been focused on the isolation, structural determination, biosynthesis and total synthesis of this large family of natural products. These efforts can be attributed principally to their central role in the agrochemical, pharmaceutical and perfumery industries. Originally, the word 'terpene' was applied to unsaturated acyclic and cyclic hydrocarbons of the formula $C_{10}H_{16}$ (now referred to as monoterpenes). However as more compounds were isolated use of the term 'terpene' was extended to include, for example, the sesquiterpenes ($C_{15}H_{24}$), the diterpenes ($C_{20}H_{32}$) and the triterpenes ($C_{30}H_{48}$). Spongian diterpenoids are an important class of naturally occurring diterpenoid compounds and a wide variety are available from marine organisms.[1] Spongian diterpenoids can be further classified as degraded tricyclic spongians (eg. Aplytandione **1.1**[2]), the recently found haumanamide **1.2**[3] forms another class of nitrogenous spongian and oxygenated spongians which include pentacyclic spongians (eg. 15,17-oxido spongian 16-one **1.3**[4]) and tetracyclic spongians with a D-ring lactone (eg. spongian 16-one[5] **1.4**) or a D-ring furan (eg. Spongiadiol **1.5**[5]).

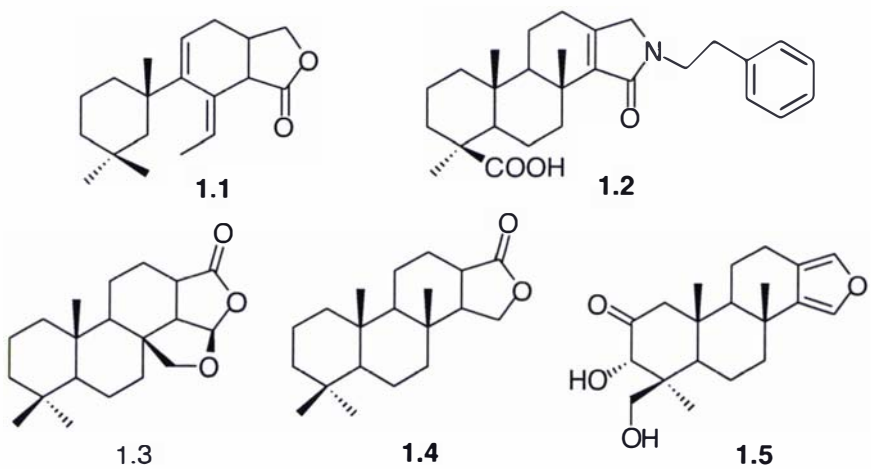
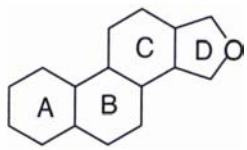


Figure 1.1

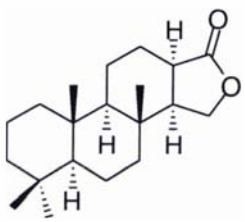
A common variety of spongian diterpenoids with a common tetracyclic skeleton show structures based on the hypothetical spongian skeleton **1.6** (**Figure 1.2**) and can be isolated from marine sponges and nudibranches.[6,7]



1.6

Figure 1.2

Many of these naturally occuring tetracyclic diterpenoids have important antimicrobial activity whilst others have shown activity against the Herpes simplex virus, type 1 and P 388 murine leukemia cells and hence, great potential for medicinal applications. Examples of such potentially useful diterpenoids are spongian-16-one **1.4** and spongiandiyl acetate **1.7** which were isolated from the marine sponges *Dictyodendrilla cavernosa*[8] and *Chelonaplysilla violacea*[9] found off the coasts of Australia and New Zealand. Representative members of this family of diterpenes include spongianenones **1.8** and **1.9** produced by the Canary Island sponge *Spongia officinalis*,[10] and the furano diterpenes spongiadiol **1.10** and isospongiadiol **1.5** isolated from the deep water Caribbean sponge *Spongia linnaeus*.[11]



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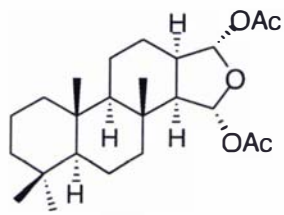

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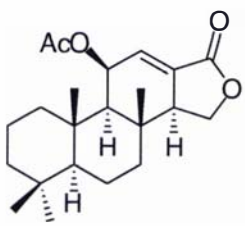

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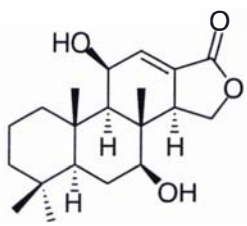


1.9

Figure 1.3

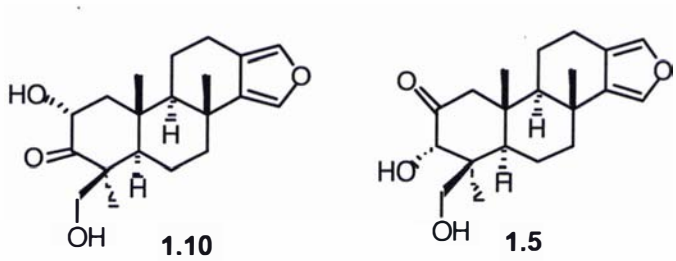
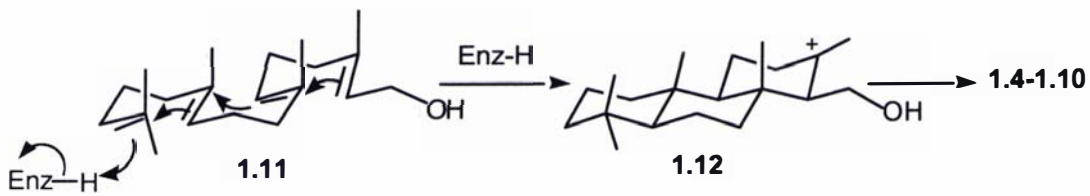


Figure 1.4

In a similar manner to the way that nature elaborates the steroid ring system *via* enzyme mediated electrophilic cyclisation of squalene oxide, the carbocyclic backbones in metabolites **1.4** –**1.10** are produced in nature via an electrophilic tandem cyclisation of a preorganised geranylgeraniol substrate as indicated in **Scheme 1.1**.



Scheme 1.1

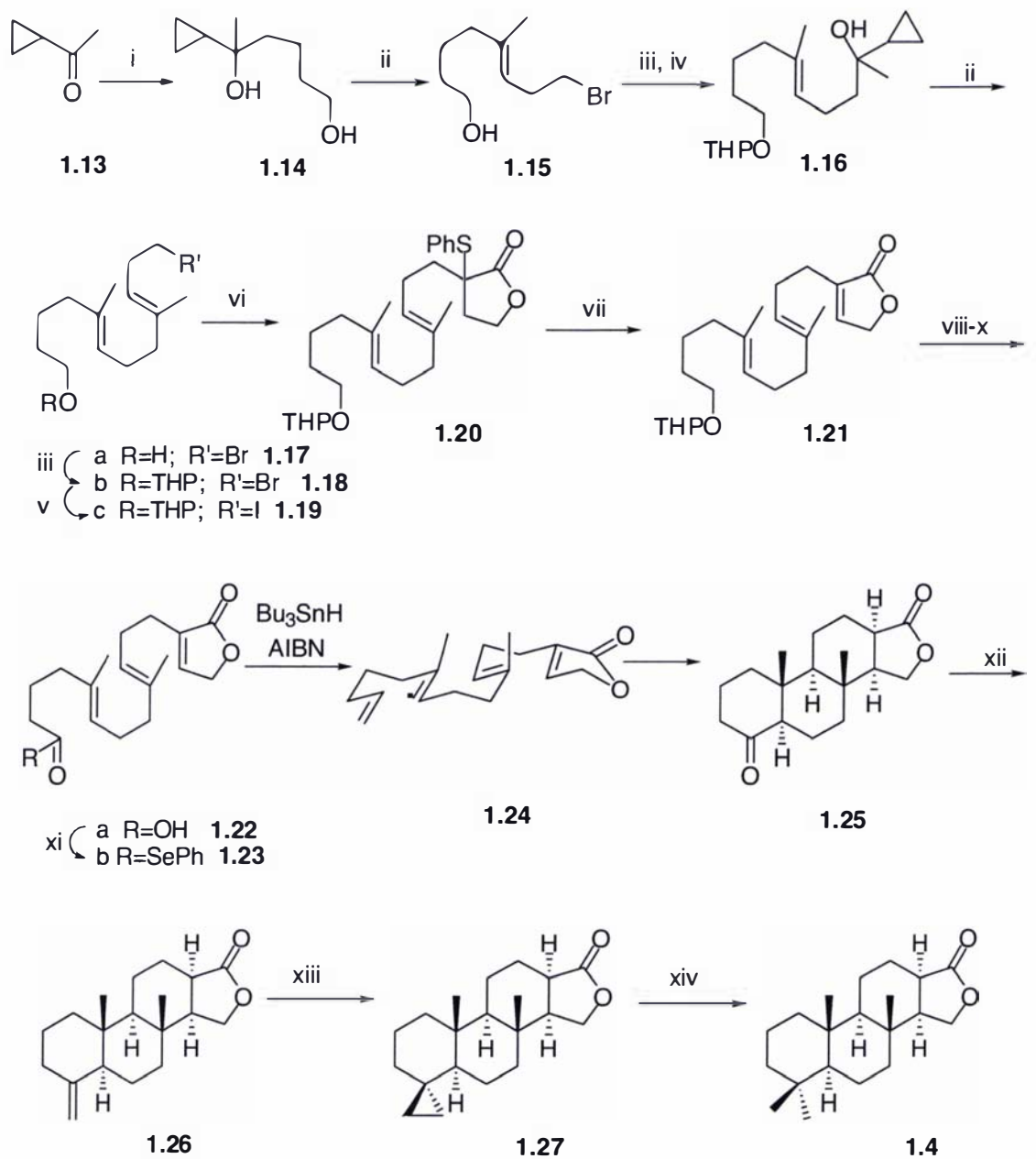
Subsequent reactions of the carbocation intermediate can give rise to a variety of tetracyclic diterpenoid compounds.

1.1.2 Previous Synthesis Of Spongian-16-One (1.4)

Previous reported synthesis of spongian-16-one is limited to only one paper published by Pattenden and Roberts in 1996[5] during the course of our research. In an elegant display of the total synthesis of spongian-16-one they have demonstrated how acyl/alkyl radical cyclisations of polyene selenoates, in the presence of Bu_3SnH-AIBN, can lead to linear and angular six-membered fused polycycles *via* cascade radical processes.

The synthesis started with commercially available cyclopropyl methyl ketone **1.13** using the strategy of Takano *et al.*[12] Reaction of **1.13** with the Grignard reagent prepared from 4-chlorobutan-1-ol, gave the diol **1.14** which, upon treatment with

hydrobromic acid according to Julia's method,[13] afforded the bromo alcohol **1.15**. Protection of **1.15** as its tetrahydropyranyl ether followed by lithiation and reaction with cyclopropyl methyl ketone gave the substituted cyclopropylmethanol **1.16**. Ring opening of **1.16** using 48% HBr at -20°C led to the homoallylic bromide **1.17**, which was then reprotected as its tetrahydropyranyl ether **1.18** and converted to the corresponding iodide **1.19** under Finkelstein conditions. Addition of the iodide **1.19** to the lithium enolate derived from 2-phenylthiobutyrolactone next gave the substituted butyrolactone **1.20**, which on oxidation and elimination of the elements of phenyl sulfinic acid was converted into the corresponding butenolide **1.21**. A series of functional group interconversions then converted the tetrahydropyranyl ether group in **1.21** into the carboxylic acid **1.22**, which on treatment with N-phenylselenopthalimide-Bu_3P finally gave rise to the central diene butenolide selenoate intermediate **1.24**.



Reagents and Conditions : *(i) $ClMg(CH_2)_3OMgBr$, THF, -25^{0}C, 81%; (ii) 48% HBr, -18^{0}C, 87%; (iii) DHP, PPTS, 25^{0}C, 95%; (iv) Li, THF, O^{0}C, Methyl cyclopropyl ketone, 80%; (v) NaI, Me_2CO, 25^{0}C, 89%; (vi) 2-Phenyl thiobutyrolactone, LDA, HMPA, -78^{0}C (vii) m-CPBA, -78^{0}C to 0^{0}C then Δ, C_6H_5Me, $CaCO_3$, 80^{0}C; (viii) PPTS, EtOH, 55^{0}C, 94%; (ix) Dess-Martin periodinane, 89%; (x) NaH_2PO_4, tBuOH, H_2O, $NaClO_4$, 2-Methylbut-2-ene, 82%, (xi) N-phenyl seleno pthalimide, Bu_3P, -30^{0}C, 88%; (xii) Zn, $TiClO_4$, CH_2Br_2, 0^{0}C, 79%; (xiii) CH_2I_2, Zn (Cu), Et_2O, Δ, 95%; (xiv) PtO_2, H_2, AcOH, 25^{0}C, 80%.*

Scheme 1.2

The diene butenolide selenoate **1.23** in dry degassed benzene was refluxed with Bu_3SnH addition over 8 h using a syringe pump in the presence of AIBN to give three consecutive 6-*endo*-trig radical cyclisations from the acyl intermediate **1.24** to give a single diastereoisomer tetracyclic keto-lactone **1.25**. The keto-lactone **1.25** was then methylated and converted to the cyclopropane intermediate **1.27**, which on hydrogenolysis gave the final product spongian-16-one. This is clearly a complex but elegant reaction scheme. The key intermediate is **1.24** which is designed for tandem radical cyclisation (**Section 1.1.5.3**) and the lactone ring is already incorporated being part of the tandem system.

There are many similar biologically active natural products with great potential for medicinal and other uses. In order to make these compounds easily available for testing and subsequent clinical use, simple, general and efficient syntheses need to be designed which can also utilise tandem cyclisations. Before discussing an alternative synthetic strategy it is necessary to review the standard cyclisation methods.

1.1.3 The Diels-Alder Reaction*

The potential of the Diels-Alder (DA) reaction has become realised as part of synthetic organic chemistry, since its inception in 1928.[15a] While a literature survey revealed few examples of such reactions, the majority of researchers remained unaware of the significant wide applicability of DA reactions in synthesis.[15b] The important siginificance of the diene synthesis was recognised by the award of the 1950 Nobel Prize in chemistry jointly to Diels and Alder "for their discovery and development of the diene synthesis". [15b] The DA reaction is today one of the very widely used methods for the synthesis of the six membered ring systems found in many natural products possessing bioactivity.[16]

The DA reaction involves the formation of a cyclohexene ring by 1,4 addition of an alkene (dienophile) to a conjugated diene *via* a thermally allowed pericyclic process (**Figure 1.5**). The diene can exist in a *cisoid* and a *transoid* conformation, but only *cisoid* form can undergo a DA reaction. If the dienes does not have or cannot adopt a *cisoid* conformation no DA reaction takes place for steric reasons. In general the reaction takes place easily, simply by mixing the components at room temperature or by

* Parts of this section are based on the review of Williamson.[14]

gentle warming in a suitable solvent. When the diene or dienophile has suitable functionality up to four new stereogenic centers can be obtained in a single reaction, allowing complex structures to be constructed very quickly.

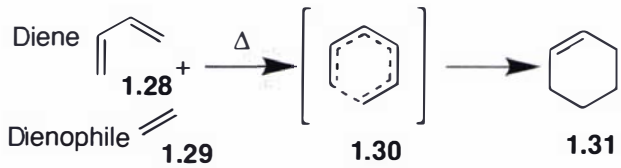


Figure 1.5

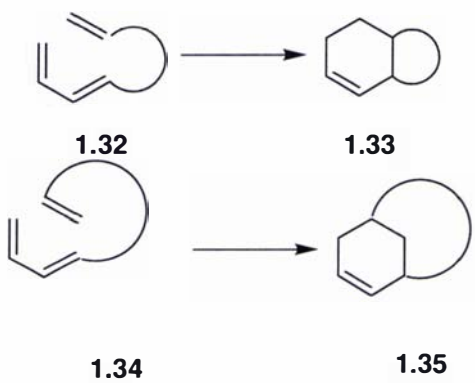
The essential feature is that the two components should have complementary electronic character. The vast majority of DA reactions involve an electron rich diene and an electron deficient dienophile. The energy difference between the LUMO (lowest unoccupied molecular orbital) of the dienophile, and the HOMO (highest occupied molecular orbital) of the diene is thus reduced. This interaction of the HOMO and LUMO raises the energy of the frontier orbitals and reduces the amount of thermal energy required for a successful DA reaction.[17]

1.1.3.1 The Intramolecular Diels-Alder Reaction

The application of an intramolecular DA (IMDA) reaction is an elegant and valuable synthetic strategy for the construction of complex natural products.[14,26] The increased reactivity (due to a favourable entropy factor) and heightened regioselectivity (due to constraints posed by the connecting chain) accounts for the explosive growth of IMDA reactions in natural product synthesis. The DA reaction proceeds through a highly ordered transition state which is reflected in large negative activation entropies.[18] In the IMDA reactions, some of the ordering has been accomplished in advance by covalently linking the two reacting functionalities (the diene and the dienophile) as part of the same molecule.[18] This results in less negative activation entropies and increased reaction rates under often surprisingly mild conditions. The length of the chain connecting the diene and dienophile also has considerable influence on the rate of the IMDA reaction, with any entropic advantage of the reaction nearly vanishing in substrates with five or more atoms in the chain.[18,19] Trans dienes containing chains of three or four atoms constitute the majority of the substrates known to undergo the

IMDA reaction. The nature of the substituents in the chain is also an important factor in influencing the rate of the reaction. Careful design of the linker might give a direct entry into the spongian 16-one type skeleton.

The most significant aspect of the IMDA reaction is its potential efficiency.[14] The increased reactivity and heightened regioselectivity as well as potential for the synthesis of stereochemically complex polycyclic molecules account for the growth of applications of IMDA reactions in organic and particularly natural product synthesis. IMDA reactions are conveniently divided into two main classes based upon the mode of connection of the diene to the dienophile[19]. *Type* 1 reactions involves trienes in which the connecting chain is attached to the diene terminus (e.g triene **1.32**) while in *Type* 2 reactions, the dienophile tether is attached to an internal diene position (e.g triene **1.36**). The number of atoms present in the diene and dienophile moieties are very important as this factor has a dramatic effect on the regio and stereo selectivity of IMDA reactions. Although both regio isomers are possible, leading to a fused product (**1.33**) and a bridged product (**1.35**), with three to five atoms in the tether, the fused isomer (**1.33**) is highly preferred. For trienes with tethers of less than 3 atoms no reaction occurs due to highly strained transition states leading to either product. With tethers containing ten or more atoms the reaction is more likely to form bridged products (**Figure 1.6**).[19]



Type 1 IMDA Reaction

Figure 1.6

Type 2 IMDA reactions are restricted to only bridged products, as shown in **Figure 1.7**. The reaction can form a *meta*-bridged adduct (**1.37**) and a *para*-bridged

adduct (**1.39**) with tethers of up to five atoms favouring *meta*-bridged isomer exclusively.[18] The focus of this chapter is on **Type 1** IMDA reactions.

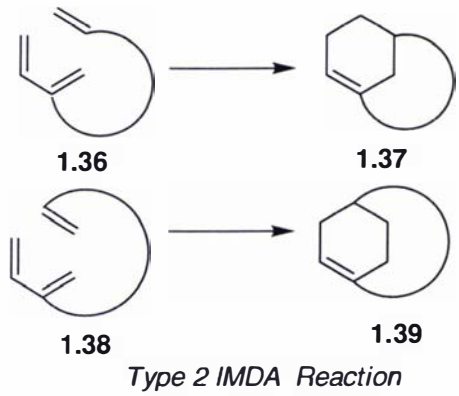


Figure 1.7

There is unanimous agreement that the IMDA reaction proceeds through a concerted mechanism. The few exceptions where the stereo chemical integrity of the diene or the dienophile do not remain intact are isomerisation of substrates prior to cyclisation or to epimerisation of the final product.[18] The cyclisation of *E* -dienes in IMDA reactions is more common than for *Z* -dienes due to its reactivity which allows an *E* –diene to form a *trans* adduct *via* an *exo* transition state whereas *Z* -dienes form a *cis* -adduct *via* an *endo* -transition state as shown in **Figure 1.8**.[18] Substrates containing *E* -dienes can undergo the IMDA reaction even when the chain contains only one or two atoms.

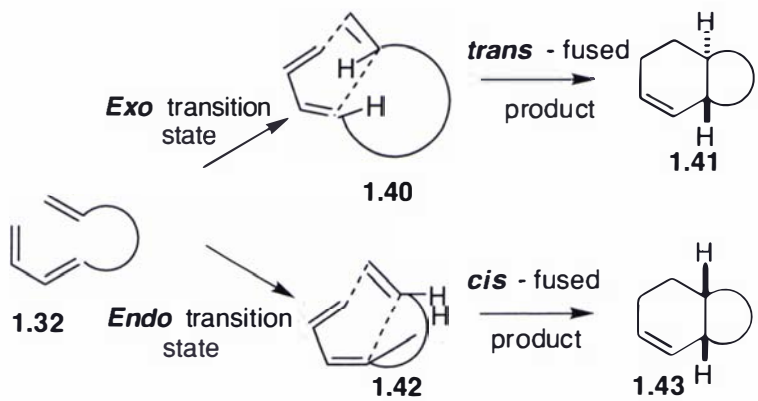


Figure 1.8

In this chapter the prefixes *Z* and *E* are used in connection with the diene to denote the stereochemistry of the double bond attached to the tether while the prefixes

endo and *exo* are used to acknowledge the position of tether group. Similarly, when the linkage between the diene and dienophile is oriented towards the cyclohexene ring it is termed an *endo* transition state and if it is facing away it is termed an *exo* transition state.

1.1.3.2 Ester-Tethered IMDA Reactions

The linkage present between the diene and dienophile can influence the rate and stereochemical course of the IMDA reaction. Several factors such as the nature of any substituents present on the tether, ring strain. flexibility and electronic and steric effects can adversely effect the rate and selectivity of the reaction.[14,20] The versatility of the DA reaction is more obvious in ester tethered DA reactions. The ester group can be oriented in several different ways. In general the structure **1.44** can be obtained from a reaction between a dienoic acid and an alkenol, whereas structure **1.45** is formed from condensation of a dienol with an alkenoic acid. Structure **1.44** is of special relevance to the synthesis of spongian 16-one which requires that the carbonyl functionality to be adjacent to the diene moiety.

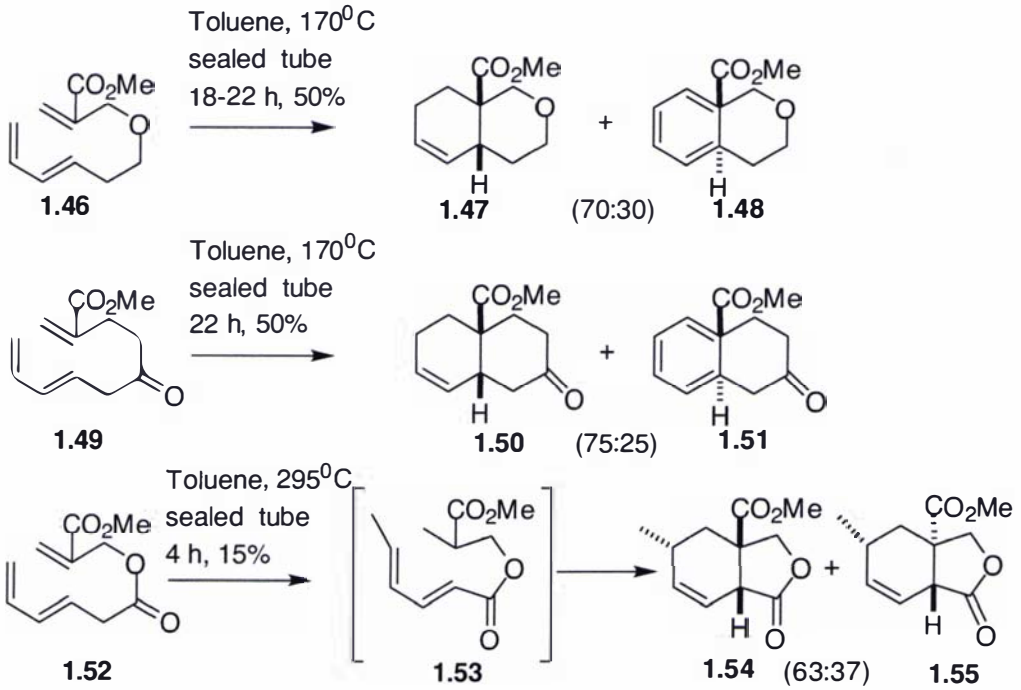
P, q, r, s = 1, 2, 3...

1.44 **1.45**

Figure 1.9

In general, incorporation of an ester linkage into the chain often has an adverse effect on the rate of the IMDA reaction. This is evident by the requirement of higher temperatures or in some cases even failure to obtain any cyclised product. Boeckman and Demko[22] investigated a series of IMDA reactions as shown below (**Scheme 1.3**) and demonstrated that the presence and location of heteroatoms in the connecting chain and overlap requirements of these groups can have a substantial influence on the reaction rate and the nature of the products resulting from intramolecular cycloaddition. When the ether linked trienone ester (**1.46**) and ketone linked trienone ester (**1.49**) were subjected to thermolysis in toluene at 170^{0}C in a sealed tube for 18-22 h (not optimized) the reaction gave exclusively unrearranged bicyclic products. However thermolysis of

triene **1.52** at a variety of temperatures up to 220^{0}C resulted in only a slow degradation of the dienophile segment in **1.52**. Reinvestigation of the cyclisation of triene **1.52** by thermolysis in toluene at 295^0 C for 4 h in a sealed tube afforded only rearranged γ-butyrolactone adducts **1.54** and **1.55** in 15% yield.[22]



Scheme 1.3

The reason for the low reactivity of IMDA precursors bearing ester tethers can be explained in terms of *transoid* effects. The unfavourable dipole-dipole interactions in the s-*cis* conformation (which has the dipoles aligned and hence is the higher energy isomer) causes the ester to adopt the s-*trans* conformation, which does not dispose the molecule towards intra molecular cycloaddition.[22] This is true irrespective of whether the carbonyl or oxygen functionality is adjacent to the dienophile.

Figure 1.10

These facts suggest that the high kinetic barrier to cyclisation is the result of the electronic demands of the ester linkage in the transition state and hence for effective cyclisation, the ester linkage in the molecule must be rotated during formation of the transition state to give the less favourable s-*cis* conformer **1.56**. (**Figure 1.11**).

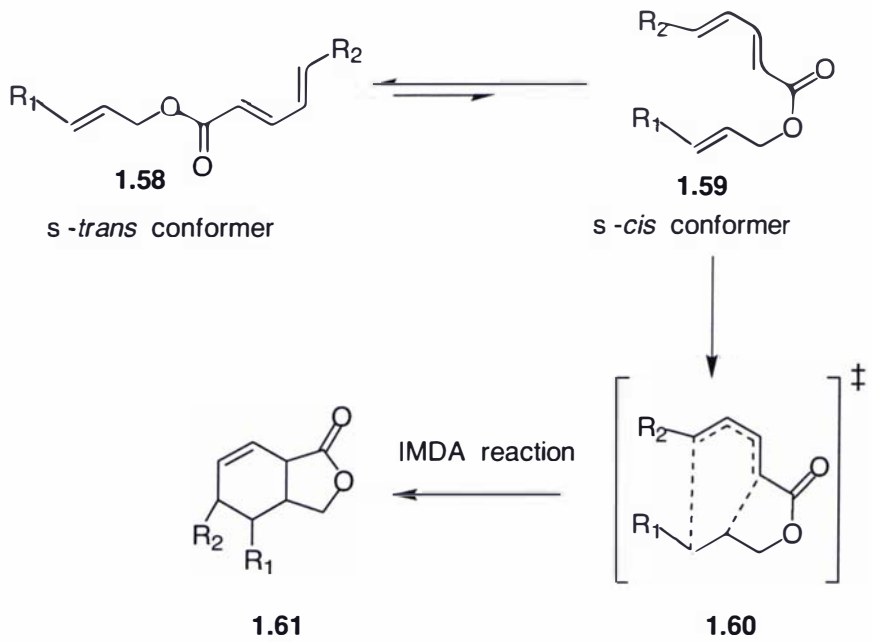
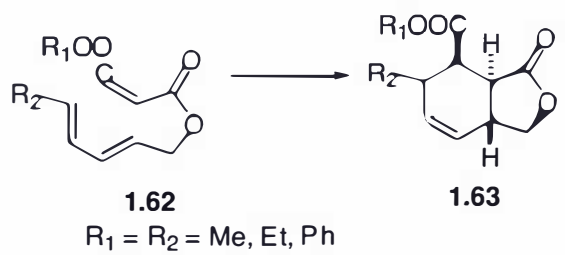


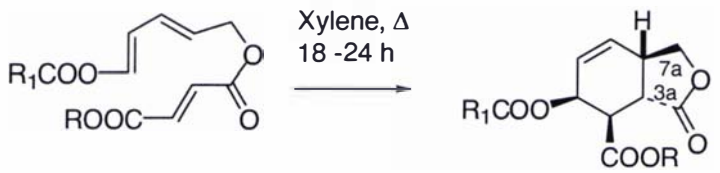
Figure 1.11

Although the ester tethered substrates have low reactivity, the literature reveals a number of successful examples of ester tethered IMDA reactions.[14] It has been well presented in the literature that precursors with three carbon atom tethers such as **1.62** effectively cyclise to form the *trans* -fused adduct **1.63**. It should be noted that these precursors have the carbonyl functionality adjacent to the dienophile, which is further activated by the $-CO_2R$ group.



Scheme 1.4

In a study by Becher and co-workers a series of triene esters **1.64**, **1.66**, and **1.68** were exposed to refluxing (**Scheme 1.5**) to yield the highly strained *trans* fused products. The NMR spectra of all products showed a significantly large coupling constant J (3a7a) = 13.3 Hz indicating a *trans* -fused ring system.[25,26]

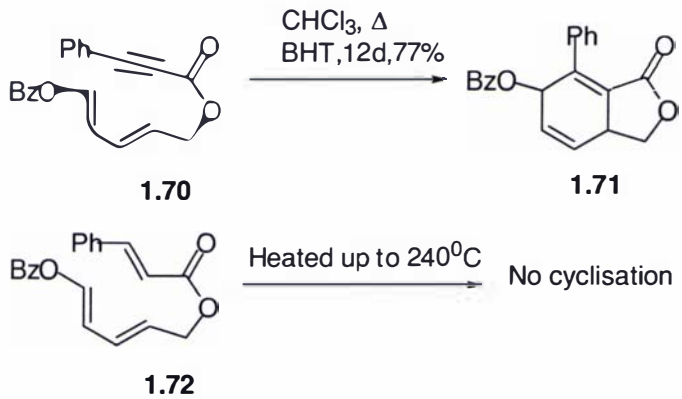


1.64 R_1 = Ph, R = Et
1.66 R_1 = PhCH=CH, R = Et
1.68 R_1 = Ph, R = Me

1.65 R_1 = Ph, R = Et 74%
1.67 R_1 = PhCH=CH, R = Et 13%
1.69 R_1 = Ph, R = Me 88%

Scheme 1.5

It is known that acetylene dienophiles are more reactive in the IMDA reaction than the corresponding olefinic dienophiles. Therefore Becher and co-workers also decided to investigate the acetylene system (**Scheme 1.6**) and found that acetylene substrate **1.70** was more reactive in the IMDA reaction of the compared with the olefinic counterpart **1.72**. Under milder conditions of refluxing in $CHCl_3$ **1.70** underwent cyclisation whereas its olefinic counterpart **1.72** failed to undergo cycloaddition even at 240^{0}C.



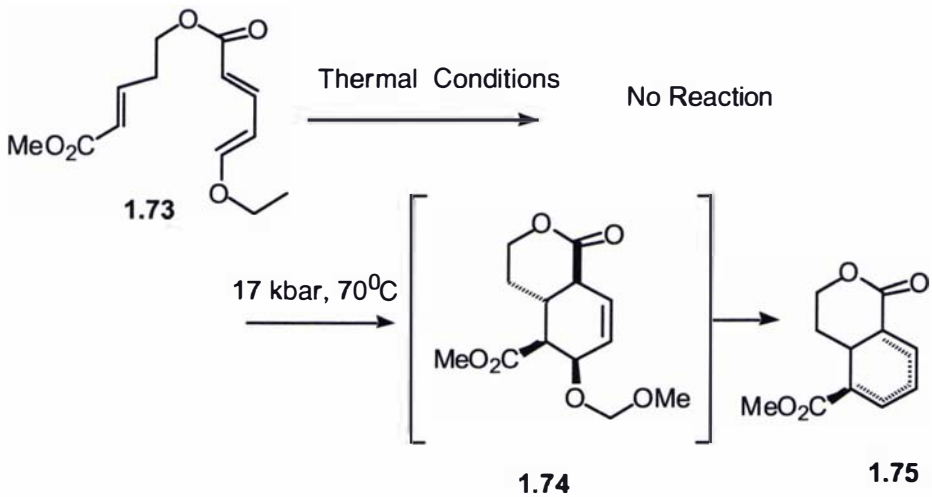
1.70 **1.71**

1.72

Scheme 1.6

When the carbonyl group of the ester tether was in conjugation with the diene and the tether linking the diene and dienophile consisted of four atoms a contrasting

result was seen. None of the expected cycloadducts were obtained under various thermal conditions (**Scheme 1.7**) [27]. Even when triene ester **1.73** was subjected to 17 kbar pressure at 20^{0}C no reaction occurred and at 70^{0}C triene **1.73** afforded a mixture of isomeric dienes **1.75**, albeit in low yield (<20%). The expected adduct probably underwent facile (1-2 diaxial) elimination of the alkoxy group, leading to isomeric dienes **1.75**.



Scheme 1.7

This result is significant since it represents the structural arrangement required for direct synthesis of the D-ring lactone in spongian-16-one.

The important aspects of successful ester tethered IMDA reactions are as follows:[14]

* They are *Type 1* IMDA reactions.

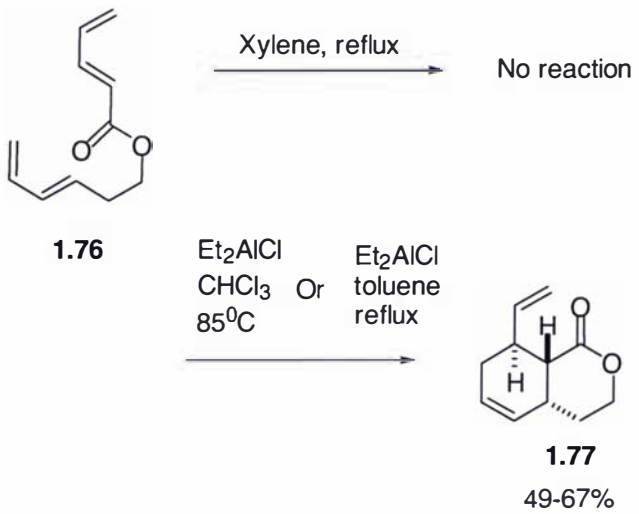
* The length of the chain linking the diene and dienophile contains three atoms.

* The carbonyl group of the ester linkage present between the diene and dienophile should be in conjugation with the dienophile.

* The diene moiety present in the substrate is either acyclic or semicyclic.

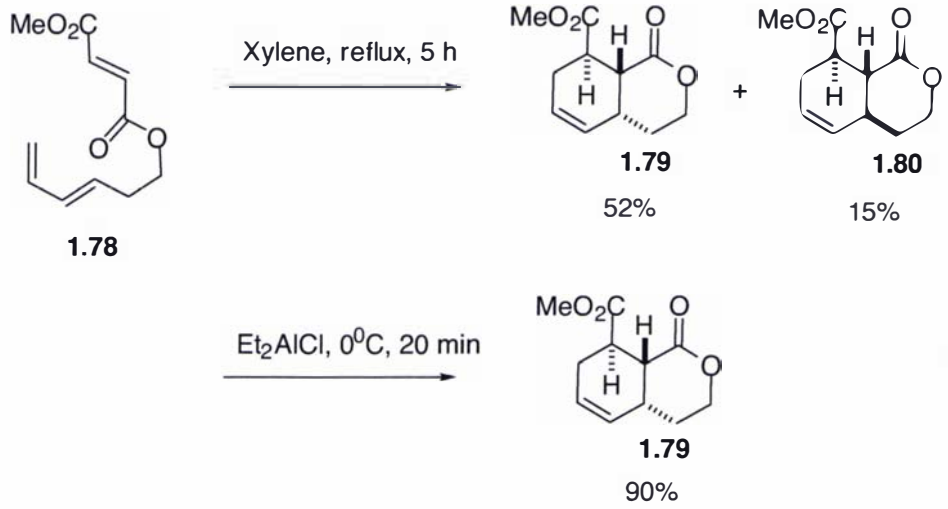
1.1.3.3 Lewis Acid Catalysis In Ester-Tethered IMDA Reactions

The influence of Leiws acids on the course of DA reactions has been known for some time, and generally greatly increased reaction rate and selectivity is observed. The literature also reveals use of Lewis acid catalysts in the ester tethered IMDA reaction.[28] It has been found that some Diels-Alder condensations are accelerated remarkably by Lewis acids such as aluminium chloride, boron trifluoride, diethyl aluminium chloride etc.[22] These effects are ascribed to complex formation between the catalyst (Lewis acid) and the polar groups of the dienophile (Lewis base) which bring about changes in the energies and orbital coefficients of the Frontier Molecular Orbitals (FMOs) of the dienophile. This results in a stabilisation of the transition state in the case of normal electron-demand DA reactions. For example thermolysis of the tetraene ester **1.76** in xylenes gave only polymer, but Lewis acids promoted the cycloaddition in chloroform at 85^{0}C or toluene at reflux to give the expected IMDA adduct **1.77** in 67% or 49% yields respectively.[29]



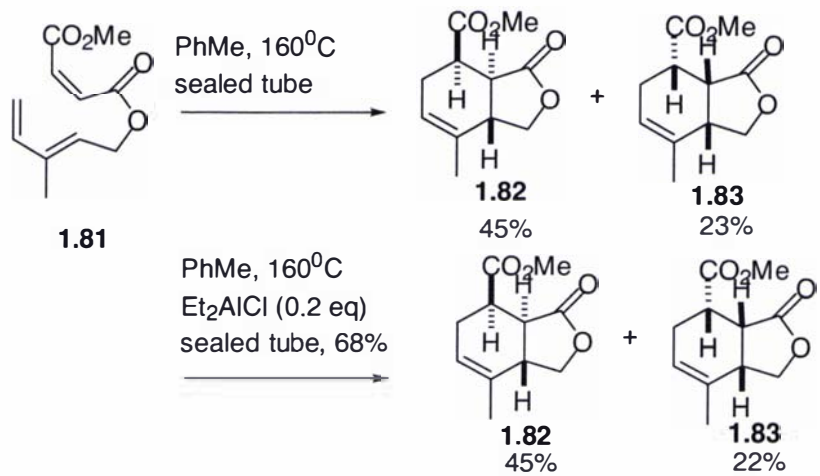
Scheme 1.8

When the tetraene ester **1.78** was heated in xylene at reflux, a mixture of cycloadducts **1.79** (52%) and **1.80** (15%) was obtained. When the reaction was repeated in the presence of Et_2AlCl only the *cis* fused product **1.79** was formed and the yield was significantly higher (90%).[29]



Scheme 1.9

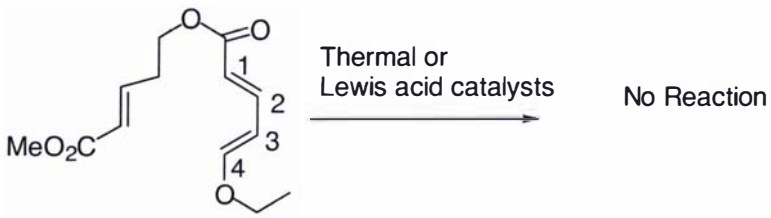
These results clearly demonstrated increased reactivity when the carbonyl group is present adjacent to the dienophile and an ester group is present at the terminus. Similar results were also obtained when the triene ester **1.81** was heated in toluene at 160^{0}C in a sealed tube yielding both *cis* and *trans* fused adducts **1.82** and **1.83** (**Scheme 1.10**). However it has been also found that the use of Lewis acids had no influence on the ester tethered IMDA reaction.[28]



Scheme 1.10

When the diene component of the precursor had an electron withdrawing substituent at position 1 and an electron donor substituent at position 4, a peculiar situation was found in a new class of dienes. Attempts were made to cyclise the triene

1.73 under various thermal or Lewis acid-catalysed conditions but the desired cyclo adduct was not obtained.[27]



1.73

Scheme 1.11

The reactions reported above clearly indicates that the use of Lewis acid catalysts in ester tethered IMDA reaction is of little benefit (if any) for effective cyclisation of the substrate with increased reactivity and selectivity. Clearly more examples of this situation need to be examined.

1.1.3.4 Summary Of Literature Work

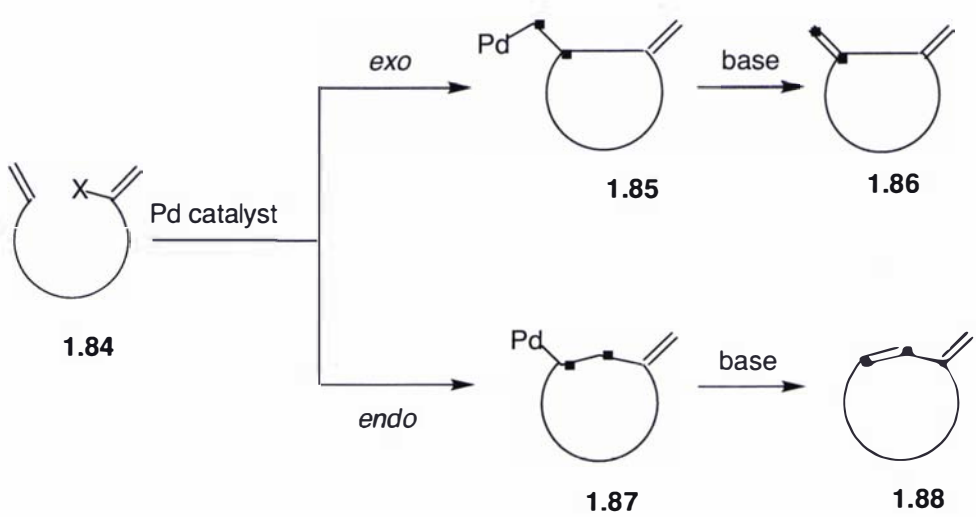
The above systematic study of ester tethered IMDA reactions provided new insights into factors affecting cyclisation. From the results presented above and elsewhere[14] it is clear that reactivity increases when the carbonyl group of the ester is in conjugation with the dienophile. The increase in reactivity was also seen for ester tethered IMDA reactions with acetylenic dienophiles compared to olefinic counterparts. When the carbonyl group of the ester tether is in conjugation with the diene it can cause a formidable challenge for the ester-tethered IMDA reaction.

1.1.4 Heck Reaction

Tandem cyclisation can also be carried out *via* the palladium-catalysed coupling of halo alkenes and halo arenes with alkenes known as the Heck reaction.[30] This has been a topic of fluctuating interest, however in the last decade activity in this area has flourished.

The Heck reaction can be represented most simply as shown in **Scheme 1.12**.[31] The reaction pathway depends on the factors such as metal catalyst used, substrate

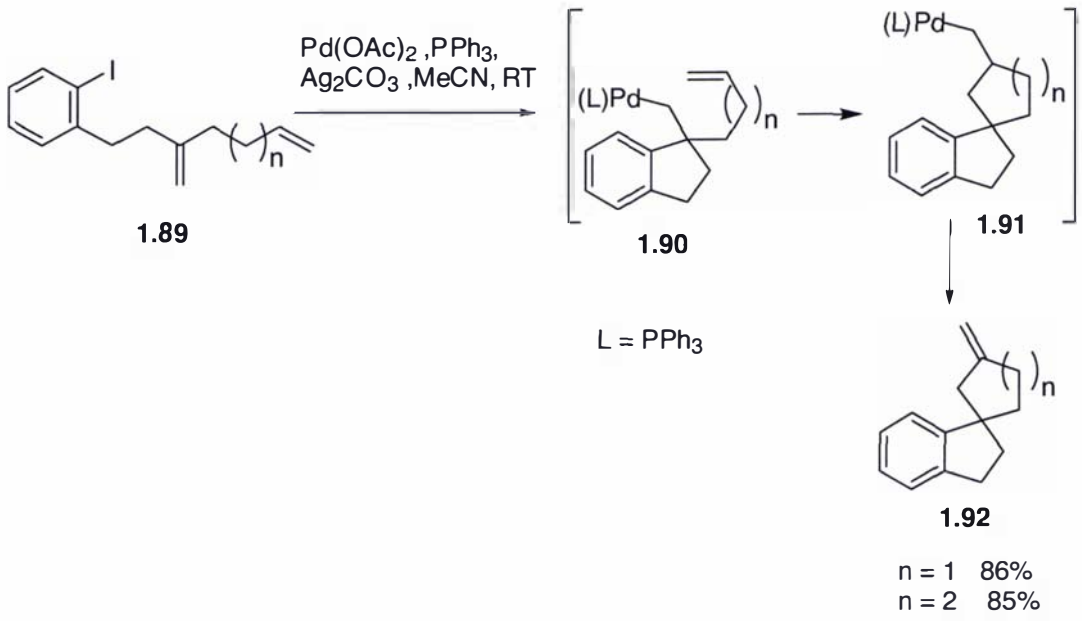
structure, and reaction conditions to give products **1.86** and/or **1.88** *via exo* and/or *endo* cyclisations, respectively.[31]



Scheme 1.12

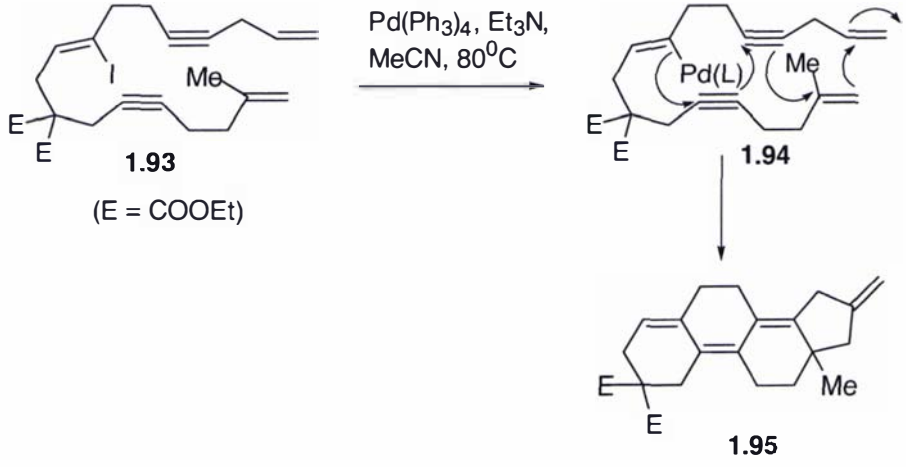
Combining multiple reaction steps in the same chemical transformation in most cases, is an elegant solution to synthetic problems. It was almost predictable, with the arrival of the Heck reaction, that its application in the construction of polycyclisation, or tandem cyclisations, would follow.[31] A tandem Heck cyclisation would allow the simultaneous construction of multiple rings in one pot *via* successive cyclisations.[31] The concept in which initially formed alkenyl palladium species are added to triple or double bonds either (re) generating an alkenyl or π allyl-palladium intermediate has proven to be particularly useful for the development of cascade reactions, as these intermediates cannot be removed, or at least not as rapidly, from the reaction by β-hydride elimination. Instead they again react with triple bonds and double bonds to give the cyclised product.[32]

A good example of the tandem Heck reaction is the cyclisation of the iodo compound **1.89** to afford spiro products **1.92** in a reasonable yields *via* two consecutive 5-*exo* cyclisations involving the organo palladium intermediates **1.90** and **1.91** (**Scheme 1.13**).[33]



Scheme 1.13

Meanwhile, increasingly higher orders of tandem Heck cyclisations are being used in to the construction of increasingly more complex polycyclic molecules.[34,35] The reaction shown in **Scheme 1.14**[36] is one version of a quadruple cyclisation involving three consecutive 6-*exo* and 5-*exo* cyclisations to give the steroid skeleton **1.95**. Interestingly, the neopentyl intermediate generated after the third cyclisation does not undergo a 3-*exo*-trig cyclisation to produce a three membered ring. Instead, a 5-*exo* - trig ring closure with participation of the terminal bond predominates.



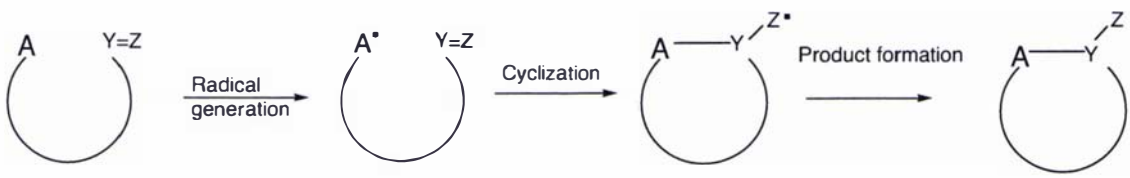
Scheme 1.14

Though research on the use of Heck reactions in organic synthesis is a hot area that has affected many investigators, a wealth of undiscovered synthetic possibilities clearly remains. It was hoped that this methodology might work if the ester tethered IMDA reaction failed and be applicable to the synthesis of spongian diterpenes and other natural products from ester-tethered precursors.

1.1.5 Free Radical Reactions

Radical cyclization reactions are amongst the most powerful and versatile methods for the construction of mono and polycyclic systems.[37,38] Interests in free radical reactions in organic synthesis has greatly increased and it is now clear that they have become an integral part of synthetic strategies in many laboratories.[39] The advantages these reactions offer to the synthetic organic chemist include high functional group tolerance and mild reaction conditions combined with a high level of regio-and stereochemical specificity.[40,41] Furthermore, recent progress in radical chemistry has led to the development of a broad range of very useful practical methods which can be used to conduct radical cyclisation reactions.

The majority of free radical reactions which are of interest to the synthetic organic chemist are chain processes and involve three basic steps: selective radical generation, radical cyclisation and conversion of the cyclised radical to the product **Scheme 1.15**.



1.96 **1.97** **1.98** **1.99**

Scheme 1.15

The success of radical reactions for a synthetic application is dependent on the "controlled generation" of the radical itself. Methods utilized for free radical generation are usually tin hydride mediated, and halides, selenides, and sulfur-containing

compounds have been used as radical precursors in most studies.[42] Many radical reactions are initiated directly using thermal or photochemical activation and in some cases initiators are also employed.[37] Although radical chain processes occur immediately at moderate temperatures, it is usually important to facilitate the chain propagation steps either by the addition of an initiator or by other means.[37] Among many, azo-bis-isobutyronitrile (AIBN) and related azo compounds are ideal initiators as they are safe, easily handled and have solvent-independent decomposition rates.[37,40,41]

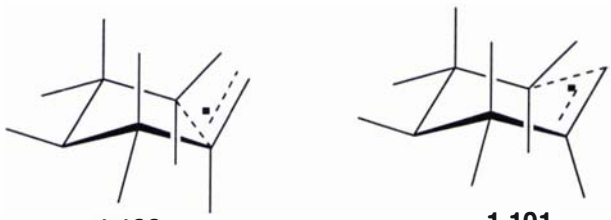
To achieve a synthetically useful radical cyclisation, several basic requirements have to be met :

1. Methods must be available that allow the selective generation of the initial radical from suitable reaction precursors and then effect the transformation of the cyclised radicals to the final products.

2. The rate constant for the ring closure reaction is of special significance as cyclisation of the initial radical must be faster than its subsequent reaction with the trapping substrate if significant yields of the product are to be obtained.

3. Each of the product forming reaction steps must be faster than the unwanted side reactions of radicals such as reaction with the solvent or radical recombination.

In principle, two competing pathways are possible - attack of the radical at the terminal end of the multiple bond (*endo* cyclization) or attack at the "inner" atom (*exo* cyclization). Radical cyclizations are usually highly regioselective, and *exo* cyclization (formation of the smaller ring) is often strongly favoured over *endo* cyclization (formation of the larger ring). The preferred formation of the thermodynamically disfavoured *exo* product from the 5-hexenyl radical is best rationalised by a stereochemically controlled cyclisation with a chair like transition state as in **Figure 1.12**.[42,43] This arrangement reflects the early transition state of the reaction with a favourable overlap between the SOMO of the radical and LUMO of the alkene. The forming C-C bond is very long (ca. 2.3-2.4 Å), and the angle of attack of the radical on the alkene (106^0) is close to the angle for a unstrained bimolecular reaction (109^0).



1.100 5-*exo*

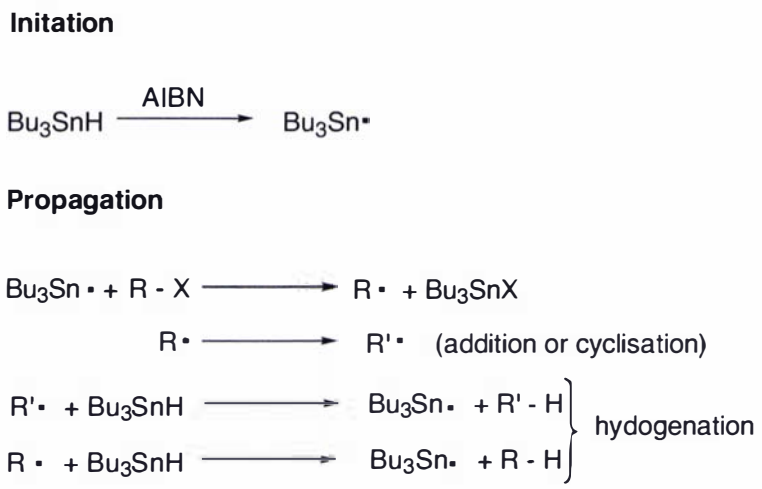
1.101 6-*endo*

Figure 1.12

The corresponding 6-*endo*-transition state is energetically less favoured because of poorer overlap of the orbitals and the higher degree of ring strain.

1.1.5.1 The Tin Hydride Method

Tributyl tin hydride (Bu_3SnH) and triphenyl tin hydride (Ph_3SnH) are the most commonly used reagents to conduct free radical reactions. Simple reduction of an organic halide by tin hydride involves a controlled chain reaction, as illustrated in **Scheme 1.16**.[39] The tin hydride method has several efficacy over other radical reaction methods. Tin hydride is extremely mild and selective, so that carbonyl groups and alcohols do not need to be protected. The laboratory simplicity and comparable kinetic understanding of the tin hydride radical reaction method also makes it accessible and easy to use.[39] Most significantlythe kinetic behavior of tin hydride is such that when a radical $R^{\bullet}$ is initiated from a reactive radical precursor in the presence of low concentration of tin hydride, it has a reasonably long life time within which to react ($R^{\bullet} \rightarrow R'^{\bullet}$) before being trapped to give final product ($R^{\bullet} \dashrightarrow R\text{-}H$).[39]

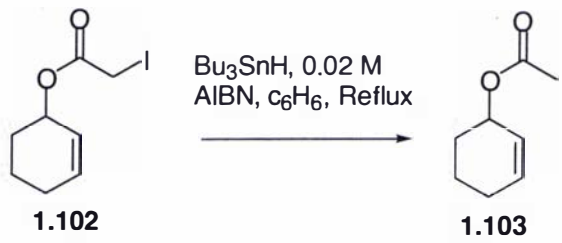


Scheme 1.16

The window of life time of a radical can be also changed over a wide range simply by changing the concentration of tin hydride. A limitation of this method is that although tin hydride allows R$^{\bullet}$ a reasonably flexible window of reactivity, slow reaction time sometimes cannot compete with direct reduction, so that the desired product R'H is often contaminated by the presence of reduced starting material RH.[39] The complete removal of tin-contaminated byproducts from the desired products can also cause a major problem.[39,44,45]

1.1.5.2 Ester-Tethered Intra Molecular Free Radical Cyclisations

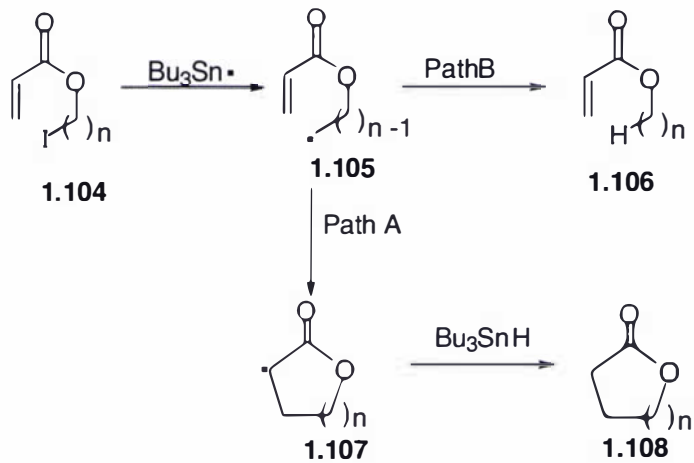
Although radical cyclisations with an ester tether have now acquired a status of immense importance in the design of the synthesis of five and six membered carbocyclic compounds, the last decade has seen an explosive growth in the synthesis of larger rings. The difficulty posed by the presence of an ester linkage has been reported in the literature by several research groups[46,47,48]. For example attempted cyclisation of the halo ester **1.102** at moderately low concentrations of Bu_3SnH gave none of the lactone, only the reduced product **1.103** being obtained as shown in **Scheme 1.17**.[39]



1.102 **1.103**

Scheme 1.17

In the first successful study of radical macrocyclisation of an ester-tethered reaction Porter and Chang identified structural criteria for successful alkyl radical macrocyclisation reactions.[49,50] Briefly they demonstrated that radical ring closure should be feasible if it occurs onto sterically unhindered, electronically activated double bonds, leading to ring sizes greater than ten. Thus 11-20 membered rings were isolated (15-76%) by means of macrocyclisation, followed by subsequent reduction (**Scheme 1.18, path A**). The cyclisation was more effective at higher ring sizes and less effective at lower ring sizes. Once again the major side product was found to be acyclic material resulting from direct reduction of the carbon-iodine bond (**path B**), which was observed even under high dilution conditions.



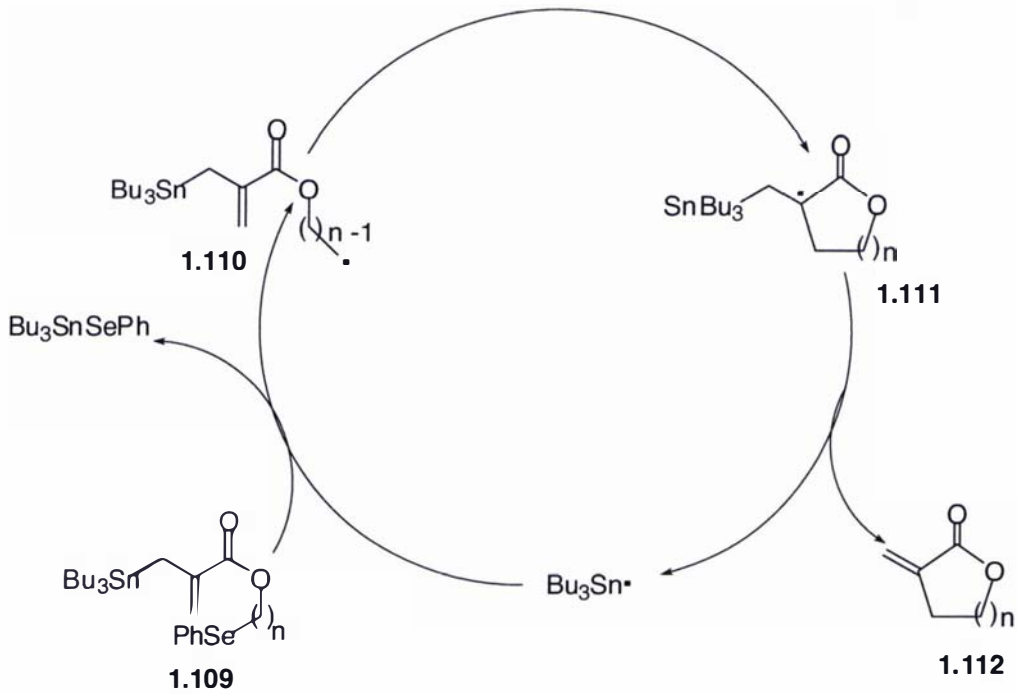
1.104 **1.105** **1.106** **1.107** **1.108**

Scheme 1.18

The cyclised radical **1.107** is favoured by the presence of an adjacent ester carbonyl functionality since some delocalisation is possible.

Baldwin *et al* reported[51,52] the synthesis of large ring (10-15 membered) α-methylene lactones **1.111** (**Scheme 1.19**) from functionalised allyl stannanes **1.109** in

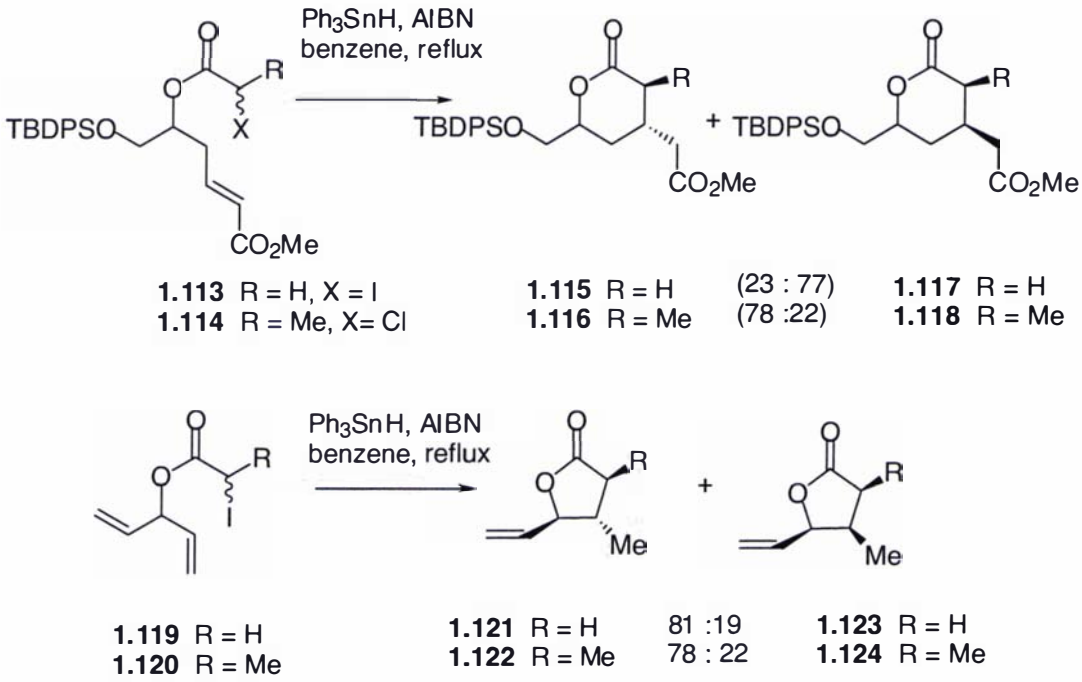
moderate to high yield under high dilutions (5 mM). Since the chain transfer agent $Bu_3Sn\bullet$ is produced by virtue of a fragmentation reaction, only a catalytic quantity of Bu_3SnH is required. The direct reduction of the radical centre was not observed. Again the cyclised radical **1.111** experiences some stabilisation of the unpaired electron by the neighbouring ester carbonyl group. Attempts to synthesise analogous 6-9 membered lactones were unsuccessful; substrates **1.109** affording dilactones in low yield and in addition a variety of minor AIBN derived adducts.



Scheme 1.19

Hanessian *et al* [47,48] in an elegant study into the intramolecular free radical cyclisation of α-ester radical intermediates (until recently considered to be unreactive in conjugate-type additions), showed that these stable radicals underwent smooth intramolecular cyclisations with activated (**1.113** and **1.114**) and unactivated olefins (**1.119** and **1.120**) in the presence of the Ph_3SnH at low concentrations (0.02-0.012 M, syringe-driven pump) (**Scheme 1.20**). The prevailing opinion was that in the presence of Bu_3SnH, such stabilised radicals underwent reduction rather than C-C bond formation. They argued that in such cases the transition state for radical ring closure probably occurs later on the reaction coordinate, compared to those for the more

nucleophilic radicals.[48] Clearly this must be the consequence of preferred orientation of the radical species in the transition state just prior to the formation of new sp^3 bond.



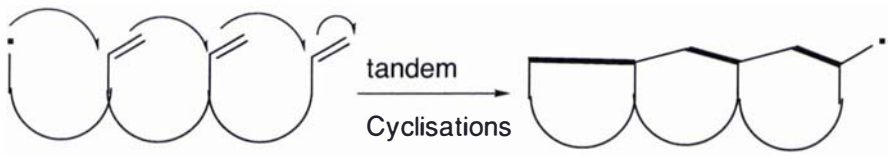
Scheme 1.20

Whereas the nature of the halogen seems to be unimportant in the case of activated olefins, α-iodo esters were preferred when cyclisation involved unactivated olefins.

1.1.5.3 Tandem Radical Cyclisation Reactions

Over the past few decades interest in tandem reactions has arisen from a need for economic and environmentally friendly methods of synthesis. These tandem reactions can be effected in a vast range of ways[53] but for the purpose of this thesis we will concentrate on those which involve free radicals. Tandem radical cyclisation reactions are important advancements in radical cyclisation methodology. Though hundreds of examples have been known and used for many years, only recently a focussed effort has been made to expand their use in organic synthesis.[40] Tandem reactions, sometimes also called domino, sequential, cascade, consecutive, iterative, zipper, and one-pot (one flask) reactions, link several transformations together in a single synthetic step. In recent years synthetic chemists have examined the scope for an

extensive range of complementary radical-mediated cascade processes from polyene precursors in the synthesis of a variety of polycyclic ring systems, including taxoids and steroids.[45] In a general approach to the tandem cyclisations of polyenes to obtain polycyclic products, reactions are accomplished by generating an internal radical within the bonding distance of the multiple bond to generate a new radical which again should be re-channelled to the next olefinic bond (**Scheme 1.21**).

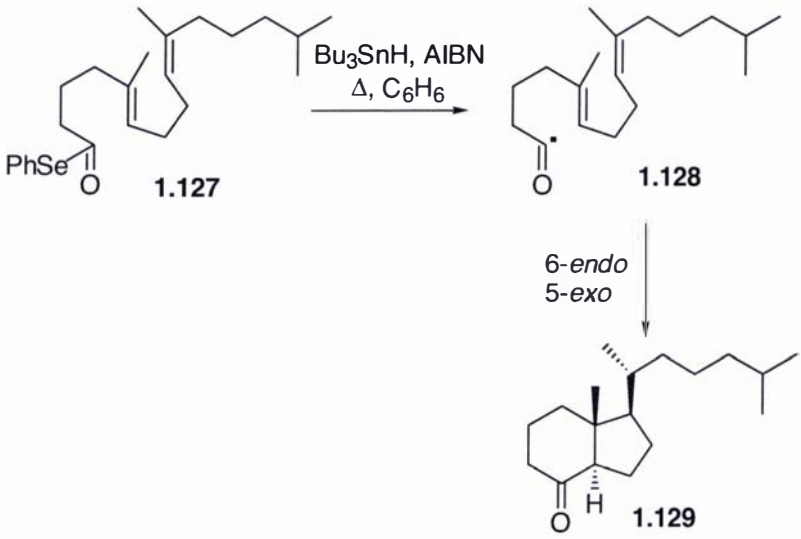


1.125 **1.126**

Scheme 1.21

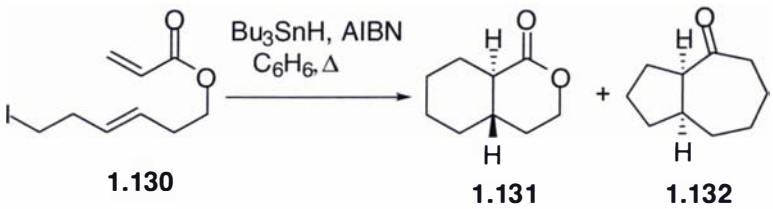
Over the past decade tandem reactions have gained wide acceptance because they increase synthetic efficiency by decreasing the number of laboratory operations required and also the quantities of chemicals and solvents used. Furthermore, they frequently permit efficient access to unique chemical structures and occasionally result in a greater reaction selectivity.

Pattenden *et al* [54,55] explored the potential of such tandem radical mediated synthesis of the C/D-portion of vitamin D_3, using a seleno precursor **1.127** and obtained the acyl radical **1.128** which then underwent consecutive 6-*endo*, 5-*exo* trig radical cyclisation to construct the C/D bicyclic portion of vitamin D_3 (**Scheme 1.22**).



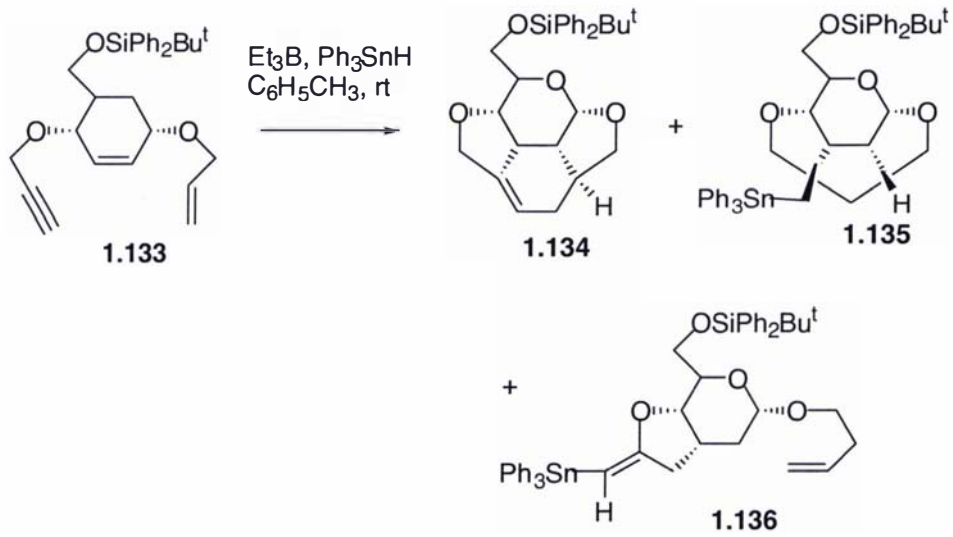
Scheme 1.22

The tandem radical macrocyclisation transannulation approach has been applied by Pattenden *et al* to the ester tethered iodo precursor **1.130** in the construction of a ring fused lactone by a 10-*endo* trig macrocyclisation reaction followed by 6-*exo*/ 7-*endo trans* annulation (**Scheme 1.23**).[56]



Scheme 1.23

Another recently reported[57] good example of a tandem intramolecular free radical cyclisation reaction is synthesis of the polycyclic molecule **1.134** *via* a [2+2+2] cyclo addition reaction of dodeca-1,6-diene-11-ynes promoted by triphenyltin hydride, in a cascade free radical process (**Scheme 1.24**). The authors reported that the particular experimental conditions were critical and precursor **1.133** was recovered unaltered when warmed in toluene or treated with Et_3B at RT and reaction with palladium complexes produced extensive decomposition.

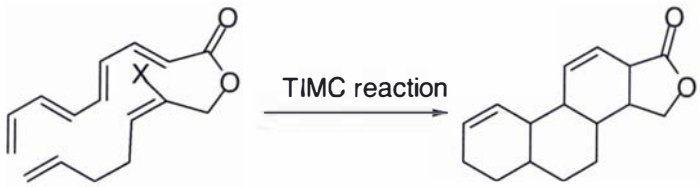


Scheme 1.24

1.1.6 Conclusion

There are many biologically active natural products with great potential for medicinal and other uses. In order to make these compounds available for testing and subsequent clinical use, efficient syntheses need to be designed. The cyclisation strategies reviewed above have long been used to construct five-membered and six-membered rings common to many natural products and now interest is focused on the application of intramolecular variants of these reactions in spongian diterpenoids. The cyclisation reactions reviewed above have the potential to generate bicyclic lactone systems common in many natural products. Attention was mainly focused on the total synthesis of spongian–16-one through a concise approach discussed in **Section 1.3.1**.

Extension of ester tethered IMDA and tandem cyclisation methodologies can allow access to even more complex natural products. Linking a *bis*-diene and dienophile together *via* an ester tether to create a tandem intramolecular cyclisation reaction could provide direct entry to the spongian skeleton which has the distinct advantage of introducing in one step 8 new sterogenic centres present in this marine natural product.



1.142 **1.144**

Scheme 1.25

1.1.7 Aims Of The Current Research

Even though the ester tethered precursors such as those prepared in this chapter are simple, convenient and facile to synthesise literature surveys revealed no preparation of these precursors. It is clear from the forgoing review that very few examples have appeared in the literature for the attempted cyclisation of unactivated ester precursors with a carbonyl group adjacent to the diene, using IMDA reactions and in fact no cyclisation has been reported on such ester tethered precursors using the alternative strategy of tandem cyclisation using Pd catalysed (Heck reaction) or Sn catalysed free radical reactions.

The aims of the current research work hence were two fold:

ii) Firstly, it was decided that a more through investigation into the cyclisation reaction pathway of ester-tethered precursors to produce a lactone ring was warranted as the literature revealed no published attempts to cyclise precursors such as those synthesised in this chapter.

i) Secondly, to investigate a new convenient synthesis of spongian–16-one *via* an unprecedented intramolecular cyclisation of an ester tethered *bis*-diene and *bis*-dienophile using the cyclisation strategies reviewed above. This was of considerable interest due to the potential use of this methodology to synthesise other tetracyclic compounds with a similar D ring lactone (see **section 1.3**, **1.4**, **1.8**, and **1.9**).

1.2 EXPERIMENTAL

1.2.1 General Experimental Details

Solvents and reagents were purified and dried according to the methods of Perrin and Amarego[80]. Benzene, toluene and diethyl ether were purified and dried by distillation from sodium benzophenone ketyl. Dichloromethane was distilled from calcium hydride. Molecular sieves (4A°) were activated at 450°C and stored in an oven at 100°C. All moisture sensitive reactions were performed by either protection with a $CaCl_2$ drying tube or under an argon (Ar) atmosphere using flame dried or oven dried (150°C) glassware.

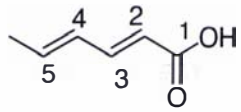
Reactions were generally monitored by thin layer chromatography (TLC) performed using pre-coated Merck Kieselgel 60 F_{254} silica plates. Visualisation was by use of a UV lamp (254 nm) and by staining with alkaline $KMnO_4$ solution, acidic alcoholic 2,4 dinitrophenyl hydrazine or acidic alcoholic vanillin followed by strong heating. Solvents were removed under reduced pressure on a Büchi rotary evaporator. The column chromatography was performed using Merck Kieselgel 60 [230-400 mesh] silicagel with the eluent mixture indicated.

Unless otherwise noted, NMR spectra were recorded in $CDCl_3$ solution at ambient temperature using a JEOL JNM–GX 270 spectrometer. ^1H NMR spectra were generally obtained at 270 MHz and chemical shifts (δ) are expressed in parts per million (ppm) downfield shift relative to $CDCl_3$ (7.27 ppm). The following abbreviations were used: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; and br, broad. Coupling constants (J) were given in Hertz (Hz). ^{13}C NMR spectra were obtained at 68 MHz and data are expressed in parts per million downfield shift relative to $CDCl_3$ (77.0 ppm). Spectral assignments were made and product structures were elucidated using the double resonance techniques COSY and HETCOR and checked where possible with literature spectra for confirmation. Both high and low resolution mass spectra were obtained using a varian VG70-250S double focusing magnetic sector mass spectrometer with an ionisation potential of 70 ev. Data was reported as m/z (relative intensity). IR absorbance spectra were collected using a Perkin Elmer PARAGON 1000 FT-IR spectrometer. Solid samples were prepared as a KBr disc and

liquid samples as thin films between NaCl plates. The data were reported in wave numbers (cm^{-1}). Melting points of crystalline materials were determined on a Reichert hot stage instrument and are uncorrected.

1.2.2 Synthesis Of Conjugated Tetraenes As A *Bis-Z*-Diene

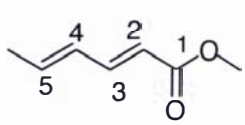
1.2.2.1 2*E*, 4*E*-Hexadienoic acid (1.157)



1.157

The title compound was prepared according to a modified procedure of Vogel[81]. A mixture of malonic acid (120 g, 1.15 mol), crotonaldehyde (80 g, 1.14 mol) and pyridine (120 g) was heated to reflux with vigorous stirring. The completion of the reaction was monitored by TLC (4-6 hr). After this time the evolution of carbon dioxide had ceased. The reaction mixture was cooled and acidified with dilute HCl (2 N, 52 ml) and stirred vigorously in ice for 3-4 hrs. The title compound was then filtered off and washed with ice-cold water (20 ml). Recrystallisation from boiling water (2.5 l), gave 2*E*, 4*E*- hexadienoic acid (R_f = 0.52, 1:1 CH_2Cl_2/EtOAc) as long white needles (34.9 g, 27 %). m.p.136°C (sharp), (Lit.[81] mp, 132-134°C. ^{1}H NMR δ/ppm 7.29-7.38 (1H, m, H3), 6.14- 6.28 (2H, m, H4, H5), 5.77 (1H, d, J = 15.4 Hz, H2), 1.88 (3H, d, -CH_3). ^{13}C NMR δ/ppm 172.6 (-COOH), 147.2 (C3), 140.7 (C4), 129.5 (C5), 118.0, (C2), 18.8 (-CH_3). I.R. (KBr disc) ϑ_{max} 2498-3200, 2926, 1701, 1685, 1654, 1617, 1267 and 1154 cm^{-1}.

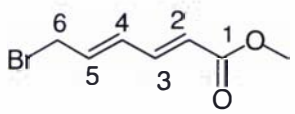
1.2.2.2 Methyl 2*E*, 4*E*-hexadienoate (1.159)



1.159

2*E*, 4*E*- hexadienoic acid (49.9 g, 0.54 mol) and methanol (250 ml) were stirred to obtain a homogeneous solution. Sulphuric acid (5.0 ml) was added and the reaction mixture was stirred vigorously at 40°C for 20 h to give methyl 2*E*, 4*E*- hexadienoate. (R_f = 0.72; 3:2 Hex:EtOAc). The excess methanol was removed under reduced pressure and the residual syrup was diluted with ethyl acetate (100 ml). The reaction mixture was washed with water (2 x 50 ml), saturated aqueous $NaHCO_3$ (2 x 50 ml) and brine (50 ml), and then dried over sodium sulphate. The solvent was removed under reduced pressure to afford a pale yellow oil of methyl hexa-2*E*, 4*E* - dienoate (51.0 g, 75 %). The ^{1}H nmr spectrum of methyl 2*E*, 4*E*- hexadienoate was clean thus the crude product was used in the next step without further purification. ^{1}H NMR δ/ppm 7.21 (1H, dd, *J* = 15.4, 4.1 Hz, H3), 6.11- 6.16 (2H, m, H4, H5), 5.74 (1H, d *J* = 15.4 Hz, H2), 3.70 (3H, s, -OCH_3), 1.82 (3H, d J = 4.6 Hz, -CH_3). ^{13}C NMR δ/ppm 167.1 (-*C* O_2 CH_3), 144.7 (C3), 138.9 (C4), 129.4 (C5), 118.3 (C2), 51.33 (-CO_2*C* H_3), 18.44 (-CH_3). I.R. (thin film) ϑ_{max} 2951, 1723, 1645, 1618, 1435, 1246 and 1141 cm^{-1}.

1.2.2.3 Methyl 6-bromo-2*E*, 4*E*-hexadienoate (1.155)



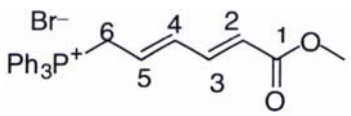
1.155

Methyl 2*E*, 4*E*-hexadienoate (44.5 g, 0.35 mol) in anhydrous chlorobenzene (300 ml) was stirred vigorously and *N* -bromo succinimide (64.4 g, 0.36 mol) was added over 10 minutes. The reaction mixture was heated to 100°C and then the radical initiator benzoyl peroxide (3.91 g, 0.01 moles) was added in small portions. The reaction mixture was heated to reflux for 2 h. The reaction mixture was then cooled and the solvent was removed under reduced pressure. The residual syrup was diluted with ether, filtered to remove solid impurities and washed with 2N NaOH (3 x 40 ml) until the washings were colourless. The ether solution was then washed with water (2 x 50 ml) and brine (50 ml) and dried over $MgSO_4$. The ^{1}H NMR analysis indicated the presence of two compounds. The solvent was removed under reduced pressure and the resultant oily liquid, on fractional distillation (40-42°C/0.1 mm Hg) gave unreacted

methyl 2*E*, 4*E*- hexadienoate (3.21 g, R_f = 0.66; 2:1:0.1 Hex:EtOAc:Me_2CO) and at 87-90°C/0.1mm Hg gave methyl 6-bromo 2*E*, 4*E*- hexadienoate (42.0 g, 65 %, R_f = 0.58; 2:1:0.1 Hex:EtOAc:Me_2CO). ^{1}H NMR δ/ppm 7.19 (1H, dd, *J* = 15.4, 4.3 Hz, H3), 6.08-6.45 (2H, m, H4, H5), 5.89 (1H, d, J = 15.3 Hz, H2), 4.21 (2H, d, J = 0.88 Hz, H6), 3.70 (3H, s, -OCH_3). ^{13}C NMR δ/ppm 167.3 (-*C* O_2 CH_3), 144.8 (C3), 139.1 (C4), 136.5 (C5), 118.3 (C2), 51.2 (-CO_2 *C* H_3), 18.5 (C6). I.R. (thin film) ϑ_{max} 2950, 1715, 1644, 1614, 1435, 1250 and 1157, 668 cm^{-1}.

1.2.2.4 5-Methoxycarbonylpenta-*2E, 4E*-dienyl triphenyl phosphonium bromide (1.156)

The title compound (4.51g, 57%) was prepared from methyl 6-bromohexa-2*E*, 4*E*-dienoate (2.85 g, 13.89 mmol) and triphenylphosphine (5.00 g, 19.0 mmol) in dry benzene at reflux temperature according to the procedure of Weedon *et al.*[67] Recrystallization from dichloromethane-ethyl acetate (4 : 1) gave yellow crystals, m.p. 185-188°C (lit.[67] m.p.188°C). The title compound was also prepared in mild conditions and better yield in the following way using the same starting materials.

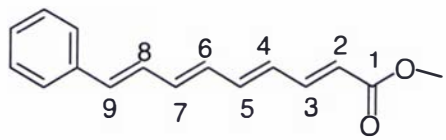


1.156

To a solution of triphenylphosphine (35.1 g, 0.13 mol) and anhydrous ether (115 ml) was added methyl 6-bromohexa-2*E*, 4*E*-dienoate (26.1 g, 0.12 mol) in anhydrous ether (13 ml) over 0.5 hrs. The reaction mixture was stirred vigorously at room temperature (16°C) in the dark under dry conditions for 20 hrs. The precipitated phosphonium salt was then filtered, washed with cold, anhydrous ether (10 ml) to give the title compound **1.156** (R_f = 0.46; 1:1 CH_2Cl_2:EtOAc) as fine crystals (46.5 g, 76%). m.p. 171.2-171.5°C, sharp. (lit.[67] m.p.188°C). The I.R. and ^{1}H nmr spectrum were in agreement with those reported in the literature. ^{1}H NMR δ/ppm 7.55-7.96 (15H, m, 3 x phenyl), 7.11 (1H, dd J = 15.4, 4.17 Hz, H3), 6.48-6.78 (1H, m, H5), 5.86-5.89 (1H, m, H4), 5.83 (1H, dd, J = 15.4, 5.3 Hz, H2), 5.15 (2H, dd, J = 16.2, 7.6 Hz, H6), 3.68 (3 H, s, -CO_2 *C* H_3). ^{13}C NMR δ/ppm 164.5 (-*C* O_2CH_3), 139.5 (C1), 139.4 (C2), 134.9,

134.7, 132.3, 131.2, 131.0, 129.6, 129.4, 127.6, 127.4, 126.1, 125.9, 123.3, 123.1, 120.1 (3 x phenyl), 130.0 (C3), 115.5 (C4), 114.3 (C5), 48.97 (-CO_2 *C* H_3), 18.6 (C6). I.R. (KBr disc) ϑ_{max} 3005, 2849, 1707, 1636, 1438, 1253 and 1157 cm^{-1}.

1.2.2.5 Methyl 9-phenylnona-2*E*, 4*E*, 6*E*, 8*E*-tetraenoate (1.162)



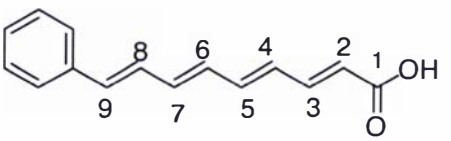
1.162

Aqueous NaOH (2N, 20 ml) was added rapidly dropwise, to a suspension of 5-methoxycarbonylpenta-2*E*, 4*E* -dienyl triphenyl phosphonium bromide (2.11 g, 4.51 mmoles) in CH_2Cl_2 (10 ml) under an atmosphere of Ar. The reaction solution was stirred gently over 15 min with the ylide solution developing an intense dark blood-red colour. After addition of an antioxidant BHT (12 mg, 0.05 mmoles) the ylide solution was added over 15 min to a solution of cinnamaldehyde (0.28 g, 2.14 mmoles) in CH_2Cl_2 (10 ml) and was stirred at 40^0C under an atmosphere of argon in the dark until no starting material was detectable (TLC, 5 h). The reaction mixture was diluted with Et_2O (75 ml) and then washed with saturated brine (25 ml) and dried over Na_2SO_4. The solvent was removed under reduced pressure and the crude solid was purified by column chromatography (3:1 Hex/EtOAc) to yield a mixture of *E/Z* methyl 9-phenyl-2, 4, 6, 8-nonatetraenoate (R_f = 0.38) as a yellow solid (0.37 g, 73%). Thio phenol (48 µL, 0.47 mmol) and AIBN (41mg, 0.25 mmol) were added to a solution of the *E/Z* isomeric mixture of tetraenoate (0.37 g, 1.55 mmol) in anhydrous benzene (10 ml), in the presence of BHT (25 mg, 0.12 mmol). The reaction mixture was refluxed under Ar and the solution was irradiated with UV/Visible light (250 W tungsten filament sun lamp) for 1 h before being recharged with thiophenol (48 µL, 0.47 mmol) and AIBN (41 mg, 0.25 mmol) twice until the reaction was judged completed by ^{1}H NMR spectroscopy. The solvent was removed under reduced pressure and the crude solid was purified by column chromatography eluting with Hex:EtOAc (1:1) to afford the title compound (R_f = 0.32) as pale yellow solid. The solid was recrystallised from acetone to give yellow needles (0.22 g, 42%), m.p 148-149°C. ^{1}H NMR δ/ppm 6.83-6.93 (6H, m, phenyl and

H3), 6.88 (1H, dd, J = 10.3 Hz, 5.6 Hz, H7), 6.58-6.70 (3H, m, H5, H6, H8), 6.33-6.55 (2H, m, H4, H9), 6.02 (1H, d, J = 15.1 Hz, H2). ^{13}C NMR δ/ppm 167.4, (C=O), 144.5 (C3), 140.3 (C5), 137.2 (C-8), 135.0 (C6), 131.8 (C9), 128.9 (C4), 128.5 (C7), 128.2, 127.8, 126.3 (phenyl), 119.7 (C2), 49.5 (-CO_2C H_3). I.R. (thin film) ϑ_{max} 1712, 1630, 1602, 1286 and 1168 cm^{-1}.

Complete *trans* isomerisation was also achieved by the following method. An *E, Z* isomeric mixture of methyl 9-phenyl-2, 4, 6, 8-nonatetraenoate (0.37 g, 1.55 mmoles) in dichloromethane (10.0 ml) was stirred with resublimed iodine (55.0 mg, 0.21 mmoles) under Ar in the light for 2.5 hr. The reaction mixture was diluted with dichloromethane (25.0 ml) and washed with 2N aqueous sodium thiosulphate (3 x 25 ml) and water (2 x 25 ml), then dried and the solvent removed under reduced pressure. The title compound was recrystallised from acetone to give yellow needles. (0.19 g, 36 %). m.p 148-149°C. All spectroscopic data were the same as for isomerisation using the thiophenol method.

1.2.2.6 9-Phenylnona-2*E*, 4*E*, 6*E*, 8*E*-tetraenoic acid (1.163)



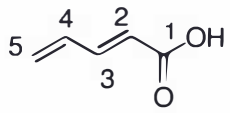
1.163

Methyl 9-phenylnona-2*E*, 4*E*, 6*E*, 8*E*-tetraenoate (0.205 g, 0.854 mmoles) was added to a solution of potassium hydroxide (0.140 g, 2.50 mmol) in dry degassed ethanol (4.1 ml) and was refluxed for 2 hrs under N_2. The solvent was removed under reduced pressure and the reaction mixture was quenched with water (50.0 ml) and then extracted with ether (3 x 20 ml). The yellow aqueous phase was acidified to pH 1.76 with HCl, and was extracted with ethyl acetate (3 x 20 ml). The solution was dried over Na_2SO_4 and the solvent was removed under reduced pressure to give the title compound (R_f = 0.26, 100% EtOAc) as yellow crystals (0.190 g, 0.799 mmol, 99 %). m.p 221-222°C. m/z (EI, 70 eV) 226 (M^+, 100%). 181 (M-COOH, 68), 165 (M-36), 154 (16), 141 (25), 115 (31), 91 (37), 77 (15) and 65 (6). ^{1}H NMR δ/ppm 7.31-7.65 (6H, m, phenyl and

H3), 7.15 (1H, dd, J = 15.6 Hz, 5.1 Hz, H7), 6.73-7.00 (3H, m, H5, H6, H8), 6.53-6.73 (2H, m, H4, H9), 6.02 (1H, d, J = 15.1 Hz, H2). ^{13}C NMR δ/ppm 167.3, (C=O), 145.4 (C3), 138.4 (C5), 137.5 (C8), 135.5 (C6), 133.2 (C9), 130.8 (C4), 129.6 (C7), 129.5, 128.8, 128.6, 127.4 (phenyl), 120.7 (C2). I.R. (thin film) ϑ_{max} 3300-2500, 1683, 1617, 1271 1157 and 1010 cm^{-1}.

1.2.3 Synthesis Of Ester-Tethered -Iodo And –Yne Precursors

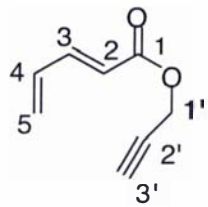
1.2.3.1 Penta 2*E*,4-dienoic acid (1.175)



1.175

The title compound was prepared using a modification of the procedure described by Jessup *et al* .[69] Pyridine (10.3 g, 0.130 mol) was stirred on an ice-salt bath and malonic acid (10.4 g, 0.100 mol) was added in portions over 10 mins. The reaction mixture was gently refluxed with vigorous stirring to get a homogeneous solution. Acrolein (6.32 g, 0.113 mol) was added with vigorous stirring over 15 mins and the reaction mixture was refluxed for 2 hrs until the evolution of CO_2 had ceased. The reaction mixture was poured in to ice-cold water (50 ml) and acidified with concentrated H_2SO_4 (13 ml). The aqueous layer was extracted with CH_2Cl_2 (4 x 15 ml). The organic extract was washed with brine (20 ml) and then dried over magnesium sulfate. The CH_2Cl_2 solution was concentrated to *ca*. 20.0 ml under reduced pressure. Cooling on ice gave long white needles of penta 2*E*,4-dienoic acid (1.82 g, R_f = 0.42 1:1 CH_2Cl_2:EtOAc, 18 %). m.p. 72-73°C (lit.[69] m.p.73-75°C). Further concentration of the mother liquor gave off-white crystals of *trans*-2,4-pentadienoic acid (2.52 g, 0.0257 mol, 25 %) m.p.70-72°C with a low level of polymeric material. ^{1}H NMR δ/ppm 11.8 (1H, s, -OH) 7.36 (1H, dd, J = 15.4, 11 Hz, H3), 6.57-6.43 (1H, m, H4), 5.93 (1H, d, J = 15.4 Hz, H2), 5.71-5.52 (2H, m, H5). ^{13}C NMR (67.8 MHz, CDCl3) δ/ppm 171.7 (-COOH), 146.7 (C3), 134.4 (C4), 126.4 (C2), 121.1 (C5). IR (thin film) ϑ_{max} 3200-2700, 1701, 1636, 1601, 1275 and 1010 cm^{-1}.

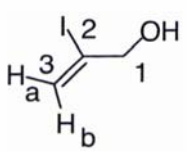
1.2.3.2 Prop-2'-ynyl *trans* -2,4-pentadienoate (1.176)



1.176

Penta 2*E*,4-dienoic acid (1.12 g, 11.5 mmol), DCC (1.89 g, 9.20 mmol) and DMAP (20 mg, 0.16 mmol) were added sequentially to a solution of 2-propyn-1-ol (0.43 g, 7.68 mmol) in CH_2Cl_2 (15.0 ml) under an atmosphere of Ar at RT. The reaction mixture was stirred for 3 h before being diluted with Et_2O (50 ml) and filtered through celite. The filtrate was washed successively with aqueous HCl (10%, 25 ml) saturated aqueous $NaHCO_3$ (2 x 25 ml), H_2O (25 ml) and brine (25 ml), dried over $MgSO_4$ and then filtered. The solvent was removed under reduced pressure to give the crude product which was purified by column chromatography using Hex:EtOAc (4:1) as eluent to give the desired, propyonate ester **1.176** (R_f = 0.56) as a pale yellow oil (0.50 g, 3.35 mmol, 44 %). ^{1}H NMR δ/ppm 7.28 (1H, dd, *J* = 15.6, 10.8 Hz, H3), 6.58-6.36 (1H, dt, H4), 5.90 (1H, d, J = 15.6 Hz, H2), 5.57 (2H, dd, J = 17.1, 10.1 Hz, H5), 4.73 (2H, br.s, H1'), 2.48 (1H, br.s, H3'). ^{13}C NMR δ/ppm 165.6 (-COOR), 145.7 (C3), 134.4 (C4), 126.2 (C2), 120.8 (C5), 74.8 (C3'), 77.5 (C2'), 51.8 (C1'). IR (thin film) ϑ_{max} 3296,2129,1721,1642,1599,1266 and 1139cm^{-1}

1.2.3.3 3-Hydroxy-2-iodo-1-propene (1.186)

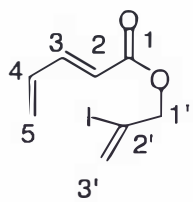


1.186

The title compound was prepared according to the procedure of Ishii *et al* [76] with some modifications. NaI (30.0 g, 0.201 moles) was added to CH_3CN (60 ml) and stirred under Ar in the dark. $ClSiMe_3$ (18.1 g, 0.167 moles) was added slowly and the reaction

mixture turned milky. Water (1.80 g, 0.100 moles) and then propargyl alcohol (5.30 g, 94.5 mmoles) were added and the reaction mixture was stirred for 2 hrs at 30°C under Ar in the dark to give crude 2-iodo propen-1-ol. The reaction mixture was quenched with water (50 ml) and washed with sodium thiosulphate solution (10%, 3 x 25 ml), water (50 ml), brine (50 ml) and was dried over $MgSO_4$. The solvent was removed under reduced pressure to give a dark yellow liquid which was then purified by column chromatography using Hex:EtOAc (4:1) as eluent to give 3-hydroxy-2-iodo-1-propene (11.6 g, R_f = 0.52, 67%). ^{1}H NMR δ/ppm 6.37 (1H, dd, J = 1.76, 1.68 Hz, H3a), 5.84 (1H, dd, J = 1.66, 1.48 Hz, H3b), 4.14 (2H, br s, H1), 3.61(1H, s, -OH). ^{13}C NMR δ/ppm 124.0 (C3), 110.2 (C2), 70.5 (C1). IR (thin film) ϑmax 3334, 1626 and 1034 cm^{-1}.

1.2.3.4 2'-Iodo prop-2'-enyl 2*E*, 4- pentadienoate (1.176a)

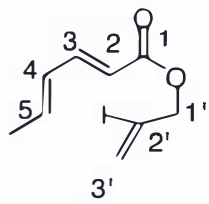


1.176a

2-Iodo propen-1-ol (2.79 g, 15.1 mmol) in CH_2Cl_2 (15 ml) was stirred at 0^0 C under an atmosphere of Ar. Pyridine (2.15 g, 27.2 mmol) was added over 5 mins and the 2*E*, 4-pentadienoyl chloride (2.02 g, 17.3 mmol) in CH_2Cl_2 (15 ml) was added slowly to the reaction mixture over 15 mins and the solution was stirred under argon for 3 hrs at RT. The reaction mixture was further diluted with CH_2Cl_2 (50 ml) and washed with aqueous 2M HCl (3 x 25 ml), saturated aqueous $NaHCO_3$ (2 x 25 ml), water (50 ml), brine (50 ml) and then dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude mixture was then purified by column chromatography eluting with Hex:EtOAc (19:1) to afford the title compound 2'-iodoprop-2'-enyl 2*E*, 4- pentadienoate (1.89 g, R_f = 0.37, 47 %) as a pale yellow liquid. ^{1}H NMR δ/ppm 7.34 (1H, dd, J = 15.4, 11 Hz, H3), 6.55-6.36 (2H, m, H4 and H3'), 6.00-5.90 (2H, m, H2 and H3'), 5.68-5.52 (2H, m, H5), 4.76 (2H, br.s, H1'). ^{13}C NMR δ/ppm 165.8 (-COOR), 144.7 (C3),

134.5 (C4), 132.1 (C2), 125.4 (C3'), 121.7 (C5), 117.9 (C2'), 64.9 (C1'). IR (thin film) ϑ_{max} 2927, 1719, 1629, 1598, 1264 and 1139 cm^{-1}.

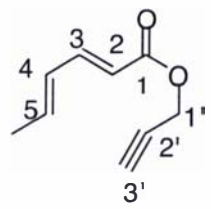
1.2.3.5 2'-Iodo prop-2'-enyl hexa-2*E*, 4*E*-dienoate 1.189



1.189

2-Iodo propen-1-ol (2.99 g, 18.3 mmol) in CH_2Cl_2 (25 ml) was stirred under an atmosphere of Ar. The solution was kept on ice and pyridine (1.94 g, 24.5 mmol) was added over 5 minutes. The solution was stirred at RT under Ar and hexa-2,4-dienoyl chloride (2.00 g, 15.3 mmol) in CH_2Cl_2 (15 ml) was added slowly to the reaction mixture over 0.5 hrs and the solution was stirred under argon for 2.5 hrs at RT. The reaction mixture was diluted further with CH_2Cl_2 (30 ml) and washed with HCl (1M, 2 x 25 ml), NaOH (2 x 25 ml), water (25.0 ml), brine (25.0 ml) and then dried over $MgSO_4$. The solvent was removed under reduced pressure to give a yellow solid which was then purified by column chromatography eluting with Hex:EtOAc (6 : 4) to give, 2'-iodo prop-2'-enyl hexa-2*E*, 4*E*-dienoate (3.88 g, R_f = 0.36, 77 %) as a pale yellow liquid. ^{1}H NMR δ/ppm 7.34-7.26 (1H, m, H3), 6.35 (1H, br.s, H3'), 6.21-6.17 (2H, m, H4-H5), 5.91 (1H, br.s, H3'), 5.81 (1H, d, *J* = 15.3 Hz, H2), 4.73 (2H, br.s, H1'), 1.85 (3H, d, *J* = 5.3 Hz, $-CH_3$). ^{13}C NMR δ/ppm 165.7 (-COOR), 146.0 (C3), 140.1 (C4), 129.5 (C5), 126.7 (C2),117.8 (C3'), 102.4 (C2'), 70.4 (C1'), 18.7 ($-CH_3$). IR (thin film) ϑ_{max} 2933, 1714, 1643, 1616, 1239 and 1133 cm^{-1}.

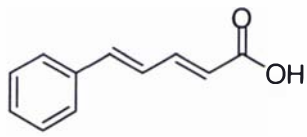
1.2.3.6 Prop-2'-ynyl hexa-2*E*, 4*E*-dienoate (1.178)



1.178

2-Propyn-1-ol (2.15 g, 38.4 mmol) in CH_2Cl_2 (20.0 ml and pyridine (3.10 g, 39.2 mmol) were stirred under an atmosphere of Ar. Hexa-2*E*, 4*E*-dienoyl chloride (4.22 g, 32.3 mmol) in CH_2Cl_2 (20.0 ml) was added slowly over 15 min and the reaction mixture was stirred for 3 hrs at 35°C. The reaction mixture was diluted with dichloromethane (100 ml) and washed with aqueous HCl (2M, 4 x 25 ml). The reaction mixture was then washed with saturated aqueous $NaHCO_3$ (2 x 25 ml) water (50.0 ml), brine (50.0 ml) and dried over Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure to give crude prop-2'-ynyl hexa-2*E*, 4*E*-dienoate. Purification by column chromatography using Hex:EtOAc (4:1) as eluent afforded the title compound (R_f = 0.32) as a pale yellow liquid (5.07 g, 88 %). ^{1}H NMR δ/ppm 7.34-7.26 (1H, m, H3) 6.20-6.16 (2H, m, H4, H5), 5.78 (1H, d, J = 15.8 Hz, H2), 4.73 (2H, br.s, H1'), 2.47 (1H, br.s, H3'), 1.85 (3H, d, J = 5.6 Hz, -CH_3). ^{13}C NMR δ/ppm 165.6 (-COOR), 145.6 (C3), 139.6 (C4), 129.3 (C2), 117.4 (C5), 77.6 (C3'), 74.5 (C2'), 51.3 (C1'), 18.4 (-CH_3). IR (thin film) ϑ_{max} 2933, 1714, 1643, 1616, 1239 and 1133 cm^{-1}.

1.2.3.7 5-Phenyl-2,-4 pentadienoic acid (1.177)

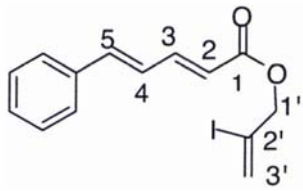


1.177

A mixture of malonic acid (15.7 g, 0.15 mol) and cinnamaldehyde (20.0 g, 0.15 mol) in pyridine (16 ml, 0.199 mol) was heated at 60-70^{0}C for 3.5 h. The reaction was monitored by TLC and the evolution of CO_2. The reaction mixture was cooled and

acidified with aq. sulfuric acid (50%, 25 ml). Most of the 5-phenyl- 2,4- pentadienoic acid separated out immediately. The reaction mixture was kept in the cold over night to obtain more complete separation. The crude mixture was then filtered off and washed with ice-cold water (15 ml) and then recrystalised in aq. EtOH (50%) to give the title compound (21.8 g, R_f = 0.56 (100% EtOAc), 83%) as yellow crystals, m.p; 166-168^{0}C (Lit.[82] mp, 165-166°C). ^{1}H NMR δ/ppm 7.85 (1H, dd, *J* = 11.2, 2.42 Hz, H3), 7.23-7.68 (5H, m, Phenyl), 6.88-7.14 (1H, m, H4), 6.67 (1H, d, *J* = 15.6 Hz, H5), 6.20 (1H, d, *J* = 14.7 Hz, H2). The title compound was also prepared by the method of Gill *et al* [71] using sulphuric acid as the condensing agent.

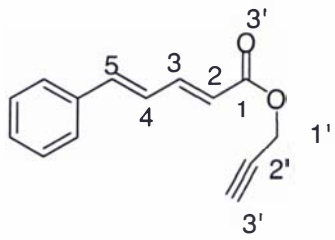
1.2.3.8 2'-Iodo prop-2'-enyl 5-phenylpenta-2*E*, 4*E*-dienoate (1.190)



1.190

To a solution of 3-hydroxy-2-iodo-1-propene (3.16 g, 17.2 mmol) and pyridine (1.95 g, 24.7 mmol) in CH_2Cl_2 (30 ml) was added 5-phenyl-2,4- pentadienoyl chloride (3.10 g, 16.1 mmol) in CH_2Cl_2 (30 ml) and the reaction mixture was stirred under Ar for 3 hrs at RT. The reaction mixture was further diluted with dichloromethane (50 ml) and washed with aq. HCl (2M, 3 x 25 ml). The reaction mixture was then washed with saturated aqueous $NaHCO_3$ (2 x 25 ml), H_2O (50 ml), brine (50 ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude mixture was then purified by column chromatography using Hex:EtOAc (19:1) as eluent to afford the title compound 2'-iodo prop-2'-enyl 5-phenylpenta-2*E*, 4*E*-dienoate (3.5 g, 60 %) as a yellow liquid and an inseparable isomeric mixture of 2'-iodoprop-2'-enyl 5-phenyl pentadienoate (0.38 g, 7 %). ^{1}H NMR δ/ppm 7.75 (1H, d, *J* = 16.0 Hz, H5) 7.34-7.57 (5H, m, phenyl), 7.18 (1H, d, *J* = 16.0 Hz, H4), 6.47 (1H, d, *J* = 15.2 Hz, H3), 6.40 (1H, d, *J* = 15.1 Hz, H2), 5.95 (1H, m, H3'), 4.91 (2H, t, H1'). IR (thin film) ϑ_{max} 2932, 1715, 1615, 1587, 1281 and 1150 cm^{-1}.

1.2.3.9 Prop-2'-ynyl 5-phenyl-2*E*, 4*E*-pentadienoate (1.179)



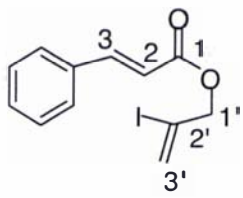
1.179

2-Propyn-1-ol (0.512 g, 9.14 mmol) in CH_2Cl_2 (5 ml) was cooled on an ice-salt bath and pyridine (1.26 g, 15.9 mmol) was added. 5-Phenyl-2,4-pentadienoyl chloride (1.59 g, 8.26 mmol) in CH_2Cl_2 (15 ml) was added slowly over 15 mins and the reaction mixture was stirred under Ar for 2.5 hrs at 35°C. The reaction mixture was diluted with CH_2Cl_2 (25 ml) and washed with aq. 2M HCl (2 x 25 ml), saturated aq. $NaHCO_3$ (2 x 25 ml), H_2O (25.0 ml), brine (25.0 ml) and dried over Na_2SO_4. The solution was filtered and the solvent was removed under reduced pressure to give the crude product, which was then purified by column chromatography using Hex:EtOAc (19 : 1) as eluent to give a white crystalline solid of prop-2'-ynyl-5-phenyl-2*E*, 4*E* -pentadienoate (1.23 g, R_f = 0.42, 64 %), m.p.; 31-33°C, and a pale yellow amorphous solid as an inseparable mixture of *E/Z* isomers prop-2'-ynyl-5-phenyl-2, 4-pentadienoate (0.365 g, 19 %).

The title compound was also prepared in the following way using an active ester method. To a solution of 5-phenyl-2,4-pentadienoic acid (2.00 g, 11.4 mmol) in CH_2Cl_2 (35 ml) was added 2-propyn-1-ol (0.96 g, 17.1 mmol), DCC (2.4 g, 11.6 mmol) and DMAP (0.20 g, 1.57 mmol) sequentially. The solution was stirred at RT under an atmosphere of Ar for 3.5 h. The reaction mixture was filtered and the solvent was removed under reduced pressure. The crude product was diluted with EtOAc (100 ml) and washed with $NaHCO_3$ (3 x 25 ml), dilute aqueous HCl (25 ml), H_2O, saturated brine and dried over $MgSO_4$ to give a semisolid compound, which was then purified by column chromatography using Hex:EtOAc (9 : 1) as eluent to afford the title compound as a white crystalline solid (1.40 g, R_f = 0.56, 58 %), m.p.; 32-36°C. ^{1}H NMR δ/ppm 7.65 (1H, d, *J* = 8.6 Hz, H5) 7.32-7.56 (5H, m, phenyl), 6.78-6.95 (2H, m, C3, H4), 6.0 (1H, d, J = 15.8 Hz, H2), 4.77 (1H, br.s, H1'), 2.54 (1H, br.s, H3'). ^{13}C NMR δ/ppm

165.8 (-COOR), 145.7 (C3), 141.0 (C6), 135.7 (C5), 129.1 128.7, 127.2 (phenyl), 125.8 (C4), 119.8 (C2), 77.9 (C2'), 75.0 (C3'), 51.9 (C1'). IR (thin film) ϑmax 3292, 2128, 1715, 1625, 1594, 1234, and 1129 cm^{-1}.

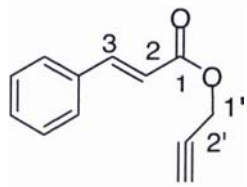
1.2.3.10 2'-Iodoprop-2'-enyl 3-phenyl-2-propenoate (1.206)



1.206

2-Iodopropen-1-ol (2.5 g, 15.3 mmol) in CH_2Cl_2 (25 ml) and pyridine (1.83 g, 23.2 mmol) was stirred under an atmosphere of Ar. After cooling in an ice bath 3-phenyl-2-propenoyl chloride (2.51 g, 15.1 mmol) in CH_2Cl_2 (25 ml) was added over 0.5 h. The solution was stirred further under Ar for 3 hrs at RT and then diluted further with dichloromethane (25 ml) and washed with aqueous dilute HCl (1M, 2 x 25 ml), NaOH (1M, 25 ml), water (25 ml), brine (25 ml) and then dried over $MgSO_4$. The solvent was removed under reduced pressure to give a crude yellow solid. Purification by column chromatography eluting with Hex/EtOAc (9:1) gave the pure white crystalline solid 2'-iodoprop-2'-enyl 3-phenyl-2-propenoate (3.6 g, R_f = 0.25, 75 %), m.p. 51^0- 52^0 C. The title compound was also synthesised in 78 % yield in the presence of triethylamine as a base in a similar fashion. ^{1}H NMR δ/ppm 7.75 (1H, d, J = 16.0 Hz, H3) 7.34-7.57 (5H, m, phenyl), 6.48 (1H, d, J = 16.0 Hz, H2), 6.41 (1H, m, H3'), 5.95 (1H, dt, H3'), 4.81 (2H, t, H1'). ^{13}C NMR δ/ppm 165.3 (-COOR), 145.6 (C3), 134.0 130.4, 128.8, 128.1 (phenyl), 127.0 (C3'), 117.0 (C2), 102.4 (C2'), 70.7 (C1'). IR (thin film) ϑmax 2932, 1702, 1635, 1617, 1308 and 1154 cm^{-1}.

1.2.3.11 Prop-2'-Ynyl 3-Phenyl-2-Propenoate (1.209)

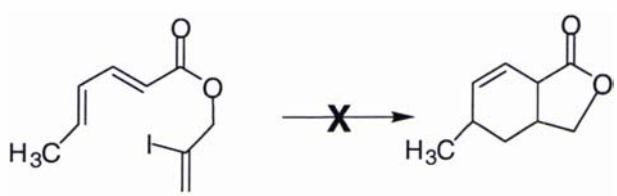


1.209

To a solution of 2-propyn-1-ol (1.02 g, 18.2 mmol) in CH_2Cl_2 (50 ml) and pyridine (2.34 g, 29.7 mmol) was added cinnamoyl chloride (3.20 g, 19.2 mmol) in CH_2Cl_2 (50 ml) over 15 min and the reaction mixture was stirred under Ar for 2.5 hrs at 35°C. the completion of the reaction was monitored by TLC (1:1 Hex:EtOAc, R_f = 0.52) to give a yellow oil. The oil was diluted further with CH_2Cl_2 (10.0 ml) and washed with HCl (1M, 2 x 25 ml), saturated aqueous $NaHCO_3$ (2 x 25 ml), water (25.0 ml), brine (25.0 ml) and then dried over $MgSO_4$. The solvent was removed under reduced pressure and the crude yellow oil purified by column chromatography eluting with Hex:EtOAc (9 : 1) to give prop-2'-ynyl 3-phenyl-2-propenoate (2.78 g, 81 %) as a colorless oil. ^{1}H NMR δ/ppm 7.75 (1H, d, J = 16.0 Hz, H3) 7.34-7.57 (5H, m, phenyl), 6.48 (1H, d, J = 16.0 Hz, H2), 4.83 (2H, d, H1'), 4.71-4.74 (2H, t, H3'). ^{13}C NMR δ/ppm 165.8 (-COOR), 145.7 (C3), 134.0 130.4, 128.8, 128.0 (phenyl), 117.0 (C2), 76.5 (C2'), 74.9 (C1'), 52.0 (C3'). IR (thin film) ϑmax 3292, 2128, 1716, 1636, 1280 and 1161 cm^{-1}.

1.2.4 Attempted Intramolecular And Tandem Intramolecular Cyclisation Reactions

1.2.4.1 Attempted IMC reaction of 2'-iodoprop-2'-enyl 2*E*, 4*E* - hexaadienoate (1.189) using $[PdPh_3]_4$

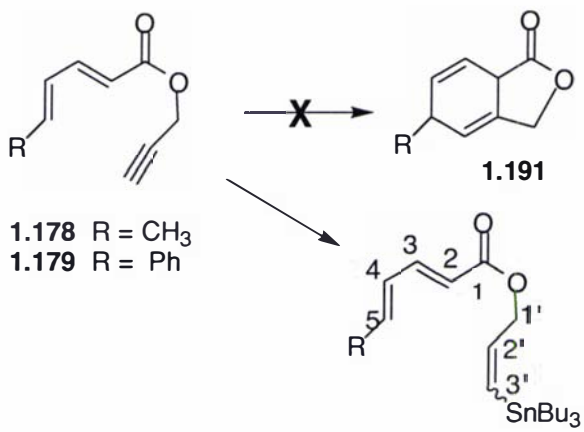


1.189 **1.190**

To 2'-iodo prop-2'-enyl 2*E*, 4*E* - hexaadienoate **1.189** (112 mg, 0.40 mmol) and Et_3N (0.04 g, 0.41 mmol) in CH_3CN (7.2 ml) were added tetrakis (triphenyl phosphine) palladium (12.5 mg, 0.01 mmol) under Ar. The reaction mixture was stirred at RT overnight in the dark. Both TLC and the ^1H NMR spectrum indicated the presence of the starting material with no traces of the required bicyclic adduct **1.190**.

In a further attempt the reaction mixture was refluxed for several hours using essentially the same substrate and reagent concentrations as described above. Both TLC and ^1H NMR spectra indicated decomposition of the starting material and hexa 2*E*, 4*E*- dienoic acid was seen with no traces of the bicyclic adduct.

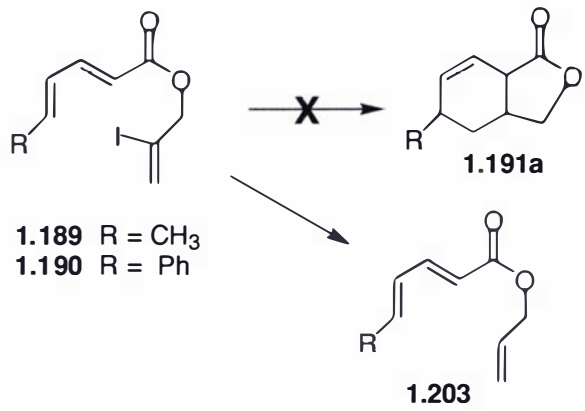
1.2.4.2 Attempted intramolecular free radical mediated cyclisation of prop-2'-ynyl hexa-2*E*, 4*E* – dienoate (1.178) and 5-phenyl prop-2'-ynyl penta-2*E*, 4*E* – dienoate (1.179)



A solution of prop-2'-ynyl hexa-2*E*, 4*E* – dienoate (0.25 g, 1.66 mmol) in dry benzene (31 ml) was degassed, and treated with AIBN (27 mg, 0.17 mmol) and heated to reflux under an atmosphere of Ar. A solution of Bu_3SnH (45 μl, 0.049 g, 0.17 mmol, 0.1 eq) in benzene (2.0 ml) was added dropwise over 5 mins. The reaction mixture was heated at reflux for a further 16 h under argon. At the end of this time both TLC and ^1H NMR showed unreacted starting materials and traces of the hydrostanylated product. The cyclisation reaction was also attempted on 5-phenyl prop-2'-ynyl penta-2*E*, 4*E* – dienoate under the same conditions described above but gave largely unreacted starting materials.

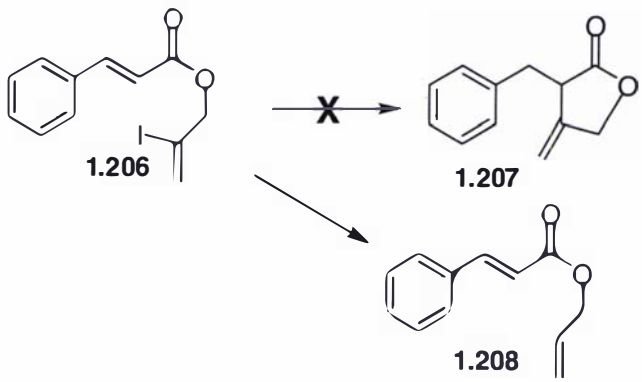
In a further attempt a solution of prop-2'-ynyl hexa-2*E*, 4*E* – dienoate (0.5 g, 3.32 mmol) in dry benzene (6 ml) was degassed, and treated with AIBN (55 mg, 0.34 mmol), and heated to reflux under an atmosphere of Ar. A solution of Bu_3SnH (90 μl, 0.098 g, 0.34 mmol, 0.1 eq) in benzene (1 ml) was added dropwise over 15 mins. The reaction mixture was heated at reflux for a further 16 h under argon. The ^1H NMR spectrum of the crude reaction mixture revealed a greater amount of hydrostanylated product but no traces of the bicyclic adduct.

1.2.4.3 Attempted free radical mediated TIMC reaction of 2'-iodo prop-2'-enyl hexa-2*E*, 4*E*-dienoate (1.189) and 2'-iodo prop-2'-enyl 5-phenylpenta -2*E*, 4*E*-dienoate (1.190)



A solution of 2'-iodo prop-2'-enyl hexa-2*E*, 4*E*-dienoate (0.5 g, 1.79 mmol) in dry benzene (175 ml) was degassed, and treated with AIBN (50 mg, 0.30 mmol), and heated to reflux under an atmosphere of Ar. A solution of Bu_3SnH (550 μl, 0.17 mmol) was added and the reaction mixture was heated to reflux for a further 3 h under Ar. Purification by column chromatography eluting with hexane followed by Hex:EtOAc (5%) gave largely unreacted starting materials and the acyclic reduced product (0.04 g, R_f = 0.46, 14%). The above reaction was repeated for 16 hrs and the progress of the reaction was monitored by ^1H NMR spectroscopy but indicated the same result as described above.

1.2.4.4 Attempted free radical mediated IMC reaction of 2'-iodoprop-2'-enyl 2*E*, 4- pentadienoate (1.206)



A solution of 2'-iodoprop-2'-enyl 3-phenyl-2-propenoate (0.10 g, 0.318 mmol) in dry benzene (33.0 ml) was degassed, and treated with AIBN (10.9 mg, 0.006 mmol) and warmed to reflux. A solution of Bu_3SnH (0.11 ml, 0.12 g, 0.413 mmol, 1.1 equiv) in benzene (2.0 ml) was added dropwise over 5 min. The reaction mixture was refluxed for 2-5 hr under Ar. The solvent was removed under reduced pressure and sample submitted for ^1H NMR, which showed unreacted starting material and small amount of the reduced product.

A solution of 2'-iodoprop-2'-enyl 3-phenyl-2-propenoate (0.10 g, 30.9 mmol) in drybenzene (58.0 ml) was stirred at reflux under argon. A solution of Bu_3SnH (104 mg, 35.7 mmol, 1.15 equiv) and a catalytic amount of AIBN (14.6 mg, 0.089 mmol) in dry benzene (2.0 ml) were added over 2 hr using a syringe pump. The reaction mixture was stirred for a total of 28 h with ^1H NMR used to study the crude reaction mixture. The ^1H NMR spectrum of the crude reaction mixture showed a mixture of the reduced product and starting material (*ca*. 30:70 %).

In a further attempt a solution of the 2'-iodoprop-2'-enyl 3-phenyl-2-propenoate (0.201 g, 63.8 mmol) in dry benzene (34.0 ml) was stirred at reflux under an atmosphere of Ar with a catalytic amount of AIBN (12.0 mg, 0.007 mmol). A solution of Ph_3SnH (407 mg, 1.15 mmol) in dry benzene (20.0 ml) was added over 5.5 h using a syringe pump. Upon completion of the addition, the reaction was allowed to cool at RT, and sample submitted for ^1H NMR, which showed the acyclic reduced product and starting material. The reaction mixture was stirred for a total of 24 h with ^1H NMR used to

study the crude reaction mixture every hour. After 24 h the ^{1}H NMR spectrum of the crude reaction mixture showed a mixture of the reduced product and starting material (*ca*. 30:70 %). A further 0.2 eq. of Ph_3SnH and 0.1 eq. of AIBN were added and the reaction mixture was refluxed for 2 h. The reaction was allowed to cool at RT, and the benzene was removed under reduced pressure. The residue was dissolved in ether (15.0 ml) and treated with 10% (w/v) aqueous KF (15.0 ml), and stirred vigorously at RT over 1 h. The white suspension which formed was filtered through celite®. The organic layer was washed with water (15.0 ml), brine (15.0 ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure to give a colorless oil, which was then purified by flash chromatography (hexane-ether-dichloromethane, 80:10:10) to give the acyclic reduced product as a colourless liquid (87 mg , 71%, R_f = 0.58) with no traces of the bicyclic adduct.

1.2.5 Free Radical Mediated Reactions Of Iodo And –Yne Precursors At Higher Concentration

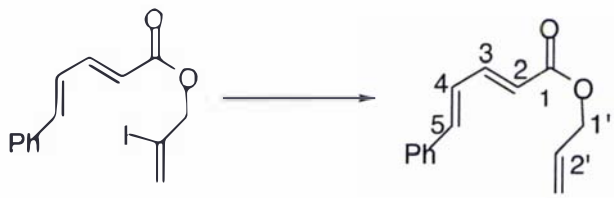
1.2.5.1 Free radical mediated reaction of 2'-iodoprop-2'-enyl hexa-2*E*, 4*E*-dienoate (1.189)

1.189 R = CH_3 **1.203**

A solution of Bu_3SnH (0.40 ml, 0.43 g, 1.48 mmol, 92% pure by ^{1}H NMR) and AIBN (0.04 g, 0.29 mmol) was added to 2'-iodoprop-2'-enyl 2E, 4E-hexadienoate (0.50 g, 1.80 mmol) in dry and degassed benzene (14.5 ml) under reflux over 10 min. The reaction mixture was stirred at reflux for 1.5 h and further portions of Bu_3SnH (0.11 ml, 0.110 g, 0.37 mmol) and AIBN (0.01g, 0.07 mmol) in benzene (1.0 ml) were added over 2 min. After a further 1 h at reflux the mixture was allowed to cool to room temperature and the solvent removed *in vacuo*. The residue was dissolved in ether (100 ml) and washed with NH_3 solution (6 X 80 ml of 15 % solution), dried over $MgSO_4$ and the solvent was removed *in vacuo* to give an oil, which was then quickly purified by flash

chromatography (Hex, Hex: EtOAc, 9:1) to give a pale yellow liquid (0.21 g, 1.38 mmol, 76 %). ^{1}H NMR δ/ppm 7.34-7.23 (1H, m, H3) 6.21-6.16 (2H, m, H4-H5), 5.9-6.0 (1H, m, H2'), 5.77 (1H, d, *J* = 15.4 Hz, H2), 5.27 (2H, dd, *J* = 17.4 Hz, 10.5 Hz, H3'), 4.63 (2H, d, *J* = 6.3 Hz, H1'), 1.85 (1H, d, *J* = 5.6 Hz, CH3). ^{13}C NMR δ /ppm 165.4 (C=O), 145.6 (C3), 139.2 (C4), 135.4 (C2'), 129.7 (C2), 117.2 (C5), 118.1 (C3'), 65.0 (C1'). IR (thin film) ϑ_{max} 938, 1717, 1645, 1618, 1242 and 1137 cm^{-1}.

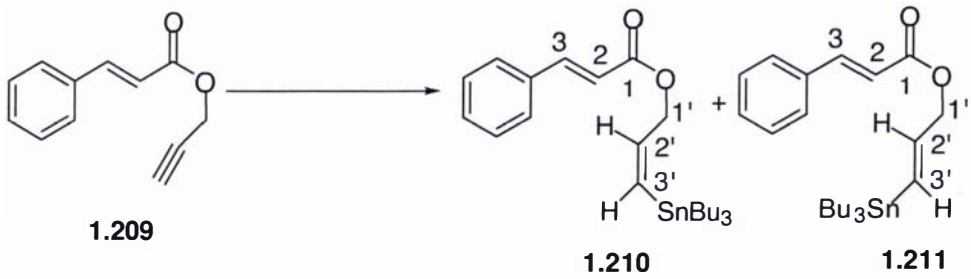
1.2.5.2 Free radical mediated reaction of 2'-iodoprop-2'-enyl 5-phenylpenta -2*E*, 4*E*-dienoate



1.190 R = Ph **1.203**

A solution of Bu_3SnH (0.40 ml, 0.43 g, 1.48 mmol, 92% pure by ^{1}H NMR) and AIBN (0.04 g, 0.29 mmol) was added to 2'-iodoprop-2'-enyl 5-phenyl-2E,4E-pentadienoate (0.50 g, 1.46 mmol) in dry and degassed benzene (14.5 ml) at reflux over 10 min. The reaction mixture was stirred at reflux for 1.5 h and further portions of Bu_3SnH (0.11 ml, 0.110 g, 0.38 mmol) and AIBN (0.01g, 0.07 mmol) in benzene (1.0 ml) were added over 2 min. After a further 1 h at reflux the mixture was allowed to cool to room temperature and the solvent removed *in vacuo*. The residue was dissolved in ether (100 ml) and washed with NH_3 solution (6 X 80 ml of 15 % solution), dried over $MgSO_4$ and the solvent was removed *in vacuo* to give an oil, which was then quickly purified by flash chromatography (Hex, Hex: EtOAc, 9:1) to give a pale yellow liquid (0.21 g, 67 %). ^{1}H NMR δ /ppm 7.75 (1H, d, *J* = 16.0 Hz, H5) 7.35-7.54 (5H, m, phenyl), 6.90-6.93 (2H, m, H3-H4), 6.0 (1H, d, *J* = 15.6 Hz, H2), 5.9-6.0 (1H, m, H2'), 5.32 (1H, dd, *J* = 16.4 Hz, 10.2 Hz, H3'). 4.70 (2H, d, H1'). ^{13}C NMR δ /ppm 166.4 (C=O), 144.8 (C3), 140.5 (C6), 135.8 (C2'), 132.2 (C5), 134.2 (C4), 128.9, 128.7, 127.1 (phenyl), 126.0 (C4), 120.7 (C2), 118.0 (C3'), 65.0 (C1'). IR. ϑ_{max} (thin film) 2945, 1714, 1616, 1589, 1233 and 1151 cm^{-1}.

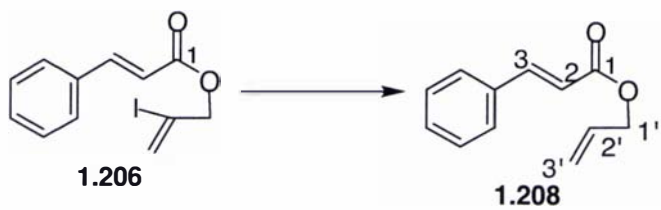
1.2.5.4 Free radical mediated reaction of (prop-2'-yne)-3-phenyl 2-propenoate) (1.209)



1.209 1.210 1.211

(Prop-2'-yne)-3-phenyl 2-propenoate (0.110 g, 0.591 mmol) was dissolved in benzene (5.0 ml). The solution was stirred under Ar and heated to reflux. The Bu_3SnH (0.094, 0.34 mmol) and AIBN (0.019g, 0.118 mmol) were added and the reaction mixture was refluxed for 3.5 h to ensure completion of the reaction. The reaction mixture was cooled to RT and the solvent was removed under reduced pressure. The residual yellow liquid was dissolved in Et_2O (25 ml), washed with aq. NH_3 (20%, 6 x 25ml) and dried over $MgSO_4$. The solvent was removed under reduced pressure to give a pale yellow oil which was then purified by column chromatography eluting with hexane followed by Hex:EtOAc (9:1) to give an inseparable mixture of **1.210** and **1.211** in a 88:12 ratio (0.16 g, 57%, Rf=0.46 as a colourless liquid. The ^1H NMR of the major isomer is indicated by the following peaks. ^1H NMR δ /ppm 7.78 (1H, d, J = 16.2 Hz, H3), 7.35-7.58 (5H, m, phenyl), 6.48 (1H, d, J = 9.5 Hz, H2), 6.36-6.42 (1H, m, H3'), 4.78 (2H, dd, J = 17.2, 10.3 Hz, H1').

1.2.5.5 Free radical mediated reaction of 2' iodoprop-2'enyl 3-phenyl-2-propenoate 1.206



1.206 1.208

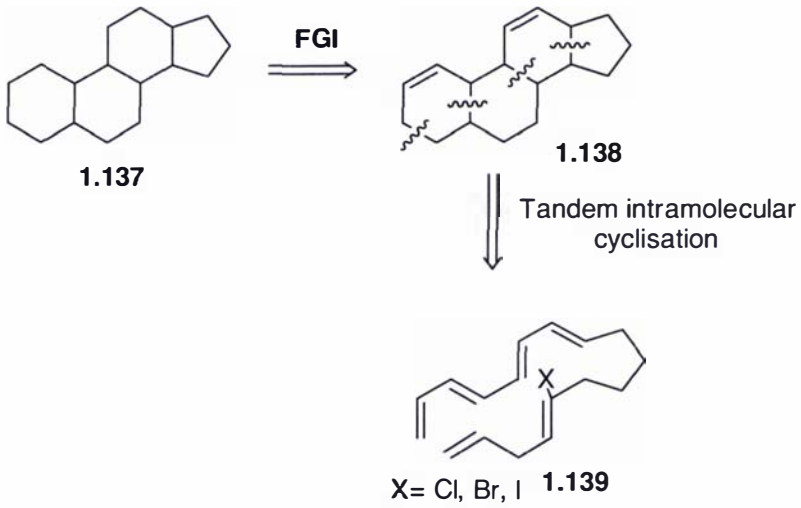
A solution of Bu_3SnH (0.24 ml, 0.26 g, 0.89 mmol) and AIBN (0.02 g, 0.12 mmol) in dry benzene (3.7 ml) was added to 2'-iodoprop-2'-enyl 3-phenyl-2-propenoate (0.304 g, 0.97 mmol) in benzene (5.0 ml) at reflux over 5 min. The reaction mixture was stirred

under reflux for 3 h and further portions of Bu_3SnH (0.1 ml, 0.108 g, 0.37 mmol) and AIBN (0.01 g, 0.07 mmol) in benzene (1.0 ml) were added over 5 min. After a further 1 h at reflux the mixture was allowed to cool to room temperature and the solvent was removed *in vacuo*. The residue was dissolved in Et_2O (50.0 ml) and washed with NH_3 solution (6 x 50 ml of 15 % solution), dried over $MgSO_4$ and then the solvent was removed *in vacuo*. The residue was quickly purified by column chromatography (Hex, Hex:EtOAc, 9:1) to give a pale yellow liquid (0.116 g, 0.61 mmol, 63 %). ^{1}H NMR δ/ppm 7.75 (1H, d, J = 16.0 Hz, H3) 7.34-7.57 (5H, m, phenyl), 6.48 (1H, d, J = 16.0 Hz, H2), 5.94-6.06 (1H, m, H2'), 4.71-4.74 (2H, dd, J = 17.4 Hz, 10.5 Hz, H3'), 4.81 (2H, d, J = 5.72 Hz, H1'). ^{13}C NMR δ /ppm 166.4 (C=O), 144.9 (C3), 134.2 (C4), 132.2 (C2'), 130.2, 128.8, 128.0 (phenyl), 118.1 (C2), 117.8 (C3'), 65.2 (C1'). IR. ϑ_{max} (thin film) / cm^{-1} 2932, 1702, 1635, 1617, 1308 and 1154 cm^{-1}.

1.3 RESULTS AND DISCUSSION

1.3.1 A New Synthetic Strategy Towards Formation Of The Spongian/Steroid Type Framework

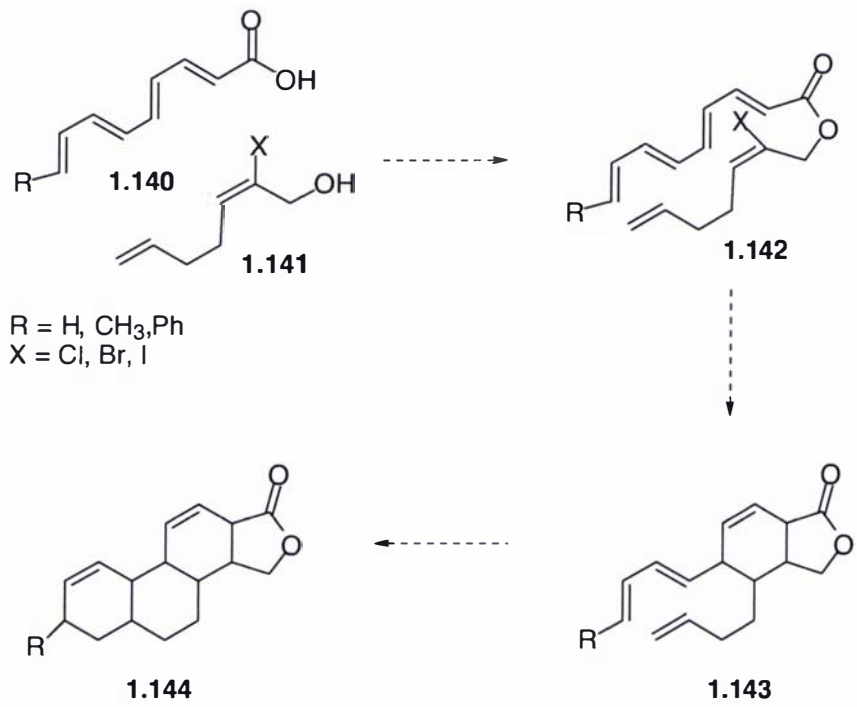
In spite of their interesting biological properties, until quite recently very few synthetic studies amongst this class of compound had been described.[14,58,59] Efficient and flexible syntheses are required if these complex natural products are to be commercially useful. The pharmalogically useful compounds can be synthesised by either modifying precursors or by total synthesis, however the synthesis must be short giving enantiomerically pure products and reactions must be easily carried out on a large scale. A novel approach of disconnection analysis of **1.137** of the non phenolic steroid meets this necessary requirement and hence is a convenient way to obtain the spongian/steroid skeleton. If successful this strategy could create up to four new C-C bonds and eight new stereogenic centers in one step. Since esterification is a convenient way of attaching a *bis* diene to a *bis* dienophile moitiey and it can be synthesised using shorter synthetic routes and inexpensive starting materials (**Scheme 1.28** and **1.30**) it is an ideal approach for the spongian/steroid type frame work.



Scheme 1.26

Given that the synthetic target is the spongian 16-one **1.4** with a D-ring lactone a synthetically facile method of connecting the *bis*-diene and *bis*-dienophile components of a tandem intramolecular cyclisation precursor is with an ester tether. The general

feasibilty of the cyclisation reactions of ester-linked precursors has been demonstrated by several groups (**Section 1.1.3.2**, **1.1.5.2** and **1.1.5.3**) but little work has been reported in which the ester carbonyl group is conjugated with the bis-diene. To investigate the synthesis of the spongian 16-one skeleton by tandem intramolecular cyclisation on an ester tethered iodo precursor **1.142** the following retrosynthetic plan was developed.



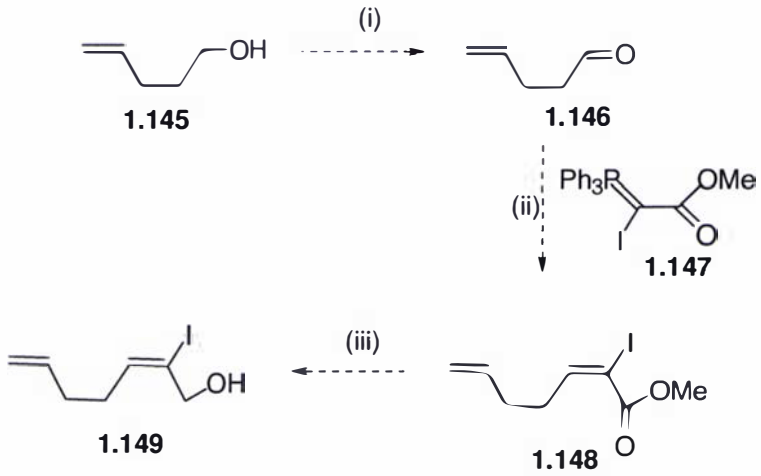
Scheme 1.27

Once the tetracyclic lactone **1.44** is obtained it could be transformed easily into the steroid skeleton. Hence it was decided to apply this efficient convergent tandem intramolecular methodology for practical synthesis of a wide range of tetracyclic natural products such as spongian diterpenoids and steroids.

1.3.1.1 Synthesis Of Bis-Z-Dienophile And Bis-Z -Diene

Owing to the difficulty of preparing suitable precursors, there are very few examples of tandem cyclisation reactions reported in the literature. It was hoped that the *bis-Z*-dienophile unit could be synthesised from the commercially available starting material 4-penten-1-ol. The synthetic strategy (**Scheme 1.28**) included the oxidation of 4-penten-1-ol (**1.145**), using Dess-Martin periodinane[60] followed by a Wittig reaction of

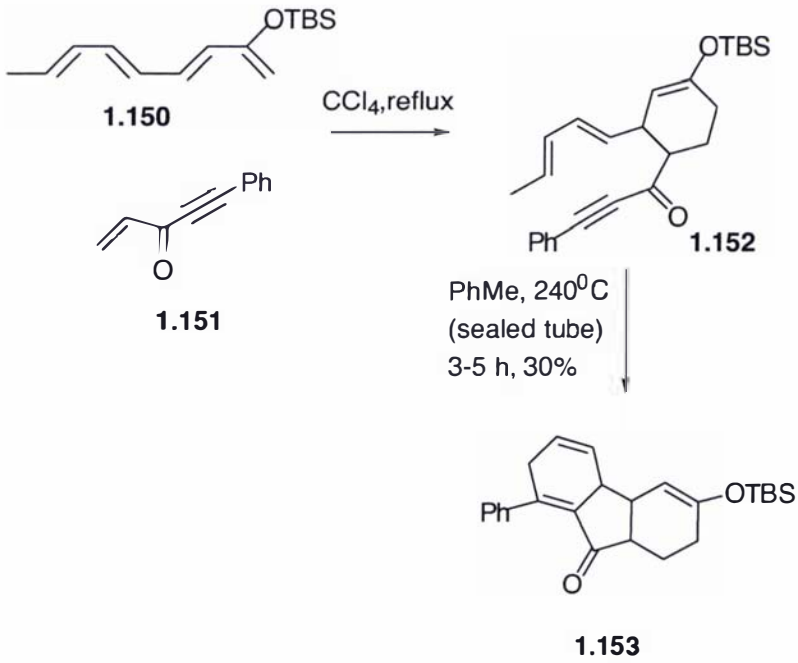
the resulting aldehyde (**1.146**) with the stable phosphonium salt[61] (**1.147**) to obtain the iodo ester (**1.148**). The subsequent selective reduction[62] using di isodibutyl aluminium hydride (DIABLH) could give a desired iodo alcohol **1.149**.



Reagents and conditions *: (i) Dess-Martin periodinane, CH_2Cl_2, RT, (ii) CH_2Cl_2, RT, BHT, (iii) DIBALH, , CH_2Cl_2, -78 ^0C*

Scheme 1.28

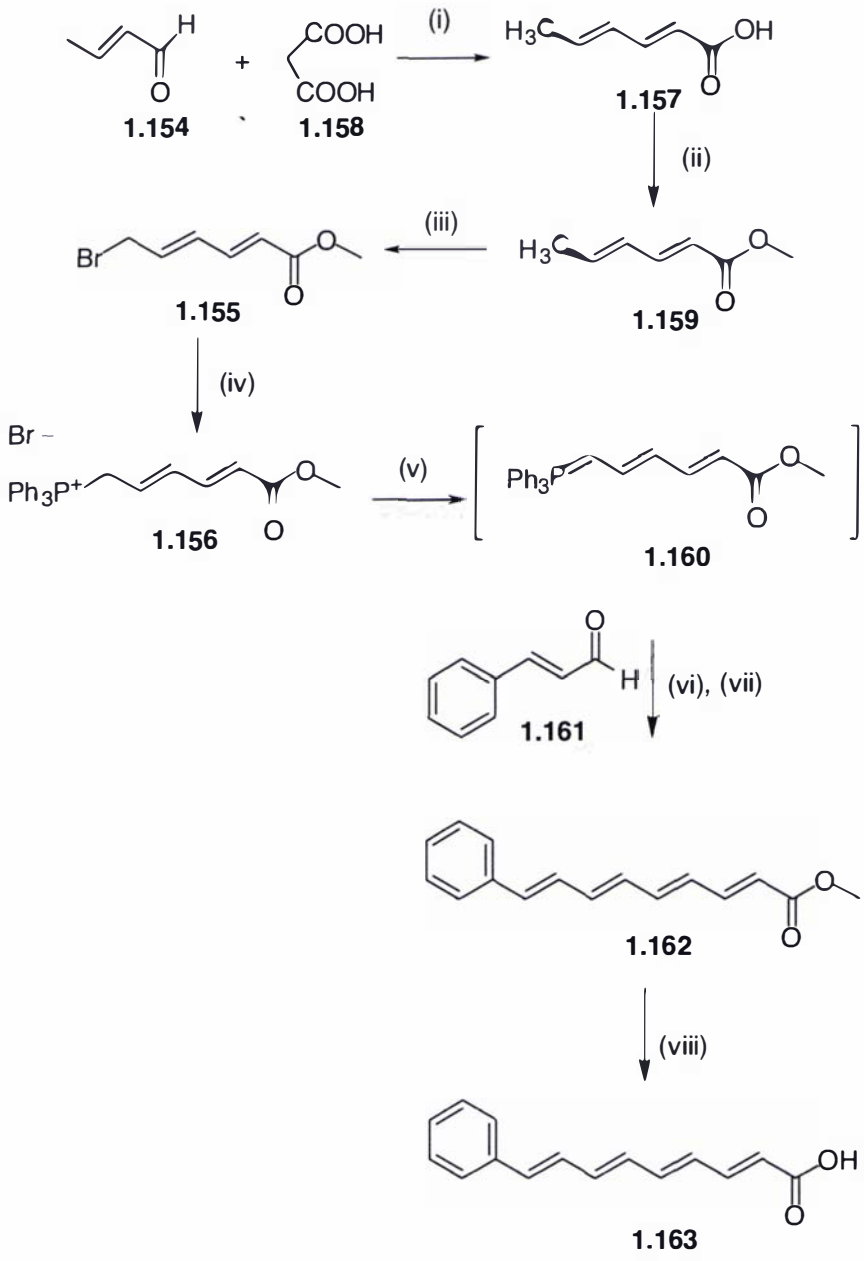
The *bis* –diene unit **1.140** (**Scheme 1.27**) consisted of a conjugated tertraene. Kraus and Taschner[63] disclosed a unique approach of use of a tetraene as the *bis* -diene in the synthesis of the tricyclic fluorenone ring system **1.153**. Thus *bis* -diene **1.150** and *bis* –dienophile **1.151** were allowed to undergo an intermolecular DA reaction followed by an IMDA reaction to form the tricyclic ring in a single reaction.



Scheme 1.29

Kraus and Taschner commented that a delicate balance between relative reactivity and the directing effects of substituents on the *bis* --diene is involved for successful cycloaddition.[63]

The conjugated tetraene as *bis* --diene **1.161** synthesised in the present work was able to be stored for a few days at 0^0C under an inert atmosphere. In general such dienes were found to be unstable at RT when stored for longer time.[14] Polyolefinic units of different geometric configurations and lengths are found in a number of biologically relevant natural and non natural products. Although much effort has been devoted to the synthesis of polyenes over the years, problems concerned with stereo selectivity, stability, accessibility to geometrically uniform long chain units, and incorporating diverse functionality have fostered a number of recent studies in this area.[64,65] Nevertheless, improved methodology is in much demand. In the present study aimed at a practical and versatile approach to the conjugated tetraene as a *bis*-diene moiety 9-phenyl-nona-2*E*, 4 *E*, 6 *E*, 8 *E*-tetraenoic acid has been synthesised from the inexpensive starting material hexa 2*E*, 4*E*-dienoic acid as shown below in **Scheme 1.30**.



Reagents and conditions : *(i) Pyridine, reflux, 4-6 h, 27%, (ii) CH_3OH, H_2SO_4, 40 ^0C, 20 h, 75%, (iii) NBS, Bz_2O, Chlorobenzene, reflux, 2 h, 65%, (iv) Ph_3P, Et_2O, RT, 20 h, dark, 76%, (v) 2N NaOH, CH_2Cl_2, 15 minutes, (vi) BHT, CH_2Cl_2, 40 ^0C, dark, 5 h, 73%, (vii) PhSH, AIBN, BHT, reflux, UV/Visible light, 1 h, 36% (viii) KOH, EtOH, reflux, 2 h, 100%.*

Scheme 1.30

The important aspects of this **Scheme 1.30** include the synthesis of hexa 2*E*, 4*E*-dienoic acid by condensation of malonic acid **1.158** and crotonaldehyde **1.154** followed by acid catalysed esterification with CH_3OH which is best effected at 35^{0}C to give the

methyl ester of hexa 2*E*, 4*E*-dienoic acid **1.157** in good yield (75%). When the reaction was attempted at higher temperatures, it gave a mixture of decomposed material and the yield of the methyl ester of hexa 2*E*, 4*E*-dienoic acid was low. The crude **1.159** was used in the next step without further purification. This on allylic bromination with NBS in dry chlorobenzene under reflux gave a mixture of the unreacted starting material and the required 6-bromo-hexa 2*E*, 4*E*-dienoic acid methyl ester **1.155**. Fractional distillation of the crude reaction mixture at 87-90^{0}C/0.1 mm Hg gave pure 6-bromo-hexa 2*E*, 4*E*-dienoic acid methyl ester. Kinoshita *et al* [66] reported a synthesis of this compound **1.159** from methyl 4-bromo crotonate in three steps using expensive reagents and chemicals and without any spectroscopic evidence to characterise the product. Because of the extremely hygroscopic nature of the phosphonium salt the remaining steps have been carried out in the dark and under a strictly inert atmosphere. Nucleophilic substitution by triphenyl phosphine (Ph_3P) on the allylic bromide **1.159** according to the procedure of Weedon *et al* [67] (refluxing in C_6H_6) gave the required 5-methoxy carbonyl penta-2*E*-4*E*-dienyl triphenyl phosphonium bromide as a brown solid in 53% yield. When the same reaction was attempted under mild conditions (diethyl ether, RT) 5-methoxy carbonyl penta-2*E*-4*E*-dienyl triphenyl phosphonium bromide was obtained as a white amorphous solid and in much better yield (76%) than previously reported (57%).[67] The de-protonation reaction of the 5-methoxy carbonyl penta-2*E*-4*E*-dienyl triphenyl phosphonium bromide **1.156** in $CH_2Cl_2$with 2N NaOH resulted in the *in situ* formation of the ylide in 15 minutes. The ester linkage present in compound **1.160** remained intact, probably because of the relatively lower concentration of hydroxide or the small reaction time which permitted very little (if any) hydrolysis. Butylated hydroxy toluene (BHT) was added as an oxidant to prevent the oxidation of the unsaturated bonds. Nucleophilic attack by the ylide **1.160** on the carbonyl carbon of cinnmaldehyde by refluxing in toluene produced a mixture of the *E*, *Z*-isomers of methyl 9-phenyl-2, 4, 6, 8-nonatetraenoate and unreacted starting materials. Careful crystallisation in acetone/petroleum ether at 0^{0}C gave the required *trans* isomer methyl 9-phenyl-2*E*, 4*E*, 6*E*, 8*E*-nonatetraenoate as a yellow solid in poor yield (12%). A better yield (36%) was obtained when the same Wittig reaction was tried with 2 equivalents of the phosphonium salt in CH_2Cl_2 at RT. When purification was attempted on silica gel slight decomposition was observed. Isomerisation of the geometrical isomers of methyl 9-phenyl-2, 4, 6, 8-nonatetrenoate in the presence of iodine in CH_2Cl_2 at RT in light gave methyl 9-phenyl-2*E*, 4*E*, 6*E*, 8*E*-nonatetraenoate in 36% yield. The isomerisation

reaction was also successfully tried in the dark in the presence of I_2 in CH_2Cl_2 at RT to obtain methyl 9-phenyl-2, 4, 6, 8-nonatetrenoate without any improvement in yield. When the isomerisation was attempted in the presence of thiophenol and AIBN in benzene at reflux methyl 9-phenyl-2*E*, 4*E* 6*E*, 8*E*-nonatetraenoate was obtained in 32% yield as yellow needles. The isomeric mixture of methyl 2, 4, 6, 8 –deca tetraenoate was also obtained in a similar fashion using crotonaldehyde (1.0 equ.) and the phosphonium salt (1.5 equ.) but the isomerisation reaction to all *trans* was not attempted.

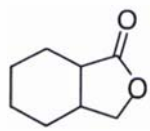
The IR spectrum of methyl 9-phenyl-nona-2*E*, 4*E*, 6*E*, 8*E* -tetraenoate showed a characteristic band at 1712 cm^{-1} (-C=O stretch for the -COOMe) and 1630 cm^{-1} (-C=C stretch) indicative of the unsaturated carbonyl group. The ^1H NMR spectrum of methyl 9-phenyl-nona-2*E*, 4*E*, 6*E*, 8*E* -tetraenoate showed a multiplet at δ 7.25-7.44 ppm with integral values for 6 protons indicative of the presence of a phenyl group and another unsaturated proton. It also shows a doublet at 5.90 ppm indicative of proton αto the carbonyl group (H2). The *trans*-diaxial relationship (β-orientation) of these protons was demonstrated by the large coupling constant of the H-2 (J = 15.2 Hz) and H-7 (J = 10.3 Hz) protons. The other olefinic protons of the tetraenoate **1.162** appear to be all in the *trans*-diaxial relationship due to the large coupling constants for all the neighbouring protons (J > 10 Hz) in the clean ^1H NMR spectrum. In the ^1H-^1H 2D cosy spectrum of **1.162** (**Figure 1.16**) five correlated protons due to aromatic protons and H-3 proton appeared as a multiplet in the region 6.83-6.93 ppm, since this proton (H-3) was corelated with doublet due to H-2 proton (6.02 ppm). The correlation of H-4 and H-9 protons (6.33-6.55 ppm) correspondingly with H-3 and aromatic region (6.83-6.93 ppm) was also obvious in the spectrum. The correlation of H-7 (dd, 6.88 ppm) with H-8 and correlation of H-2 (dd, 5.90 ppm) with H-3 were also obvious in the spectrum. The H-5 and H-6 protons was hidden due to protons H-5, H-6 and H-8 all appeared in the same region (6.58-6.70 ppm) were also established based on correlation with corresponding protons. In addition ^1H-^{13}C HETCOR experiment (**Figure 1.17**) was also carried out to correlate assigned protons directly with carbons to support the assignments of carbon peaks.

Various attempts to hydrolyse methyl 9-phenyl-2*E*, 4*E* 6*E*, 8*E*-nonatetraenoate **1.162** using LiOH in THF/MeOH/H_2O resulted in only partial hydrolysis but when hydrolysis was attempted in KOH/EtOH at reflux the target *bis*-diene methyl-9-phenyl-

2*E*, 4*E*, 6*E*, 8*E*-nonatetraenoic acid was achieved in 100% yield. The IR spectrum of 9-phenyl-nona-2*E*, 4*E*, 6*E*, 8*E* -tetraenoic acid showed the characteristic band at 1683 cm^{-1} (-C=O stretch) and 1617 cm^{-1} (-C=C stretch) indicative of the unsaturated carbonyl group. The presence of the acid was also confirmed by the characteristic band at 3200-2500 cm^{-1} (-OH of -COOH) and 1010 cm^{-1} (-OH bending). The ^1H NMR spectrum of 9-phenyl-nona-2*E*, 4*E*, 6*E*, 8*E* -tetraenoic acid showed the disappearance of the $-CH_3$ group at δ 3.76 ppm from the starting material.

1.3.2 Model Studies On Spongian C/D Ring System

The hydro benzofuranone nucleus **1.164** is a desirable target due to its prevalence in many naturally occurring biologically active compounds such as spongian-16-one. The purpose of the work presented in this chapter therefore was to investigate the possibility of synthesising this bicyclic lactone **1.164** by utilising the intramolecular cyclisation reaction of an ester linked triene using standard DA reaction conditions (with or without Lewis acids) before embarking the complete syntheses of tetracyclic skeleton such as spongian 16-one.



1.164

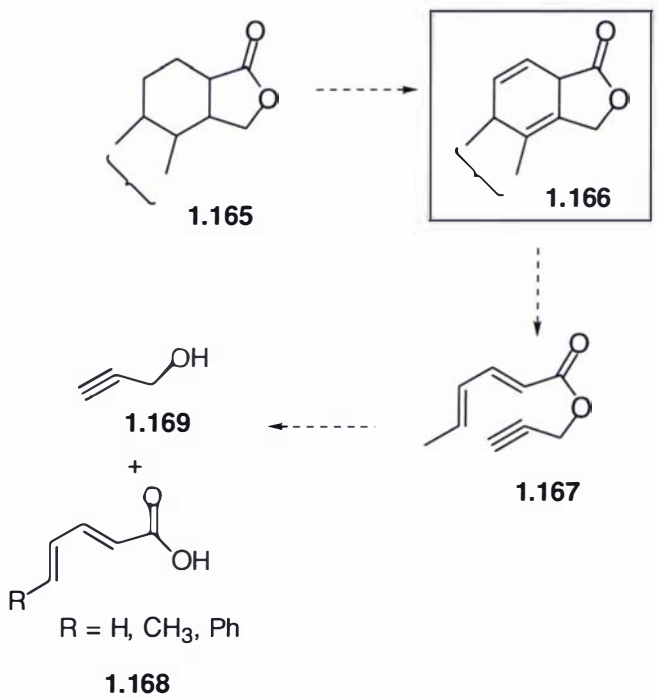
Figure 1.13

The utilisation of an ester linkage between the diene and dienophile often has adverse effects on the reaction rate, however the effect is less pronounced when the ester group is placed such that it activates the dienophile. Utilisation of an ester linkage resulted in a decreased reaction rate, however synthesis of an ester linkage between diene and dienophile is convenient and high yielding. The feasibilty of a stereoselective intramolecular cyclisation reaction of ester linked triene **1.167** in producing enantiopure bicyclic lactones **1.166**, was investigated for two main reasons.

Firstly, it can provide an efficient entry into the construction of the benzofuranone ring system commonly found in many naturally occurring bioactive

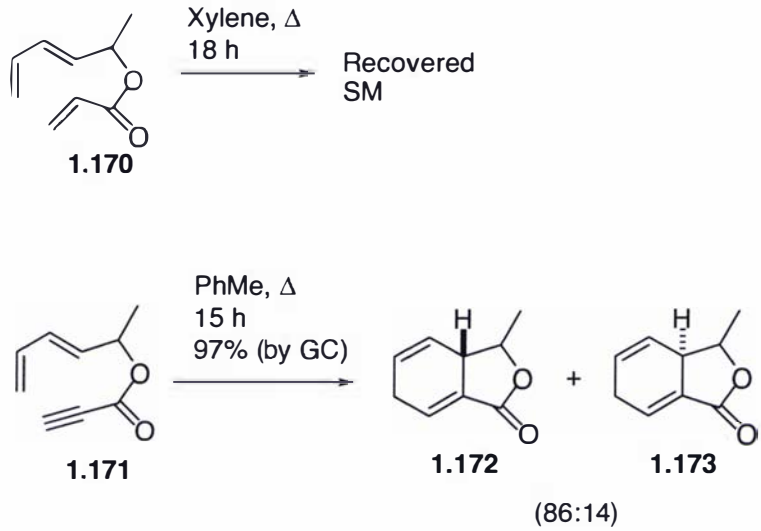
natural products such as the spongian C/D ring system. Secondly, if successful it can act as a model study for the initial intramolecular cyclisation step in a tandem intramolecular cyclisation reaction.

The system chosen for this model study was the propyonate ester **1.167** (**Scheme 1.30**) containing an unactivated diene and dienophile linked by an ester group. The propyonate ester **1.167** was chosen due to the rate increase seen previously with acetylenic dienophiles relative to the corresponding alkenic dienophiles (**Section 1.1.3.2**).



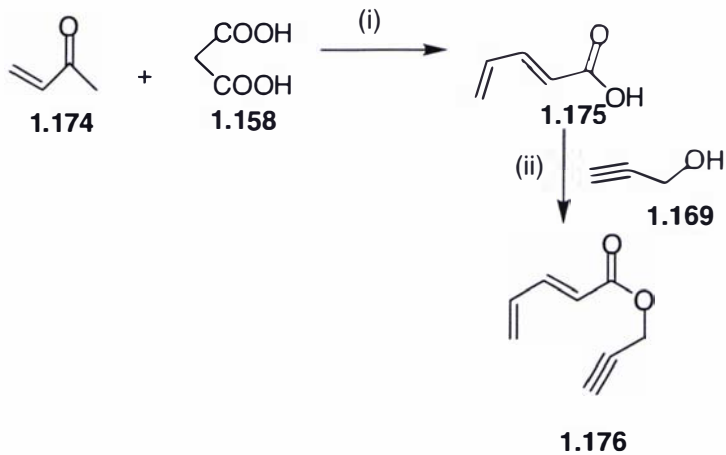
Scheme 1.31

Previous attempts to cyclise a similar structure, the singly activated acrylate ester **1.170**, by Birtwhistle *et al* [68] in refluxing xylene met with failure, whereas the more reactive propynoate ester **1.171** underwent an IMDA reaction in refluxing toluene to give mixture of bicyclic lactones **1.172** and **1.173** in 97% yield. Thus it was anticipated that an acetylenic dienophile might lead to cyclisation even when the dienophile is unactivated as in the case of **1.176**, **1.178** and **1.179**.



Scheme 1.32

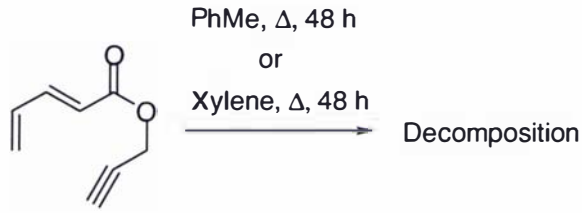
For the convenient entry into the synthesis of the bicyclic lactone by the unprecedented IMDA reaction we chose to synthesise the simple propargyl ester precursor of 2,4-penta dienoic acid. It is apparent that propargyl alcohol and 2,4-penta dienoic acid (obtained by the condensation of acrolein and malonic acid[69]) are ideal starting materials and hence the IMDA precursor propynoate **1.176** was conveniently prepared by coupling of penta 2*E*, 4-dienoic acid and propargyl alcohol using dicyclohexyl carbodimide (DCC) and 2, 6-dimethyl amino pyridine (DMAP) as activating reagents by the method originally developed by Steglich[70] (**Scheme 1.32**).



***Reagents and conditions**: (i) Pyridine (1.3 eq), 60 ^{0}C, 1h, 42%; (ii) DCC (1.2 eq), DMAP (0.1 eq), CH_2Cl_2, RT, Ar, 3h, 44%.*

Scheme 1.33

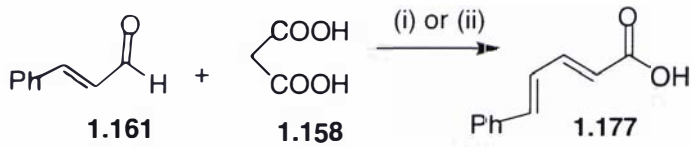
However the attempt to cyclise the propynoate **1.176** in refluxing toluene or xylene for two days proved to be unsuccessful. The reaction mixture was monitored by TLC and ^{1}H NMR spectroscopy which initially showed only unreacted starting material and started to give decomposed polymeric material on prolonged heating (**Scheme 1.33**).



1.176

Scheme 1.34

To increase the stability of the precursors at high temperature it was decided to synthesise the propynoates **1.178** and **1.179** hence 5-phenyl 2*E*, 4 -pentadienoic acid was prepared by condensation of malonic acid **1.158** with cinnamaldehyde **1.164**. 5-Phenyl 2E, 4-pentadienoic acid was also synthesised using the method reported by Gill et al[71] using concentrated H_2SO_4 as condensing agent in almost the same yield.

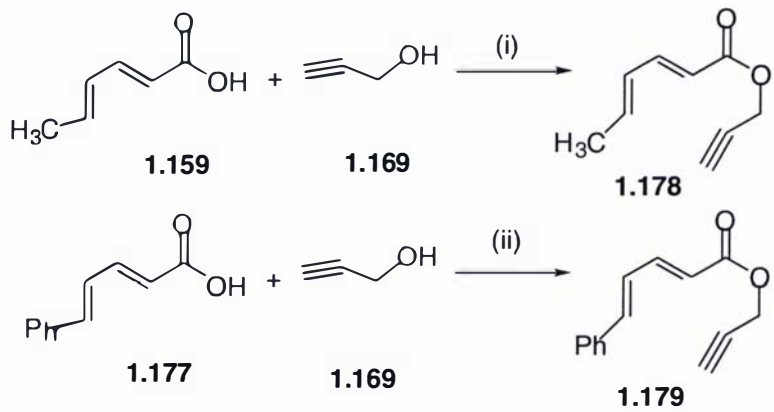


1.161 **1.158** **1.177**

Reagents and conditions : *(i) Pyridine (1.3 eq), 60 ^{0}C, 3.5 h, 83%; (ii) Concd. H_2SO_4, 60 ^{0}C, 1h, 82%.*

Scheme 1.35

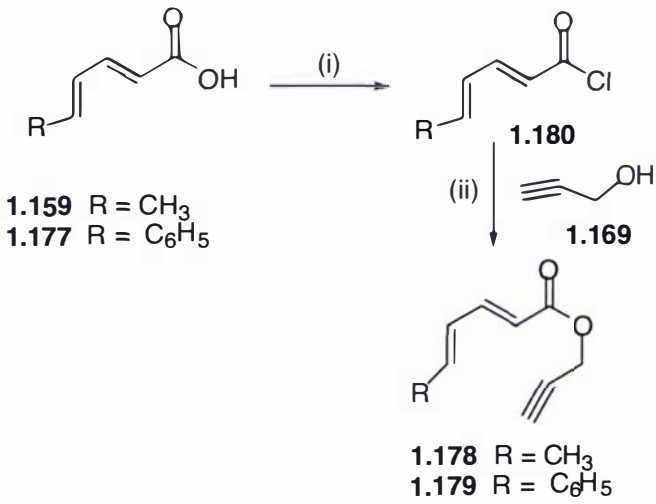
5-Phenyl 2E, 4-pentadienoic acid and the earlier synthesised hexa 2E, 4-dienoic acid were then condensed separately with propargyl alcohols using DCC and DMAP as activating reagents to give the more stable propynoates **1.178** and **1.179**.



Reagents and conditions : *(i) DCC (1.3 eq), DMAP (0.1 eq), CH_2Cl_2, RT, Ar, 3.5 h, 59-62% (ii) DCC (1.3 eq), DMAP (0.1 eq), CH_2Cl_2, RT, Ar, 3 h, 54-56% .*

Scheme 1.36

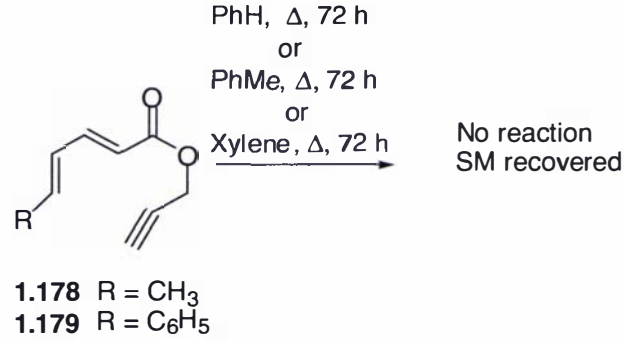
However preparation of these esters using this method gave low and variable yields and hence these IMDA precursors were also synthesised in pure form and good yield as shown in **Scheme 1.36** by condensation of the dienoyl chlorides and propargyl alcohols. This method is particularly convenient since products formed beside the dienoyl chlorides are gases and easily separable from the dienoic acids. The dienoyl chlorides were prepared by the reaction of the dienoic acid with thionyl chloride by refluxing in benzene or toluene. The refluxing in benzene required a longer reaction time of up to 16 h and slight polymerisation was observed for **1.179** while refluxing in toluene required only 3-4 h. Hexa 2*E*, 4-dienoyl chloride was purified by fractional distillation under reduced pressure (75^0C, 20 mm) whereas 5-phenyl penta 2*E*, 4-dienoyl chloride was obtained as a pale yellow solid and used in the next step without further purification.



Reagents and conditions : *(i) Toluene, $SOCl_2$, 2.5 h-3 h, 86%-90%, (ii) DCC (1.3 eq), DMAP (0.1 eq), CH_2Cl_2, 35 ^0C, Ar, 2 h, 88% (R = Ph, 64%).*

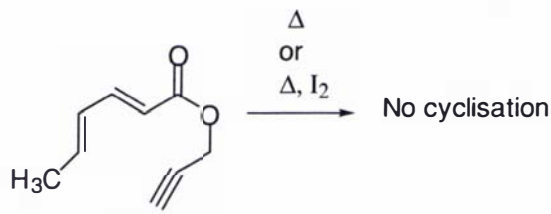
Scheme 1.37

Attempts were then made to cyclise the diene and dienophile moiety of propargyl dienoate *via* a thermal intramolecular Diels-Alder reaction. Propargyl dienoates **1.178** and **1.179** were heated to reflux in various solvents such as benzene, toluene, and xylene at different intervals of time to get the cyclic adducts. This intramolecular Diels-Alder reaction also met with failure, resulting only in the recovery of unreacted starting materials (**Scheme 1.37**).



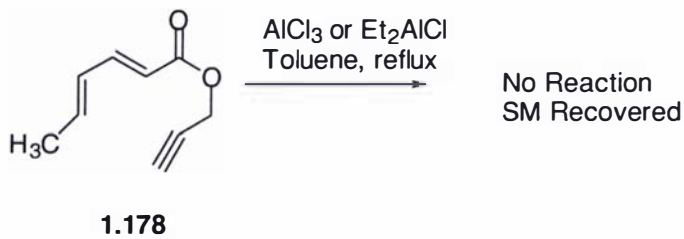
Scheme 1.38

This result is in agreement with the various attempts to cyclise propargyl sorbate by heating alone or in the presence of iodine by Johnson *et al* [72] (**Scheme 1.38**).



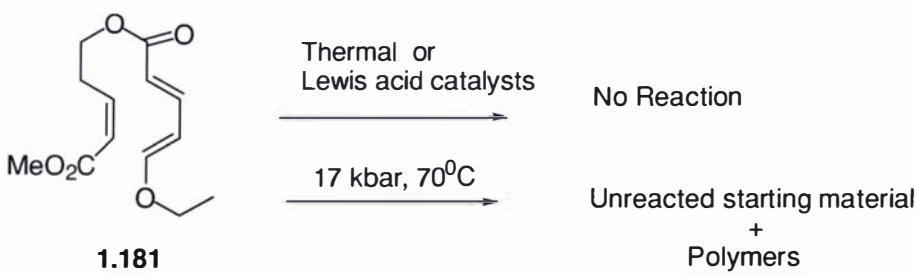
Scheme 1.39

Attempts to accelerate the rate of the IMDA reaction using Lewis acids such as $AlCl_3$ and Et_2AlCl in toluene at reflux or 160°C in a sealed tube to yield a cyclic product from propargyl dienoate **1.178** were also unsuccessful.



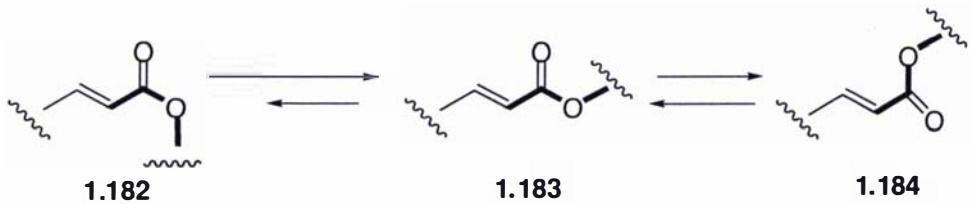
Scheme 1.40

d'Angelo[27] reported similar results during the attempted intramolecular cyclo addition with the precursor triene containing the carbonyl of the ester tether in conjugation with the diene. When the triene **1.181** was subjected to various thermal and Lewis acid catalysed conditions the starting material was recovered unchanged. Even when triene **1.181** was subjected to 17 Kbar pressure at 20°C no reaction occurred and at 70°C only unreacted starting material and polymers were recovered.



Scheme 1.41

Irrespective of the experimental conditions employed, none of the expected adducts were obtained from the propynoates **1.178** and **1.179**, a vexing result in view of the fact that such intramolecular processes are favored entropically[73]. It was assumed as do others[74] that the loss of reactivity of these propynoates is due to the dipolar intractions which strongly destabilize the cisoid conformation of the ester portion of the diene system, thereby resulting in an increase in the energy of the activation of the intramolecular transition state.



1.182 **1.183** **1.184**

***Cisoid* (disfavoured)** ***Transoid* (favoured)**

Reactive conformation Non-reactive conformation

Figure 1.14

The lack of success in the cyclisation reaction is compounded by the fact that synthesis of the benzofuranone system requires a precursor in which the dienophile is deactivated further increasing the overall activation energy for the cyclisation reaction.

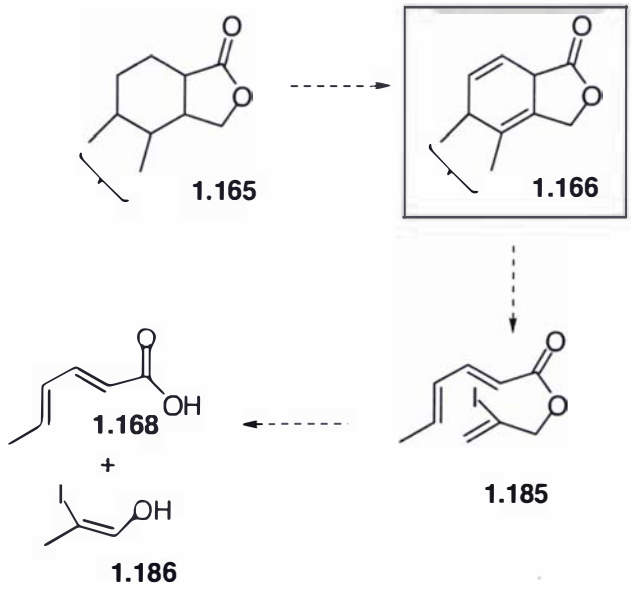
1.3.3 Summary

Thus utilisation of an ester linkage between the diene and deienophile has an adverse effect on the reaction rate as in the present work and which cannot be overcome by the structural features of the precursors used here. The effect is clearly less pronounced when the ester group is placed such that it activates the dienophile (as in **Section 1.1.3.2** and **1.1.3.2**) but is more pronounced when the required (*E*,*E*) diene component bears an electron withdrawing substituent (carbonyl group) at position 1.

1.3.4 Alternative Approaches To The Spongian C/D Ring System

To overcome the problems associated with the decreased reaction rate when using a ester tether which exists mainly in the non reactive confirmation (**Figure 1.14**)

an alternative methods such as the Pd catalysed (Heck reaction) or Sn catalysed tandem intramolecuar reaction on propynoate precursors and iodo alkene precursors (**Scheme 1.41**) were also attempted.



Scheme 1.42

1.3.4.1 Synthesis Of Iodo-Substituted Precursors

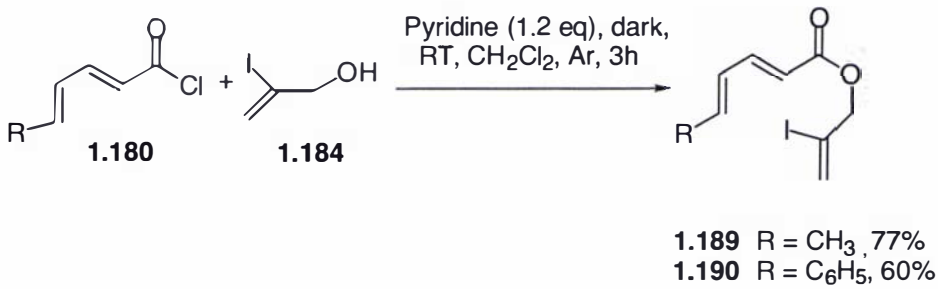
The nature of the leaving group (halogen X) can affect the cyclo-addition rate (RI > RBr >> RCl, R= alkene), because the oxidative addition of halo alkenes to Palladium(0) is in many cases the rate determining step for the Heck reaction.[34] Along this line the detailed study carried out by Curran *et al* [41,49,] and Porter *et al* [50] also showed that in Sn catalysed reactions the overall reaction is driven by an exchange of the Sn-H bond for a relatively strong Sn-X bond. They demonstrated that the transferability of various atoms and groups X to tin radicals is generally in the order I > Br > SePh ≈ OC(S)SMe > Cl > SPh. For these reasons, it was decided to synthesise internal alkenyl iodides as precursors ahead of precursors with other possible leaving groups. Alkenyl iodides, which are important starting materials in organic synthesis, are usually prepared by conventional methods but internal alkenyl iodides are scarcely obtained by these methods. For this purpose haloboration of 1-alkynes by B-iodo-9-borabicyclo[3.3.1] nonane has been developed.[74] It has been reported also that an alkyne in the presence of Bu_3SnH and a Pd complex in THF followed by addition of

iodine gave internal alkenyl iodides in reasonable yield.[75] To avoid complex reagents, the tedious work up and low yields associated with these methods it was decided to use the modified procedure described by Ishii et al.[76] Hence 2-iodo-propene-1-ol was obtained by the hydroiodination of propargyl alcohol *via* a facile *in situ* generation of hydrogen iodide from Me_3SiCl/NaI in the presence of H_2O in acetonitrile under mild conditions. In contrast to the reaction of allylic alcohols with HI which produced alkyl iodides by substitution rather than addition reaction of propargyl alcohol with HI afforded the internal addition product (**Scheme 1.42**).

Scheme 1.43

The ^{1}NMR spectrum clearly indicated a broad singlet at 4.10 ppm due to two protons suggesting the internal addition of iodine to give product **1.186** and not **1.188**. The allylic coupling constants (~0-3 Hz) in **1.186** were also confirmed as coupling constants for H-3 protons were ~1.5-1.8 Hz. The ^{13}C NMR also indicated terminal carbon (C-3) in **1.186** was further down field at 124 ppm then would be expected for **1.188**.

Reaction of 2-iodo-propen-1-ol with various dienoyl chlorides prepared using thionyl chloride (**Scheme 1.36**) in the presence of pyridine gave the required iodo ester precursors. These iodo esters were purified by flash chromatography eluting with Hexane:EtOAc in good yield as shown in **Scheme 1.43**. 2-Iodo prop-2-enyl 5-phenylpentadienoate showed some isomeric impurity and hence was purified again by column chromatography using CH_2Cl_2:EtOAc to isolate the desired product in good yield along with an inseparable complex mixture.

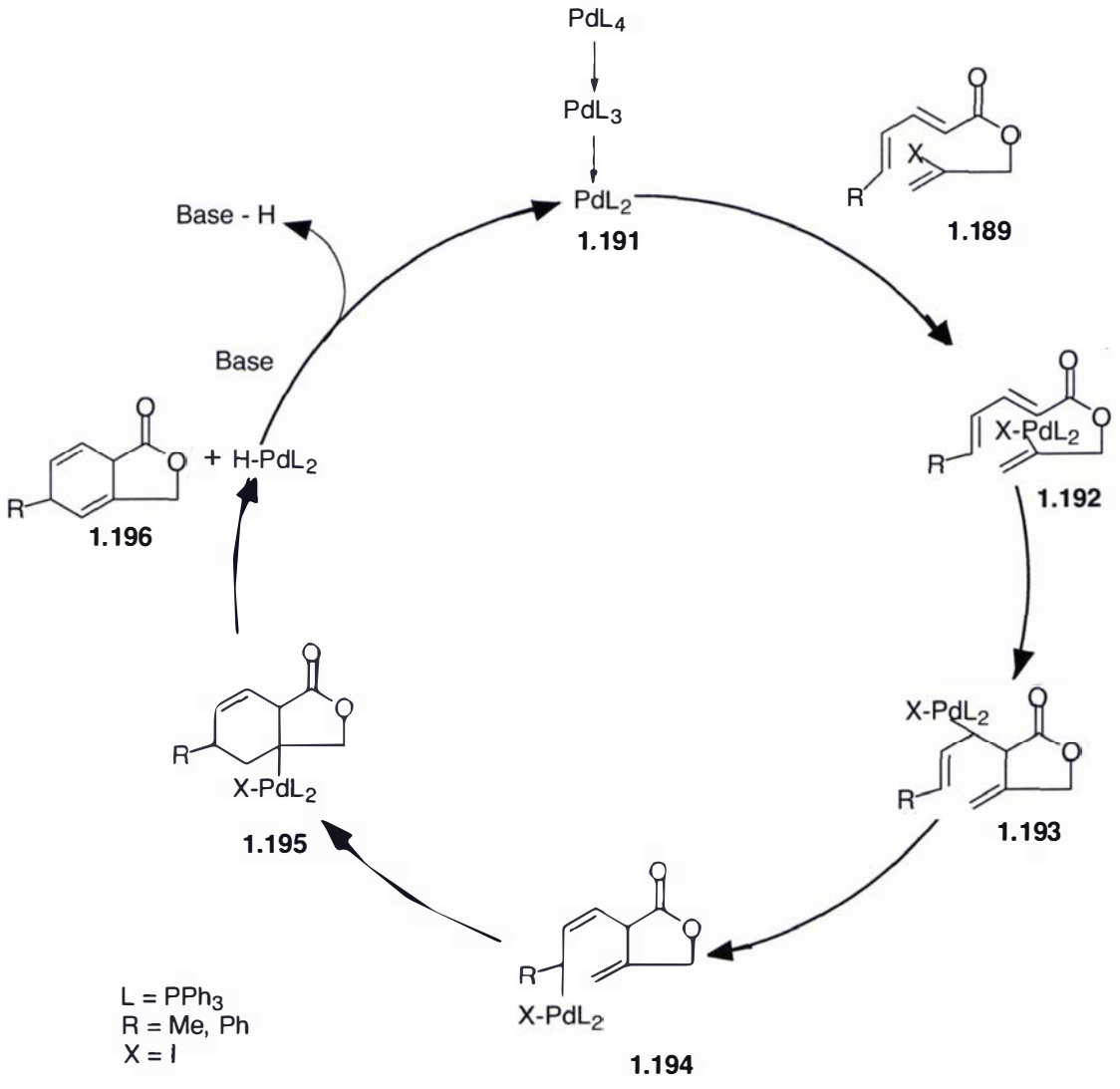


1.180 **1.184**

1.189 R = CH_3, 77%
1.190 R = C_6H_5, 60%

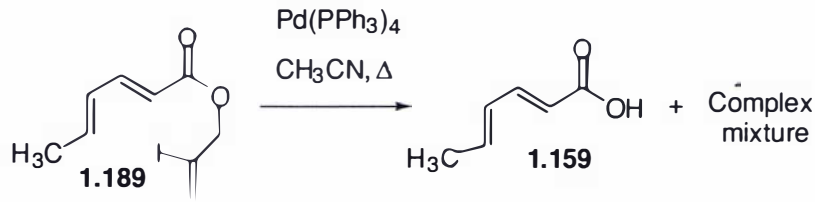
Scheme 1.44

Due to the success reported by other groups in achieving tandem intramolecular cyclisations with internal alkenyl iodides using the Heck reaction (**Section 1.1.4**) cyclisation of **1.189** was considered as following the proposed mechanism (**Scheme 1.44**). This involved oxidative addition of the allylic iodo compound **1.189** to a palladium(0) compound **1.191** to give **1.192** followed by carbo palladation to give the five membered lactone **1.193.** This would be followed by two further carbo palladation steps and subsequent elimination of palladium hydride from the intermediate **1.195** to give the required bicyclic lactone **1.196**.



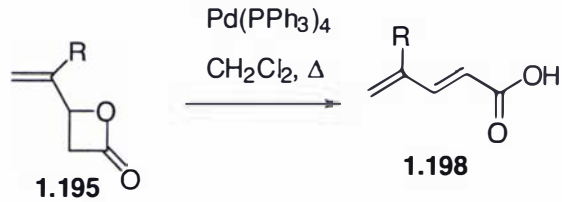
Scheme 1.45

Unfortunately the attempted cyclisation of the iodo triene ester **1.189** using tetrakis (triphenylphosphine)palladium(0) $Pd(PPh_3)_4$ also met with failure. When the cyclisation of **1.189** was tried in the presence of $Pd(PPh_3)_4$ and Et_3N in CH_3CN at RT for 16 hrs no product was seen. Both the TLC and the ^1H NMR spectrum indicated the presence of the starting material with no traces of the bicyclic adduct. Similar attempts at reflux resulted in decomposition of the starting material. ^1H NMR of the reaction mixture indicated the presence of hexa 2*E*, 4*E* –dienoic acid along with other impurities.



Scheme 1.46

It is presumed that during the formation of the π -allylic palladium intermediates allylic carbon-oxygen bond cleavage occurs as reported by Nolk *et al* [77] in the synthesis of 2,4-pentadienoic acid by allylic cleavage of β-pent-4-enolactone.



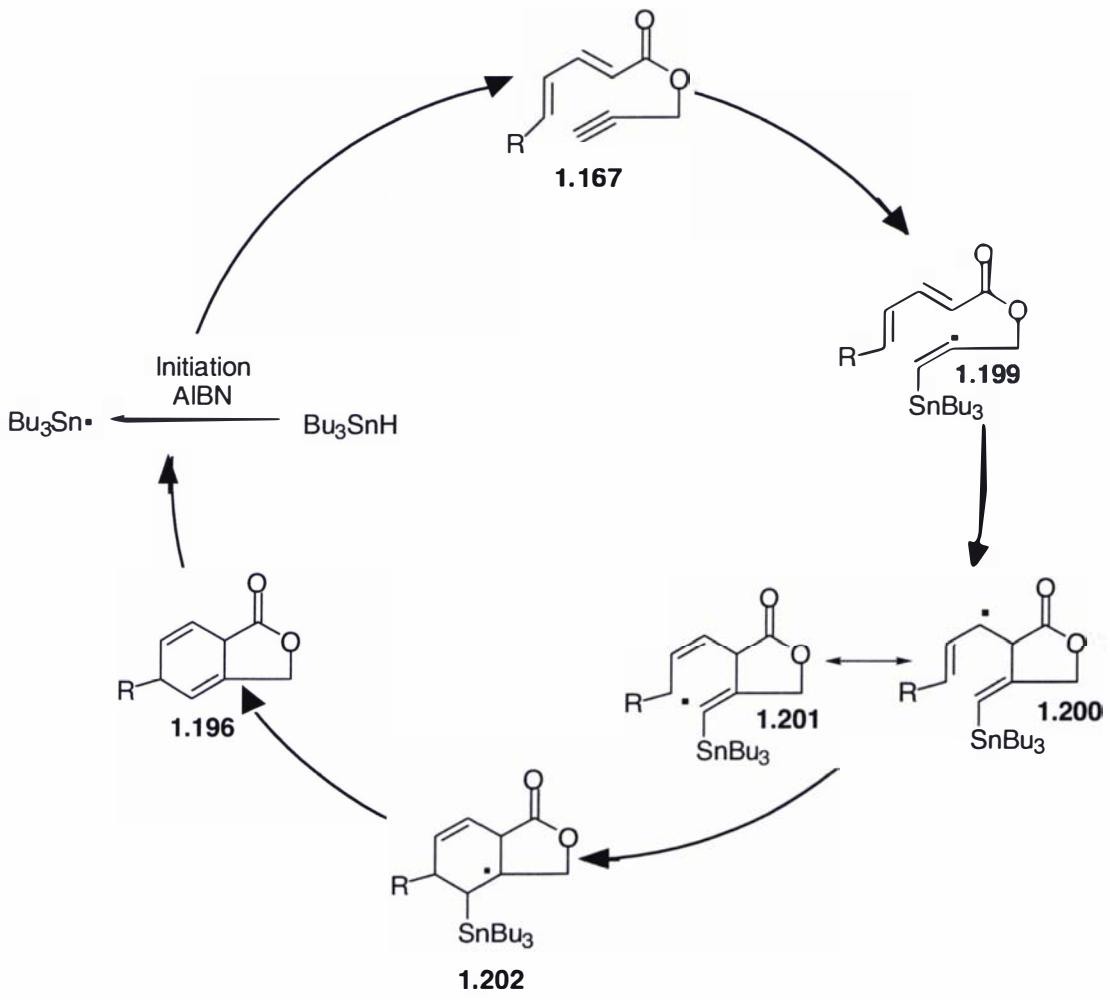
Scheme 1.47

Presumably the first step of the reaction is occuring but cyclisation is again hindered by the instabiliyty of s-cis conformer.

1.3.5 Attempted Intramolecular Free Radical Cyclisation Of Ester-Tethered Precursors

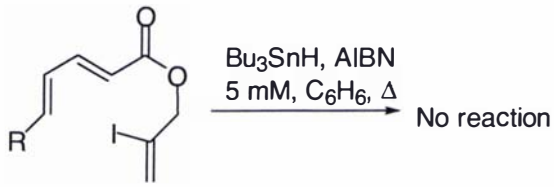
As discussed in **Section 1.1.5**, free radical methods are becoming increasingly important in organic synthesis, and successful methods for the construction of five and six membered rings by radical cyclization are now an established part of the synthetic repertoire. However, not a single successful reaction has been reported for cyclization with ester tethered precursors such as those attempted in this chapter. In the current research we aimed to investigate further this new type of tandem intramolecular free radical reaction using ester-tethered precursors. The strategy we attempted for the spongian C/D ring system was based on elaboration of the ester moiety followed by 5-*exo*-trig radical cyclisation initiated from the acyl butyl tin radical intermediate. This would be followed by generation of another acyl radical intermediate and subsequent 6-

endo-trig cyclisation. **Scheme 1.47** represents the proposed mechanistic route of this reaction using propargyl ester precursor **1.167**.



Scheme 1.48

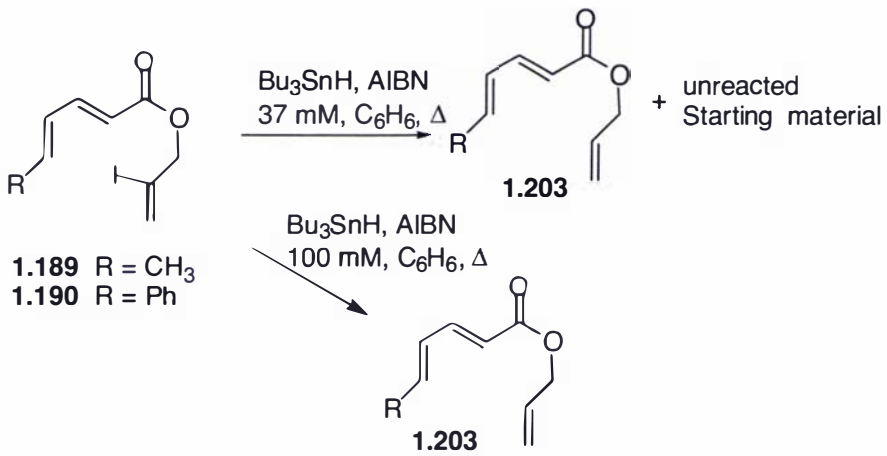
A similar mechanistic route can be also be drawn for iodo ester precursors. Attempted cyclisation of the iodo precursors **1.189** and **1.190** to synthesise the required bicyclic lactone using the tandem intramolecular free radical strategy at low concentrations (5 mM) of substrate in benzene at reflux resulted in no reaction. The progress of the reaction was monitored by ^{1}H NMR spectroscopy. The ^{1}H NMR spectrum of the crude reaction mixture showed the presence of unreacted starting material. The reaction mixture was refluxed in benzene for a longer time only to get very little of the acyclic reduced product **1.203** and none of the expected cyclic product was obtained.



1.189 R = CH_3
1.190 R = Ph

Scheme 1.49

When the concentration of the substrate was increased to 37 mM the reaction mixture showed an increased yield of the acyclic material resulting from direct reduction of the carbon-iodine bond and unreacted starting material. At higher concentrations (100 mM) of substrate and in presence of Bu_3SnH and AIBN the product obtained was entirely the acyclic reduced material **1.203** and no unreacted starting material or cyclic product was seen.

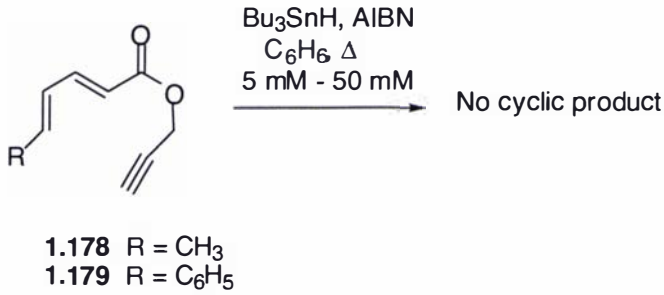


Scheme 1.50

The ^1H NMR spectrum of the acyclic reduced product as eluted from the column, revealed the presence of organotin contaminants. A literature review of Bu_3SnH reactions showed problems associated with the work up. It is also reported in several papers[39,44,45] that it is very difficult and in some cases it is impossible to remove tributyltin impurities from the product. Unfortunately, the organotin halide from these reactions shows a tendency to hydrolyse slowly on silica gel which makes purification difficult. Few approaches were made to circumvent the problems associated with purification of the crude reaction mixture contaminated with organotin residues.

Several attempts have been made to remove organotin residues in the presence of NH_3 and $NaBH_3CN$ as reported in the literature.[39,44] The $NaBH_3CN$ method did not show good results and met with failure. Initial attempts to remove organotin residues by co-ordination with NH_3 showed low levels of tributyltin impurities, probably because tributyltin residues were forming unstable amine complexes. This problem was overcome by quick column chromatography after NH_3 treatment and analytically pure products were obtained.

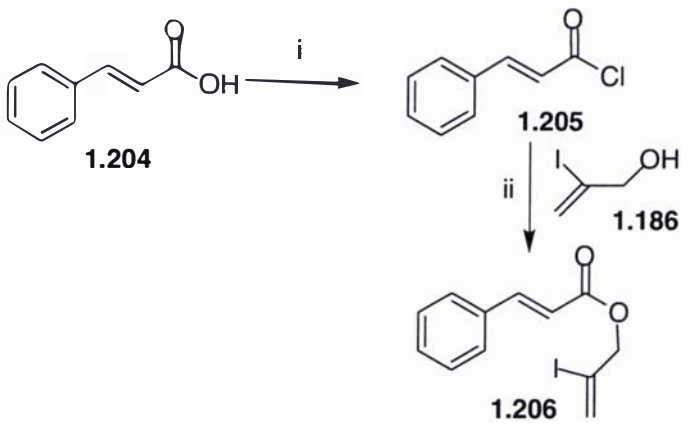
Similar attempts to synthesise the bicyclic adduct using tandem intramolecular cyclisations of propargyl esters **1.178** and **1.179** using various concentrations by Bu_3SnH and AIBN also met with failure. Both the TLC and the ^1H NMR spectrum of the crude reaction mixture of propargyl sorbate **1.178** with Bu_3SnH at 5 mM and 50 mM concentration of substrate gave traces of the hydrostanylated product and unreacted starting material. The tin hydride reaction of propargyl styryl acrylate **1.179** under similar conditions also gave largely unreacted starting material. This showed that the compounds were slow to react with tin hydride.



1.178 R = CH_3
1.179 R = C_6H_5

Scheme 1.51

With the numerous attempts of tandem radical cyclisations of the above precurors failing attention was turned to intramolecular cyclisation of the simple model precursor **1.206**. The precursor **1.206** was prepared in a similar fashion as earlier precursors by condensation of cinnamoyl chloride and 2-iodo propen-1-ol to give a pure white crystalline solid.

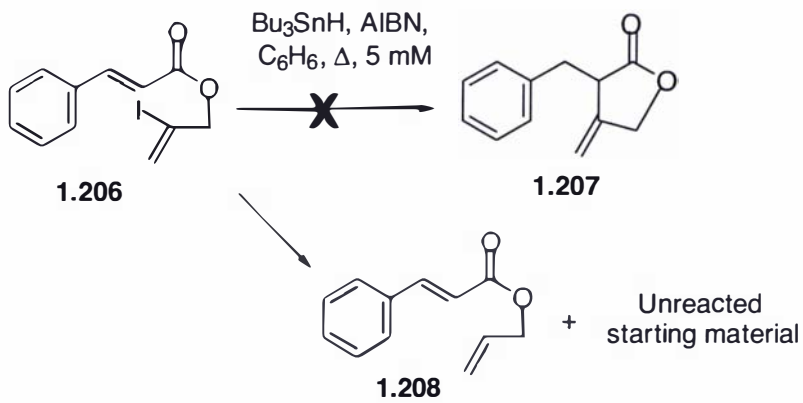


Reagents and conditions: *(i) $SOCl_2$,Toluene, Reflux, 1.5 h, 98%; (ii) Et_3N (1.5 eq), CH_2Cl_2, RT, Ar, 3h, 78%.*

Scheme 1.52

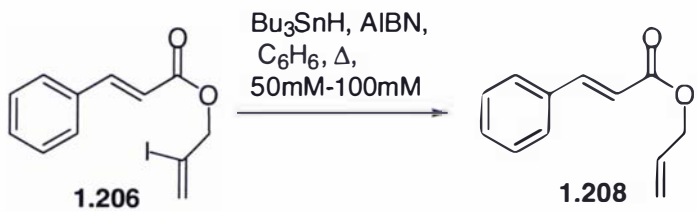
The reason behind the synthesis of the phenyl substituted precursor was to generate a resonance stabilised radical, which can make the radical forming reaction faster and hence activate the system towards radical cyclisation. This reaction would show whether the ester tether in this orientation is suitable for radical cyclisation reactions. In common with other intramolecular techniques using Bu_3SnH-AIBN at high dilution conditions (5 mM) to get the cyclised product by lowering the probability of bimolecular processes (most notably the direct reduction of the initially formed radical) resulted in a very poor yield of the cyclic product if formed at all. The product obtained was acyclic material resulting from the direct reduction of the carbon-iodine bond together with the unreacted starting material. This showed that the first step, generation of the radical was occurring as anticipated, but the first cyclisation step was not.

In a further attempt, a 5 mM solution of the iodosubstrate **1.206** in dry, degassed refluxing benzene was treated slowly (by use of a syringe pump allowed the addition of Bu_3SnH to be performed over several hours) with a solution in the presence of catalytic amounts of Bu_3SnH. The reaction mixture was heated under reflux for 3 h at which point TLC analysis showed a new spot. The ^1H NMR of the crude reaction mixture showed a multiplet at 5.99-5.87 ppm indicating a mixture of the unreacted starting material and the acyclic reduced product. The reaction was stirred for 28 hrs to ensure completion of the reaction without any change in the result.



Scheme 1.53

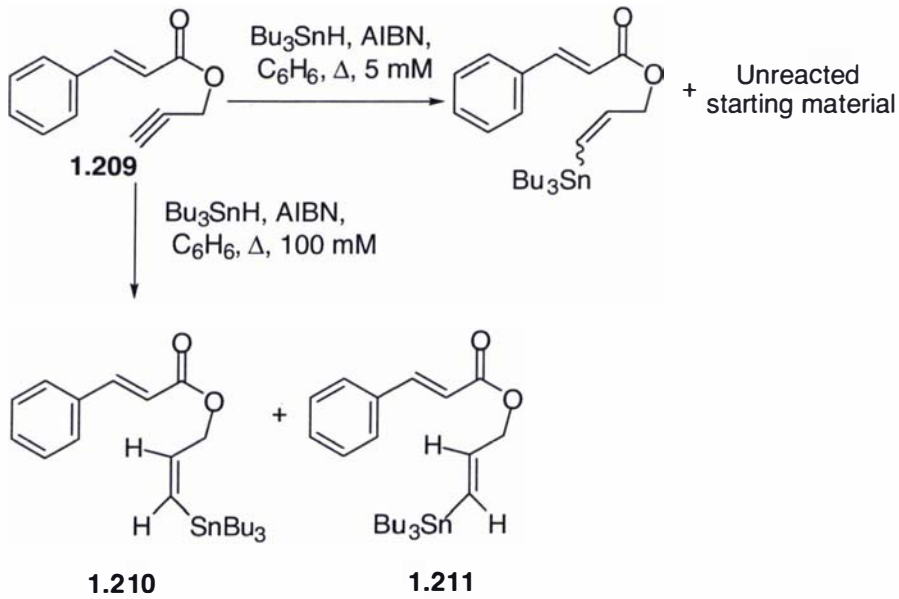
The concentration of substrate was increased slowly in order to see whether any cyclisation reaction occurred but this free radical mediated reaction of the precursor **1.206** at high concentrations of iodo substrate gave entirely the reduced product (prop-2'-enyl)-3 phenyl-2-propenoate **1.208** as shown in **Scheme 1.53.**



Scheme 1.54

Intramolecular cyclisation was also attempted on the more reactive acetylenic precursor (prop-2'-yne)-3-phenyl-2-propenoate with Bu_3SnH-AIBN at low concentrations (5 mM) of acetylenic substrate. The reaction mixture was then refluxed for 7 h to ensure completion of the reaction. The TLC result showed a single spot and hence the reaction was monitored by ^1H NMR spectroscopy which showed unreacted starting material and the peaks due to the acyclic hydrostannylated product. This was probably due to identical R_f values of the starting material and the hydrostanylated product. At higher concentrations (100 mM) of acetylenic substrate and in the presence of Bu_3SnH-AIBN this precursor gave the expected hydrostannylated product as a pale yellow liquid. The ^1H NMR spectrum of the hydrostannylated product as eluted from the column after single work up with aqueous NH_3 (20%) revealed the presence of an organotin contaminant. The reaction mixture was stirred with aqueous NH_3 (20%) for

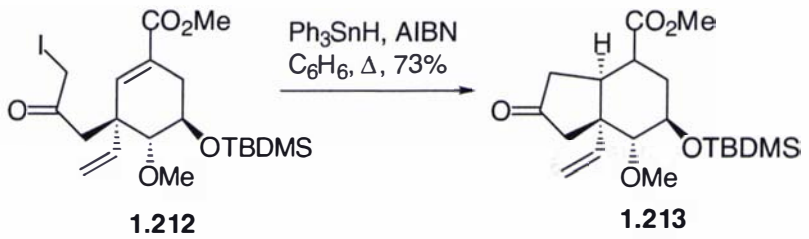
30 minutes to ensure the complete removal of organotin residues and then purified again by column chromatography to give an inseparable mixture of isomeric hydrostannyled products **1.210** and **1.211** in a 88:12 ratio as a colorless liquid.



Scheme 1.55

This result again showed that the first step in the reaction sequence is occurring but the cyclisation step is failing in all of the precursors tried, even when a radical stabilising substrate was attempted (as in **1.209**).

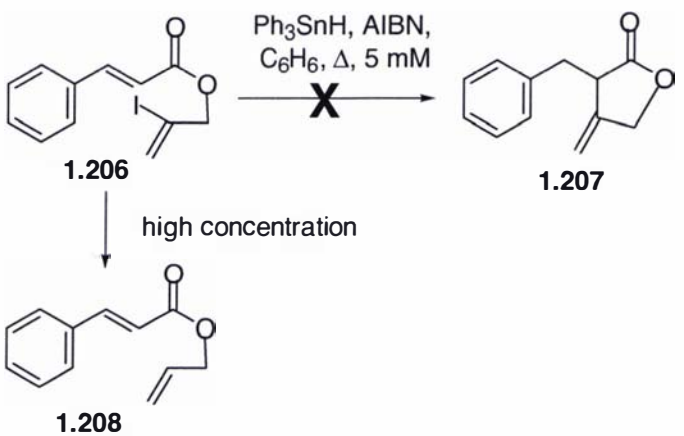
Hanessian et al recently reported[48] a study of the intramolecular free radical cyclization of α -halo acetates of δ-hydroxy-α,β-unsaturated esters to give the corresponding γ-lactones. The prevailing opinion was that in the presence of tributyltin hydride, the resultant stabilised radicals underwent reduction rather than C-C bond formation. The studies done in their laboratory show that efficient and highly stereo-controlled intramolecular cyclization of α,β- and β,γ-unsaturated α-haloacetates and propionates occurs under conditions of slow additions of triphenyltin hydride, to give appropriately substituted γ-and δ-lactones.



1.212 **1.213**

Scheme 1.56

This reaction shows the elegance of intramolecular free radical cyclisation. This example encouraged us to apply this methodology to our precursor as until recently, α-acyloxy carbon radicals were considered unreactive in conjugate type additions.[78] Hence free radical mediated cyclisation was attempted on 2'-iodo-prop-2'-enyl-3-phenyl 2-propenoate **1.206** under slow addition of Ph_3SnH as the radical propagator. A solution of Ph_3SnH in dry benzene was added over 6 hrs using a syringe pump to a solution of iodo vinyl ester **1.206** in dry, degassed benzene at reflux. Unfortunately in a system developed to favour *exo* cyclisation, no cyclisation was observed whatsoever under the standard conditions of Hanessian. Upon completion of addition the crude reaction mixture was monitored for 24 hrs by ^1H NMR spectroscopy which showed evidence of the simple iodide reduction product. The yield of the acyclic reduced product was observed to be more than when using similar conditions with Bu_3SnH. With no cyclic product in sight a further aliquot of Ph_3SnH-AIBN was added and the reaction mixture was stirred for 2 hrs. The crude reaction mixture was then treated with aq. KF (10%) thus removing the organotin contaminant as white fluffy Ph_3SnF residues to isolate the acyclic reduced product in much better yield than obtained using Bu_3SnH.



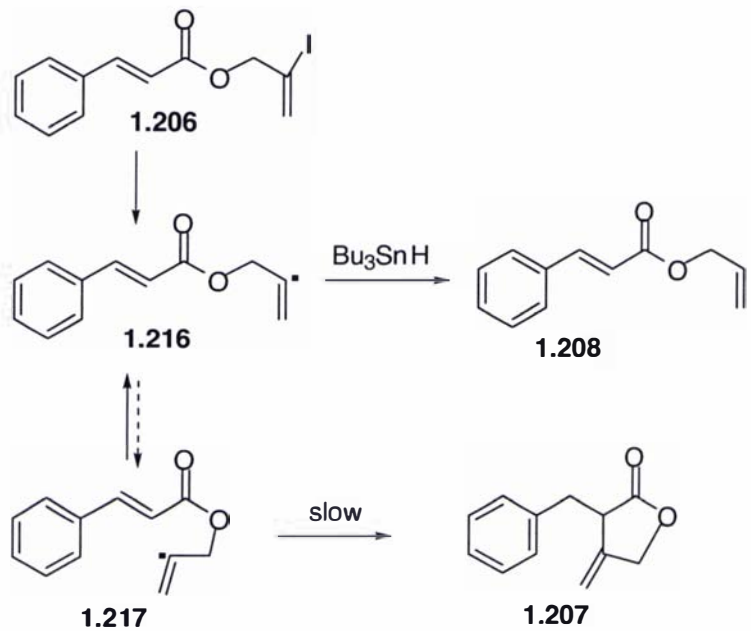
1.206 **1.207** **1.208**

Scheme 1.57

This again clearly demonstrates the relative ease of formation of the initial radical species.

1.3.6 Summary

From the above results it can be suggested that for cyclisation of ester tethered precursors having conjugation of the diene moiety to the ester carbonyl group, the lifetime of any derived radical such as **1.216** needs to be long enough to allow rotation of the ester bond into the unfavourable s-*E* conformer (**1.217**). The relative activation energies for hydrogen atom abstraction and cyclisation should be at least equal for there to be any chance of annulation to occur. The fact that no evidence for cyclised products such as **1.207** was obtained indicates that the cyclisation step must be too slow to compete with hydrogen atom abstraction, or that the starting material exists virtually completely as the s-*transoid* conformer.



Scheme 1.58

Thus the total failure to effect these ring closures can be accounted for in terms of the traditional problem of a slow rate of cyclisation for lactones with smaller ring sizes such as five membered and six membered lactones used in this work due to considerable Pitzer and transannular strain in the cyclised product. Also the requirement that iodo-substrates (**1.89, 1.90** and **1.206**) and –yne substrates (**1.178,**

1.179 and 1.209) must adopt the unfavorable s-*E* conformation to accommodate the required radical transition state geometry works against the cyclisation pathway (**Scheme 1.58**). Apparently, only for the longer alkyl chains presumably n>4, can the desired disposition of reactive centers be adopted while the preferred s-*Z* conformation is maintained as shown in **Figure 1.15**.

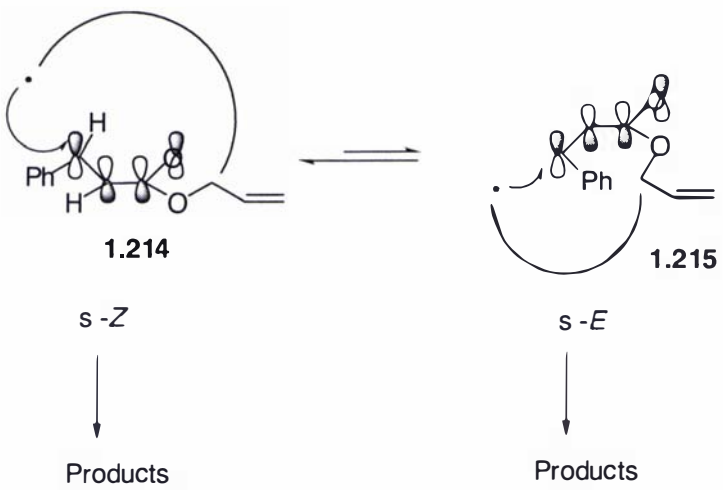


Figure 1.15

Thus, despite the attraction of using ester tethered dienes and dienophiles to produce D ring lactone of spngian 16-one (Such as **1.164**) none of the procedures tried here (IMDA, Heck reaction or free radical coupling) were able to effect cyclisation.

1.3.7 Future Directions

These preliminary studies carried out indicate that the construction of five membered or six membered lactone rings *via* cyclisation of suitable precursors is clearly not a simple task. Previous literature studies have shown the lack of reactivity towards cyclisation of dienes containing *Z* alkenes, especially if the diene and dienophile moieties are inactivated and the results reported in this chapter reinforce this. It is not known for certain why these ester tethered –iodo and –yne precursors were reluctant to cyclise by slow addition of the free radical catalysts using a syringe pump. However much information has been gained from these attempted cyclisation reactions.

Whilst the cyclisation reactions studied in this chapter were not successful, the elegance of the strategy to form tetracyclic spongian or steroid skeletons *via* TIMC

reactions is still compelling. The most promising area of the further work for the successful cyclisation of ester tethered precursors could be activation of at least the diene or dienophile moieties. Attention therefore must be focused on the synthesis of other precursors such as **1.216** and **1.218** for the effective cyclisation.

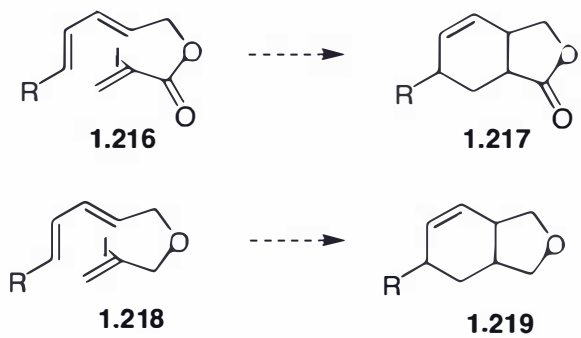


Figure 1.16

Once success has been achieved, this approach can be extensively utilised for the construction of the tetracyclic skeleton, particularly in the synthesis of a variety of tetracyclic spongian diterpenoids. The time constraints in this thesis prevented reactions on these new precursors from being attempted. However, a number of strategies are still available for directing efforts towards a successful TIMC reaction in the future.

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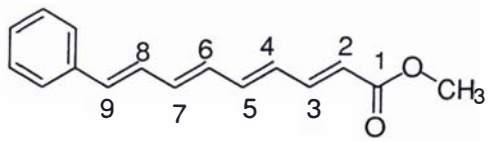
Figure 1.16 COSY Spectrum Of **1.162**



1.162

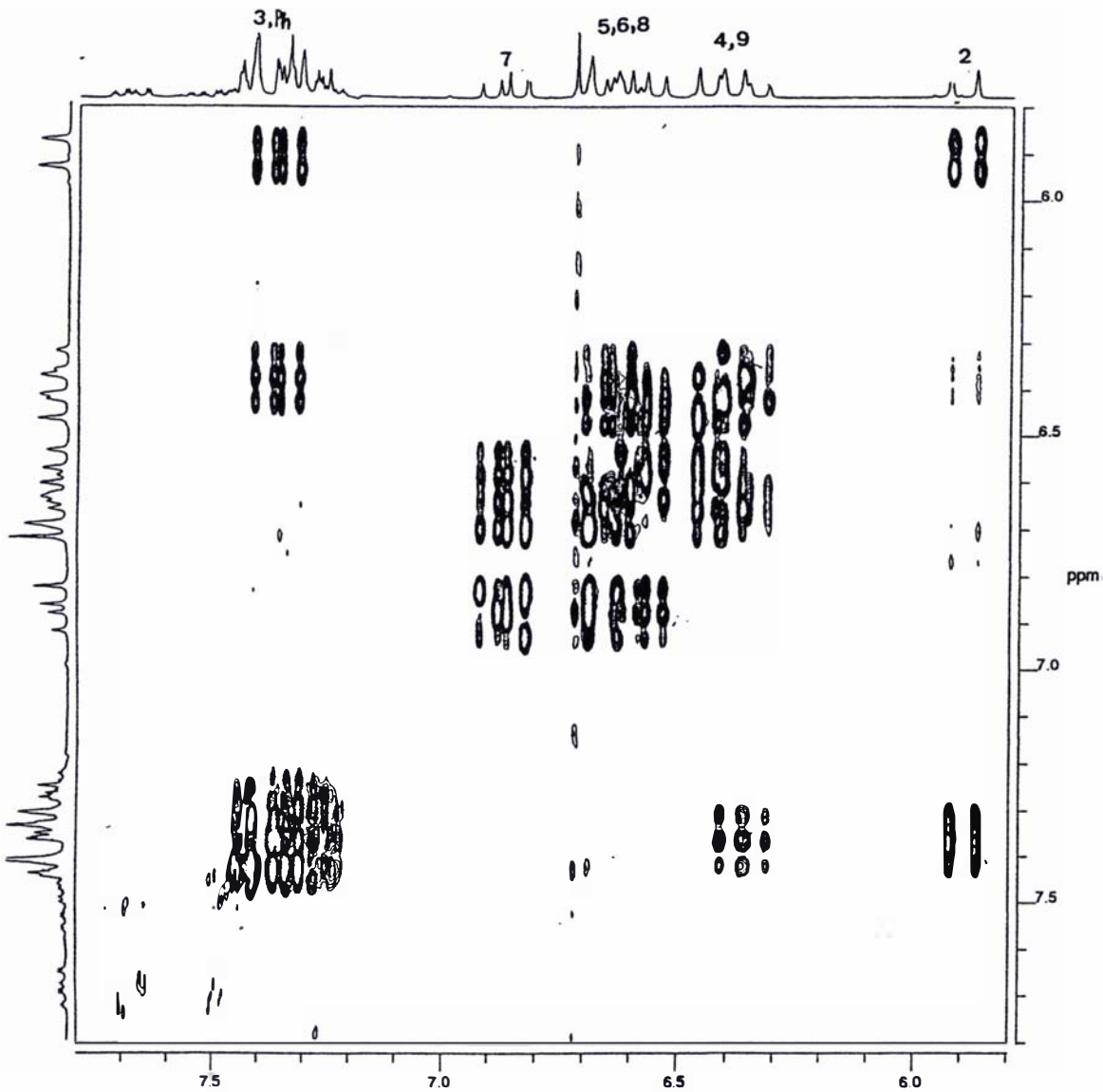
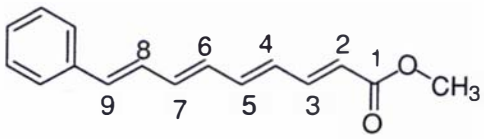


Figure 1.17 HETCOR Spectrum Of **1.162**



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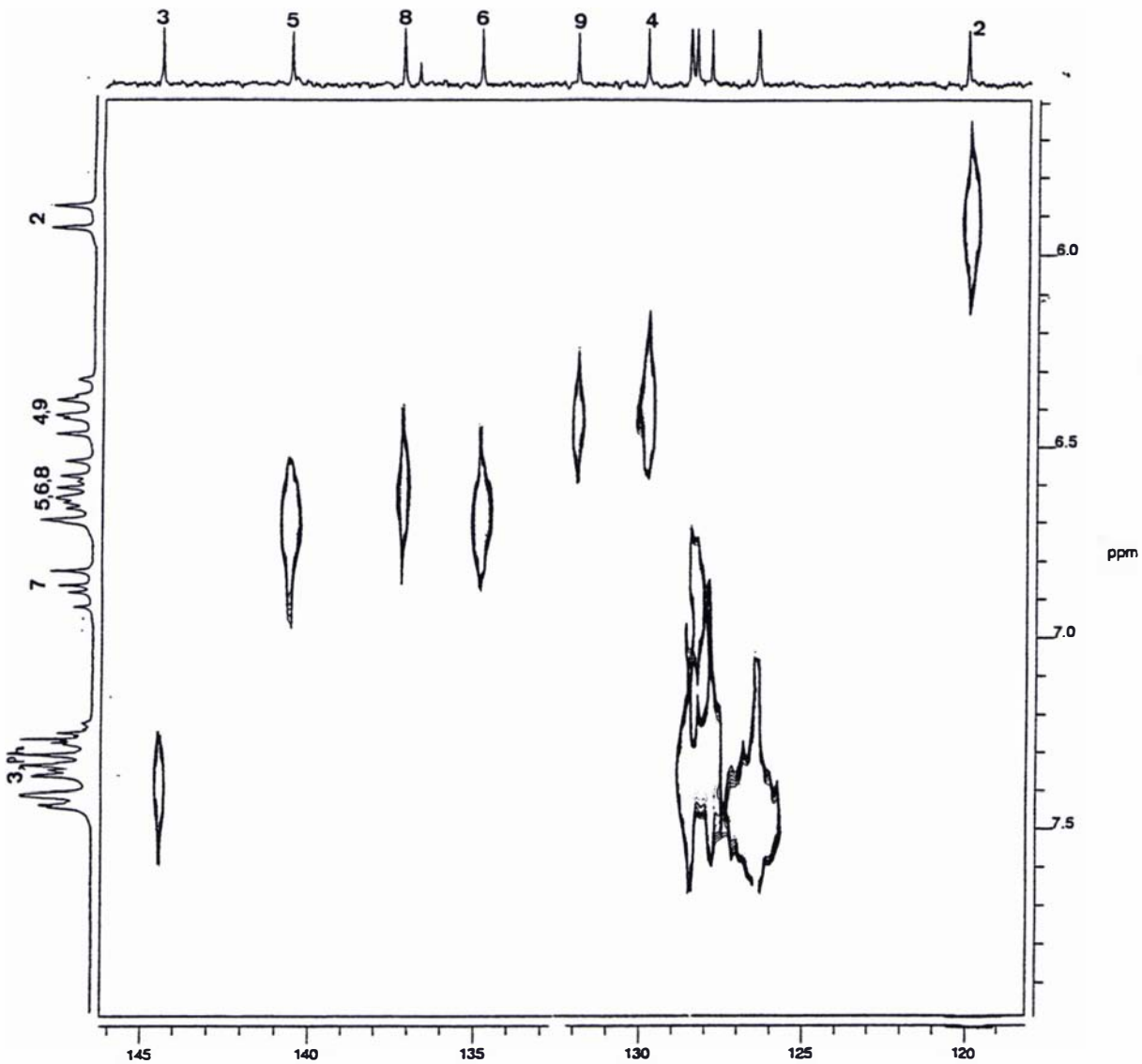


CHAPTER 2 DEVELOPMENT OF A MULTI-PURPOSE HOME OR ON-SITE ASSAY SYSTEM BASED ON THE HOME OVARIAN MONITOR

2.1 BACKGROUND TO THE STUDY

2.1.1 Biomarkers Of Health And Disease

A biomarker can be defined as any substance, structure or process that could be monitored in tissues or bodily fluids that predicts or influences health or assesses the incidence of biological behavior of disease.[1] Identification of biomarkers that are on a biochemical pathway have a high probability of reflecting the state of an individuals health or progression to clinical disease. For example biomarkers are needed to evaluate reliably the intake of specific foods or drugs that might influence health or increase disease risk and access one or more biological effects and hence effectively predict individual susceptibility.[2,3]

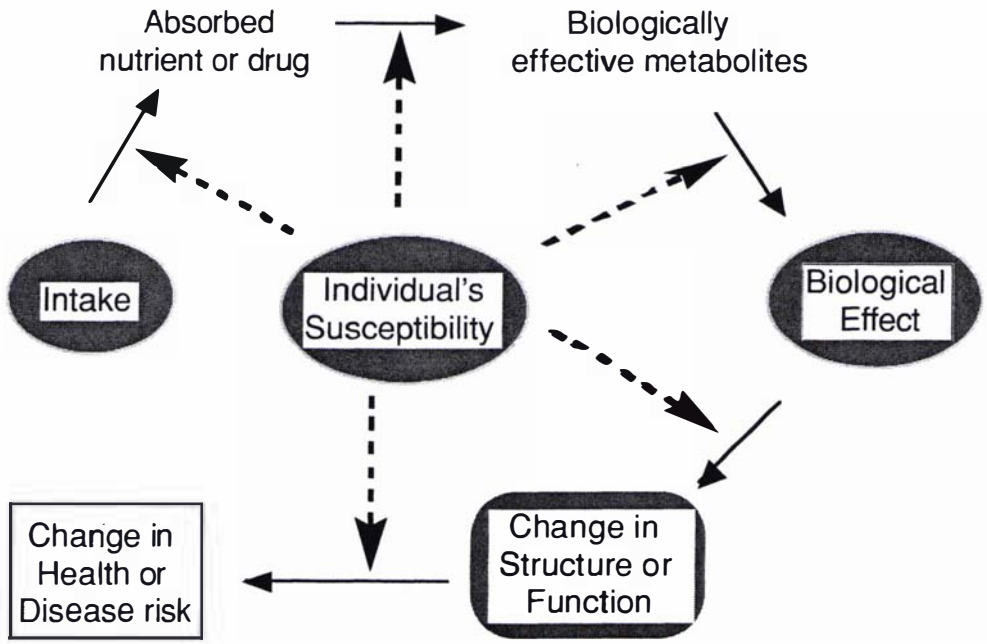


Figure 2.1

Figure 2.1 shows the interelationship between intake of functional foods, effect and suceptibility of an individual. Rapid, accurate, and inexpensive methods for assessing the intake of specific nutrients and drugs, both essential and non essential remains a challenge to health professionals.[4] Errors in estimating and controlling nutrient and drug intakes, possible interactions among the nutrient or drug components and incomplete data on the content of ingested nutrients or drugs limits the usefulness of many biomarkers. The absorption process may depend on the drug's dissociating from its dosage form, and then diffusing across biological membrane barriers into the blood stream. In addition to the amount absorbed, the rate of absorption is also important. The absorption of a drug or nutrient is considered a first-order process, and the absorption rate constant of a drug is usually much greater than its elimination rate constant.[5] Combining assessments of functional foods with tissue or fluid concentrations of components or metabolites may offer special insights into how genetics and other factors can influence absorption and metabolism of active ingredients and thus be useful in qualitatively determining an individual's susceptibility and benefits. The use of biomarkers can ideally assist in the development of better definitions of risk profiles and thereby in developing appropriate strategies for prevention including modification of dietary habits.

The utility of biomarkers capable of predicting a disease state can be more valuable if they confer a long lead time relative to the onset of the disorder. Although much of the focus of today's health care revolves around the detection of early events during the development of disease states there is also a critical need to identify biomarkers that can be used to assess health and promote the quality of life. A biomarker can be measured qualitatively or quantitatively by chemical, immunological or molecular biological methods to identify the presence of disease. There is a growing realisation that non-invasive home or point-of-care devices capable of delivering laboratory accurate clinical data to lay individuals and health professionals on levels of biomarkers of health and disease can revolutionise medical and health practice.

The best known biomarker is probably blood glucose for which there are already many semiquantitative devices available and which are used by diabetic patients to determine their blood glucose levels as a measure of control and immediate insulin requirements. Tumor markers have been also widely used for diagnosis, prognosis and

monitoring effects of therapy as well as for organ targets for localization for therapy. The biomarker value at the time of diagnosis can be used as a prognostic indicator for likely disease progression and patient survival. Unfortunately most known tumor markers are neither specific nor sensitive enough for such purposes. Hormones have been recognised as tumor markers for over a half century while other tumor markers including catecholamines, polyamines, lipid associated sialic acid and receptors have been also used clinically with varying degrees of success.[6] Receptors are probably the most successful targets of this group of biomarkers. For example, estrogen and progesterone receptors have been used in breast cancer as predictors of response to hormonal therapy.[6] Receptor levels greater than 10 fmol/mg of cytosol protein having been considered positive. The importance of this measurement is that patients with positive levels of estrogen and progesterone receptors tend to respond to hormonal therapy, whereas those with negative levels are best treated by other therapies (e.g. chemotherapy). Hormone receptors can also serve as prognostic factors in breast cancer since patients with positive receptor levels tend to survive longer.[5,6]

Clinical laboratories are continuously changing to meet the needs of the public and medical profession. Because of advances and discoveries made in basic medical sciences, they are constantly faced with implementing new tests that are more sensitive, general and effective in monitoring health and disease. In addition new measurement technologies are being developed and commercialised that make possible wider use of these and older methods. Most of the important accessible biomarkers are products of metabolism and often also excreted in urine as glucuronides or as sulphates and carboxylic acid conjugates. In principle these urinary metabolites constitute a battery of biomarkers which can be utilised in non-invasive home or point-of-care urine tests. If a suitable methodology can be developed for rapid quantitative measurement of urinary glucuronides at home then this will form the basis of a family of biomarker tests. The following requirements for such an assay can be suggested based on the results by Brown et al who have developed a home test for estrone glucuronide (E1G) as a marker for fertility.[7]

(i) Be able to distinguish between small daily increases in levels of the analyte glucuronide.

(ii) It must be able to measure an analyte glucuronide whose concentration in urine is equal or greater than 25-50 nmol/ 24 hrs.

(iii) It must be simple, rapid and robust.

(iv) Its results must be easily interpretable.

(v) Its cost must be low.

(vi) It must not contain any hazardous chemicals.

(vii) Its results must be as good as the best laboratory assays.

(viii) It must be absolutely reliable: errors can be fatal.

These requirements have been met in a device known as the Ovarian Monitor which measures biomarkers for the fertile period in humans.

2.1.2 Metabolism Of Xenobiotic Compounds

When a foreign substance or drug (known as a xenobiotic) enters the body, the immune system produces antibodies to interact with and destroy it. Small molecules, however generally do not stimulate an antibody response. The principal mechanism to protect the human body from low molecular weight environmental toxins is the use of non-specific enzymes that transform the toxins (often highly non polar molecules) into polar molecules which are excreted by the normal bodily processes. Although this prophylactic mechanism to rid the body of xenobiotics is highly desirable, it can cause problems when the toxin is a drug the body needs to enter and be retained long enough for it to be effective.[8] Since many drugs or xenobiotics have structures similar to those of endogenous compounds, they may be metabolized by specific enzymes for related natural substrates as well as by non specific enzymes.[9] The principal site of drug metabolism is the liver. Its role is to convert lipophilic non polar molecules to more polar water soluble forms.

Enzymes such as cytochrome P_{450} present in the liver modify drug molecules by phase I reactions and alter the chemical structure by oxidation, reduction or hydrolysis. For example there are enzymes which can demethylate a methyl ether to reveal a more polar hydroxyl group. There is also a series of metabolic reactions classed as phase II reactions. These are conjugation reactions such as glucuronidation or sulfation, whereby a polar molecule is attached to a suitable polar 'handle' which is already present on the drug or has been placed there by a phase I reaction (**Figure 2.2**).[8,10] The resulting conjugate has increased polarity thus increasing its excretion rate in urine or bile even further. Renal excretion is a major pathway for the elimination of drugs and their metabolites.

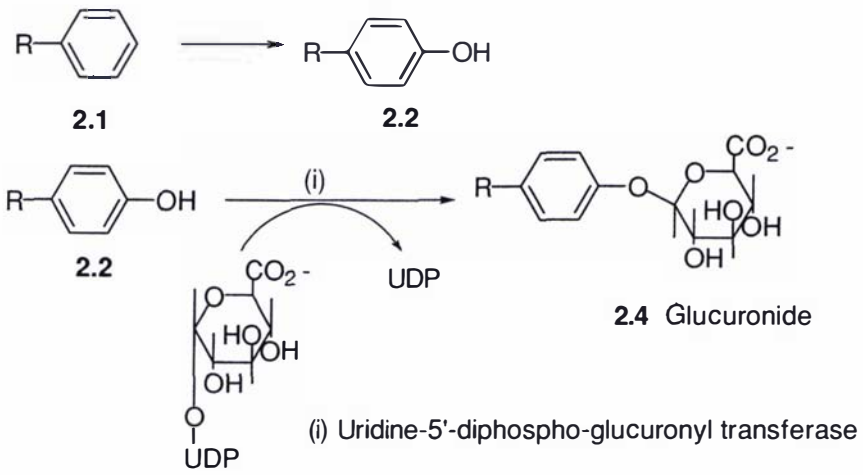


Figure 2.2

Phenols and alcohols form *O*-glucuronides by reaction with UDP-glucuronic acid such that the highly polar glucuronic acid molecule is attached to the drug. Glucuronides may have significant biological activity themselves and hence are potential biomarkers. In such instances these metabolites should also be measured because they are contributing to the effect of the drug on the patient.

2.1.3 Methods For Measurement Of Urinary Estrogen Glucuronides As Biomarkers

Measurement of estrogen glucuronides in urine has been a continuing challenge since the 1930's when estrogens were first discovered by Marrian *et al.*[11] Despite the

many difficulties encountered by previous workers, a number of useful experimental methods have been developed over the years for the quantitative measurement of estrogens as biomarkers of female fertility.

Initially, a biological assay for estrogens was described by Astwood in the1930's,[12] in which the procedure was dependent on an estrogen induced increase in the uterine weight of immature rats. Since the method was only semi-quantitative, not specific and a large number of animals were needed to obtain significant results, this biological method is now considered to be too lengthy to enjoy general acceptance, and to lack sensitivity, reproducibility and specificity.

Chemical methods for the measurement of estrogens were developed in the 1950's by Professor J.B.Brown and his contemporaries at the university of Edinburgh.[13] These were carried out by heating urine with concentrated sulphuric acid and ethanol after hydrolysis of the glucuronides and extraction into organic solvents resulting in the formation of characteristic orange-yellow coloured derivatives (the Kober reaction).[14] These derivatives exhibited an intense green fluorescence, which was utilised in the development of highly specific colorimetric and fluorimetric methods. Another chemical method, which was originally developed by Professor J B Brown,[13] employed initial hydrolysis of the glucuronide conjugates in urine samples, extraction of the free estrogens with ether and methylation with dimethyl sulphate, purification by alumina column chromatography and final measurement by the Kober colour reaction. The chemical assay was very sensitive since 100 ml or more of the urine sample could be extracted but this method is obviously impractical for a home or on-site test. Although chemical methods are accurate, reliable and provided the vital background information for the clinical application of the test, they are time-consuming and their use was restricted to only a few centres. Possibly the most important landmark in the measurement of steroid hormone concentrations and in endocrinology was the advent of the radioreceptor and radio immunoassays developed in the late 1960's.[15] These methods resulted in unprecedented sensitivity (pmol/L), were highly specific and were relatively easy to perform. As a result immunoassay became the way of the future for measuring picomolar or lower concentrations of steroid hormones, drugs and many other small analytes in both plasma and urine samples.

2.1.4 Immunoassay Methods

The immunoassay is an analytical method that depends upon antigen-antibody binding reactions. The term immunoassay usually refers to a quantitative method for analyzing the immunological properties of analytes.[16] For example, radio-labelled immunoassays use reagents incorporating radioisotopes as tracers to monitor the distribution of free and bound antigen in radioimmunoassays (RIA). Radioimmunoassays for determining steroid hormone glucuronide concentrations have been reported since 1975.[17] Since steroid glucuronides are too small to elicit an antibody response, they have to be attached to a larger molecule to achieve this. The resulting unit of the steroid glucuronide and the carrier protein (often bovine serum albumin (BSA) or thyrogobulin) which is known as a conjugate functions as the immunogen. Several steroid glucuronides were linked directly to a carrier protein by the carboxyl group of the glucuronides. The resultant steroid-glucuronide-BSA complexes were used as antigens in rabbits, goats or sheep. The antiserum produced bind the steroid glucuronides with high specificity and affinity and hence can be used to determine the concentrations of steroid glucuronides directly without a preliminary hydrolysis step. Recently a crystal structure of an anti-E1G antibody has been published[18] and this shows clearly, for the first time the mode of a binding of a steroid glucuronide to the antibody binding site. In an immunoassay protocol the non-radioactive steroid glucuronide (analyte) and the radio-labelled steroid glucuronide (radioligand) can bind to a limited amount of antiserum in a competitive manner. The concentration of the free steroid glucuronide (analyte) is calculated from scintillation counting after a separation step and reference to a standard curve which relates the ratio of bound to free label with the concentration of free hormone. **Figure 2.3** below describes the principle reactions involved in radioimmunoassay.

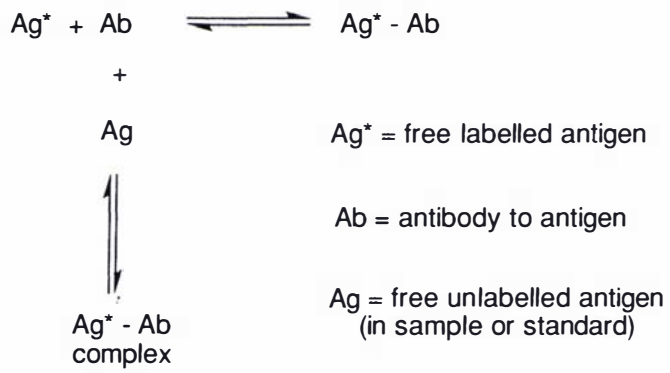


Figure 2.3

An advantage of radioimmunoassays relative to biological or chemical assays is the high degree of sensitivity obtainable with radio-labelled compounds. The steroid glucuronides may occur in human body fluids in concentrations which range, in small aliquots, from picomoles per litre to micromoles per litre. Radioimmunoassays have the capacity to detect compounds at levels of picomoles per litre or less, well beyond the sensitivity of biological or chemical assay systems. However, the greatest disadvantage of the radioimmunoassay format is the need to physically separate the bound and free labelled antigens. This step is necessary since bound and free antigen cannot be distinguished from each other by radio active methods. Other problems with radioimmunoassays include the short half-life of the radioactive label, the requirement for special detection equipment and limitations in labelling certain antigens. In addition, regulations regarding disposal of radioactivity have made the process costly. More recently, many other immunoassay methods have been developed for the measurement of analyte glucuronides in urine, and are now widely used in research and in the clinical laboratory (**Table 2.1**).

Table 2.1 Immunoassay methods for measuring analyte glucuronides in urine

		Ref.
Radioimmunoassay (RIA)	Antigen is labelled with a radionuclide for use as the tracer.	19-22

Enzyme Immunoassay (EIA)	An assay procedure based on the reversible and non-covalent binding of an antigen by a specific antibody, in which one of the reactants is labelled with an enzyme.	7, 23-25
Chemiluminescence Immunoassay (CLIA)	Antigen is labelled with a chemiluminescent molecule for use as the tracer.	26, 27
Fluoroimmunoassay (FIA)	Antigen is labelled with a fluorophore for use as the tracer	28
Metal immunoassay (MIA)	Antigen is labelled with gold sol, selenium sol or silver sol for use as the tracer	29

The use of nonisotopically labelled compounds in an immunoassay system has been developed to avoid many of the problems inherent in radioimmunoassays. Among the nonisotopic immunoassay methods, enzyme immunoassay (EIA) has provided the most convenient means by which the sensitivity and specificity of radioimunoassay (RIA) can be more generally applied. The reagents and equipment used are relatively cheap, and not associated with special hazards and disposal problems. The enzymes employed for labelling may be stored for long periods of time and enzyme assays are easy to perform often giving colorimetric end-points which can be visually assessed.

Enzyme immunoassays are based on two important biological phenomena. (i) the extraordinary discriminatory power of antibodies, and (ii) the extremely high catalytic power and specificity of enzymes, which may quite often be detectable with great ease at low levels. Enzyme immunoassays consist thus of a two-pronged strategy: the reaction between the immunoreactants (antibodies with the corresponding antigen) and the detection of that reaction using enzymes, attached to the reactants (hapten-enzyme conjugates) or antibodies (protein-enzymes conjugates), as indicators. There are two general assay types: one in which free and bound label are separated before

detection, and one in which the free label can be detected in the presence of the bound label. The assays are termed "Heterogeneous" Enzyme Immunoassays and "Homogeneous" Enzyme Immunoassays respectively.

2.1.4.1 Homogeneous Enzyme Immunoassay And The Ovarian Monitor

A "homogeneous' enzyme immunoassay is one which requires no physical separation of bound and free label and was first reported by Rubenstein *et al* in 1972,[30] as an alternative to radioimunoassays (RIA) and "heterogeneous" enzyme immunoassays. Since it requires no purification, extraction or separation of the antibody-bound antigen from the unbound antigen, this revolutionary technique gave the immunoassay much simplicity and the potential to be utilised in home assays.

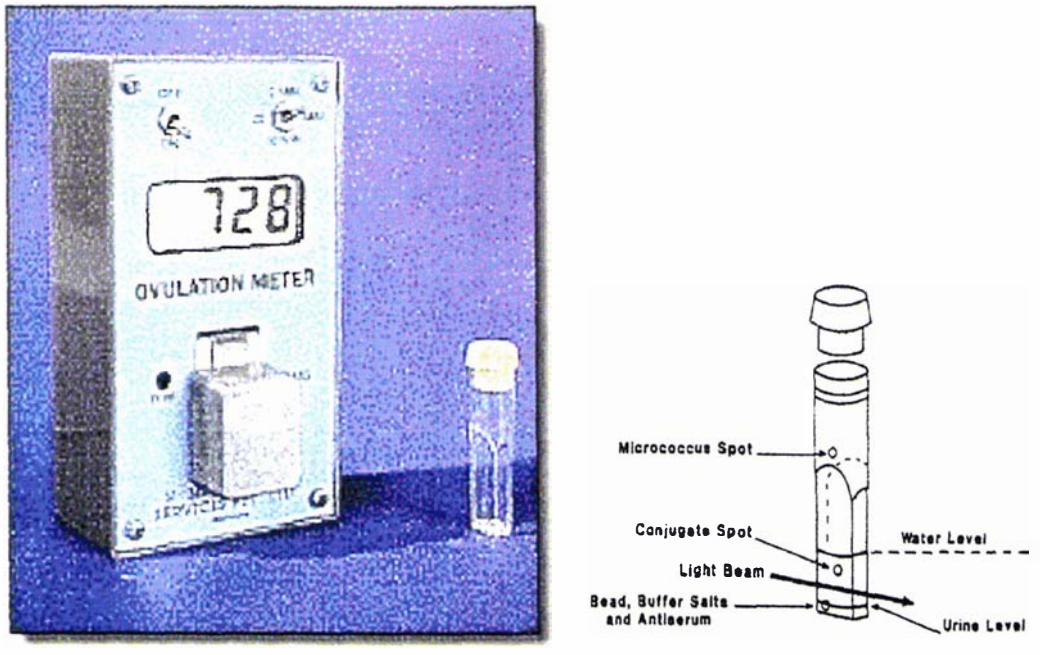
One of the important features in "homogeneous: enzyme immunoassay systems is that the enzyme activity of the enzyme-hapten conjugates is extensively inhibited by addition of the relevant antibodies. This inhibition is usually inversely proportional to the amount of free hapten in a stochiometric manner. Hence, a measurement of the enzyme activity of a mixture of enzyme and antibody is directly related to the amount of free antigen introduced from a test sample.

2.1.5 Biochemical Basis Of The Ovarian Monitor

Although a large number of immunoassay formats have been published now for the measurement of ovarian steroid hormones and their metabolites, the first system which is both laboratory accurate and currently developed for use in the home situation is the Ovarian Monitor. The Ovarian Monitor has been developed and refined by Brown and colleagues[7, 23] for over 20 years as a device for the home monitoring of the cyclic levels of the urinary metabolites of ovarian estradiol (estrone glucuronide) and progesterone (pregnanediol glucuronide) during a woman's menstrual cycle. This information can be gathered and used by women in the home to identify cyclic periods of fertility and infertility. This system has been validated extensively by many studies including a World Health Organisation (WHO) trial and is currently used around the world in many countries, both developed and undeveloped. The basis of the test is a homogeneous enzyme immunoassay system in which an anti-steroid estrone

glucuronide or pregnanediol glucuronide antibody extensively inhibits the lytic activity of a corresponding lysozyme-steroid glucuronide conjugate. The mechanism of this inhibition has been studied by Smales and is believed to be mainly due to a combination of a steric blocking of the active site of the enzyme[31] and physical obstruction of the approach of the large of *Micrococcus lysodeikticus* cell to the much smaller area of the lysozyme active site. The conjugated E1G is bound at lysine residue 33 which is situated at the left hand side of the active site. Clearly attachment of the antibody will prevent approach of the cell wall of *Micrococcus lysodeikticus* so that it can bind in the active site. Hence lysis cannot occur and the lytic activity of the conjugate is extensively inhibited (>90%). If the antibody binding site is already occupied by a free E1G molecule from a urine sample then it cannot attach itself to the E1G moiety of the lysozyme conjugate and the full lytic activity of the conjugate is observed. As a result, the concentration of E1G or PdG in a urine sample can be determined directly from a simple rate assay without the need for a separation step (**see Scheme 2.1**).

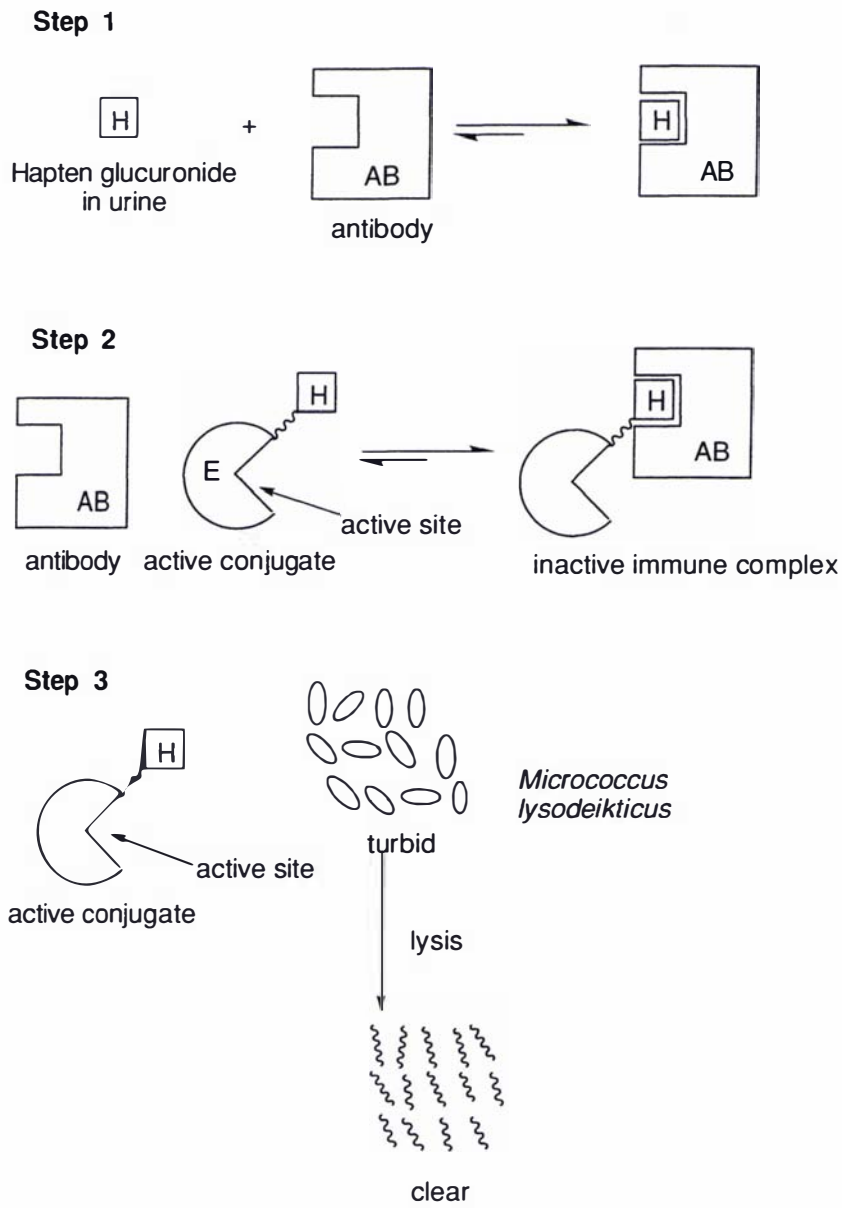
Figure 2.4 A diagram of the Ovarian Monitor, assay tube and its constituents



The Ovarian Monitor consists of two important parts; an assay tube and a monitor (**Figure 2.4**). In the assay tube, a total of three reactions are performed in the

one plastic colorimeter tube which contains the antiserum, enzyme-hormone conjugate and *Micrococus lysodeikticus* (enzyme substrate) placed in exactly measured amounts at different levels on the sides of the tube. This allows the three reactions to be performed serially in a single tube. The Ovarian Monitor has three key functions; (i) as a miniature spectrophotometer measuring and displaying the transmission result; (ii) using an in-built thermostat to control the assay temperature at 40°C; (iii) an in-built timer to control the reaction time of the three discrete steps.

The assay process is performed as shown in **Scheme 2.1**. The first step is the antigen-antibody reaction; on the time scale of the reactions, the free estrone glucuronide or pregnanediol glucuronide in the urine binds irreversibly to the appropriate anti-steroid glucuronide antibody (for El G or PdG) which is present in the tube in a slight excess. Thus, the proportion of antibody binding or neutralisation is determined by the E1G or PdG levels in the urine.



Scheme 2.1

In the second step, the remaining excess antibody (which was not bound by E1G or PdG in the previous step) binds again effectively and irreversibly on the time scale of the experiments to the steroid glucuronide-lysozyme conjugate. The steroid glucuronide-lysozyme conjugate is prepared by the coupling of E1G or PdG *via* their carboxyl groups with amino groups of lysine residues of hen egg white lysozyme (HEWL).[7] The amount of free conjugate remaining at the end of the second step is determined by the amount of free antibody left at the end of the first step, which is determined in turn by the level of E1G or PdG in the urine.

The third step is the *Micorcoccus lysodeikticus* lytic reaction. In this step, the free steroid glucuronide-enzyme conjugate remaining after the second step is active to lyse its substrate, *Micrococcus lysodeikticus*, by breaking the peptidoglycan bonds and turning the initially turbid solution clear. The rate of this reaction is measured by the amount of clearing of the turbid solution over fixed time periods. A high level of E1G (or PdG) in the urine gives rise to a high level of free lysozyme-steroid glucuronide conjugate since the antibody is neutralised in the first step of the assay (**Scheme 2.1**) and hence to a high rate of lysis. Conversely, a low concentration of E1G (or PdG) gives a low level of free conjugate since there is sufficient antibody in the assay to bind up to 95% of the enzyme conjugate in the absence of exogenous steroid glucuronide. Hence there is a low rate of lysis. Thus there is a direct relationship between the rate of lysis of the turbid solution and the concentration of free steroid glucuronide in solution. The difference in the transmission of the turbid solution between time zero (T_1) in the measurement phase of the lytic reaction and at 5 minutes for a PdG measurement and 20 minutes for an E1G measurement (T_2) is a measure of the rate of lysis ($\Delta T = T_2 - T_1$). This is shown in a typical standard curve for $E1G^{32}$ in **Figure 2.5** where ΔT is the transmission change over 20 minutes in step 3 of **Scheme 2.1**.

Figure 2.5 Typical standard curve for estrone glucuronide (E1G)

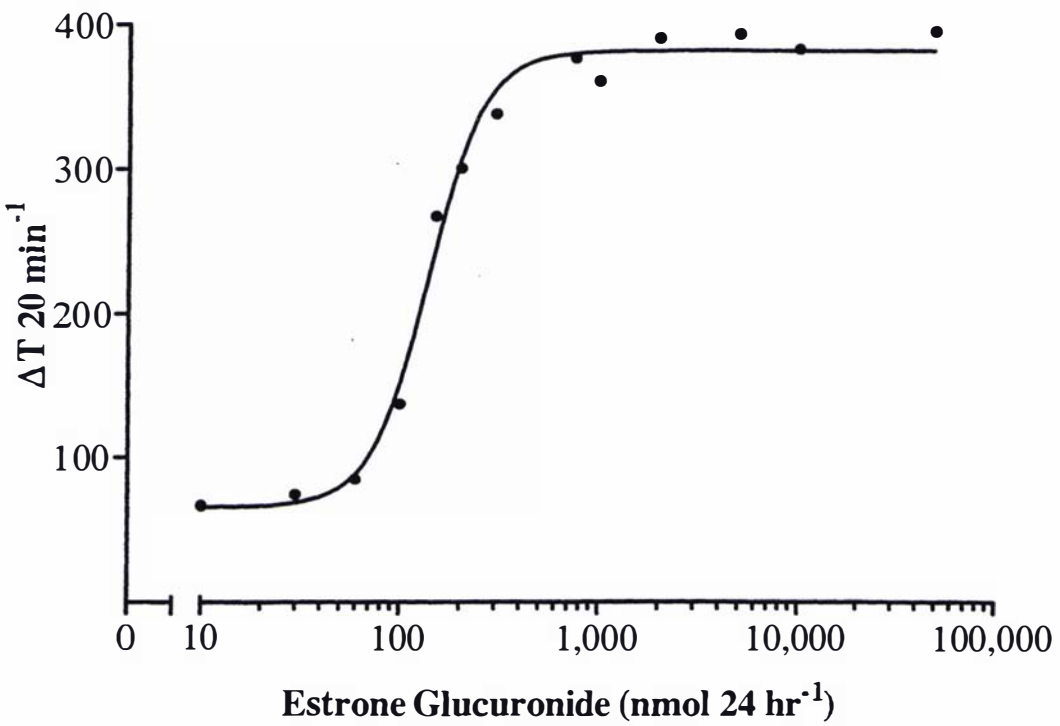


Figure 2.5 shows the typical sigmoidal relationship between assay response and analyte concentration. The user of the monitor may describe the changing pattern of hormone excretion by daily changes in ΔT or use a standard curve to derive the actual concentrations. For E1G the working range of the assay is from 50 nmol/24 h of E1G to about 400 nmol/24 h. Thus any analyte present in urine at concentrations greater than 50 nmol/24 h can be measured in this way. It should be noted that the standard curve for the pre-coated assay tubes is highly reproducible thus a single urine assay can be carried out to give quantitative results without recourse to a fresh standard curve for each measurement. This allows one-off or single urinary assay to be carried out and important information on an individual's fertility to be gained.

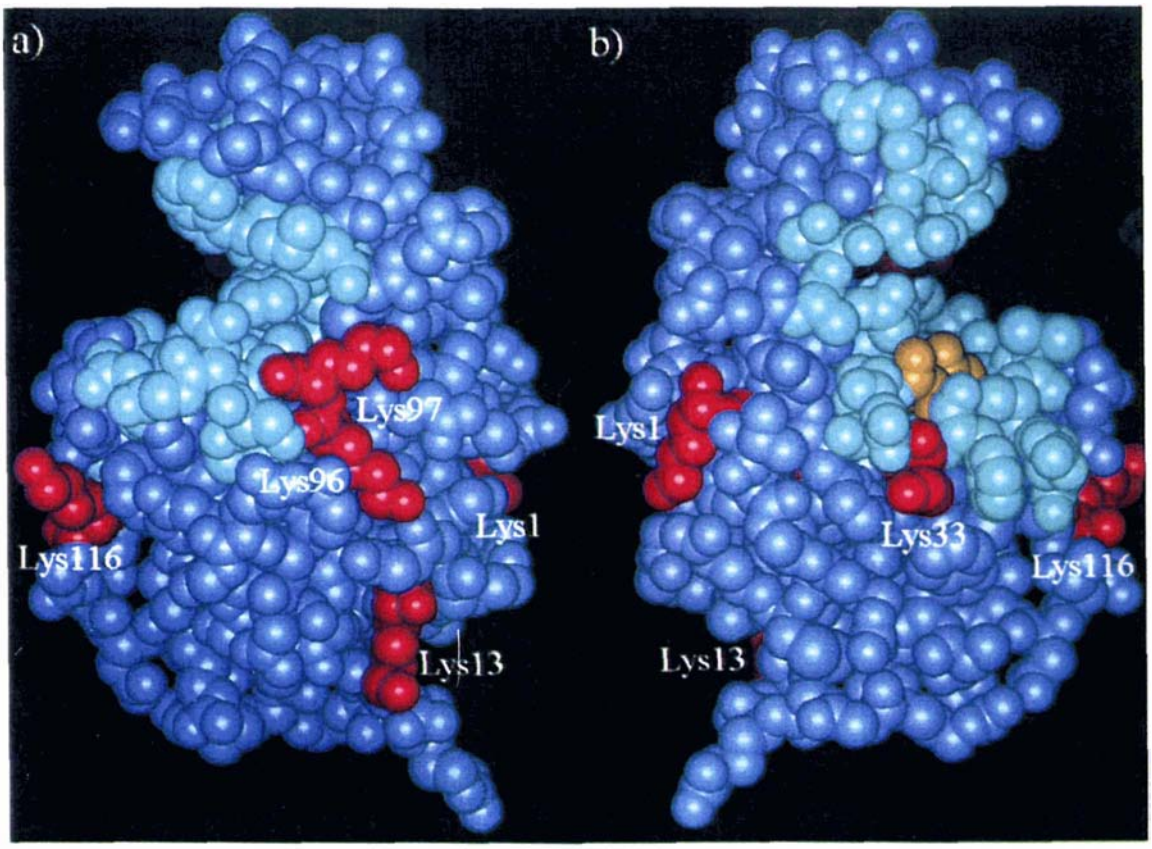
2.1.6 Preparation Of An Antibody And Pure Lysozyme-Hapten Conjugates

One of the key requirements for the monitor assay is the ability to prepare and purify lysozyme conjugates which are free of non conjugated lysozyme. This is necessary so that the difference in lysis rate between the zero standard and the top standard is maximised. Clearly any unconjugated lysozyme will not be inhibited by anti-steroid glucuronide antibodies and will therefore contribute a high back ground lysis rate. Another essential requirement is a high affinity–high specificity antibody to the steroid glucuronides to avoid unwanted cross reactions with similar steroid or steroid glucuronide structures.

The hapten-glucuronide antibodies are raised in sheep, and this is achieved by inoculating the sheep with a synthetic analyte glucuronide coupled to a carrier protein. The carrier protein, which is usually bovine serum albumin (BSA) or thyroglobulin is required to raise an immune response by the sheep which are unable to recognise the small steroid glucuronides on their own. Its conjugation is achieved by either the mixed anhydride method or active ester method.[33] Antibodies of the required affinity and specificity to the steroid glucuronide are usually generated after a minimum time of three months. The sheep are than bled and the serum obtained after clotting is of sufficient quality to be used directly in the assay. The lysozyme-hapten conjugates are formed also when a steroid glucuronide is attached to an ε-amino group (or the N-terminal amino group) of the lysine residue of a hen egg white lysozyme molecule *via* the carboxylic acid moiety on the glucuronide carbohydrate ring by the mixed

anhydride or active ester methods. There are six lysine residues within the amino acid sequence of hen egg white lysozyme[34], all of which, including the N-terminal α-amino group, are found near the surface of the molecule.[35]

Figure 2.6 CPK Space-filling representation of hen egg white lysozyme showing six lysine residues (highlighted in red) in the enzyme with active site cleft oriented from the right hand side (a) and left hand side (b) of the molecule



Hence the conjugation reaction may in principle acylate any one or more of these amino acid groups to form a stable conjugate via an anhydride linkage. It has been suggested that the site of acylation of haptens in enzyme conjugates is important in determining the recognition and strength of binding of the hapten by anti-hapten antibodies.[115] Although the mixed anhydride method is the usual method of conjugation, lysozyme can be acylated readily by a variety of acylating reagents[33] to give stable conjugates which retain lytic activity only if the total number of lysine residues acylated is less than four.[34] It is therefore necessary to use conjugation

conditions which maximise the concentration of conjugates with low degrees of steroid substitution.

Smales *et al* [31] have investigated the factors which control the reactivity of the acylating agents and identified the sites of acylation under near stoichiometric ratios of the acylating reagent. At a molar ratio of 1.5:1 the only lysine residues acylated are 33, 97 and 116 and a mixture of mono-, di- and tri-substituted conjugates is obtained. The kinetics of the various conjugates and their application in homogeneous enzyme immunoassays for E1G has also been investigated.[31] An inevitably low stoichiometry means that significant amounts of unreacted lysozyme will remain after completion of the conjugation reactions.

The sensitivity and precision of a homogeneous enzyme immunoassay is largely dependent on the purity of the enzyme-hapten conjugate used in the system.[39] This requirement necessitates that after conjugation any unreacted lysozyme is completely separated from the conjugated material.

To obtain the maximum assay discrimination between similar hormone concentrations, as is required for the delineation of the fertile period by a home assay,[7] it is necessary to isolate an enzyme conjugate with a high degree of inhibition in the presence of excess anti-hapten antibodies (<90%) to discriminate between similar hormone concentrations with the accuracy required. Ideally the enzyme activity of the conjugate in the immune complex should be zero since the accuracy, working range and gradient of the standard curve are proportional to the difference in enzyme activity between the bound and free conjugate. Thus enzyme conjugates which are both free from unconjugated material and show a high level of inhibition (>90%) in the presence of excess anti-hapten antibody must be prepared and purified for homogeneous enzyme immunoassay.

There have been few reports of chromatographic purification procedures for protein products after conjugation with small molecules apart from the use of a gel filtration step.[40] While such a step removes unreacted haptens or coupling reagents from the protein products, it does not remove unconjugated protein. The serial dialysis of conjugated material in 7 M urea solutions[41] is the only reported purification

procedure for the separation of lysozyme conjugates from unconjugated lysozyme. While this procedure gives conjugates with high degrees of inhibition (>90%), they are uncharacterised and the majority of the conjugated material is unsuitable for use in immunoassays either because it still contains lysozyme or the specific activities are too low.

The Ovarian Monitor system currently uses lysozyme-steroid glucuronide conjugates produced by acylation of lysozyme with estrone glucuronide or pregnanediol glucuronide by the mixed anhydride procedure.[42] The resulting conjugates are then purified by ion exchange chromatography on a CM Sepharose column at pH 6.0. Both the mixed anhydride acylation procedure and the ion-exchange purification are difficult to undertake and are poorly reproducible in inexperienced hands. Although this system is not ideal, it has produced good quality lysozyme-steroid glucuronide conjugates which are currently used in the Ovarian Monitor homogeneous enzyme immunoassay system. The conjugated material is purified as the trailing edge of a single large peak eluted from a CM-Sepharose cation-exchange column at pH 6.0 with the conjugate fractions identified by their inhibition (>90%) in the presence of excess anti-steroid glucuronide antibody. This single peak also contains unreacted lysozyme and other conjugated material which cannot be easily separated from the unreacted lysozyme material. As a result the conjugate material must be rechromatographed several times in order to produce conjugates with high levels of inhibition (>90%) in the presence of excess anti-hapten antibody.[42]

More recently Smales and Cooke[43] have reported a two column large scale purification procedure using an S–Sepharose fast flow cation-exchange column in 7M urea and a hydrophobic interaction column (butyl Sepharose). This procedure gives good yields of pure conjugates which may be used for research purposes and conjugate characterisation. In principle the procedures reported can be applied to any steroid glucuronide-lysozyme conjugate.

2.1.7 Steroid Glucuronides As Biomarkers Of Fertility

17β-Estradiol is the major steroid produced by the ovary during the follicular phase of the human menstrual cycle and is the result of aromatization of C19 androgens

within the granulosa cells of the growing follicle. The major androgen produced by the thecal cells is androstenedione, which is first aromatized to estrone, before being reduced by a 17β-hydroxysteroid dehydrogenase to estradiol. Smaller quantities of androstenedione in the ovary can also be reduced to the androgen testosterone, which can then be directly aromatized to estradiol.[44] **Figure 2.7** illustrates these interconversions.

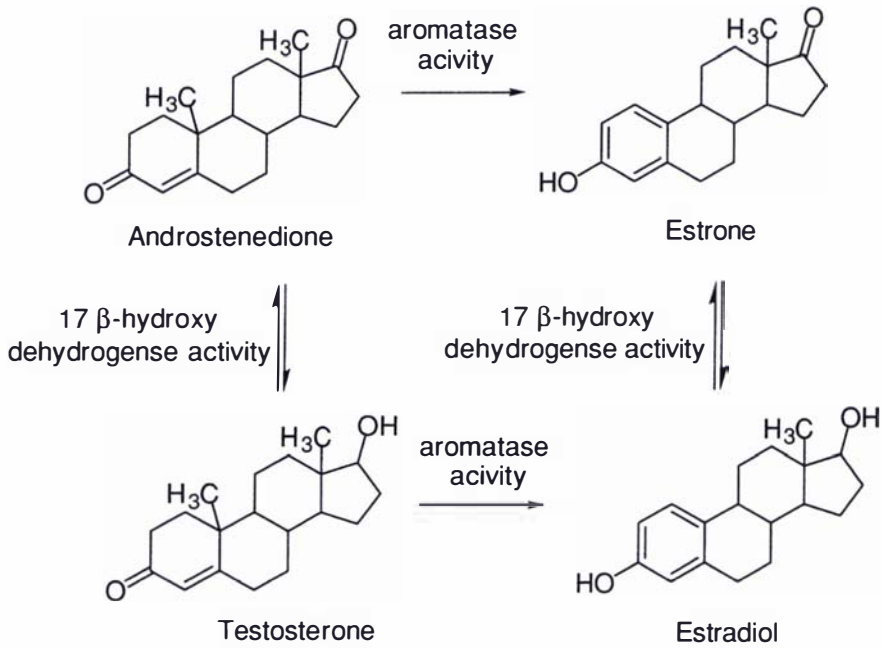


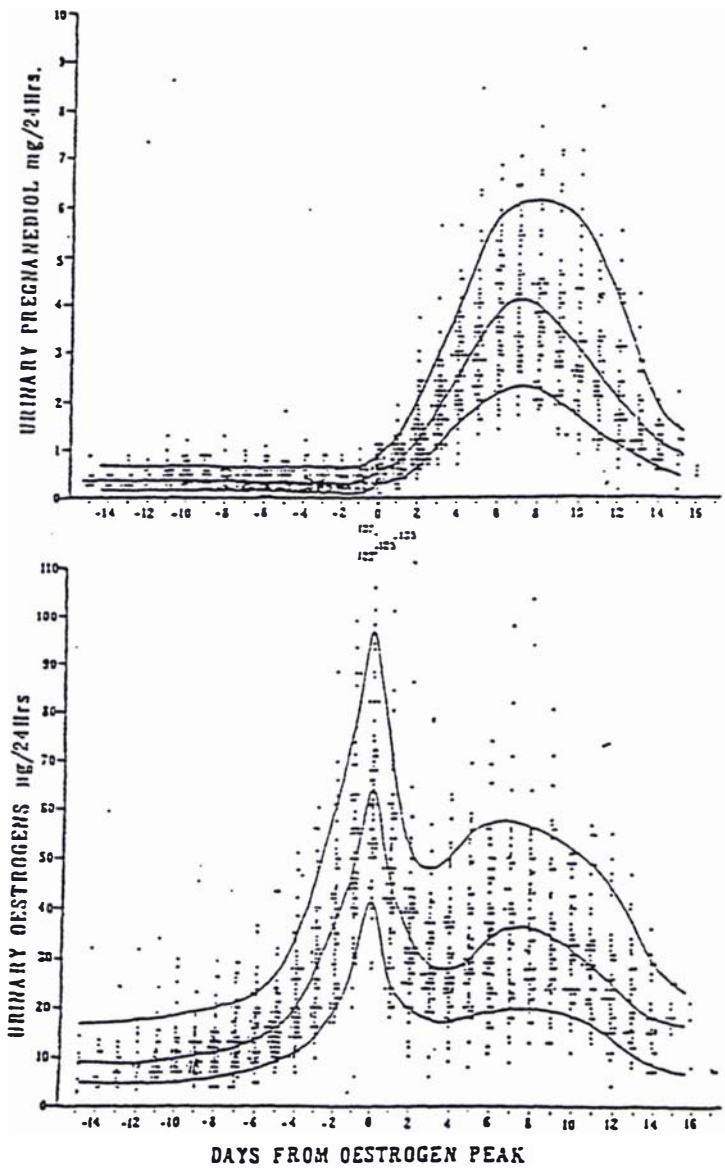
Figure 2.7

The first significant increase either in plasma estradiol or its urinary metabolites from early base line values is now accepted as the biochemical marker for the beginning of the potentially fertile phase of the human menstrual cycle.[45] For example, when a reference database of urinary total estrogens (the sum of estrone-, estradiol- and estriol-conjugates in a 24 hour urine sample) was analysed for the first rises as a marker of the beginning of the potentially fertile period,[45] the early estrogen baseline values fluctuated between 3 and 16 μg/24 hours. Once the estrogen values exceeded 20 μg/24 hours, they increased progressively without interruption to the peak value and then decreased rapidly. The peak values ranged from 40 μg/24 hours to 100 μg/24 hours.

When a time series analysis algorithm (Trigg's tracking signal) was applied to these data[45] the first rise in urinary total estrogens from the baseline was unambiguously

recognised in all complete cycles. The mean warning of impending ovulation given by the first rises was 6.5±1.4 days. This was sufficient warning for all but the longest sperm survival times. This analysis agrees with the 50th perecentile given in **Figure 2.8**[7] for the data from 61 ovulatory menstrual cycles which gives a value for the first rise day of about 4 days before the total estrogen peak. Since this is day one of the fertile period and the total estrogen peak day occurs on average 36 hours before ovulation, this also corresponds to a mean warning of ovulation of 6-6.5 days. Thus, the first rise in total urinary estrogens serves as a good marker for the beginning of potential fertility with a high degree of certainty and gives sufficient warning for al but the longest sperm survival times.

Figure 2.8 Daily urinary estrogen and pregnanediol values through out 61 ovulatory menstrual cycles



The amount of pregnanediol (detected in the form of its glucuronide) in a woman's urine rises rapidly (note the second peak in **Figure 2.8**) just after the time of ovulation, indicating the end of fertility, and declines rapidly at the time of menstruation (**Figure 2.8**).[7,45] It can be seen from **Figure 2.8** that a pregnanediol excretion rate of 1.4 mg/24 hours signals the end of fertility from 1 to 4 days after the total estrogen peak. This threshold level has been used successfully in the Ovarian Monitor test for PdG (set at 6.3 μmole/24 hours, which is the equivalent of 1.4 mg Pd/24 hours).[7] If conception has occurred, the pregnanediol level remains high thus constituting an early pregnancy test.

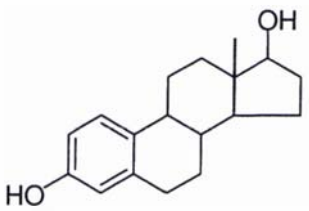
Therefore, measurement of E1G (or the corresponding estriol metabolites of estradiol, E3-16G and E3-3G) and PdG levels in urine is very useful for achieving, preventing or diagnosing of pregnancy. The measurement of urinary ovarian steroids is thus very helpful for people to gain a knowledge of their natural periods of fertility and infertility, and in particular as an aid to natural family planning. It is clear that the method described for the home measurement of E1G and PdG as markers of the fertile period has the potential to be adopted for use in other areas.

Hence urinary glucuronides have the potential to be utilised as important biomarkers in a variety of fields. The goal in this research therefore was to develop a simple, accurate cheap home and laboratory multi-purpose monitoring system for steroids and other urinary glucuronides. The decision was made to investigate the possibility that urinary metabolites of plant estrogens could serve as a valuable biomarkers for a variety of health conditions.

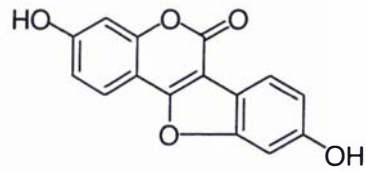
2.1.8 Phytoestrogens In Health And Disease

Phytoestrogens (or plant estrogens) are biologically active plant chemicals found in human and animal foods that resemble steroidal estrogens and mimic many of their actions including an ability to activate or block the estrogen receptor. They are of interest in human health because they occur in substantial quantities in human urine and have been proposed as having protective and preventive biological properties.[46] Progress in understanding the significance of plant estrogens has been slow, in part due to the scientific isolation of what was thought to be an idiosyncratic animal husbandry

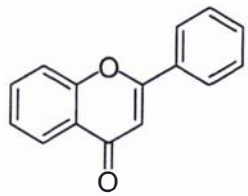
problem. Phytoestrogens are known to be diverse in their chemical structures as well as their origins. The two major classes, the coumestans and plant flavonoids (**Figure 2.9**) each have a number of representatives with different estrogenic potencies; they may have different patterns of biological activities as well.[47]



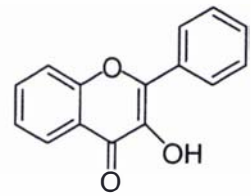
2.6 17β-Estradiol



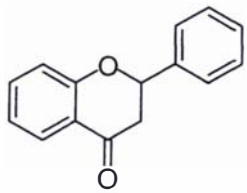
2.7 Coumestan



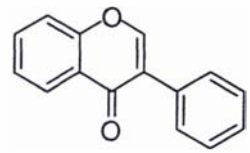
2.8 Flavone



2.9 Flavonol



2.10 Flavanone



2.11 Isoflavone

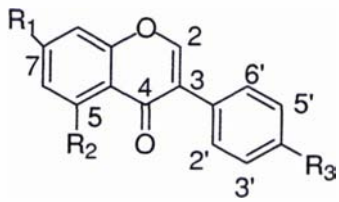
Figure 2.9

It is generally assumed that nonsteroidal estrogens exert their stimulatory effect on the estrogen receptor by binding to the same site as that occupied by steroidal estrogens such as 17-β-estradiol.[47] Thus while general statements regarding phytoestrogen effects can be made additional properties may be associated with specific phytoestrogens.

2.1.8.1 Isoflavones, Isoflavone Glucuronides And Their Health Effects

Flavonoids are usually found in plants, vegetables, and flowers, but isoflavones such as daidzein, formononetin and geninstein (**Figure 2.10**) are found in just a few

botanical families because of the limited distribution of the enzyme chalcone isomerase which is found largely in tropical legumes. This enzyme converts a flavone precursor into 2-hydroxy daidzein. Because of the limited distribution of the enzyme chalcone isomerase, the modern American or British diets contain very small amounts of isoflavones.[48] The general structure and numbering system of isoflavones is shown in **Figure 2.10**.



2.12 R_1 = OH, R_2 = H, R_3 = OH Daidzein
2.13 R_1 = OH, R_2 = H, R_3 = OMe Formononetin
2.14 R_1 = OH, R_2 =O H, R_3 = OH Genistein

Figure 2.10

Isoflavones play a dual biochemical role as estrogen and antiestrogen regulators in women's bodies. They are weak non steroidal estrogens meaning that they possess some of the characteristics of hormones but are not true hormones. Daidzein, formononetin, geninstein and other isoflavones work in part by attaching to estrogen receptors in cells and influencing cell behaviour. Their chemical structure is enough like estrogen to promote calcium absorption, increase bone density and protect against osteoporosis.[129] By attaching to estrogen receptors isoflavones also prevent the more potent hormone 17-β-estradiol from attaching to the receptors and stimulating cell growth in cancer. Many researchers believe an antiestrogen effect may be one of the reasons why diets high in isoflavones protect against hormonally related cancers such as breast cancer, prostate cancer and endometrial cancers.[50] There is also some evidence that isoflavones block the carcinogenic effect of synthetic estrogen analogues which includes many pesticides.[51] Evidence has been produced that isoflavones could prevent breast and endometrial cancer by inhibiting tyrosine kinase an enzyme that promotes growth of cancer cells.[52] Other researchers have also shown that isoflavones function as protease inhibitors, substances that block the breakdown of proteins. For example protease inhibitors have been found to be useful in treating AIDS and some types of cancers.[53]

Isoflavonoids have been reported also as a potentially important link between diet and cancer risk. Epidemiological studies suggest that soybean consumption is one of several factors that may have contributed to the lower rates of breast, colon and sex hormone dependent prostate cancers in Asian countries. Dietary supplementation is being proposed as a means to increase consumption of isoflavonoids particularly in western diets. Urinary excretion of isoflavones and their metabolites was 20-30 times higher among Japanese women and men consuming a traditional diet than in people living in western countries.[54,55] Compared with vegetarian and omnivorous healthy controls, breast cancer patients excreted about 21% less of the isoflavone metabolite equol in urine.[56] The recommended diet contains an average intake of 40-60 mg isoflavones on a daily basis. This is equivalent to approximately 120 g of tofu, 1.3 litres of soy milk or 350 g of soy flour. Red clover extract has a high isoflavone concentration of daidzein, formononetin, geninstein and methoxy geninstein compared to other plants. Very recently, an Australian biotechnology company Novogen and others reported selling of red-clover extracts in the market place for its uses as an effective hormone replacement therapy. However some experiments showed that isoflavones might suppress or enhance tumor risk depending on the dose, feeding period or organ involved.

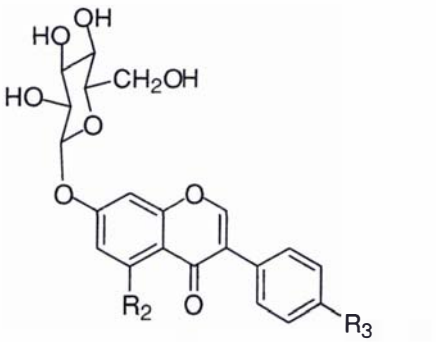
Although the evidence for beneficial effects of food containing these components is mounting, the potential for toxicity from high levels of isoflavonoids has not been adequately investigated due to the limited quantities of isoflavonoids available for research. The reported adverse effects of the synthetic antiestrogen tamoxifen used in clinical trials to prevent breast cancer are examples of the potential for toxic as well as beneficial effects of hormonal therapies.[57] The impact of dietary isoflavones such as daidzein, formononetin and geninstein upon the health of both adults and developing infants is also a growing concern of the medical profession and regulatory bodies.[58]

Ingestion of high amounts of isoflavones are also known to cause a serious infertility syndrome in sheep and cattle grazing in certain clover fields. It became apparent that the disease syndrome was triggered not only by plant isoflavonoids but also by their intestinal metabolites. According to several studies[59] all the red clover varieties studied in Nordic countries contain estrogenic isoflavones especially formononetin but the quantities of estrogenic isoflavones discovered in white clover varieties were small. Formononetin was again the main component (90%-95% of the

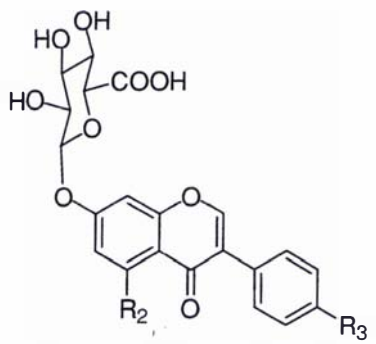
total) and a very small amount of geninstein (5%-10%) was detected. In most of the material the amount of daidzein was below detection limits. The phytoestrogenic effect of legume fodder needs to be understood also because of increasing consumer interest in so called organic farming products. This type of farming which avoids the use of artificial fertilizers relies heavily on legume fodder because of the plants ability to bind nitrogen from air for its use.

Clearly the balance among these differing actions has important implications for the medicinal and nutritional use of these phytochemicals. The analytical methods available for studying the bioavailability and metabolic fate of dietary isoflavones are based on GC-MS[60] or HPLC.[61,62] The GC-MS method requires the relatively laborious process of sampling and in the HPLC method the sample needs prepurification. Both techniques require expensive instrumentation. These facts render both methods less suitable for epidemiological studies. Immunoassay methods are a powerful alternative to those methods because of their ease, relatively low cost and availability for serial work and automation. Although non radioisotopic methods are preferred for routine work, it is usual that radio isotopic methods are developed first to pave the way for other more convenient methods. Recently Wang and co-workers[63] have developed a new RIA method for quantitative analysis of formononetin in blood plasma whereas Aldercreutz *et al* [64] developed a novel RIA for quantitative assays for daidzein in human biological fluids. This means that the appropriate antibodies and analytical standards are available.

Before deciding on a suitable analytical method for field, on-site or a home analysis of phytoestrogens it is necessary to consider the various forms in which they are formed. For example, phytoestrogen isoflavonoids occur in foods almost entirely as a complex mixture of glycosides and glycoside esters while the metabolites in human urine occur mainly as the glucuronide and sulfate conjugates.



2.15 R_2 = OH, R_3 = O H Daidzin
2.17 R_2 = O H, R_3 = OH Genistin
2.16 R_2 = OH, R_3 = OMe Formononetin glucoside



2.18 R_2 = OH, R_3 = O H Daidzein glucuronide
2.19 R_2 = OH, R_3 = OMe Formononetin glucuronide
2.20 R_2 = O H, R_3 = OH Genistein glucuronide

Figure 2.11

Glucuronidation is a major detoxification system for several potentially toxic endogenous and exogenous substances including phytoestrogens. It has been reported also that isoflavone and isoflavone glucuronides suppresses epidermal growth factor-induced prostaglandin biosynthesis by abolishing the response to calcium ionophores which is especially helpful for elderly people whose natural killer cell (NK) activity is decreased and prostaglandin production increased with age.[56] The glucuronide forms are more active over a wider concentration range than the parent isoflavone in activating NK cells. The study demonstrated that isoflavone glucuronides might not only compete with endogenous estrogen to inhibit estrogen-dependent proliferation of cancer cells, but might also activate NK cells at a site different from interleukin-2 action to potentially increase the immune defences of the body against cancer. Very recently Zhang *et al* [56] reported that daidzein and geninstein glucuronides (DG and GG), major isoflavone metabolites may be partly responsible for the biological effects of isoflavones, such as estrogen receptor binding and natural killer cell (NK) activation or inhibition.

2.1.8.2 Metabolic Transformations Of Isoflavones And Isoflavone Glucuronides

The major metabolic transformation of isoflavones is performed by micro organisms in the rumen. Formononetin is demethylated to daidzein and further *via* hydrogenation and ring fission predominantly to equol. The metabolic pathway of formononetin may also proceed under some circumstances, *via* an alternative route involving reduction without prior demethylation, where *O*-methyl-equol is the major

excretory product of formononetin. The major metabolic pathway of formononetin is *via* daidzein to equol, rather than *via* *O*-methyl equol and to desmethyl angolensin.[65]

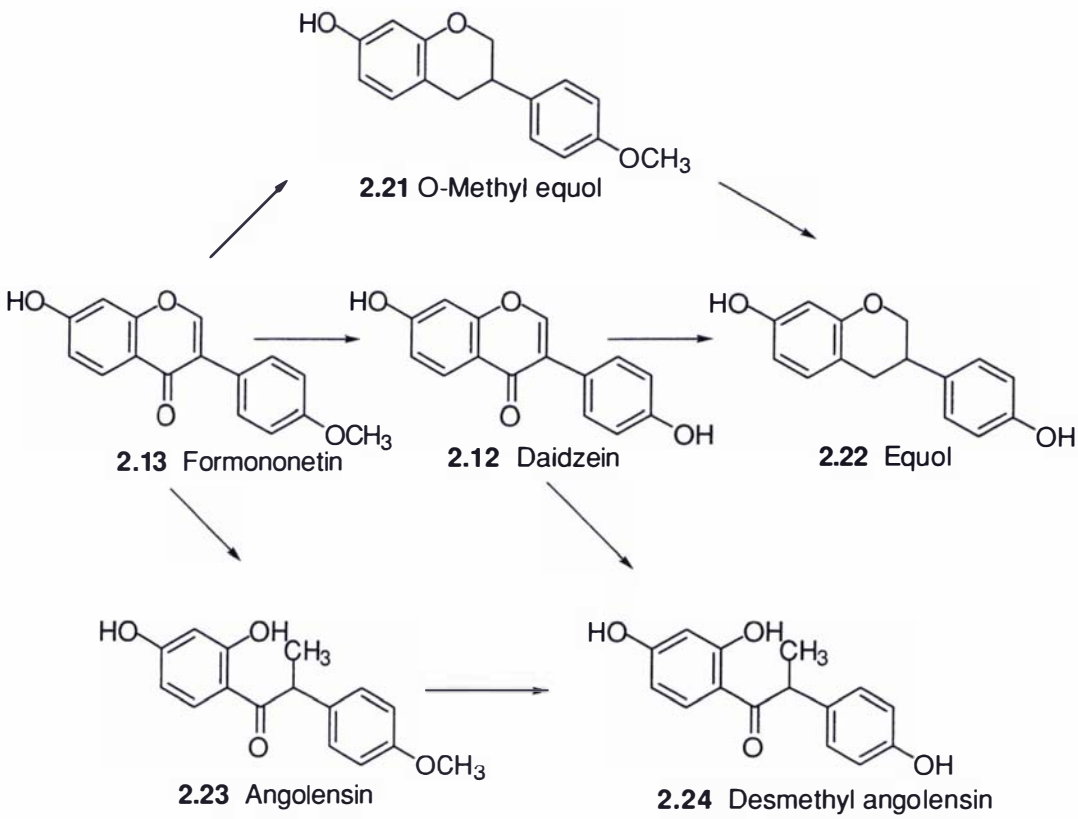


Figure 2.12

Isoflavone glucosides constitute over 97% of the isoflavones in soybeans.[66] After consumption, glucoside isoflavones reach the lower intestine and can be cleaved by bacterial glucosidases. The released free aglycones can be absorbed by the intestinal mucosa where they are glucuronidated to a large extent and transported to liver *via* the portal vein, where they can be further reacted with UDP-glucuronyl transferase and sulfotransferase to form glucuronide and sulfate conjugates.[67] Glucuronidation has low affinity but high capacity where as sulfation has high affinity but low capacity. Therefore, following administration of relatively large doses of isoflavone compounds, glucuronidation would be the major pathway of biotransformation.

2.1.9 Preparation Of Isoflavone Glucuronide By Enzymatic Synthesis

Numerous functional groups can undergo conjugations with glucuronic acids and form O-, N- and S-glucuronides, respectively. Certain nucleophilic carbon atoms

form C-glucuronides as well. As isoflavones contain hydroxyl groups in their structure: daidzein at the 7'- and 4'- positions, geninstein at the 5'- and 7'- and 4'- position, they can form *O*-glucuronides at all these positions.

Zhang *et al* [56] reported the synthesis of DG and GG using 3-methyl chloranthrene induced rat liver microsomes (a good source of glucuronyl transferase). They reported, based on UV absorption results that the hydroxyl group in the 7-position seemed to be the most active group and reacted with UDPGA to form the isoflavone glucuronide. With formononetin the site of glucuronide formation must be the 7-hydroxyl group, since no other position is available. They showed that when DG and GG dissolved in different solvents, the maximal UV absorption of the glucuronides differed. For example the maximal absorption was at 250 nm for DG and 262 nm for GG. In 100% methanol or ethanol the maximal absorption was 262 nm for both DG and GG. After acidification to pH 6.0 the maximal absorption of DG shifted to 250 nm and there was no change for GG. There was a large bathochromic shift in both DG and GG when NaOMe was added, which indicated that there was a free 4'-OH in their structure. When the weaker base of sodium acetate was used, a modest bathochromic shift indicated that the 7-poition was occupied by another group instead of the hydroxyl group which causes a strong bathochromic shift.[56] The bathochromic shift of GG in the presence of $AlCl_3$ and $AlCl_3$/HCl was due to the presence of a 5-OH group, which was absent in DG. Both the DG and GG bathochromic shift patterns were similar to daidzin (daidzein glucoside) and geninstin (geninstein glucoside) respectively.[68]

In an earlier work Dutton reported[69] that a wide variety of phenolic and alcoholic compounds form glucuronides when incubated in the presence of UDP-glucuronic acid, with microsomal fractions from animal tissues. Williamson *et al* [70] also reported that under appropriate conditions in vitro, liver and kidney tissue from rabbits and from humans can also effect the transfer of glucose, galactose and N-acetyl glucosamine from their respective uridine nucleotides to a hydroxyl group on steroid molecules. The transfer of N-acetyl glucosamine was limited to alcoholic hydroxyl groups in ring D of the steroid, but glucose and galactose may be transferred to either a ring D alcoholic or to the phenolic 3-hydroxyl group of the steroid oestrogens. In all the reported cases the sugar transferred and the acceptor group on the steroid were remarkably specific for the species studied. Along this line Labow *et al* [71] incubated the

isoflavones daidzein, formononetin, geninstein and equol in the presence of UDP-glucuronic acid, with microsomal fractions from rabbit liver tissues to prepare corresponding glucuronides. Only mono glucuronides were formed and no formation of conjugates was observed when UDP-glucuronic acid was omitted from the incubation medium. The identity of the presumptive glucuronides was further examined by subjecting the conjugates to hydrolysis by β-glucuronidase. The structure of the glucuronides were not further studied.

For the purpose of developing qualitative and quantitative analytical methods for isoflavone glucuronides authentic isoflavonoid phytoestrogens are needed. Therefore attention was focused on the synthesis of isoflavones, themselves a group of biologically active compounds of edible plants.

2.1.10 Aims Of The Present Study

The aim of the present project was to investigate whether it is possible to produce analyte glucuronides which can fulfil the requirements of an Ovarian Monitor assay and hence allow a multipurpose home monitor to be developed by adapting the platform technology developed and used in a point-of-care monitoring device for the fertile period in humans. In this thesis two new analyte classes of compound will be investigated.

1) Other steroid glucuronides such as testosterone and estrone (already in use in Ovarian Monitor) for use in athletic doping testing and fertility respectively.

2) Phenolic analyte isoflavone glucuronides (cancer prevention).

A multi purpose monitor requires the following critical steps for the new developments.

(i) Synthesis of an analyte glucuronide since commercial purchase or isolation from biological substances (such as urine) are both prohibitive.

(ii) Synthesis of lysozyme glucuronide conjugates which are free of native or unconjugated lysozyme.

(iii) Preparation of a suitable antibody to the glucuronide (raised in sheep or rabbits) by immunisation with an appropriate immunogen. The antibody produced must have high titre and of high affinity.

(iv) A standard curve with a working range corresponding to the physiological levels of the glucuronide of interest in urine.

(iii) Validation of the concentrations obtained with the assay against an acceptable reference method.

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CHAPTER 3 STEREO SELECTIVE β - GLYCOSYLATIONS OF ISOFLAVONES AND MODEL PHENOLIC COMPOUNDS

3.1 INTRODUCTION

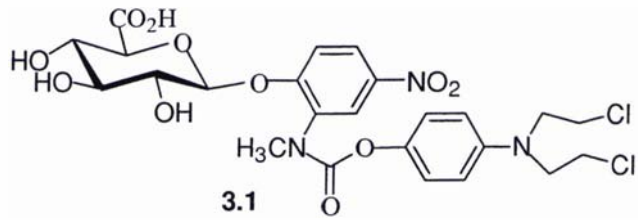
The increased understanding of metabolic processes has lead to a growing appreciation of the role and significance of glucuronide metabolites. Apart from being the final form of a drug or xenobiotic which is eliminated from the body, often performing a detoxification role, glucuronides may also have significant biological activity. Regulatory bodies are now demanding that a glucuronide (or other metabolites) should be tested thoroughly in its own right before acceptance of a xenobiotic as a potent drug.[1] While potential new drugs are frequently found as their glucuronides, high purity glucuronide samples for assay puposes are also in demand. To assist in the unequivocal identification and quantification (eg by radio assay procedures) of such conjugated metabolites it is therefore necessary to have readily available, a convenient synthesis of a wide range of β-D-glucuronides.

Provided that the aglycone portion is compatible with the base catalysed hydrolytic removal of ester groups in the final step, the readily accessible aryl glycosides and glucuronate intermediates are fully satisfactory and are indeed most commonly used as drugs. Generally a participating C-2 substituent in the sugar moiety in glucuronidation leads predominantly to the β-configuration of the glycosyl derivative which is almost invariably the natural configuration (exceptions occur however particularly with couplings of 1-hydroxy sugars). By contrast, ether intermediates typically give α/β mixtures, though varying degrees of stereo chemical control are possible.

3.1.1 Classification Of *O*-Glucuronides

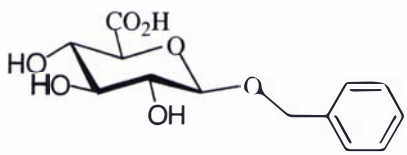
O-Glucuronides may be classified chemically into three classes depending on the nature of the acetal linkage; (i) aryl glucuronides in which the linkage to the aglycone moiety involves a phenolic OH group **3.1**; (ii) alkyl glucuronides which

involve linkage to an alcoholic OH group **3.2** and (iii) acyl glucuronides involving linkage to a carboxylic acid OH group **3.3**.

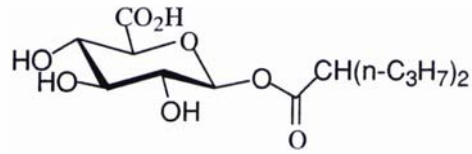


3.1

Figure 3.1



3.2



3.3

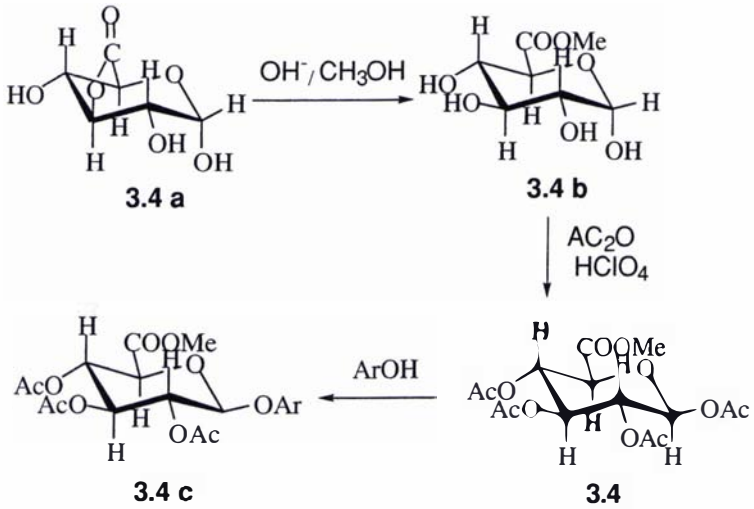
Figure 3.2

In general it may be said that both alkyl and aryl glucuronide can be prepared by the same synthetic methods. Aryl glucuronides are especially important as it is unusual for a reasonably lipophilic phenolic drug not to yield at least some glucuronide conjugates in humans. In this chapter the chemical synthesis and characterisation of aryl glucuronide esters will focus on the linkage of a glucuronic ester moiety to give β - linked aryl glucuronides. The methods appropriate for preparation of anomeric β-D-glucuronides: perester coupling method, glycosyl halide coupling method, thio derivatives as glycosyl donors and various other methods for glycosylation will be reviewed.

3.1.2 Glycosylation Using The Perester Coupling Method

The commercial availability of glucuronolactone (marketed as glucuronic acid lactone; Aldrich Chemical Company) makes this substance particularly attractive as a starting point for the synthesis of aryl glucuronic acids and the present research was aimed at exploring this reaction. The glucuronide reactions of this class are relatively easy and applicable to large scale synthesis starting from precursors which are either available commercially or can be prepared simply by acetylation of the corresponding

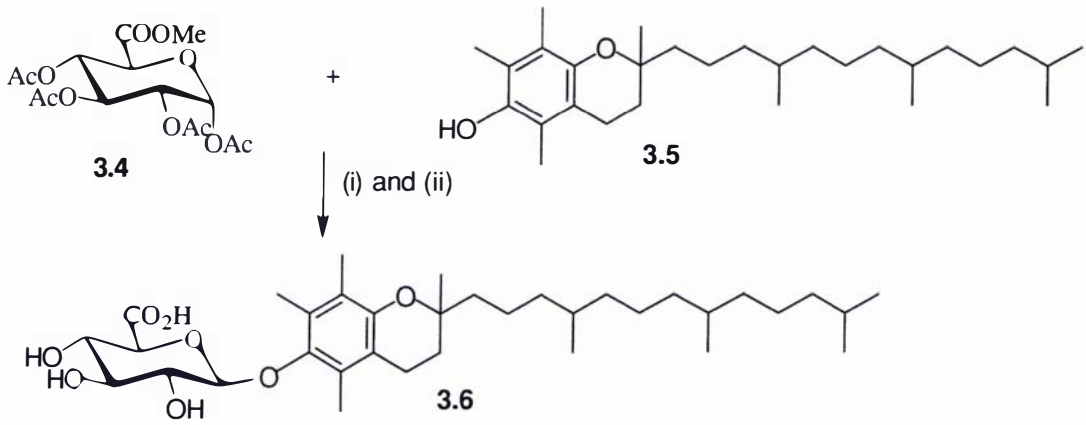
sugars. The use of methyl glucopyranuronate **3.4** avoids the use of expensive and special reagents, chemicals, solvents and promoters. The method works well for phenols and many aryl glucuronides have been prepared using this methodology.



Scheme 3.1

The reaction is stereochemically reliable, giving only the β-anomers of the conjugates. The use of the 1-β-acetate ester is essential as the 1- α- acetate ester anomer gives very little or no product.[2] This coupling method does not lead to the formation of an orthoester but the α-anomer can be obtained at higher temperature and prolonged reaction times. Many methyl (aryl tri-*O*-acetyl) β-D-glucucopyranuronates have been prepared by the fusion of methyl β-D-glucopyranuronate **3.4** using toluene 4-sulfonic acid (PTSA) or zinc chloride.[1] However it has been reported that this reaction failed with more acidic phenols; for example with *p*-nitro phenol and *o*-chloro phenol no product was obtained.

One of the interesting examples of the perester coupling method is the preparation of the 6-*O*-glucuronide of the anti diabetic drug CS-045 **3.6**.[3] This conjugate was synthesised by coupling of freshly prepared **3.4** and the aglycone **3.5** in nitrobenzene. The reaction mixture was heated under reduced pressure in the presence of an acid catalyst to give the corresponding protected glucuronide in moderate yield. This on alkaline hydrolysis gave the required glucuronide **3.6**. Interestingly the glucuronide **3.6** retained most of the parent drugs biological activity.



***Reagents and Conditions**: (i) $PhNO_2$, TSOH, 85°C, 20 mm Hg, 4 h, 23%, (ii) NaOH, MeOH, H_2O, 53%.*

Scheme 3.2

Recently the more powerful Lewis acid $BF_3.OEt_2$ has been used for the preparation of raloxifene-6-β-glucuronide **3.7** by direct coupling of the tert-butyldimethylsilyl protected aglycone raloxifene with **3.4**. in CH_2Cl_2[4] The reaction was stereochemically reliable giving the desired β-stereochemitry at the anomeric center.

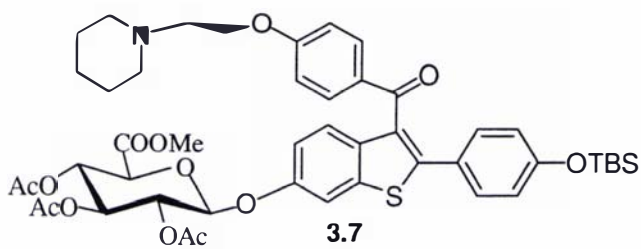
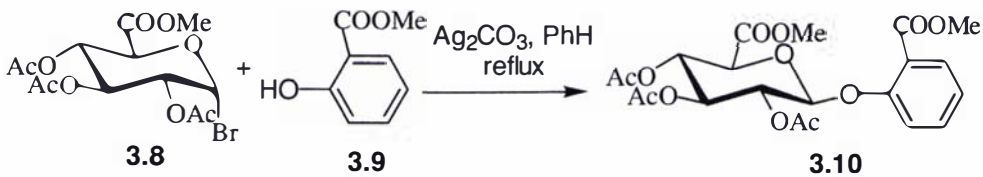


Figure 3.3

Subsequent removal of the ester groups proved problematic as treatment of **3.7** with NaOH resulted in an immediate cleavage of the sugar moiety to give back the aglycone raloxifene. There was no mention in the article why this cleavage took place. However after considerable experimentation they found that heating **3.7** with LiOH in dioxane at 60°C followed by treatment with tetrabutyl ammonium fluoride gave the required glucuronide.

3.1.3 Glycosyl Halide Couplings

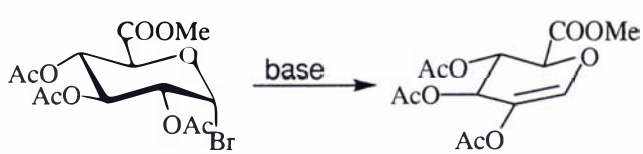
The 1-α-bromo sugar **3.8** is probably still the most popular glucuronidation intermediate. Its highly unstable reputation is not entirely justified, but it must be kept dry and below 0^0C to store it for extended periods. The coupling of this glycosyl bromide with an alcoholic or phenolic group under anhydrous conditions is described as the Koenigs-Knorr reaction This will be discussed in more detail later in **Chapter 5**. In general the method appears rather more suitable for the preparation of alkyl rather than aryl glucuronides, though it is interesting that the conjugate **3.10** from methyl salicylate was satisfactorily produced from only the bromo sugar **3.8** and silver carbonate.[5]



3.8 **3.9** **3.10**

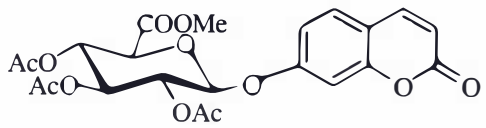
Scheme 3.3

The most common solvents used have been benzene, toluene and quinoline, removal of any water produced (by molecular sieves or azeotropic distillation) being very important. Additional base may be necessary to destroy the hydrogen bromide formed during the reaction. Glycosylation of aryl alcohols using phase transfer catalysts has been developed also for glycosyl bromides or chlorides and provides a facile, stereospecific and general method to prepare β-aryl glycosides[6,7] (this will be discussed also in more detail in **Chapter 5**). A persistent by-product from such reactions however is the 'glycoseen' or 2-acetoxy glycal **3.12** formed by hydrogen bromide elimination.[8]



Scheme 3.4

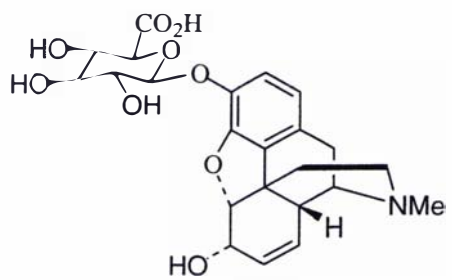
Recently the sodium salt of 7-hydroxy coumarin was reacted with the bromo sugar **3.8** using benzyltriethylammonium bromide as a phase transfer catalyst and the glucuronide ester **3.13** was produced in low (11%) yield.[9] The author failed to give any reason for such a low yield.



3.13

Figure 3.4

Another possibility with phenols is to react the bromo sugar with the alkali metal phenolate. The best procedure appears to be to use a lithium salt in methanol and morphine-3-glucuronide **3.14**, the major human metabolite of morphine in urine has been obtained in 53% yield in this way.[163]



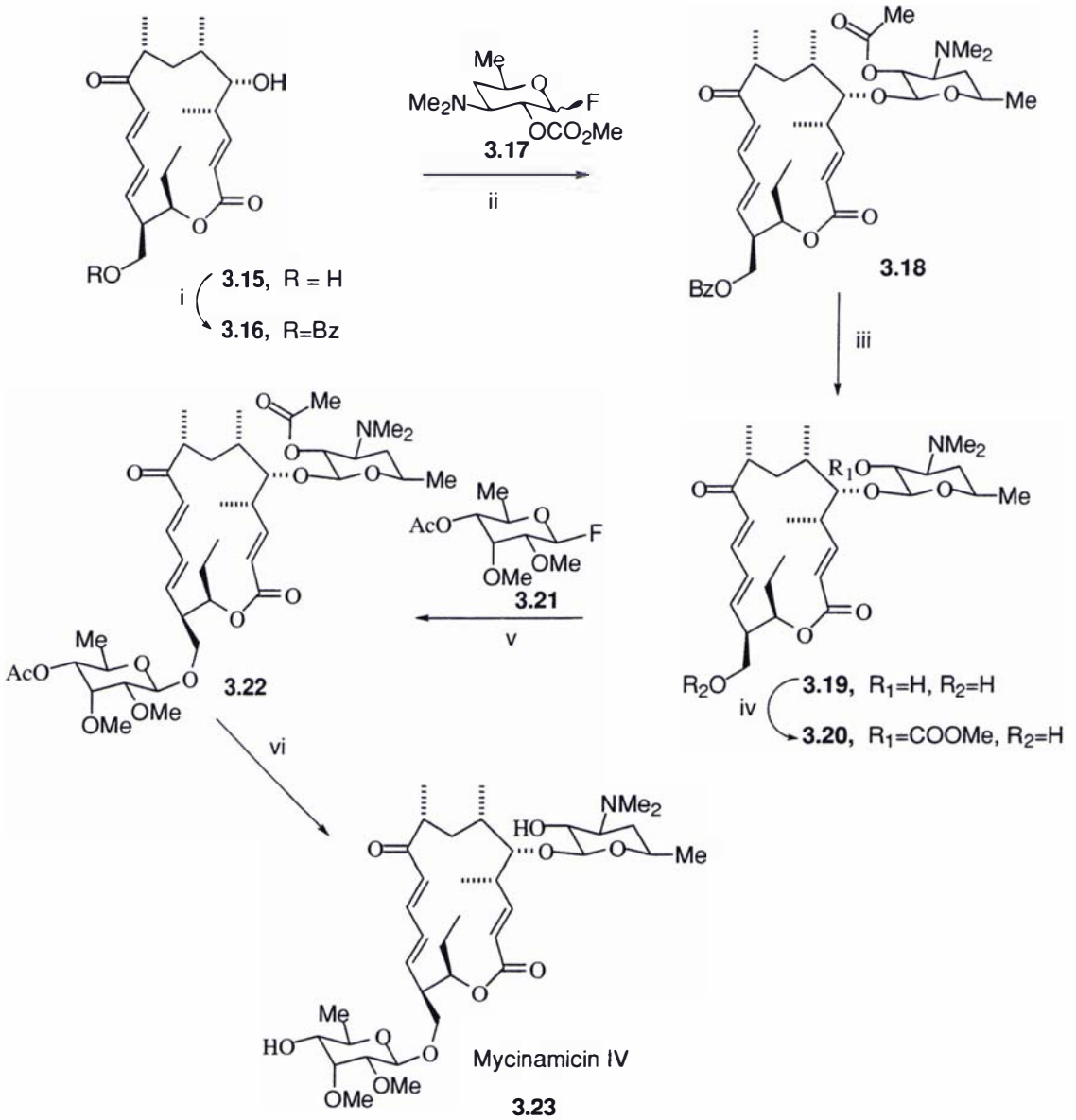
3.14

Figure 3.5

Recently, a simple thermal glycosylation of an alcohol with glycosyl chlorides using a metal salt has been developed. It has been found that a dramatic change in stereoselectivity occurs depending on the reaction temperature. A relatively low temperature favours β-selective coupling, whereas α-selective coupling occurs at higher reaction temperatures.[11] The use of glycosyl fluorides as the glycosyl halide was first introduced by Mukaiyama *et al.*[12] The major advantage of a glycosyl fluoride is its high thermal and chemical stability as compared with the low stability of other glycosyl halides. Glycosyl fluorides can be purified easily by an appropriate distillation and even by column chromatography. A number of specific promoter systems have been

developed for it. The high β-stereo selectivity was obtained by using shelf-stable glycosyl fluorides and "non Koenigs-Knorr" sugars [sugars without a participating group (acyl group) at C(2)-OH].[165]

One of the striking examples is the synthesis of mycinamicin IV by employing Cp_2MCl_2-$AgClO_4$ (Cp=Cylcopentadienyl; M=Zr, Hf) as effective activators of glycosyl fluorides to obtain highly β-selective glycoside linkages.[13] Mycinolide IV **3.15** was benzoylated under controlled conditions to give the selectively mono protected aglycone **3.16** in 96% yield. Glycosylation of **3.16** with glycosyl fluoride **3.17** in the presence of Cp_2HfCl_2-$AgClO_4$ in CH_2Cl_2 proceeded smoothly to afford **3.18** in 72% yield (α:β ratio=1/6). After separation of the α anomers, the two protecting groups of **3.18** were detached to give compound **3.19** in 75% yield.



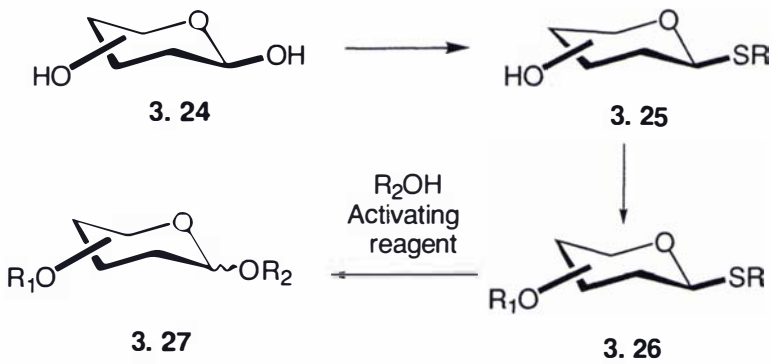
Reagents and Conditions : *(i) PhCOCl, Pyridine, CH_2Cl_2, 0 ^0C; (ii) CP_2HfCl_2, $AgClO_4$, CH_2Cl_2; (iii) Et_3N, H_2O, MeOH (1:15), 70 ^0C; (iv) $ClCO_2Me$, CH_2Cl_2, 0 ^0C; (v) CP_2ZrCl_2, $AgClO_4$, C_6H_6, RT, 1 h; (vi) Et_3N, H_2O, MeOH (1:1.5), RT, 16 h.*

Scheme 3.5

Subsequent treatment of **3.19** with methyl chloroformate cleanly afforded the alcohol **3.20** which then proceeded by means of a second glycosidation with glycosyl fluoride **3.21** in the presence of Cp_2HfCl_2-$AgClO_4$ in benzene to furnish the glycoside **3.22** in 86% yield with an excellent stereoselectivity (α:β ratio=1/26). After seperation, the hydrolytic clevage of the protecting groups of **3.22** afforded mycinamicin IV **3.23**.

3.1.4 Thio-Derivatives As Glycosyl Donors In Glycosylation Reactions

The possibility of using thio-derivatives as glycosyl donors has been known for a considerable time but it is only in recent years that it has been extensively explored.[14-18] The versatility of thioglycosides in carbohydrate chemistry stems from the fact that the sulfur atom in thioglycosides is a soft nucleophile, and is therefore able to react selectively with "soft" electrophiles, such as heavy metal cations, halogens and alkylating or acylating reagents. On the other hand, the hydroxyl and ring oxygen atom of carbohydrates are hard nucleophiles, which can be functionalised with "hard" reagents, without affecting any alkylthio or arylthio functionalities. The sulfur bearing anomeric centre can then be selectively activated with a soft electrophile, to form a reactive glycosylating species that can be used in creation of a new glycosidic bond.[19]

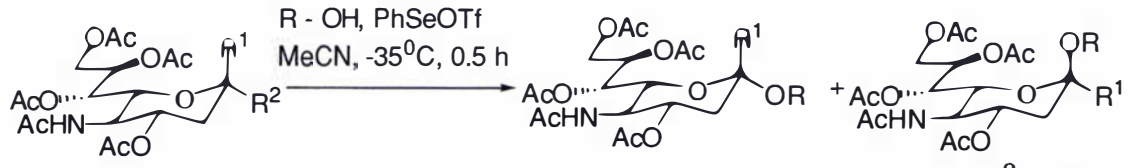


Scheme 3.6

3.1.4.1 Thiosugars As Glycosyl Donors

The thioglycosides can be prepared conveniently by efficient thiolyses of an acetyl or acetate sugar using broron trifluoride etherate as a promoter[20] or reaction of glycosyl bromide with thiols under phase transfer conditions.[21] The direct glycosylation of alcohols with thioglycosides can be accomplished using various thiophilic reagents as promoters. Since Ferrier *et al* [22] first introduced a mercury salt as a glycosylation promoter of thioglycosides, other thiophilic metal salts such as $Cu(OTf)_2$[23], $Pd(ClO_4)_2$[24] and a combination of $CuBr_2$-Bu_4NBr-AgOTf[25] appeared to give good yields of β-linked derivatives. Ogawa and his co-workers[14] developed the "super electrophilic activator" benzene selenyl triflate (PhSeOTf), from benezene selenyl chloride and silver triflate.

The glycosylation reaction proceeded with remarkable ease and was completed almost instantaneously, even at –35^{0}C. The major products were revealed to be the β-isomers.

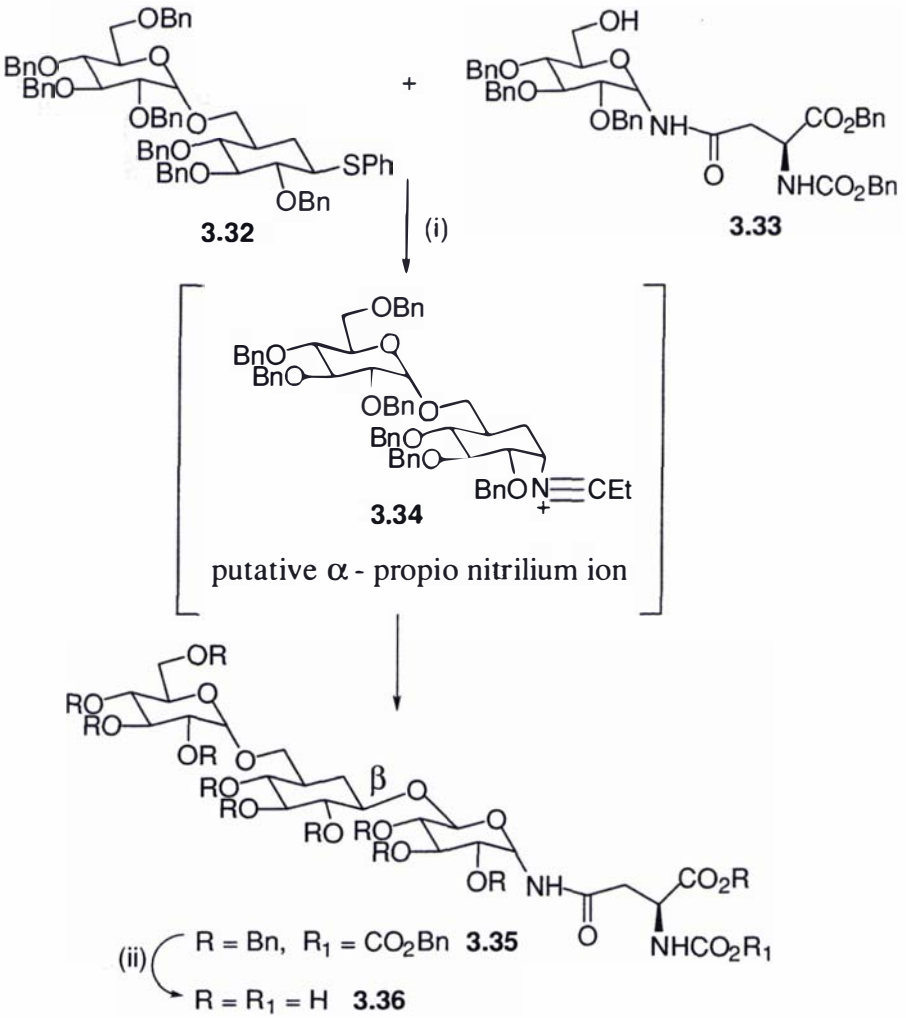


3.28 $R^1 = CO_2Me$, $R^2 = SMe$
3.29 $R^1 = SMe$, $R^2 = CO_2Me$

3.30 **3.31**

Scheme 3.7

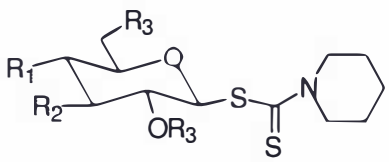
The mild and more practical glycosylation reaction using NBS as a promoter of phenyl thioglycosides having a participating group at C-2 was introduced and applications were demonstrated in the synthesis of tylosine derivatives.[26] Along this line N-iodosuccinimide/ triflic acid assisted activation of thiogycosides also having a participating group at C-2 was found to be a fast and very high yielding β-coupling reaction. Similarly, stereo controlled synthesis of the oligosacharide moiety of nephritogenoside was reported[16] using NBS/TfOH as the promoter. The key to this synthetic strategy was β-selective glycosylation without neighbouring group participation (**Scheme 3.8**).



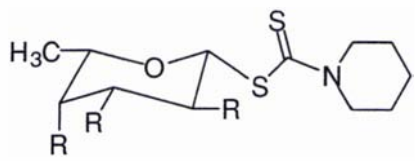
Reagents and Conditions: *(i) NBS, TFOH, CH_3CH_2CN, -78 ^0C (ii) H_2, $Pd(OH)_2/C$, THF, EtOH, H_2O, RT.*

Scheme 3.8

The coupling of **3.33** with 1.5 equivalent of phenyl thioglycoside **3.32** under the influence of NBS-TfOH in propionitrile at -78^0C proceeded smoothly *via* the putative α-propionitrilium ion **3.34** to give trisaccharide **3.35**, which was hydrogenolysed to furnish the final target compound **3.36**. Recently, a new series of glycosyl donors, acylated glycopyranosyl 1-piperidinecarbodithioates **3.37-3.41**[27] (**Figure 3.6**), have been prepared by reacting piperidine with sodium hydride and the resulting salt treated with carbon disulphide and then with an appropriate acylated bromo-sugar.



3.37 $R_1 = R_3 = OAc$, $R_2 = H$;
3.38 $R_1 = H$, $R_2 = R_3 = OAc$;
3.39 $R_1 = R_3 = OH$, $R_2 = H$;
3.40 $R_1 = R_3 = OCOPh$, $R_2 = H$



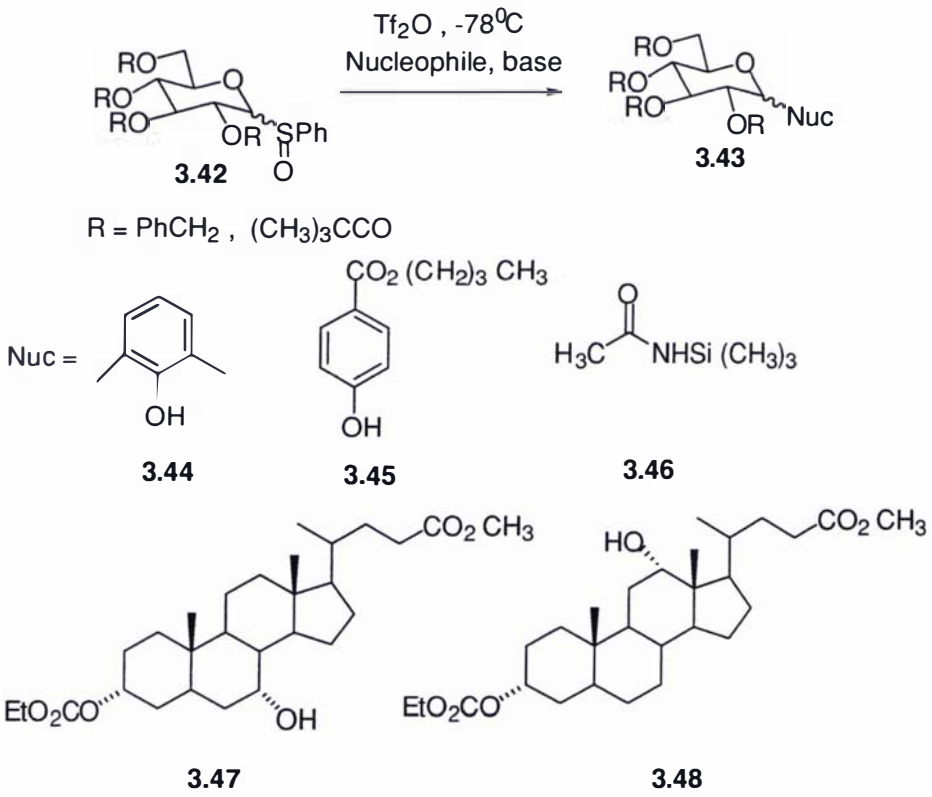
3.41 R = OAc

Figure 3.6

These new glycosyl donors are very stable, crystalline and non hygroscopic compounds which require no special care in storage or handling and are very useful in 1,2-trans-glycosidation reactions.

3.1.4.2 Sulfoxide Sugars As Glycosyl Donors

In spite of the recent developments in thio sugars as glycosyl donors, the application of phenyl sulfenyl glycosyl donors has recently gained a new impetus, also as rather inactive nucleophiles can be effectively glycosylated in the presence of triflic anhydride. The sulfenyl glycosyl donors are in turn readily accessible by oxidation of phenyl thio glycosides.[28] This sulfoxide glycosylation method has attracted attention because of its mild reaction conditions, high reactivity of its activated glycosylating agent, its generally good to excellent stereo control and compatibility with both solution and solid phase glycosylations. For example hindered alcohols and derivatives of phenols which present particular difficulties[29] for glycosylation can be reacted under mild conditions in good yields by using the sulfoxide glycosyl reagent **3.42** as shown in **Scheme 3.9**.[28]

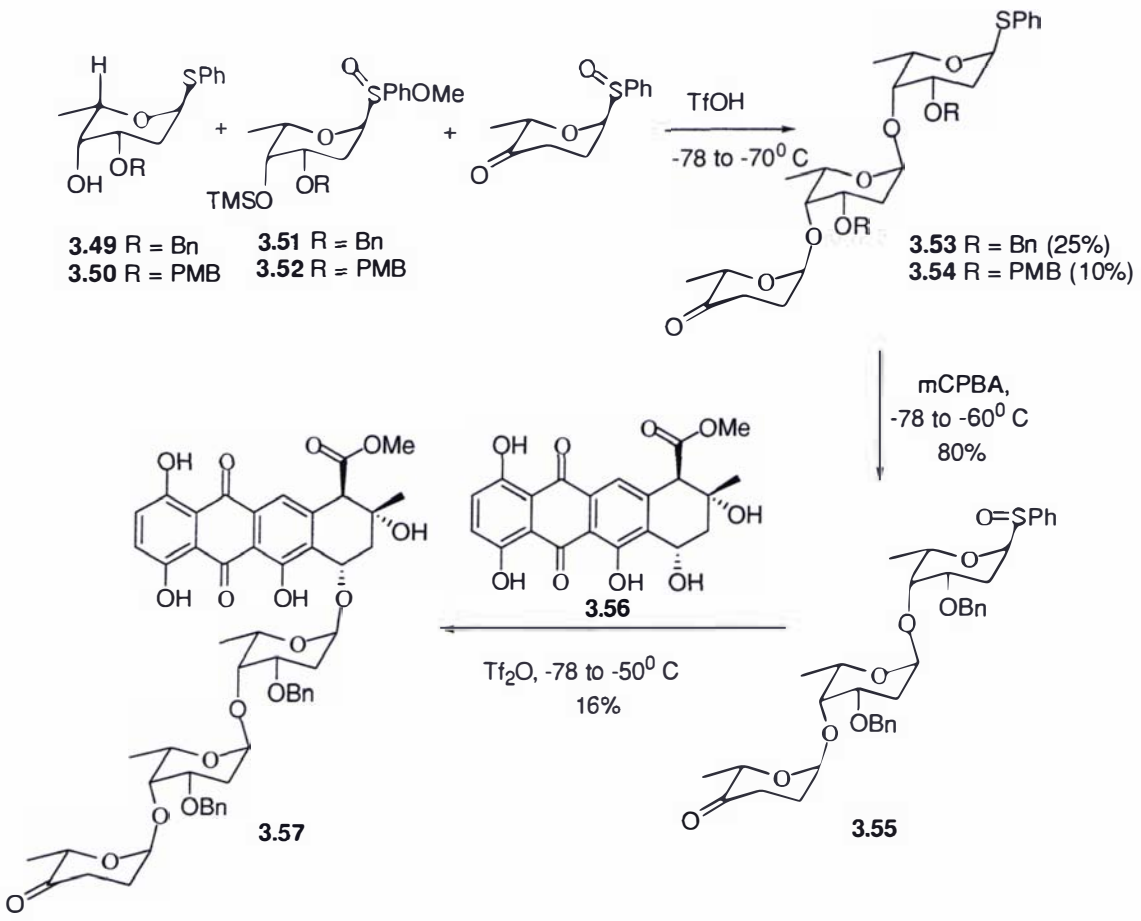


Scheme 3.9

The stereochemical outcome of this reaction in the absence of neighbouring group participation is strongly influenced by the solvent. In general the percentage of the β-glycoside produced increases with solvent polarity. The reaction works well regardless of the electron releasing or electron withdrawing properties of the sugar protecting groups. The effectiveness of this reaction is shown by the glycosylation of the 12-OH group of steroid derivatives such as compound **3.48**, which has strong steric hindrance towards glycosylation and by glycosylation of the unreactive nucleophile **3.45**.

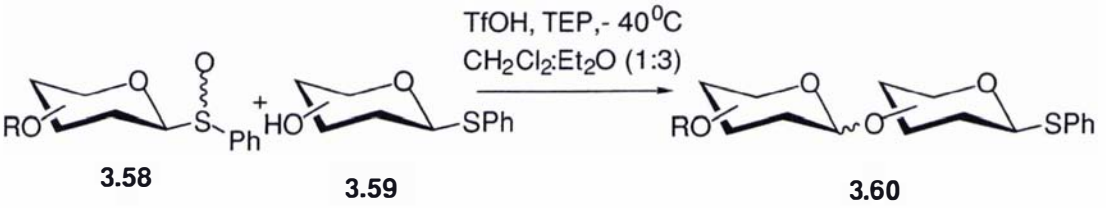
Since sulfoxide glycosyl donors are much more reactive than the thioglycosides the selective glycosidation with a sulfoxide glycosyl donor could be achieved even when both sulfoxide and thio-functional groups are present in the same compound. The most striking example of the effectiveness of this reaction is very recently shown by the stereocontrolled synthesis of the biologically active anthracycline antibiotic ciclamycin 0[30] (**Scheme 3.10**). The trisaccharide was constructed in a single reaction from three monomers by virtue of the different reactivities of thioglycosides and sulfoxide glycosyl

donors. The reactivities of the sulfoxide and nucleophiles were tuned such that the linkage between the A ring and B ring would occur first followed by formation of the linkage to the C ring. This trisaccharide was oxidised to give sulfoxide **3.55** and than coupled with the aglycone ε-pyromycinone to give ciclamycin 0.



Scheme 3.10

Recently Martin –Lomas and colleagues[31] reported that their attempt to use Kahne's methodology in a project aimed at the synthesis of an octasaccharide as a putative second messanger of the hormone failed. They doubted that this system was not effective for sulfinyl glycosides other than for 2-deoxy sugars. They therefore developed a new promoter system for the sulfoxide glycosylation reaction using TEP (triethyl phosphite) as the acid scavanger with TfOH in a catalytic amount in the preparation of several disaccharides in acceptable yields (**Scheme 3.11**).



3.58 3.59 3.60

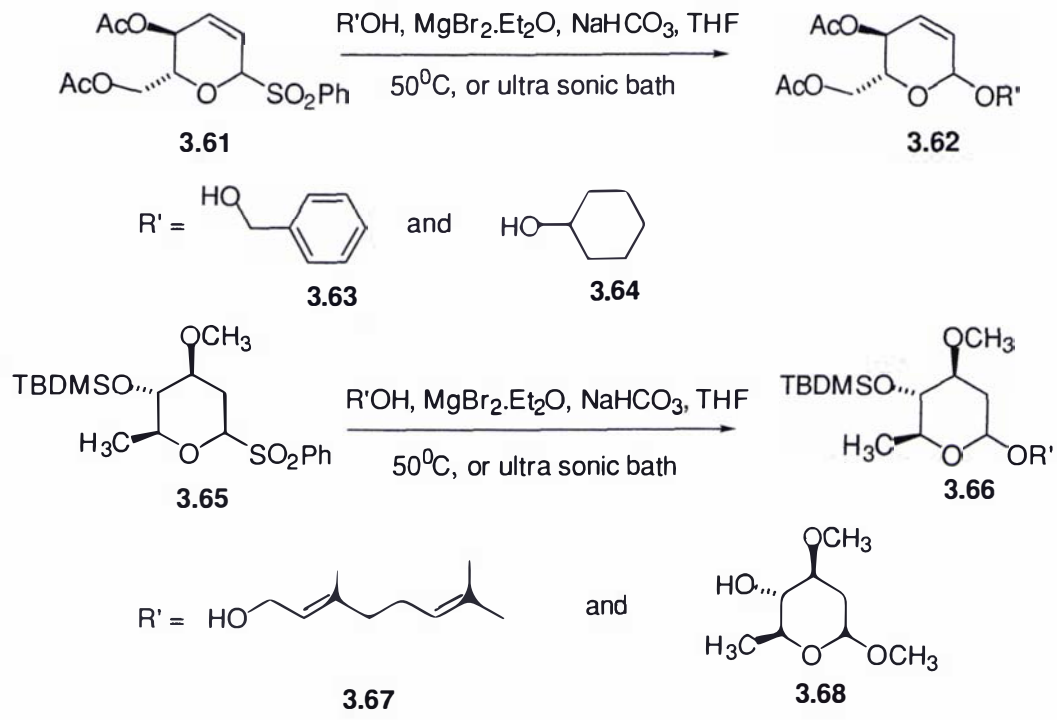
Scheme 3.11

In general, the effectiveness of the glycosylation reaction is not only dependent on the glycosyl donor used, but also and in a large extent on the promoter used to activate it.

3.1.4.3 Sulfone Sugars (Phenyl Sulfonyl Sugars) As Glycosyl Donors

As the demands of modern carbohydrate synthesis constantly require greater functional group compatibility, there is a continuing need to study alternative systems. The exciting results offered by thioglycoside and sulfoxide sugars triggered an interest in using sulfonyl sugars as glycosylating agents.

Preliminary experiments with glycosyl sulfone showed (as shown in **Scheme 3.12**) that glycosylations could be achieved with varying degrees of success.[32,33] This reaction can be carried out in the presence of magnesium bromide etherate ($MgBr_2.Et_2O$) and sodium bicarbonate in THF at room temperature, giving good yields of the coupled product for simple sulfones.[33,34] These mild conditions tolerate a wide variety of functional groups including furans, ketones, esters, silyl ethers, acetals, alkenes and alkynes.

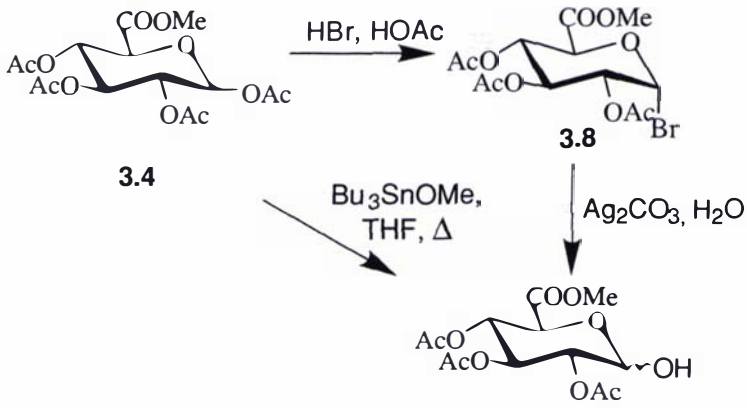


Scheme 3.12

Although most reactions went to completion overnight (by TLC analysis), for slow reactions the mixture could be warmed to 50°C, or better immersed in a small ultrasonic bath, in which case a dramatic rate enhancement was observed. This was the case for most glycosylation reactions. However the literature survey indicated no use of the sulfone sugar in the glycosylation reaction of phenols.

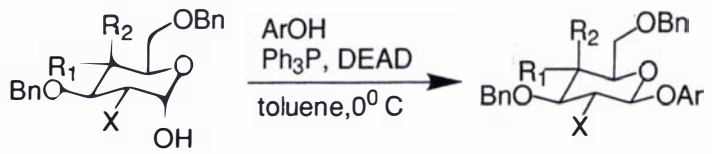
3.1.5 Hydroxy Sugars As Glycosyl Donors In Glycosylation Reactions

The synthesis of glucuronides is most frequently carried out via the Koenigs-Knorr reaction or by one of its modifications. The limited shelf life of the bromo-sugar, the need for an elevated reaction temperature and the ubiquitous formation of hemiacetals (ortho esters) as side products prompted an investigation to be made on the more stable hydroxyl derivatives. As shown in the **Scheme 3.13** either the β-tetra acetate or bromo-sugar are suitable precursors. Treatment of the acetate sugar with Bu_3SnOMe in THF at reflux[35] or silver carbonate hydrolysis of the bromo-sugar gave good yields of the hydroxy sugar.[36]



Scheme 3.13

It was possible to obtain some *O*-glucuronides directly by the Mitsunobu coupling of a 1-hydroxy sugar to the appropriate phenol. For example, with more acidic phenols such as the *p*-Br and *p*-NO_2 derivatives a 40-50% yield of the corresponding conjugate was obtained, but phenols of low acidity, for example *p*-cresol required prior complexation to $Cr(CO)_3$ to obtain useful yields. Significant amounts of the ester and some α-glucuronide were also obtained.[37] So far, the method has been little used in glycosylations and has been tried only when the perester or Koenigs-Knorr couplings failed completely or afforded very poor yields. The Lewis acid catalyst BF_3 and in particular TMSOTf,[37] have been used more often for glycosylation using this reaction rather than other catalysts. Relatively nucleophilic aglycones such as low acidity phenols and primary alcohols yield almost entirely the β-glucuronides. The literature also reveals that the use of the novel catalyst diethyl azo dicarboxylate (DEAD) under Mitsunobu reactions results in an efficient and highly stereo selective method for the synthesis of aryl 2-deoxy-β-D-glycosides as shown in **Scheme 3.14**.[38]



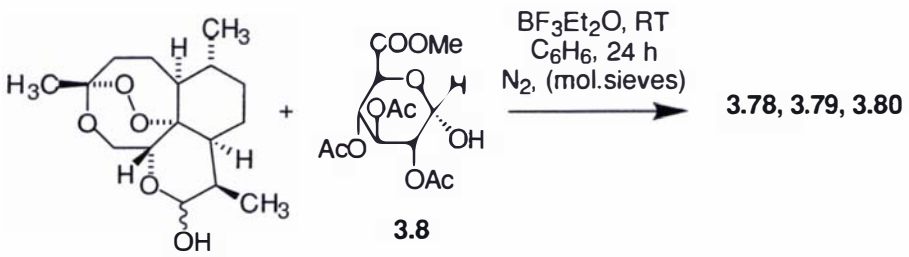
3.69, R_1=H, R_2=OBn, X=SPh
3.70, R_1=H, R_2=OBn, X=SePh
3.71, R_1=OBn, R_2=H, X=SPh
3.72, R_1=OBn, R_2=H, X=SePh

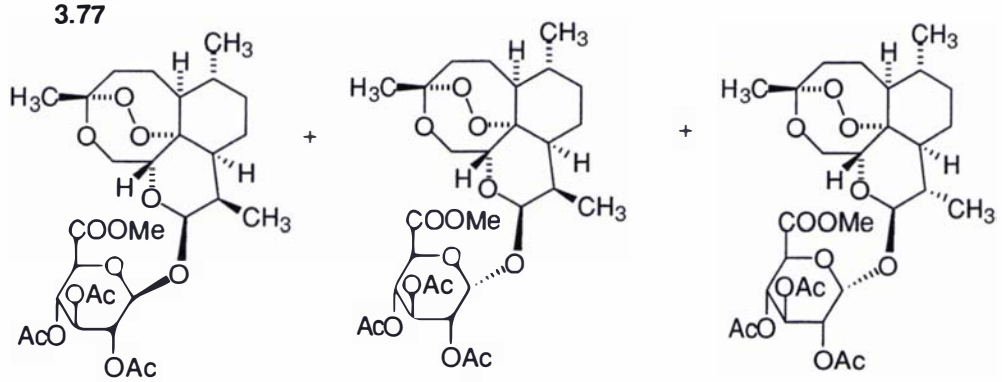
3.73, R_1=H, R_2=OBn, X=SPh
3.74, R_1=H, R_2=OBn, X=SePh
3.75, R_1=OBn, R_2=H, X=SPh
3.76, R_1=OBn, R_2=H, X=SePh

ArOH a, 2 - napthol; b, phenol; c, *o* - cresol

Scheme 3.14

In the anti malarial artemisinnin series the β-glucuronide of β-dihydroartemisinnin (DHART) was obtained only by using a hydroxy sugar in conjugation with $BF_3.Et_2O$ catalysis.[39] Minor amounts of the α-glucuronide and the 11-epimer also resulted.





3.78a α-Glucuronide (tri-OAc ester) β-DHART

3.78b α-Glucuronide (tri-OAc ester) 11-epi- α-DHART

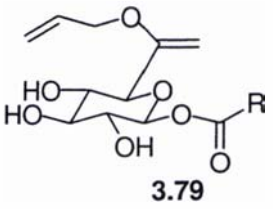
3.78c β-Glucuronide (tri-OAc ester) β-DHART

Scheme 3.15

Here the Koenigs-Knorr procedure had previously afforded only a low yield of the α-glucuronides and couplings with the acetate sugar **3.4** failed completely.

3.1.6 Other Variations Of Glycosyl Donors In Glycosylation Reactions

There has been a considerable growth of interest in the syntheses of *O* -Acyl glucuronides which show very low hydrolytic stability and hence conventionally protected derivatives of this series are liable to be of very limited use. Recently the allyl ester of D-glucuronic acid has been coupled to a range of carboxylic acids using Mitsunobu conditions to afford conjugates.[40] These were later deprotected by Pd^0 in the presence of pyrolidine to afford good yields of the *O* -acyl β-glucuronides.

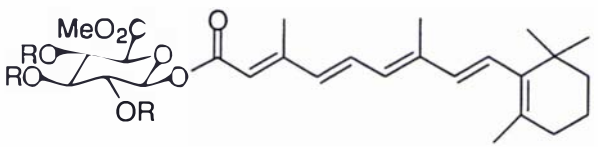


3.79

R = Various

Figure 3.7

The highly biologically active (cell growth control, teratogenicity) retinoyl glucuronides have been prepared in high yield by coupling the siver salt of all-*trans* retinoic acid and the bromo sugar **3.8**.[41] The polyene conjugation of the retinoyl moiety was responsible for improved hydrolytic stability, so that the required methyl ester **3.80b** was obtained using Zemplen deacylation of the conjugate **3.80a**

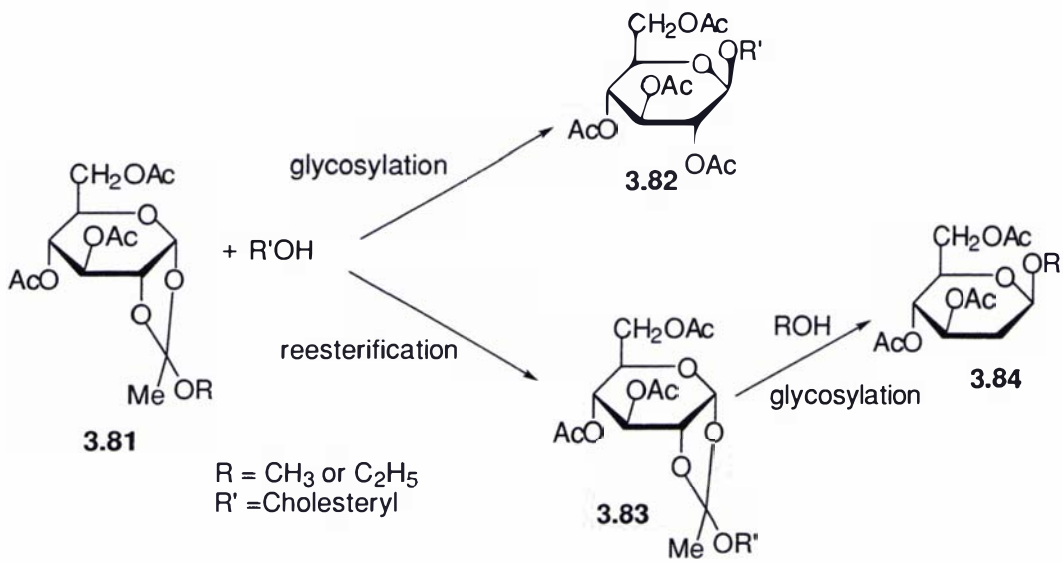


3.80a R = Ac
3.80b R = H

Figure 3.8

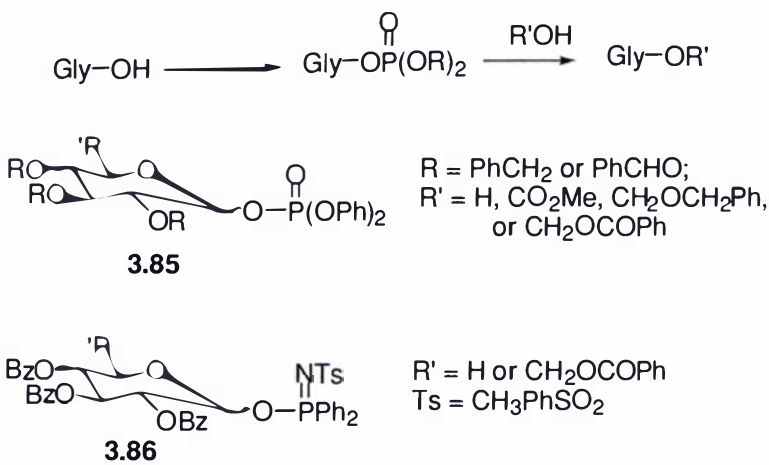
Besides the use of the above glycosyl halides, thio derivatives and hydroxy sugars used as as glycosyl donors, there have been other types of glycosyl donors used in Koenigs-Knorr reactions. Among the known 1,2-*trans*-glycosylations the traditional orthoester methods suffers from the disadvantage that, besides the desired glycoside **3.82** from the direct glycosylation form the starting ortho ester **3.81** a second isomeric

glycoside **3.84** is also obtained by rearrangement (resterification) of the starting orthoester **3.81** to give a new orthoester **3.83** followed by glycosylation (**Scheme 3.16**).[42]



Scheme 3.16

Several glycosyl donors possessing a phosphorous atom in the leaving group at the anomeric center have also been investigated in glycosylation reactions.



Scheme 3.17

Hashimoto *et al* [43] successfully used different glycopyranosyl phosphates **3.85-3.86** as glycosyl donors for glycosylation reactions in the presence of BF_3OEt_2 or TMSOTf as promoter and CH_2Cl_2 as solvent. The reactions gave extremely rapid

glycosylation with high β-selectivity with or without neighbouring group participation. Some steroid compounds such as estrone, formed β-glycoside conjugates in very good yield.

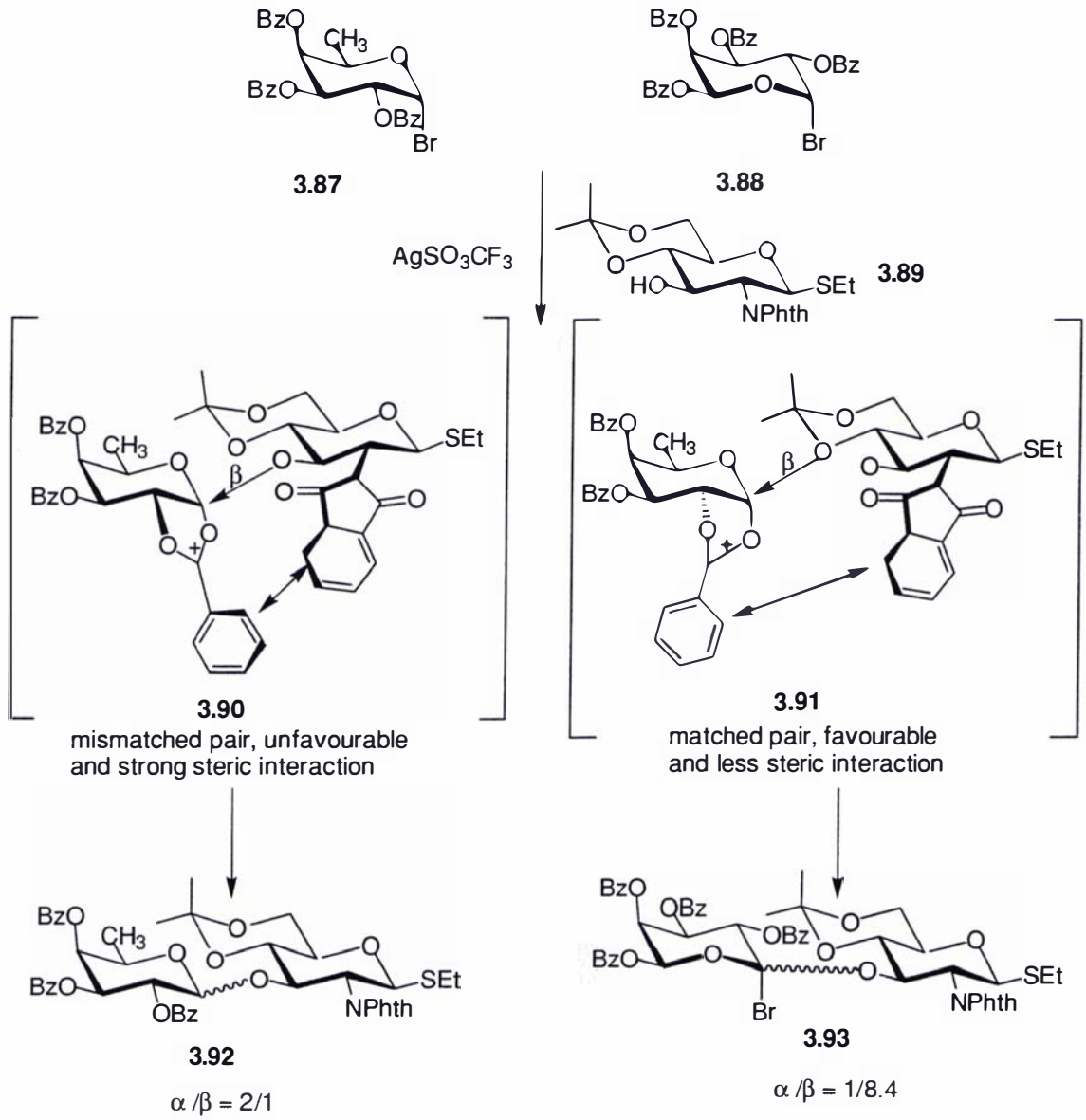
Recently a highly stereocontrolled 1,2-trans-β-glycosidation reaction without neighbouring group participation has been developed by the above authors.[44] They used benzyl-protected glycopyranosyl phosphite as a glycosyl donor and BF_3OEt_2 as a promoter. This method was claimed to exhibit the highest level of 1,2-trans-β-selctivity known to date for glycosidation without neighbouring group participation on C-2.

The trichoroimidate glycosyl donor is another important type of glycosylation protocol which will be discussed in detail in **Chapter 5**. The thermally and chemically stable trichloroimidate glycosyl donor was easily synthesised from the corresponding 1-hydroxy sugar by treatment of trichloroacetonitrile in presence of a base. Up to now, the trichloroimidated glycosylation has been found to have wide applications in the synthesis of natural products.[40]

There have been other types of glycosyl donors reported for the β–glycosydations such as glycosyl 2-pyridinecarboxylate[45] and a 1-*O*-silylated sugar.[46]

3.1.7 Matched-Mismatched Glycosylation Reactions

In general the glycosyl donor possessing an acyloxy group with a participating function at C-2 position exclusively gave the corresponding 1,2 trans glycoside with quite high stereo selectivity. However the stereo selectivity of glycosylation can be changed dramatically by the structure of the glycosyl acceptor. The steric interaction between a glycosyl donor and an acceptor also strongly influences the stereoselectivities of the glycosylation reactions. For example, for the glycosylation of the glycosyl donor 2,3,4-tri-*O*-benzoyl-6-deoxy-α-D-galactopyranosyl bromide **3.87** or its L-isomer **3.88** with the glycosyl acceptor **3.89**, the stereo chemical outcome (α/β) ratio of the two glycosylation reactions were quite different (**scheme 3.18**).[47]



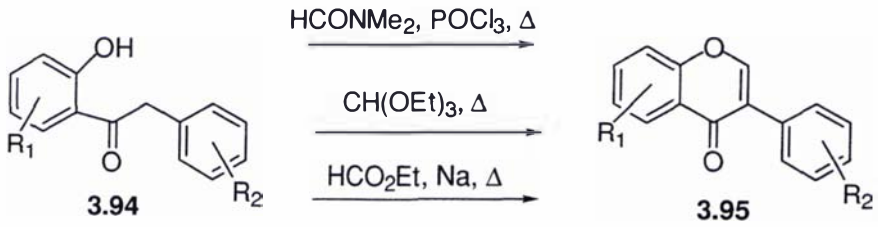
Scheme 3.18

Although a participating group is present at C-2 of the glycosyl donor, which should be expected to give mainly the β-coupling product from the glycosylation according to the Koenigs-Knorr reaction mechanism, the actual glycosylation product **3.92** was predominated by the α-coupling reaction (α/β ratio =2/1). On contemplating this unusual result we can explain the unexpected stereochemical outcome of the coupling reaction between **3.87** and **3.89** in terms of a sterically mismatched-pair for the β-glycoside transition state. During the formation of the β-glycoside, considerable steric hindrance arises between the phthalimido group of **3.89** and the benzoyl ester of (sterically mismatched-pair) **3.90**, but not between the phthalimido group of **3.89** and the benzoyl ester of the L-isomer **3.88** (sterically matched pair, **3.91**). Hence the

glycosylation reaction by the sterically matched-pair **3.88** and **3.89** gave a much higher β-coupling product than that obtained by sterically mis-matched pair **3.87** and **3.89**.

3.1.8 Previous Synthesis Of Isoflavones

In the past, synthetic isoflavonoids have been mainly needed as comparison samples to confirm the new isoflavonoid structures isolated from nature. The classical methods of synthesis of isoflavones require several steps and a need for protection of any free phenolic hydroxyl groups by oxidative transformations of the chalcone intermadiate or by various ring closures of deoxybenzoin. Chalcones are readily obtained by condensation of acetophenones and aromatic aldehydes and are thus more accessible than deoxybenzoins. Earlier methods for synthesis of isoflavones involved the addition of a one carbon unit by a variety of C1 reagents such as phosphorous oxy chloride and DMF,[48,49] triethyl orthoformate[50] or ethyl formate and sodium[51] reagents to benzyl *O*-hydroxy phenyl ketones.

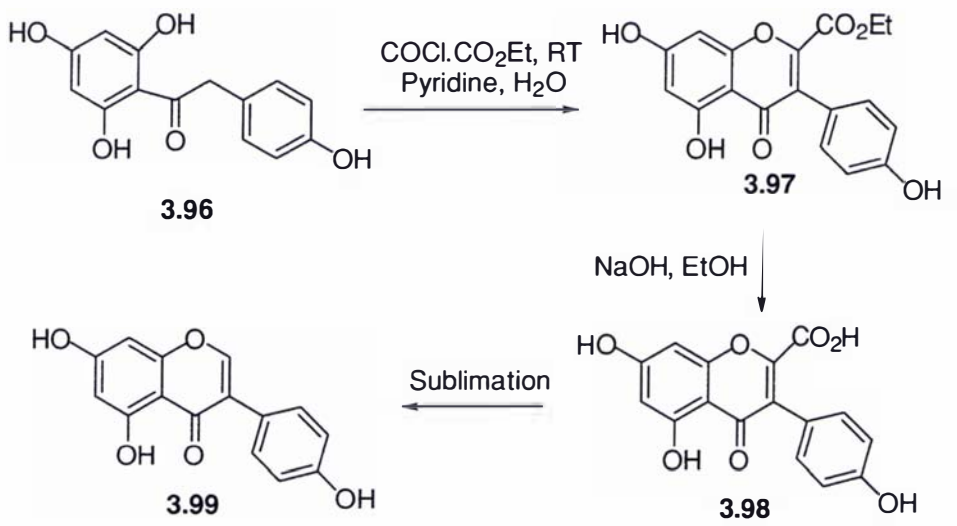


Scheme 3.19

In early applications elevated temperatures were used and the yields of isoflavones were poor but at or below RT improved yields were obtained. The reactions proceed best when all hydroxyl groups in the benyl phenyl ketones **3.94** except that involved in cyclisation were protected, and failed when three or more free hydroxyl groups were present. The protection of functional groups other than the hydroxyl group is necessary also to make the required pyrone ring (ring C).

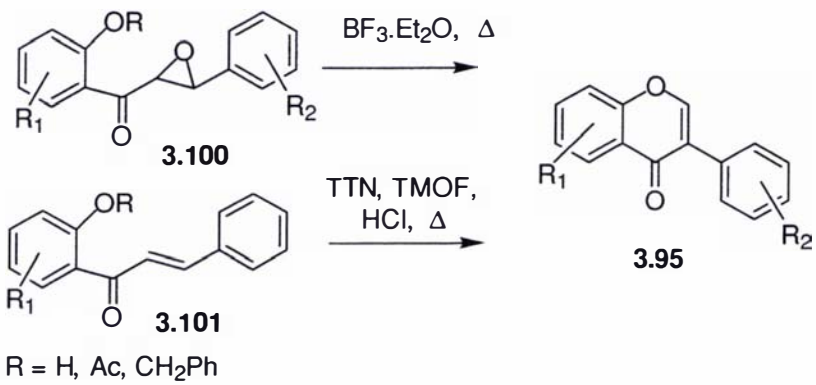
Baker *et al* [52] investigated a new synthesis of isoflavones from the same starting materials which, unlike the above procedures proceeded equally well even in the presence of three or more hydroxyl groups. Thus benzyl *o*-hydroxy phenyl ketones **3.96** containing all n free hydroxyl groups were treated with (n+1) equivalents of

ethoxalyl chloride in pyridine at RT, followed by addition of water. Mild alkaline hydrolysis and subsequent decarboxylation gave the required isoflavone in moderate yield.



Scheme 3.20

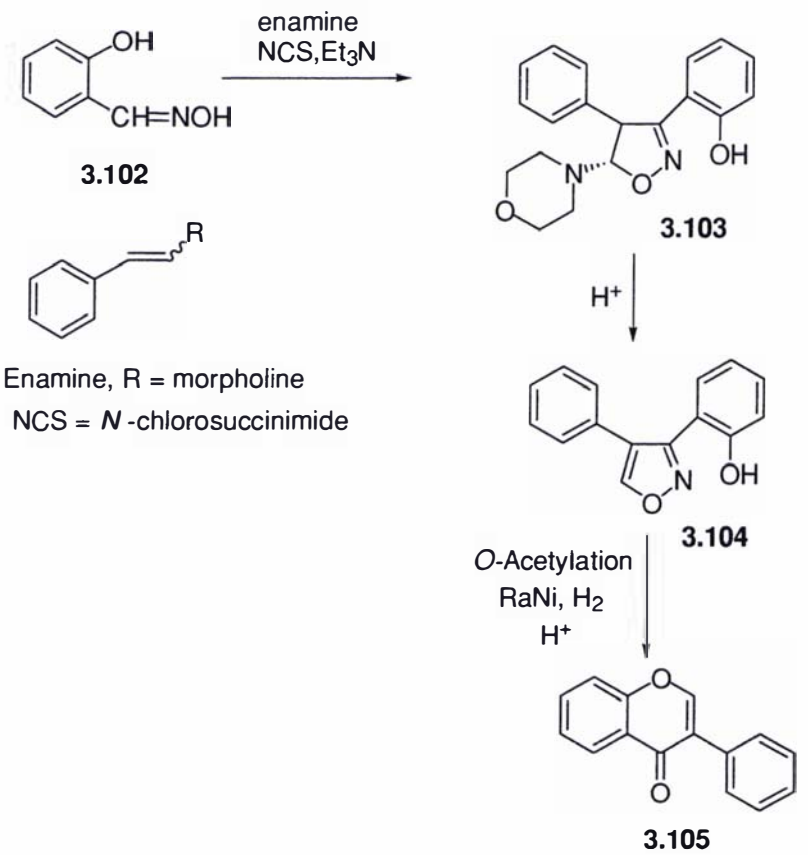
More frequently isoflavones were synthesised by an oxidative rearrangement of chalcones (**Scheme 3.21**). Rearrangement of the chalcone epoxide **3.100** catalyzed by $BF_3.Et_2O$ is still used as a method but product yields tend to be poor.[53] An alternative oxidative chalcone method by thallium nitrate (TTN) in methanol or trimethyl orthoformate has been widely used also.[49,54]



Scheme 3.21

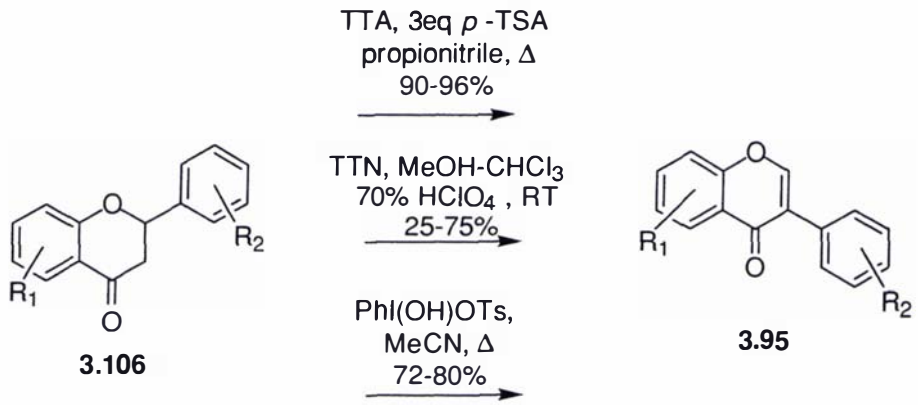
Another new route to isoflavones starts from oxidation products obtained by boiling n-styryl morpholine and salicylaldehyde with CrO_3–pyridine in benzene to give

4-phenyl substituted isoxazoles.[55] The isoxazoles are acetylated, reduced and cyclized in the presence of acid to yield isoflavones. The acylation step is essential as Raney Ni reduction in methanol gives partial over-reduction giving, among other products, isoflavones in low yields of 10%-15%.



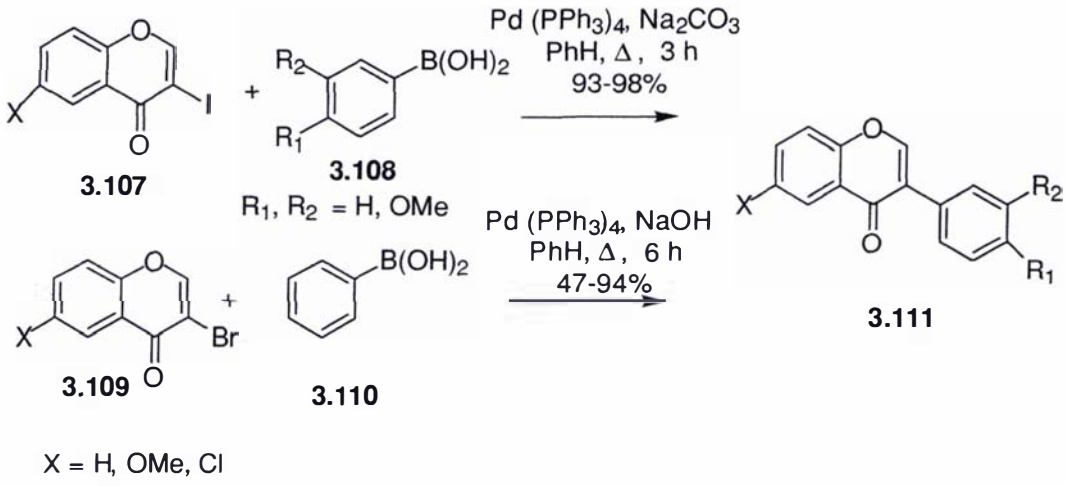
Scheme 3.22

Biomimetic oxidative aryl rearrangement from flavanones using reagents such as thallium(III) acetate, thallium(III) nitrate and thallium(III) *p*-tolyl sulfonate have been developed also.[56--58]



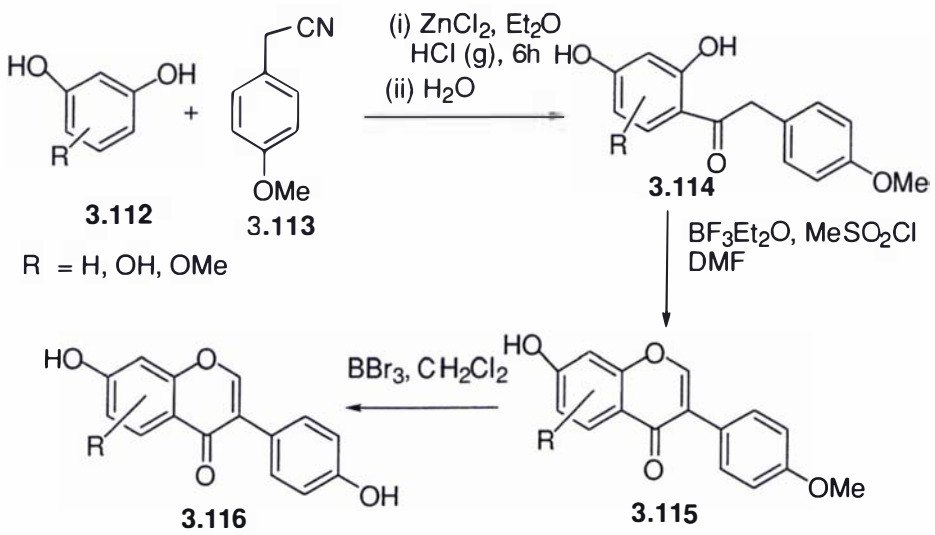
Scheme 3.23

Another new route to isoflavones includes direct arylation on a 3-bromo or 3-iodo chromone frame work by a palladium catalyzed cross coupling reaction of aryl boronic acid or its butyl esters.[59]



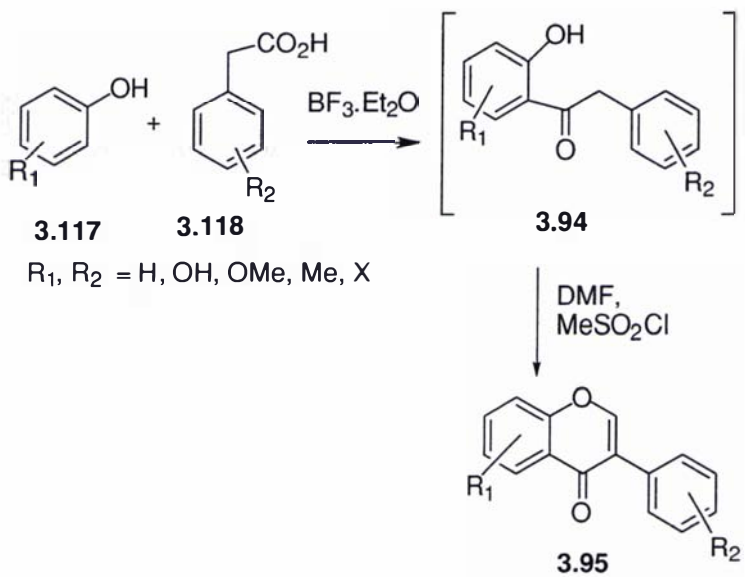
Scheme 3.24

Isoflavones were also prepared from the corresponding methoxy substituted deoxy benzoins, prepared by the Hoesch condensation in 40-50% yield (**Scheme 3.25**).[60] The Bass cyclisation method in which only the unprotected phenolic hydroxy groups are in ring **A** was found to be useful for various hydroxy methoxy substituted isoflavones. These hydroxy methoxy isoflavones were later on deprotected using the selective reagent BBr_3 in CH_2Cl_2.



Scheme 3.25

In 1983 Luk and co-workers reported the preparation of deoxybenzoins using Friedel-Crafts acylation of resorcinol with methoxy substituted phenyl acetic acid in $BF_3.Et_2O$.[64] An application of this method was applied by Hase and Wahala[61] recently in a direct one pot synthesis of polyhydroxy isoflavones from appropriately substituted phenols and phenyl acetic acids without resorting to any of the often employed protection-deprotection sequences.



Scheme 3.26

3.1.9 Aims Of This Investigation

It is clear from the forgoing review that the literature reveals numerous glycosyl donors used in the effective stereoselective *O*-glycosylation synthesis for various phenols. The literature also revealed several possible pathways for the synthesis of phytoestrogens, isoflavones and isoflavanes, but the use of various glycosyl donors for the glycosylation of the various phytoestrogens is relatively new. The aims of the work described in this chapter were two fold:

(i) Reinvestigate the simple, convenient and effective synthesis of phytoestrogens isoflavones and isoflavanes for their use in effective stereoselective *O*-glycosylation reactions.

(ii) To investigate the stereoselective *O*-glycosylation of various activated and deactivated phenols as model studies into the stereoselective glucuronidation of phytoestrogen metabolites (isoflavones and isoflavans).

3.2 EXPERIMENTAL

3.2.1 General Experimental Details

See **Section 1.2.1** for more **General Experimental Details**.

Additionally the molecular sieves (4 A^0) were activated at 450°C and stored in an oven at 100°C before used in the reactions. All the NMR spectra were recorded in $CDCl_3$ except for the isoflavones which were recorded in DMSO-d_6. Thin layer chromatography (TLC) plates for molecules with sugar moieties were visualised by UV lamp (254 nm) and spraying with 10% concentrated H_2SO_4 in EtOH and heating at 100°C for 2 minutes.

3.2.2 Preparation Of Glucuronide Ester Derivatives As Glycosyl Donors

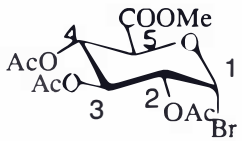
3.2.2.1 Methyl tetra-*O*-acetyl glucopyranuronate (3.4)

3.4

The title compound was prepared from glucuronolactone **3.4a** according to the procedure described by Bollenback *et al* [62], except that Na was used in the reaction instead of NaOMe. Glucuronolactone **3.4a** (17.62 g) was added over 0.5 h to the above solution with vigorous stirring and the reaction mixture was stirred for a further 0.5 h. Methanol was then removed under reduced pressure and the resulting yellow syrup was dissolved in acetic anhydride (68 ml). Perchloric acid (70%, 0.3 ml) in acetic anhydride (10 ml) was then added over 15 mins to the above solution at 20-25°C. Care was taken to keep the temperature between 20°C and 30°C. The reaction mixture was allowed to stand at RT overnight following which another aliquot of $HClO_4$ (0.1 ml) was added. The reaction mixture was stirred for 0.5 h and then allowed to cool at 5°C overnight to obtain white crystals. The crystals were washed with ether and then recrystallised in hot EtOH. The product **3.4** was obtained in 50% yield as pure white needles, mp, 176-

177^{0}C, (Lit. mp, 176.5-178^{0}C)[218]; ^{1}H NMR δ/ppm 5.77 (1H, d, J = 7.69 Hz, H1), 5.12-5.16 (3H, m, H2-H4), 4.18 (1H, d, J = 9.16 Hz, H5), 3.75 (3H, s, -CO_2CH_3), 2.12 (3H, s, -OC(O) CH_3), 2.05 (6H, s, 2 x -OC(O) CH_3), 2.04 (3H, s, -OC(O) CH_3).

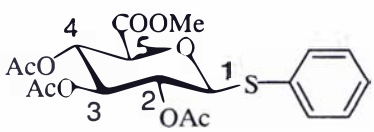
3.2.2.2 Methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate (3.8)



3.8

This was prepared according to the method of Bollenback *et al.*[62] Methyl tetra-*O*-acetyl glucopyranuronate **3.4** (5.02 g, 13.3 mmol) was dissolved in HBr (45% in HOAc, 20 ml). The solution was stirred at RT in the dark for 15 mins and allowed to stand below 5^{0}C overnight. The excess HBr and HOAc were removed under reduced pressure below 35^{0}C to get a thick yellow syrup. The resulting syrup was diluted with $CHCl_3$ (25 ml) and washed with aqueous saturated $NaHCO_3$, H_2O, brine and then dried over Na_2SO_4. The solvent was removed under reduced pressure to afford an oil, which became crystalline on addition of absolute EtOH (15 ml). The crude solid was recrystalised from absolute EtOH to give the pure product methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate **3.8** as colourless crystals (4.23 g, 80%). Mp, 106-107^{0}C, (Lit. mp, 106-107^{0}C)[62]; ^{1}H NMR δ/ppm 6.65 (1H, d, J = 4.03 Hz, H1), 5.62 (1H, t, H4), 5.24 (1H, t, H3), 4.85 (1H, dd, J = 4.17, 3.96 Hz, H2), 4.58 (1H, d, J = 10.25 Hz, H5), 3.77 (3H, s, -$COOCH_3$), 2.11 (3H, s, -OC(O) CH_3), 2.06 (3H, s, -OC(O) CH_3), 2.05 (3H, s, -OC(O) CH_3).

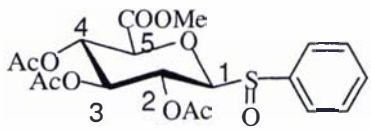
3.2.2.3 Methyl 1-thiophenyl-1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate (3.119)



3.119

The title compound was prepared according to following procedure.[63] Methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate **3.8** (300 mg, 0.765 mmol) and tetrabutyl ammonium hydrogen sulfate (Bu_4NHSO_4, 260 mg) were dissolved in EtOAc (3 ml) and the solution was stirred vigorously at RT. After addition of Na_2CO_3 (1 M, 3 ml) and thiophenol (PhSH, 15 drops, excess), the solution was continuously stirred for another 30 min until the TLC showed the completion of the reaction (Hex/EtOAc 1:1, R_f = 0.55). The reaction mixture was diluted with EtOAc (50 ml) and the organic phase was washed with Na_2CO_3 (1 M, 30 ml x 3), water (30 ml x 2), brine (30 ml) and dried over $MgSO_4$ overnight. The EtOAc was removed under reduced pressure to afford an oil, which was precipitated by adding EtOH (20 ml). The crude solid was recrystallised twice from EtOH to give pure product methyl 1-thiophenyl -1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate **3.119** as white crystals (308 mg, 95% yield). mp, 117-118^{0}C; ^{1}H NMR δ/ppm 7.33-7.53 (5H, m, phenyl), 4.97-5.28 (3H, m, H2-H4), 4.74 (1H, d, J = 10.1 Hz H1), 4.04 (1H, d, J = 9.7 Hz H5), 3.77 (3H, s, -$COOCH_3$), 2.09 (3H, s, -OC(O) CH_3), 2.02 (3H, s, -OC(O) CH_3), 2.00 (3H, s, -OC(O) CH_3).

3.2.2.4 Methyl 1-phenylsulfenyl-1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate (3.120)



3.120

Methyl 1-thiophenyl-1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate **3.119** (0.5 g, 1.17 mmol) was disolved in CH_2Cl_2 (170 ml) and was cooled to 5^{0}C. A solution of the magnesium salt of m-perphthalic acid (MMPP) (335 mg, 0.67 mmol) in distilled water (170 ml) was added slowly. The reaction mixture was stirred gently for 7 hours until TLC showed completion of the reaction (Hex/EtOAc 1:1, R_f= 0.33). The reaction mixture was washed with saturated aqueous $NaHCO_3$ (50 ml x 3), water, (50 ml x 2), brine (30 ml) and dried over Na_2SO_4 for several hours. The solvent was removed under reduced pressure to afford a white solid, which was purified by short column chromatography on neutral Al_2O_3 with an eluting solvent of Hex/EtOAc (1:1) to give the desired methyl 1-phenylsulfenyl-1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyran-

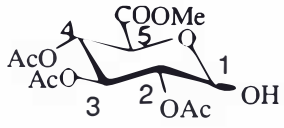
uronate as a white amorphous solid (397 mg, 79% yield). Mp, 140-142^{0}C. Found: (M-Ac$^+$) 399.0732, $C_{17}H_{19}O_9S$ requires 399.0749. ^{1}H NMR δ/ppm 7.58-7.97 (5H, m, phenyl), 4.91-5.33 (3H, m, H2-H4), 4.54 (1H, d, J = 9.7 Hz, H1), 4.04 (1H, d, J = 9.9 Hz, H5), 3.72 (3H, s, -COOCH$_3$), 2.14 (3H, s, -OC(O) CH$_3$), 2.00 (3H, s, -OC(O) CH$_3$), 1.99 (3H, s, -OC(O) CH$_3$).

3.2.2.5 Methyl 1-phenylsulfonyl-1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate (3.121)

3.121

Methyl 1-phenylthio-1-deoxy-2,3,4-tri-*O*-acetyl-β-D-glucopyranuronate **3.119** (0.5 g, 1.17 mmol) was dissolved in EtOH (30 ml) and was cooled to 5^{0}C. A solution of the magnesium salt of m-perphthalic acid (MMPP) (283 mg, 0.57 mmol) in distilled water (35 ml) was added slowly. The reaction mixture was stirred vigorously for 4 hours until TLC showed completion of the reaction (Hex/ EtOAc 1:1, R_f= 0.22). The reaction mixture was washed with saturated aqueous $NaHCO_3$ (30 ml x 3), water (30 ml x 2), brine (30 ml) and dried over Na_2SO_4 for several hours. The solvent was removed under reduced pressure to afford a white solid; (475 mg, 88 % yield). Mp, 153-154^{0}C. Found: MH$^+$ 459.0889, $C_{19}H_{23}O_{11}S$ requires 459.0882, 591 (M+ Cs)$^+$ requires 590.99328. ^{1}H NMR δ/ppm 7.61-7.97 (5H, m, phenyl), 4.99-5.43 (3H, m, H2-H4), 4.58 (1H, d, J = 9.9 Hz, H1), 4.14 (1H, d, J = 9.9 Hz, H5), 3.72 (3H, s, -COOCH$_3$), 2.14 (3H, s, -OC(O) CH$_3$), 2.01 (3H, s, -OC(O) CH$_3$), 2.00 (3H, s, -OC(O) CH$_3$).

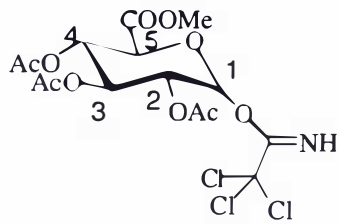
3.2.2.6 Methyl 1-hydroxy-2,3,4-tri–*O*–acetyl–α,β –D-glucopyranuronate (3.69)



3.69

To methyl 2, 3, 4-tri-*O*-acetyl-1-bromo-α-D-glucopyranuronate **3.8** (1.23 g, 3.1 mmol) in acetone (7.37 ml), H_2O (0.5 ml) was added freshly prepared Ag_2CO_3 (0.85 g) and the mixture was gently stirred at RT for several hours. Further additions of H_2O were made until there were no signs of further reaction. The reaction was monitored by TLC and after completion (6 h) the reaction mixture was filtered through a short celite pad and washed with warm acetone. The filtrate and washings were combined and the solvent was removed under reduced pressure to give a syrup which solidified on standing. The crude solid was recrystallised from EtOH to give the desired methyl 1-hydroxy-2, 3, 4-tri-*O*-acetyl- D-glucopyranuronate **3.69** (R_f = 0.6, 1:1; EtOAc/Hexane) as white crystals (0.89 g, 86%). Mp, 95-101^{0}C, (Lit. mp, 91-92^{0}C).[36]

3.2.2.7 Methyl 2,3,4-tri-*O*–acetyl-1-*O*-(trichloroacetamidoyl)-α-D-glucopyranuronate (3.116)



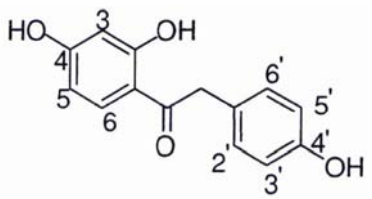
3.116

The title compound was prepared according to the procedure of Brown *et al.*[65] A solution of the 1-hydroxysugar **3.116** (0.84 g, 2.5 mmol) in anhydrous CH_2Cl_2 (12.0 ml) and trichloroacetonitrile (1.8 ml) was stirred with K_2CO_3 (0.19 g, 1.37 mmol) over 4A^0 molecular sieves at RT. The reaction mixture was stirred until no starting material was detectable (14 hrs, TLC). The reaction mixture was then filtered through a short silica gel pad and eluted first with Et_2O and then with Hex/EtOAc (1:1). Appropriate fractions were pooled and the solvent was removed under reduced pressure to give a yellow gum. This was recrystallised in Hex/EtOAc to give the desired methyl 2,3,4-tri-*O*-acetyl-1-*O*-(trichloroacetamidoyl)-α -D-glucopyranuronate as white crystals (1.01 g, 85%, R_f =0.51, 1:1, Hex:EtOAc). Mp, 108-109^{0}C, (Lit. mp, 108^{0}C).[65] ^{1}H NMR δ/ppm 8.73 (1H, br s, =NH), 6.62 (1H, d, *J* = 3.52 Hz, H1), 5.62 (1H, t, H4), 5.22-5.30 (1H, m, H3), 5.12-5.18 (1H, dd, H2), 4.48 (1H, d, *J* = 10.3 Hz, H5), 3.74 (3H, s, -CO_2CH_3), 2.045 (3H, s, -OC(O) CH_3), 2.025 (3H, s, -OC(O) CH_3), 2.011 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 169.5, 169.5, 169.2 (3 x –O*C* (O) CH_3), 166.9 (-*C* O_2CH_3), 160.3 (-

C(O)CCl_3), 92.5 (C1), 70.4(C3), 69.4 (C5), 69.0 (C2), 68.8 (C4), 53.0 (-CO_2CH_3), 20.7, 20.5, 20.4 (3 x -OC(O) *C* H_3).

3.2.3 Synthesis Of Deoxy Benzoins, Isoflavones and Isoflavans

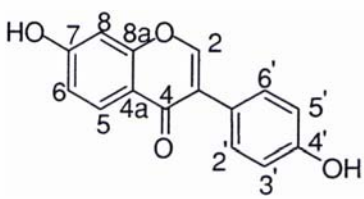
3.2.3.1 2, 4, 4' - Triydroxy deoxybenzoin[61] (3.122)



3.122

Resorcinol (5.46 g, 0.05 mol) and 4-hydroxy phenyl acetic acid (7.57 g, 0.05 mol) were dissolved in freshly distilled $BF_3.Et_2O$ (50 ml) under an atmosphere of Ar. The reaction mixture was stirred and heated at 75^0-80^{0}C for 1.5 h. The solid canary yellow precipitate separated out from the reaction mixture. The reaction mixture was then cooled at RT, filtered, washed with ice cold water and dried to obtain 2,4,4' trihydroxy deoxybenzoin as a canary yellow solid (7.09 g, 60%, R_f = 0.6, 7:2 CH_2Cl_2/EtOAc). The filtrate was again heated to 75^0-80^{0}C for 1 h to obtain a further crop of 2,4,4' trihydroxy deoxybenzoin (3.13 g, 26.1%). The combined solid was recrystallised from aqueous EtOH to give 2,4,4' trihydroxy deoxybenzoin **3.122** as off white crystals (9.23 g. 77.8%); mp, 186-187^{0}C. ^{1}H NMR δ/ppm 8.09 (1H, d, *J* = 8.79 Hz, H6), 7.23 (2H, d, *J* = 8.35 Hz, H2', H6'), 6.85 (2H, d, *J* = 8.35 Hz, H3', H5'), 6.54 (1H, dd, *J* = 8.79, 2.2 Hz, H5), 6.40 (1H, d, *J* = 2.2 Hz, H3), 4.29 (2H, s, -CH_2).

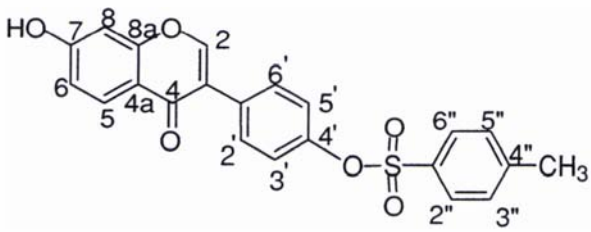
3.2.3.2 Daidzein (3.123)



3.123

2,4,4'-Trihydroxy deoxybenzoin **3.122** (2.0 g, 8.19 mmol) and $ZnBr_2$ (1.88 g, 8.35 mmol) were dissolved into dry DMF (24 ml) and the mixture was heated to 60^0-70^0C under Ar for 1 h. A solution of $MeSO_2Cl$ (4.8 ml) in dry DMF (4.0 ml) was added slowly over 15 mins and the reaction mixture was stirred at 80^0C for 3 h. The reaction mixture was cooled at RT and poured into a large volume of ice cold water. The crude yellow product (R_f = 0.53, 7:2 CH_2Cl_2/EtOAc) was filtered, washed with $CHCl_3$ and recrystallised from aqueous MeOH to give fine white crystals (1.84 g 89%). Mp, 212-214^{0}C, (Lit. mp, 213.5-214.5^{0}C).[61]. ^{1}H NMR δ/ppm 8.32 (1H, s, H2), 7.98 (1H, d, *J* = 8.57 Hz, H5), 7.42 (2H, d, *J* = 8.35 Hz, H2', H6'), 6.96 (1H, dd, *J* = 8.57, 2.98 Hz, H6), 6.89 (1H, d, *J* = 2.98 Hz, H8), 6.84 (2H, d, *J* = 8.35 Hz, H3', H5'). ^{13}C NMR δ/ppm 175.6 (C4), 163.3 (C4'), 158.2 (C8a), 158.0 (C7), 153.7 (C2), 130.9 (C2', C6'), 128.1 (C5), 124.3 (C3), 123.4 (C1'), 117.5 (C4a), 116.0 (C6), 115.8 (C3', C5'), 102.9 (C8).

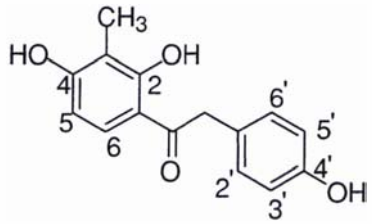
3.2.3.3 4'-Tosyl daidzein (3.124)



3.124

Daidzein **3.123** (0.71 g, 2.79 mmol) and K_2CO_3 (2.70 g) were stirred in dry acetone (70 ml) at RT under an atmosphere of Ar. *p*-Toluene sulfonyl chloride (0.64 g, 3.35 mmol) was added and the reaction mixture was heated to reflux for 2 h. The reaction mixture was filtered and the solid was washed with dry acetone (25 ml). The solvent was removed under reduced pressure to 25 ml and acidified with CH_3COOH (0.1 N) to give the desired 7-tosyl daidzein as fine white crystals (R_f = 0.61, 0.91 g, 80%); mp, 218-220^{0}C. Found, M^+, 407.0585; $C_{22}H_{15}O_6S$ requires 407.0589. ^{1}H NMR δ/ppm 8.10 (1H, d, *J* = 9.0 Hz, H5), 7.88 (1H, s, H2), 7.65 (2H, d, *J* = 8.79 Hz, H2", H6"), 7.38 (2H, d, *J* = 8.35 Hz, H2', H6'), 7.22 – 7.29 (2H, m, H3", H5"), 7.16 (1H, d, *J* = 1.98 Hz, H8), 6.96 (2H, d, *J* = 8.35 Hz, H3', H5'), 6.84 (1H, dd, *J* = 5.37, 2.42 Hz, H6). IR (KBr disc) ϑ_{max} 3449, 3095, 1651, 1507, 1442, 1350, 1195, 1162, 951, 852, 824.

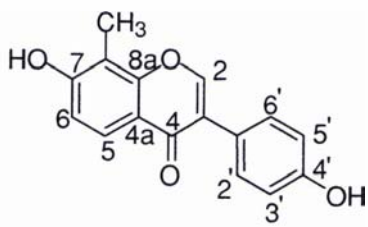
3.2.3.4 2, 4, 4'-Trihydroxy-3-methyl deoxybenzoin (3.125)



3.125

The title compound **3.125** (2.68 g, 87 %, R_f = 0.58, 7:2 CH_2Cl_2/EtOAc) was prepared from 2-methyl resorcinol (1.5 g, 12.5 mmol) and 4-hydroxy phenyl acetic acid (1.90 g, 12.5 mmol) by a procedure similar to that used in the preparation of 2, 4, 4'-trihydroxy deoxybenzoin **3.122**. mp, 192-194^{0}C, (Lit. mp, 187-188^{0}C).[61] ^{1}H NMR δ/ppm 8.09 (1H, d, *J* = 8.80 Hz, H6), 7.34 (2H, d, *J* = 8.35 Hz, H2', H6'), 6.85 (2H, d, *J* = 8.35 Hz, H3', H5'), 6.74 (1H, dd, *J* = 8.80, 2.2 Hz, H5), 4.30 (2H, s, -CH_2), 2.26 (3H, s, -CH_3). ^{13}C NMR δ/ppm 203.6 (-C(O)R), 164.8 (C2), 163.4 (C4), 158.0 (C4'), 132.3 (C2', C6'), 130.0 (C6), 126.3 (C1'), 113.6 (C3', C5'), 112.0 (C1), 108.4 (C3), 102.8 (C5), 8.9 (-CH_3), 44.6 (-CH_2).

3.2.3.5 4', 7-Dihydroxy-8-methylisoflavone (3.126)

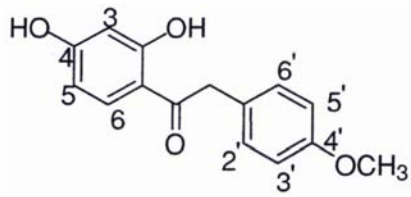


3.126

The title compound 4', 7-dihydroxy-8-methylisoflavone (1.79 g, 86%, R_f = 0.51, 7:2 CH_2Cl_2/EtOAc) was prepared by a procedure similar to that used in the preparation of daidzein by cyclization of 2, 4, 4'-trihydroxy-3-methyldeoxybenzoin (2.0 g, 7.81 mmol) in 2.5 h.; mp, 232-235^{0}C. (Lit. mp, 236-237^{0}C)[61] Found, M^+, 268.0734; $C_{16}H_{12}O_4$ requires 268.0735. ^{1}H NMR δ/ppm 8.34 (1H, s, H2), 7.99 (1H, d, *J* = 8.58 Hz, H5), 7.42 (2H, d, *J* = 8.57 Hz, H2', H6'), 6.95 (1H, d, *J* = 8.58 Hz, H6), 6.85 (2H, d, *J* = 8.36 Hz, H3', H5'). ^{13}C NMR δ/ppm 175.8 (C4), 160.6 (C7), 157.9 (C8a), 156.2 (C4'), 153.6

(C2), 130.8 (C2', C6'), 124.6 (C5), 123.8 (C1'), 123.4 (C3), 117.5 (C4a), 115.7 (C3', C5'), 114.6 (C6), 111.6 (C8), 21.2 (-CH_3).

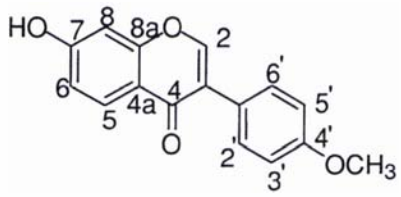
3.2.3.6 2, 4-Dihydroxy-4'-methoxydeoxybenzoin (3.127)



3.127

Resorcinol (5.30 g, 0.05 mol) and 4-methoxy phenyl acetic acid (8.0 g, 0.05 mol) were dissolved in freshly distilled $BF_3.Et_2O$ (50 ml) under an atmosphere of Ar. The reaction mixture was stirred and heated at 70^0-80^0C for 2.5 h. The homogeneous reaction mixture was cooled to RT and poured into ice cold aqueous NaOAc (60 g/ 500 ml). The aqueous suspension was extracted with CH_2Cl_2 (4 x 200 ml) and the combined CH_2Cl_2 layer was washed with $NaHCO_3$, and dried over $MgSO_4$. The solvent was removed under reduced pressure to give crude 2,4-dihydroxy-4'-methoxy deoxybenzoin as a yellow solid (R_f = 0.58, 7:2 CH_2Cl_2/EtOAc). The crude yellow solid was recrystallised from aq. EtOH to give 2,4, dihydroxy-4'-methoxy deoxybenzoin as off-white crystals (10.1 g. 82%); mp, 157-158 ^{0}C. ^{1}H NMR δ/ppm 7.65 (1H, d, J = 9.01 Hz, H6), 7.08 (2H, d, J = 8.57 Hz, H2', H6'), 6.76 (2H, d, J = 8.57 Hz, H3', H5'), 6.35 (1H, dd, J = 8.6, 2.7 Hz, H5), 6.40 (1H, d, J = 2.4 Hz, H3), 4.04 (2H, s, -CH_2). 3.69 (3H, s, -OCH_3). ^{13}C NMR δ/ppm 201.6 (-C(O)R), 165.0 (C2), 164.8(C4), 158.0 (C4'), 132.3 (C1), 130.0 (C2', C6'), 126.3 (C5), 113.6 (C3', C5'), 111.9 (C1'), 108.2 (C6), 102.8 (C3), 54.9 (-OCH_3), 43.4 (-CH_2).

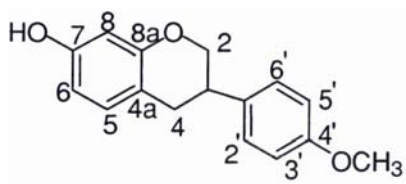
3.2.3.7 Formononetin (3.128)



3.128

The title compound formononetin (1.67 g, 80%, R_f = 0.49, 7:2 CH_2Cl_2/EtOAc) was prepared by a procedure similar to that used in the preparation of daidzein by cyclization of 2,4-dihydroxy-4'-methoxy deoxybenzoin (2.0 g, 7.81 mmol) in 2.5 h. Mp, 258-260^{0}C, (Lit. mp, 257^{0}C).[64]. ^{1}H NMR δ/ppm 8.29 (1H, s, H2), 7.97 (1H, d, *J* = 9.7 Hz, H5), 7.49 (2H, d, *J* = 9.7 Hz, H2', H6'), 6.98 (1H, d, *J* = 9.7 Hz, H6), 6.87 (1H, d, *J* = 2.3 Hz, H8), 3.76 (3H, s, -OCH_3). ^{13}C NMR δ/ppm 179.4 (C4), 166.8 (C4'), 163.1 (C8a), 161.7 (C7), 157.4 (C2), 134.3 (C2', C6'), 131.5 (C5), 128.4 (C3), 127.5 (C1'), 120.7 (C4a), 119.6 (C6), 117.9 (C3', C5'), 106.4 (C8), 59.8 (-OCH_3).

3.2.3.8 (±) 4'-Methoxy equol (7-Hydroxy, 4'-methoxyisoflavan) (3.129)

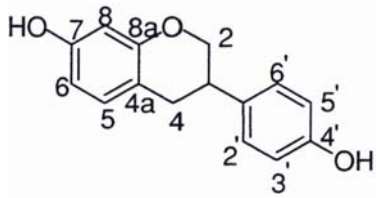


3.129

Formononetin **3.128** (0.67 g, 2.5 mmol) was hydrogenated over 10% Pd-C (130 mg) in glacial acetic acid (125 ml) at RT and atmospheric pressure under a stream of hydrogen overnight (16 h). The catalyst was then removed by filtration on a bed of celite and washed with MeOH. The solvent was removed under reduced pressure and the residue recrystallized from $CHCl_3$-Hexane to give fine white crystals of the desired 7-hydroxy, 4'-methoxyisoflavan **3.129** (0.41 g, 64%, R_f = 0.63, 7:2 CH_2Cl_2/EtOAc). Mp, 163-164^{0}C (Lit. mp, 158-160^{0}C).[64] m/z (EI, 70 eV) (256.1104, M^+, 44%), 134 (100%), 121 (15), 91 (9). ^{1}H NMR δ/ppm 9.64 (1H, s, -OH), 7.67 (2H, d, *J* = 8.79 Hz, H3', H5'), 7.33 (1H,

d, J = 7.91, H5), 7.31 (2H, d, J = 8.57 Hz, H2', H6'), 6.74 (1H, dd, J = 8.13 Hz, J = 2.42 Hz, H6), 6.69 (1H, d, J = 2.41 Hz, H8), 4.54-4.67 (1H, m, H4), 4.29-4.47 (1H, m, H4), 4.16 (3H, s, -OCH_3), 3.52-3.56 (1H, m, H3), 3.15-3.38 (2H, m, H2). ^{13}C NMR δ/ppm 158.8 (C4'), 158.7, (C8a), 157.3 (C7), 134.2 (C1'), 130.8 (C5), 129.2 (C2', C6'), 114.7 (C3', C5'), 113.2 (C4a), 108.8 (C6), 103.3 (C8), 71.0 (C4), 55.9 (-OCH_3), 38.0 (C3), 32.2 (C2).

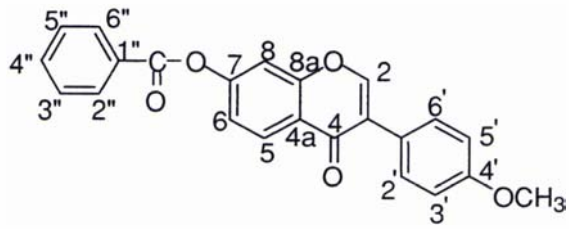
3.2.3.9 (±) Equol (4', 7-Dihydroxyisoflavan) (3.155)



3.155

The title compound 4', 7-dihydroxy isoflavan (374 mg, 52%) **3.155** was prepared by hydrogenation of daidzein (500 mg, 1.96 mmol) **3.123** according to the method described above for methoxy equol **3.129**. Mp, 182-185^{0}C (Lit. mp, 189-190^{0}C).[64] ^{1}H NMR δ/ppm 9.08 (1H, s, -OH), 8.95 (1H, s, -OH), 6.98 (2H, d, J = 8.51 Hz, H2', H6'), 6.76 (1H, d, J = 7.91, H5), 6.65 (2H, d, J = 8.47 Hz, H3', H5'), 6.21 (1H, dd, J = 8.21 Hz, J = 2.38 Hz, H6), 6.13 (1H, d, J = 2.34 Hz, H8), 4.07-4.11 (1H, m, H4), 3.79-3.83 (1H, m, H4), 2.91-2.98 (1H, m, H3), 2.69-2.78 (2H, m, H2). ^{13}C NMR δ/ppm 157.3 (C4'), 157.2 (C8a), 156.9 (C7), 132.2 (C1'), 130.6 (C5), 128.9 (C2', C6'), 116.0 (C3', C5'), 113.2 (C4a), 108.8 (C6), 103.3 (C8), 71.2 (C4), 38.1 (C3), 32.2 (C2).

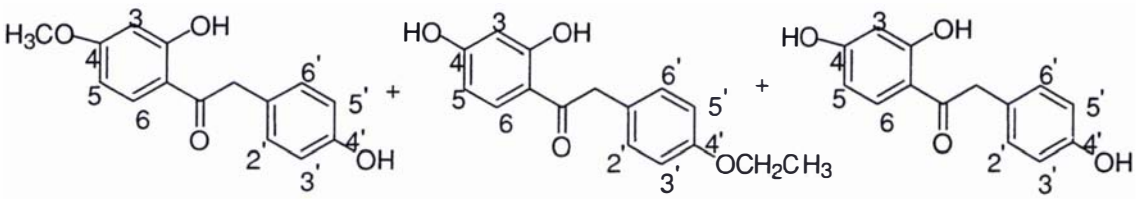
3.2.3.10 Formononetin benzoate (3.130)



3.130

To a stirred solution of formononetin (0.2 g, 0.74 mmol) in pyridine (0.68 ml) at RT was added benzoyl chloride (1.2 ml). The reaction mixture was heated to reflux for 0.5 h. The completion of the reaction was monitored by TLC. ^{1}H NMR δ/ppm 8.29 (1H, s, H2), 7.98 (1H, d, *J* = 8.8 Hz, H5), 7.94 (2H, d, *J* = 7.03 Hz, H2", H6"), 7.51-7.58, 2H, m, H3", H5"), 7.38-7.45 (1H, m, H4"), 7.31 (2H, d, *J* = 8.78 Hz, H2', H6'), 7.26 (1H, d, *J* = 2.2 Hz, H8), 7.24 (1H, d, *J* = 2.2 Hz, H6), 6.77 (2H, d, *J* = 8.78 Hz, H3', H5'), 3.62 (3H, s, -OCH_3). ^{13}C NMR δ/ppm 175.4 (C4), 170 2 (-CO(R)), 164.8 (C4'), 159.9 (C8a), 156.9 (C7), 130.9 (C2', C6'), 130.8 (C3", C5"), 129.9 (C2", C6"), 114.5 (C3', C5'), 129.2, 127.8, 124.6, 122.6, 121.0 (8 x aryl C), 112.6 (C8), 56.1 (-OCH_3). IR (KBr disc) (ν_{max} 3455, 3088, 2829, 1736, 1639, 1515, 1444, 1233, 1178, 697.

3.2.3.11 a) 2,4'-Dihydroxy-4-methoxydeoxybenzoin (3.131), b) 2,4-Dihydroxy-4'-ethoxydeoxybenzoin (3.132) and c) 2, 4, 4'-Trihydroxydeoxybenzoin (3.122)



3.131 **3.132** **3.122**

m-Methoxy phenol (3.1 g, 25 mmol) and 4-hydroxy phenyl acetic acid (3.8 g, 25 mmol) were dissolved in freshly distilled $BF_3.Et_2O$ (25 ml) under an atmosphere of Ar. The reaction mixture was stirred and heated at 70^0-80^{0}C for 2.5 h. The homogeneous reaction mixture was cooled to RT and poured into ice cold aq. NaOAc (60 g/ 500 ml). The aqueous suspension was extracted with CH_2Cl_2 (4 x 200 ml) and the combined CH_2Cl_2 layer was washed with $NaHCO_3$, and dried over $MgSO_4$. The solvent was removed under reduced pressure and the residual yellow solid was purified by column chromatography with an eluting solvent EtOAc:CH_2Cl_2 (1:7) and then EtOAc: CH_2Cl_2 (2:7). A small amount of by-product 1-(2-hydroxy-4-methoxy phenyl)-2(-4 hydroxy phenyl) ethanone **3.132** (0.18 g, 3%, R_f = 0.56) mp, 97-98^{0}C was followed by the desired 4 methoxy 2, 4', dihydroxy deoxybenzoin **3.131** (R_f = 0.53, 3.05 g, 47%) mp, 156-157 ^{0}C, then a second by-product 2, 4, 4'-trihydroxydeoxybenzoin **3.122** (R_f = 0.47, 1.45 g, 21%); mp; 75-77^{0}C.

a) 2,4-Dihydroxy-4'-ethoxydeoxybenzoin (3.132)

^{1}H NMR δ/ppm 12.8 (1H, s, -OH), 7.75 (1H, d, J = 8.87 Hz, H6), 7.18 (2H, d, 8.85 Hz, H2', H6'), 6.88 (2H, d, J = 8.85 Hz, H3', H5'), 6.42-6.53 (2H, m, H3, H5), 4.15 (2H, s, -CH_2), 4.01 (2H, q, J = 6.1 Hz, -OCH_2 CH_3), 3.80 (3H, s, -OCH_3), 1.37 (3H, t, J = 6.1 Hz, -OCH_2 CH_3). ^{13}C NMR δ/ppm 202.1 (-C(O)R), 165.9 (C2), 165.7(C4), 157.3 (C4'), 131.9 (C1), 130.2 (C2', C6'), 126.0 (C5), 114.6 (C3', C5'), 111.4 (C1'), 107.7 (C6), 100.9 (C3), 63.4 (-OC H_2CH_3), 55.5 (-OCH_3), 44.0 (-CH_2), 14.9 (-OCH_2C H_3).

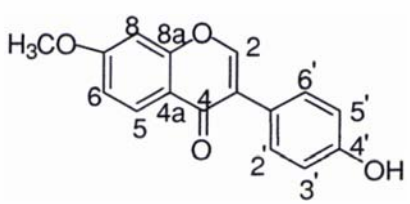
b) 2, 4'-Dihydroxy-4–methoxy-deoxybenzoin (3.131)

^{1}H NMR δ/ppm 7.75 (1H, d, J = 8.35 Hz, H6), 7.14 (2H, dd, J = 8.57, 2.2 Hz, H2', H6'), 6.81 (2H, dd, J = 8.79, 2.4 Hz, H3', H5'), 6.47 (1H, d, J = 1.98 Hz, H5), 6.43 (1H, d, J = 2.0 Hz, H3), 4.16 (2H, s, -CH_2), 3.84 (3H, s, -OCH_3). ^{13}C NMR δ/ppm 202.5 (-C(O)R), 166.0 (C2), 165.7(C4), 154.4 (C4'), 131.9 (C1), 130.4 (C2', C6'), 126.4 (C5), 115.5 (C3', C5'), 114.0 (C1'), 107.8 (C6), 100.9 (C3), 55.6 (-OCH_3), 43.9 (-CH_2).

c) 2, 4, 4'-Trihydroxydeoxybenzoin (3.122)

^{1}H NMR δ/ppm ($CDCl_3$+DMSO-d_6) 7.64 (1H, d, J = 8.45 Hz, H6), 6. 85 (2H, d, J = 8.35 Hz, H2', H6'), 6.58 (2H, d, J = 8.77 Hz, H3', H5'), 6.10-6.23 (2H, m, H5, H3), 3.92 (2H, s, -CH_2). ^{13}C NMR δ/ppm 202.7 (-C(O)R), 165.3 (C2), 164.8(C4), 155.9 (C4'), 132.4 (C1), 130.0 (C2', C6'), 124.9 (C5), 115.5 (C3', C5'), 112.1 (C1'), 108.2 (C6), 103.0 (C3), 43.7 (-CH_2).

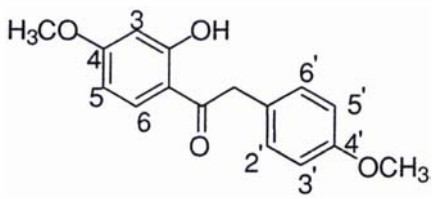
3.2.3.12 4'-Hydroxy-7-methoxyisoflavone (3.133)



3.133

The title compound (1.54 g, 74%, R_f = 0.46, 7:2 CH_2Cl_2/EtOAc) was prepared by a procedure similar to a that used in the preparation of daidzein by cyclization of 4-methoxy 2, 4'-dihydroxy deoxybenzoin (2.0 g, 7.81 mmol) in 2.5 h. Mp, 222-225^{0}C. (Lit. mp, 218-220^{0}C).[70] ^{1}H NMR δ/ppm 8.32 (1H, s, H2), 7.95 (1H, d, J = 9 .0 Hz, H5), 7.48 (2H, d, J = 8.4 Hz, H2', H6'), 6.97 (1H, s, H8), 6.86 (2H, d, J = 8.7 Hz, H3', H5'), 6.92 (1H, dd, J = 8.7, 2.2, H6), 3.76 (3H, s, -OCH_3). ^{13}C NMR δ/ppm 175.4 (C4), 164.9 (C4'), 158.2 (C8a), 158.0 (C7), 153.9 (C2), 130.8, (C2', C6'), 127.7 (C5), 124.5 (C3), 123.1 (C1'), 118.4 (C4a), 115.8 (C3', C5'), 115.5 (C6), 101.3 (C8), 57.0 (-OCH_3).

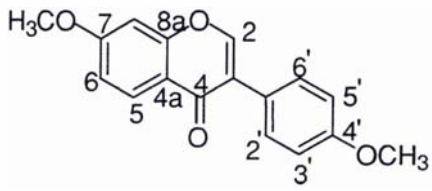
3.2.3.13 2-Hydroxy-4, 4'-dimethoxydeoxybenzoin (3.134)



3.134

The title compound (2.78 g, 82 % R_f = 0.49, 1:2 Hex/EtOAc) was prepared from 3-methoxy phenol (1.5 g, 12.5 mmol) and 4-methoxy phenyl acetic acid (2.08 g, 12.5 mmol) by a procedure similar to that used in the preparation of 4-methoxy 2, 4' dihydroxy deoxybenzoin in 3 h. The product was purified using column chromatography by eluting with the solvent CH_2Cl_2:EtOAc (7:2). ^{1}H NMR δ/ppm 7.82 (1H, d, J = 8.8 Hz, H6), 7.22 (2H, d, J = 8.4, H2', H6'), 6.86 (2H, d, J = 8.4 Hz, H3', H5'), 6.34-6.54 (2H, m, H5, H3), 4.18 (2H, s, -CH_2), 3.85 (3H, s, -OCH_3), 3.78 (3H, s, -OCH_3). ^{13}C NMR δ/ppm 202.1 (-C(O)R), 166.0 (C2), 165.7(C4), 158.5 (C4'), 131.9 (C1), 130.4 (C2', C6'), 126.2 (C5), 114.1 (C3', C5'), 113.0 (C1'), 107.7 (C6), 101.0 (C3), 55.6, 55.3 (2 x -OCH_3), 44.0 (-CH_2).

3.2.3.14 4', 7-Dimethoxyisoflavone (3.135)

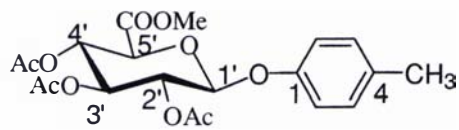


3.135

The title compound (1.57 g, 76%, R_f = 0.49, 7:2 CH_2Cl_2/EtOAc) was prepared by a procedure similar to that used in the preparation of daidzein by cyclization of 2-hydroxy 4, 4'-dimethoxydeoxybenzoin (2.0 g, 7.35 mmol) in 2.5 h. Mp; 166-168^{0}C. (Lit. mp, 162-164^{0}C).[70] Found, M^+, 282.0888; $C_{22}H_{15}O_6S$ requires 282.0892. ^{1}H NMR δ/ppm 8.42 (1H, s, H2), 8.04 (1H, d, *J* = 8.79 Hz, H5), 7.54 (2H, d, *J* = 8.57 Hz, H2', H6'), 7.16 (1H, d, *J* = 1.98 Hz, H8), 7.10 (1H, dd, *J* = 8.79, 2.2, H6), 7.00 (2H, d, *J* = 8.57 Hz, H3', H5'), 3.92, 3.80 (2 x 3H, 2 x s, 2 x -OCH_3). ^{13}C NMR δ/ppm 175.3 (C4), 164.4 (C4'), 159.8 (C8a), 158.2 (C7), 154.1 (C2), 130.8, (C2', C6'), 127.7 (C5), 124.9 (C5), 124.2 (C3), 118.4 (C1'), 115.5 (C4a), 114.4 (C3', C5'), 101.4 (C8), 3 (C8), 58.1, 57.0 (2 x -OCH_3).

3.2.4 Synthesis Of Aryl *O* -β-D-Glucuronide Esters

3.2.4.1 Methyl (*p*-tolyl)-2',3',4'-tri-*O*-acetyl-β-D-glucopyranosiduronate (3.136)

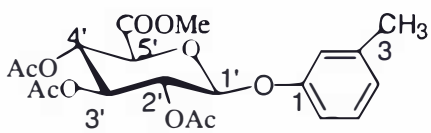


3.136

A typical experimental procedure for the coupling reaction of *p*-cresol with methyl tetra-*O*-acetyl glucopyranuronate was as follows: To a solution of *p*-cresol (0.25 g, 2.31 mmol) in CH_2Cl_2 (12 ml) and molecular sieves (4A^0, activated) was added methyl tetra-*O*-acetyl glucopyranuronate **3.4** (1.74 g, 4.62 mmol) and $BF_3.OEt_2$ (0.33 ml, 2.31 mmol). The resulting reaction mixture was protected from moisture and stirred at 25-30^{0}C for 12 hours. The reaction mixture was diluted with CH_2Cl_2 (50 ml) and washed

with aqueous KOH (2N, 25 ml x 3), water (25 ml x 2), brine (25 ml) and dried over $MgSO_4/Na_2SO_4$. The solvent was removed under reduced pressure to afford a crude pale yellow solid. The crude product was purified by column chromatography eluting with Hex/EtOAc (2:1) and then Hex/EtOAc (1:1) to give a white solid. This was recrystallised using propan-2-ol to give colourless needles of the desired methyl (*p*-tolyl - 2', 3', 4'- tri-*O*-acetyl-β-D-glucopyranosiduronate **3.136** (794 mg, 81%, R_f= 0.22, 1:1 Hex/EtOAc). The coupling reaction was also performed using a 2:1 mole ratio of *p*-cresol (0.11 g, 1.04 mmol) and methyl tetra-*O*-acetyl glucopyranuronate (0.19 g, 0.50 mmol) in presence of $BF_3.OEt_2$ (0.07 ml, 0.49 mmol). The crude product (without flash chromatography) was recrystallised in propan- 2-ol and the pure product (292 mg, 59%) was obtained as white crystals. Mp, 132-133^{0}C, (Lit. mp, 137-138^{0}C)[62]. Found: MH^+, 425.1447; $C_{20}H_{25}O_{10}$ requires 425.1443. ^{1}H NMR δ/ppm 6.88-7.11 (4H, m, phenyl), 5.11-5.35 (3H, m, H2'-H4'), 5.05 (1H, d, *J* = 7.25 Hz, H1'), 4.13-4.17 (1H, m, H5'), 3.74 (-CO_2CH_3), 2.30 (3H, s, -CH_3Ar) 2.06 (3H, s, -OC(O) CH_3), 2.05 (3H, s, -OC(O) CH_3), 2.04 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 169.9, 169.1, 169.0 (3 x –O*C* (O) CH_3), 166.72 (-*C* O_2CH_3), 154.5, 132.9, 129.9, 117.0 (4 x aryl C), 99.5 (C1'), 72.6(C3'), 71.9 (C5'), 71.0 (C2'), 69.1(C4'), 52.9 (-CO_2C H_3), 20.7 (-CH_3(Ar)), 20.6 (3 x -OC(O) *C* H_3). IR (KBr disc) ϑ_{max} 3006, 2957, 1760, 1458, 1444, 1374, 1231, 1091, 1042, 908 and 891 cm^{-1}.

3.2.4.2 Methyl (*m*-tolyl)-2',3',4'-tri-*O*-acetyl-β-D-glucopyranosiduronate (3.137)



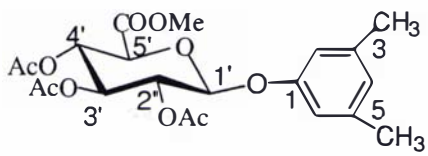
3.137

The coupling reaction was performed by a procedure similar to that used in the preparation of **3.136** except that a 2:1 mole ratio of *m*-cresol (0.25 g, 2.31mmol) and acetate sugar **3.4** (0.435 g, 1.15 mmol) was used. The product **3.137** (203 mg, 41%, R_f = 0.23, 1:1 Hex/EtOAc) was obtained as white needles from propan-2-ol. Mp, 113^{0}C (sharp), (Lit. mp, 113-114^{0}C)[62] Found: MH^+, 425.1447; $C_{20}H_{25}O_{10}$ requires 425.1443. ^{1}H NMR δ/ppm 7.13-7.25 (1H, m, H2), 6.75-6.94 (3H, m, H4-H6), 5.23-5.41 (3H, m, H2'-H4'), 5.13 (1H, d, *J* = 6.98 Hz, H1'), 4.11-4.16 (1H, m, H5'), 3.74 (-CO_2CH_3), 2.33

(3H, s, -CH_3 Ar) 2.06 (3H, s, -OC(O) CH_3), 2.05 (3H, s, -OC(O) CH_3), 2.04 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 169.9, 169.1, 169.0 (3 x –*O*C (O) CH_3), 166.7 (-*C* O_2CH_3), 156.5, 139.6, 129.2, 124.1, 117.7,113.7 (6 x aryl C), 99.1 (C1'), 72.6(C3'), 71.8 (C5'), 71.0 (C2'), 69.1(C4'), 52.9 (-CO_2*C* H_3), 21.4 (-CH_3 Ar), 20.6 (2 x -OC(O) *C* H_3). IR (KBr disc) ϑ_{max} 2957, 1761, 1458, 1444, 1374, 1231, 1091, 1041, 908 and 891 cm^{-1}.

The coupling reaction of *m*-cresol (0.25 g, 2.31mmol) and bromo sugar **3.8** (1.10 g, 2.77 mmol) was also performed using the standard Koenigs-Knorr method as described in **5.2.1.1** to obtain the desired product **3.137** (267 mg, 54%) as white solid.

3.2.4.3 Methyl (3, 5-dimethyl phenyl)-2', 3', 4'-tri-*O*-acetyl-β-D-glucopyranosiduronate (3.138)

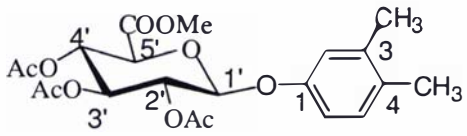


3.138

The coupling reaction was performed by a procedure similar to that used in the preparation of **3.136** using 3,5-xylenol (0.2 g, 1.64 mmol) and acetate sugar **3.4** (0.3 g, 0.82 mmol). The product (166 mg, 47%, R_f = 0.46, 1.5:1 Hex/EtOAc) was obtained as a white crystalline substance from propan-2-ol; mp, 169-170^{0}C, Found: MH^+ 439.1603; $C_{21}H_{26}O_{10}$ requires 439.1605. ^{1}H NMR δ/ppm 6.36-6.91 (3H, m, phenyl), 5.22-5.42 (3H, m, H2'-H4'), 5.12 (1H, d, *J* = 7.25 Hz, H1'), 4.15-4.22 (1H, m, H5'), 3.74 (-C O_2CH_3), 2.29 (6H, s, 2 x -CH_3 Ar) 2.067, (3H, s, -OC(O) CH_3), 2.058 (3H, s, 3 x -OC(O) CH_3), 2.053 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 169.9, 169.1, 169.0 (3 x –*O*C (O) CH_3), 166.7 (-*C* O_2CH_3), 156.5, 139.3, 125.0, 114.5 (4 x aryl C), 99.0 (C1'), 72.6(C3'), 71.8 (C5'), 71.1 (C2'), 69.1(C4'), 52.9 (-CO_2*C* H_3), 21.4 (2 x -CH_3 Ar), 20.6 (2 x -OC(O) *C* H_3), 20.5 (-OC(O) *C* H_3). IR (KBr disc) ϑ_{max} 2953, 1759, 1742, 1500, 1445, 1378, 1233, 1097, 1050, and 897 cm^{-1}.

The coupling reaction of 3,5-xylenol (0.2 g, 1.64 mmol) and bromo sugar **3.8** (0.780 g, 1.96 mmol) was also performed using the standard Koenigs-Knorr method as described in **5.2.1.1** to obtain the desired product **3.138** (162 mg, 46%) as a white solid.

3.2.4.4 Methyl (3,4-dimethyl phenyl)-2', 3', 4'-tri-*O*-acetyl-β-D-glucopyranosiduronate 3.139

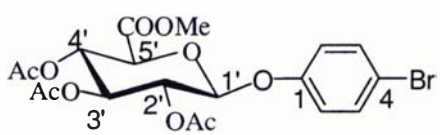


3.139

The title compound was prepared from the coupling reaction of 3,4 xylenol (0.2 g, 1.64 mmol) and the acetate sugar **3.4** (0.3 g, 0.82 mmol) according to the method described above for the preparation of methyl (3, 5-dimethyl)-2', 3', 4'-tri-*O*-acetyl-β-D-glucopyranosiduronate **3.139**. The product (162 mg, 46%, R_f = 0.42, 1.5:1 Hex/EtOAc) was obtained as a white crystalline substance from propan-2-ol. Mp; 172^{0}C (sharp). Found: MH^+ 439.1605; $C_{21}H_{26}O_{10}$ requires 439.1604. ^{1}H NMR δ/ppm 7.06 (1H, d, *J* = 7.18 Hz, phenyl), 6.67-6.86 (2H, m, phenyl), 5.21-5.31 (3H, m, H2'-H4'), 5.08 (1H, d, *J* = 7.25 Hz, H1'), 4.15-4.17 (1H, m, H5'), 3.74 (3H, s, -C O_2CH_3), 2.23, 2.20 (6H, 2 x s, 2 x -CH_3 Ar), 2.07, 2.05, 2.04 (9H, 3 x s, 3 x -OC(O) CH_3), 2.04 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 169.9, 169.1, 169.0 (3 x –O*C* (O) CH_3), 166.7 (-*C* O_2CH_3), 154.0, 137.8, 131.6, 130.2,118.5,114.0 (6 x aryl C), 99.0 (C1'), 72.6(C3'), 71.9 (C5'), 71.0 (C2'), 69.2(C4'), 52.9 (-CO_2C H_3), 20.6 (2 x -CH_3 Ar), 20.5, 20.0, 19.0 (3 x -OC(O) *C* H_3). IR (KBr disc) ϑ_{max} 2954, 1759, 1743, 1500, 1445, 1379, 1233, 1097, 1050, and 897 cm^{-1}.

The coupling reaction of 3,4-xylenol (0.2 g, 1.64 mmol) and bromo sugar **3.8** (0.780 g, 1.96 mmol) was also performed using the standard Koenigs-Knorr method as described in **5.2.1.1** to obtain the desired product **3.138** (155 mg, 44%).

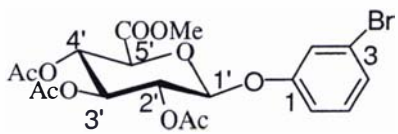
3.2.4.5 Methyl (*p*-bromo phenyl) -2', 3', 4'-tri-*O*-acetyl-β-D-glucopyranosiduronate (3.140)



3.140

The title compound was prepared from the coupling reaction of *p*-bromophenol (0.1 g, 0.58 mmol) and acetate sugar (0.1 g, 0.29 mmol) according to the method described above for the preparation of methyl (*p*-tolyl)-2', 3', 4'- tri-*O*-acetyl-β-D-glucopyranosiduronate **3.136**. The product **3.140** (64-68 mg, 45-48%, R_f = 0.42, 1.5:1 Hex/EtOAc) was obtained as a white crystalline substance from propan-2-ol. Mp; 159-160^{0}C, (Lit. mp, 162-163.5^{0}C).[62] Found: M^+ 487.0245; $C_{19}H_{20}O_{10}Br$ requires 487.0241. ^{1}H NMR δ/ppm 7.40 (2H, d, *J* = 9.01 Hz, phenyl H2, H6), 8.36 (2H, d, *J* = 9.01 Hz, phenyl H3, H5), 5.24-5.36 (3H, m, H2'-H4'), 5.01 (1H, d, *J* = 7.03 Hz, H1'), 4.16-4.19 (1H, m, H5'), 3.73 (-C O_2CH_3), 2.06 (3H, s, -OC(O) CH_3), 2.05 (3H, s, -OC(O) CH_3), 2.04 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 169.8, 169.1, 168.9 (3 x –*O*C (O) CH_3), 166.5 (-*C* O_2CH_3), 155.5, 132.4, 118.8, 116.0 (4 x aryl C), 99.0 (C1'), 72.6(C3'), 71.6 (C5'), 70.9 (C2'), 68.9(C4'), 53.0 (-CO_2*C* H_3), 20.6 x 2, 20.5 (3 x -OC(O) *C* H_3). IR (KBr disc) ϑ_{max} 2957, 1760, 1743, 1500, 1442, 1380, 1233, 1097, 1050, and 627 cm^{-1}

3.2.4.6 Methyl (*m*-bromo phenyl)-2',3',4'-tri-*O*-acetyl-β-D-glucopyranosiduronate (3.141)



3.141

The title compound was prepared from the coupling reaction of *m*-bromo phenol (0.2 g, 1.16 mmol) and the acetate sugar **3.4** (0.2 g, 0.58 mmol) according to the method described above for the preparation of methyl (*m*-bromophenyl)-2', 3', 4'-tri-*O*-acetyl-β-D-glucopyranosiduronate. The product (17 mg, 12%, R_f = 0.42, 1.5:1 Hex/EtOAc) was obtained as an off white crystalline substance from propan-2-ol. Mp 142-144^{0}C. Found: M^+ 487.0239; $C_{19}H_{20}O_{10}$ Br requires 487.0241. ^{1}H NMR δ/ppm 7.16-7.21 (3H, m, phenyl), 6.89-7.00 (1H, m, phenyl), 5.23-5.39 (3H, m, H2'-H4'), 5.14 (1H, d, *J* = 7.03 Hz, H1'), 4.15-4.21 (1H, m, H5'), 3.74 (-C O_2CH_3), 2.07 (3H, s, -OC(O) CH_3), 2.06 (3H, s, -OC(O) CH_3), 2.05 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 169.8, 169.1, 168.9 (3 x –*O*C (O) CH_3), 166.5 (-*C* O_2CH_3), 157.0, 130.6, 126.5, 122.5, 120.3, 115.6 (6 x aryl C), 98.8 (C1'), 72.6(C3'), 71.6 (C5'), 70.9 (C2'), 68.9 (C4'), 53.0 (-CO_2*C* H_3), 20.6

(2 x -OC(O) *C* H_3), 20.5 (-OC(O) *C* H_3). IR (KBr disc) ϑ_{max} 2957, 1759, 1743, 1614, 1500, 1444, 1379, 1233, 1097, 1050, and 623 cm^{-1}.

The coupling reaction of *m*-bromo phenol (0.2 g, 1.16 mmol) and bromo sugar **3.8** (0.552 g, 1.40 mmol) was also performed using the standard Koenigs-Knorr method as described in **5.2.1.1** to obtain the desired product **3.138** (14 mg, 6%) as a white solid.

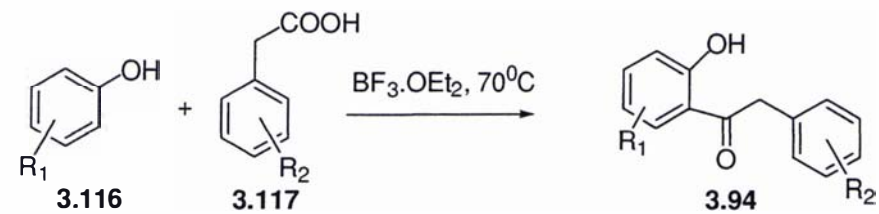
3.3 RESULTS AND DISCUSSION

3.3.1 Synthesis Of Isoflavones And Their Metabolites

An important incentive for the synthetic work to produce isoflavones is the emerging biological activities for many of them. The chemical structures of these biologically interesting isoflavone phytoestrogens are not particularly complex, yet their simplicity creates an opportunity for state-of- the art synthesis. This is particularly true when the goal is to develop an efficient macroscale preparative method for isoflavonoids. Relatively large quantities of variously substituted isoflavonoids are required for synthesis of isoflavone glucuronide conjugates and preparation of isoflavone metabolites. The demand of ready availability means that short routes would be preferred.

3.3.1.1 Synthesis Of Deoxybenzoins

Luk and co-workers[220] reported the Friedel-craft acylation of resorcinol with methoxy-substituted phenyl acetic acid catalysed by gaseous boron trifluoride (BF_3) or by $BF_3.OEt_2$. An application of this method using unprotected starting materials and the more easily handled $BF_3.OEt_2$ as both catalysts and solvent proved to be highly successful for the synthesis of various deoxybenzoins.



R_1 = OH, OMe
R_2 = H, OH, OMe

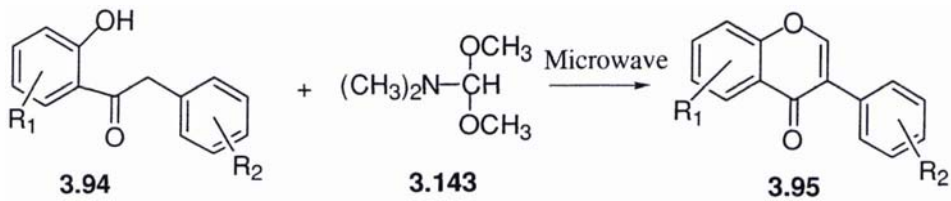
Scheme 3.27

Thus various deoxybenzoins (the precursors of isoflavones) were synthesised by refluxing appropriately substituted phenols with either 4-hydroxy or 4-methoxy phenyl acetic acid in $BF_3.OEt_2$. The ^1H NMR spectra confirmed the deoxybenzoin structures from the ABX pattern in ring A and a singlet of 2 protons from the benzylic methylene

group at 4.09 ppm expected for deoxybenzoin **3.94**. The synthesis of this ketone was also reported by Shriner and Hull[66] and Yorder *et al*[67] by saturating a solution of resorcinol and 4-hydroxyphenyl acetonitrile in ether with dry HCl over a period of 3 days.

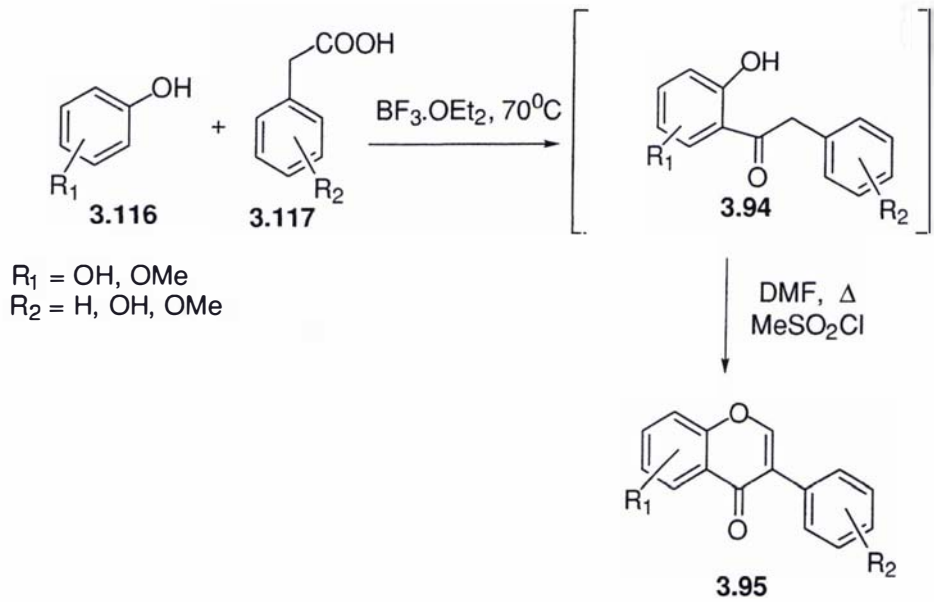
3.3.1.2 Synthesis Of Isoflavones

The best two previous methods for the convenient and facile synthesis of isoflavones were reported by Chang *et al* [68] and Wahala *et al*.[61] Chang and coworkers synthesised isoflavones using the methodology of Pelter and Foot[69] in high yields by cyclisation of the appropriate deoxybenzoins in a conventional microwave method. In this simple procedure the deoxybenzoins were reacted with N,N dimethyl formamide dimethyl acetal and THF as the solvents and the required isoflavones were obtained under medium microwave energy for 2 minutes. The yields obtained in this procedure were reasonably high (60%-80%).



Scheme 3.28

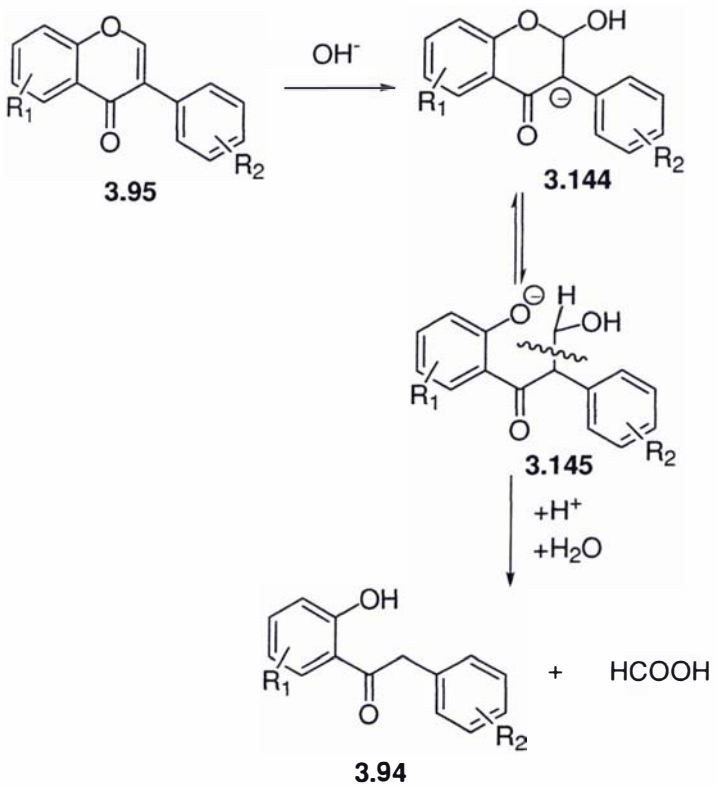
Recently Wahala and co-workers[61] reported the putative expedient synthesis of polyhydroxy isoflavones in a one pot procedure using the methodology developed by Bass[60] for the formation of a chromene ring. The intermediate deoxybenzoins were directly cyclised with DMF catalysed by $BF_3.OEt_2$ and $MeSO_2Cl$ to give the isoflavones in moderate to excellent yield (50%-98%) yields.



Scheme 3.29

The latter method was chosen here for the synthesis of the isoflavones owing to the availability of the reagents and the apparent ease with which the reaction could take place. Unfortunately, a variety of attempts to realize one pot cyclisations with resorcinol and 4-hydroxy phenyl acetic acid always resulted in a poor yield. For instance under the conditions of Wahala *et al* [61] daidzein was obtained in only 7-10% yield. Cyclisation under more forcing conditions, for example at higher temperatures resulted in even worse yields (3-5%) of daidzein. Many other conditions including long reaction times, increased concentrations of $MeSO_2Cl$, increased amount of catalysts either failed to promote any reaction or resulted in a poor yield of daidzein **3.123**. The TLC and ^1H NMR results of the crude reaction mixture indicated the presence of unreacted deoxybenzoin along with a complex mixture of unidentified products. The TLC result also indicated an additional intense yellow spot which could be visualised with and without the presence of UV light. The yellow color on TLC became more intense when the concentration of $MeSO_2Cl$ was increased. It was presumed that this persistent yellow color might be due to the formation of highly conjugated methane sulfonate esters. After the completion of the reaction the crude product was quenched into a large quantity of ice cold water or aqueous NaOAc. When the mixture was quenched into aqueous NaOAc not only did it precipitate the desired isoflavones but it also liberated a yellow color into the filtrate. This result during the work up is consistent with the fact that methane sulfonyl chloride is a good protecting group for

phenols and can be cleaved by treatment with base.[71] However liberating any methane sulfonate esters by hydrolysis with base poses a threat to the base sensitive chromene ring and ultimately causes a cleavage and degradation to yield the precursor deoxybenzoin **3.94.**



Scheme 3.30

To avoid the problems associated with the alkaline work up the crude product was quenched into a large quantity of water and extracted with EtOAc. An attempt was then made to purify the crude mixture using column chromatography. However this attempt involved a long and tedious elution with CH_2Cl_2 and the polarity was gradually increased by the addition of EtOAc to give daidzein in 7-10% yield. The complicating factor was the low solubility of daidzein compounded with the fact that a number of commercially available silica-gels contain a metallic impurity (probably iron) which caused the more polar isoflavones to adhere strongly to the column. Bottomley *et al*[72] have recommended pre-treatment of silica gel with warm concentrated HCl to remove any metallic impurities thus making the adsorbent much more useful for the separation of polar compounds. In fact this pretreatment reduced the time required to elute the

daidzein but again gave a very poor yield (3%) of daidzein. This could have been due to the instability of the chromene ring under the acidic conditions[73] as well.

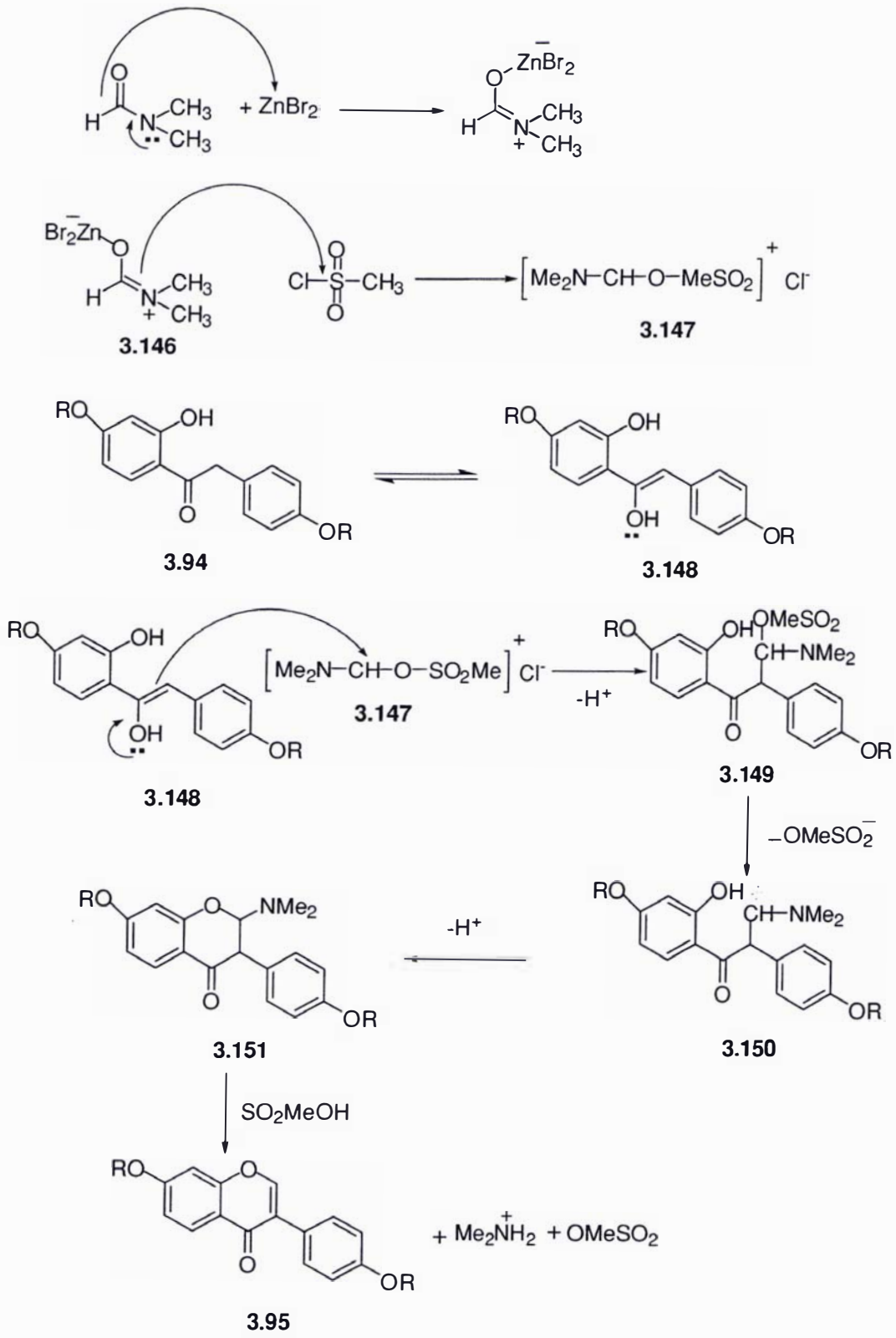
As $BF_3.OEt_2$ is a strong Lewis acid catalyst the presence of a large quantity of $BF_3.OEt_2$ as solvent and electrophilic catalyst might activate the reaction further to mask the phenolic -OH groups with $MeSO_2Cl$. It was also presumed that excess $BF_3.OEt_2$ might cause a stability problem as the chromene ring is unstable under acidic conditions. Rearranged products were also observed when the synthesis of deoxybenzoin from *m*-methoxy phenol and 4-hydroxy phenyl acetic acid was attempted in $BF_3.OEt_2$. This result was in agreement with similar results reported by Luk *et al*[64] thus making isolation of the crude deoxybenzoin essential. These problems encouraged an investigation into the cyclisation to isoflavones after isolation and purification of the deoxybenzoin and to try other milder Lewis acid catalysts.

The new methods used in this work follow the same design as older methods in that an appropriately substituted deoxybenzoin was reacted with a one carbon unit and a Lewis acid. Moderate yields of daidzein were obtained when $AlCl_3$ or $MgBr_2.OEt_2$ were used as Lewis acid catalysts. Due to the difficulties associated with column chromatography product was not isolated but the ^1H NMR spectrum of the crude reaction mixture indicated 40-50% yield of the daidzein on the basis of the doublet at 8.09 in deoxybenzoin and a singlet at 8.32 in daidzein due to H1. An attempt was also made to purify the product using HPLC according to the procedure described in the literature[74] for isoflavones obtained from soyabean. This proved to be difficult and time consuming due to the low capacity of the reverse phase column which was available hence other Lewis acids were tried. The precursor deoxybenzoin was unreacted when the cyclisation reaction was tried in the presence of the Lewis acid catalyst $SnCl_4$. Fortunately a marked difference in the cyclisation reaction was seen when the milder Lewis acid catalyst $ZnBr_2$ was used. The TLC indicated the presence of a minor yellow spot but no spot due to unreacted deoxybenzoin was seen. The reaction was easily carried out for the deoxybenzoins reported in the **Table 3.1** and worked up on a larger scale.

Table 3.1 Preparation of isoflavones using $ZnBr_2$ as Lewis acid catalyst in DMF at 80-100^{0}C

Deoxy benzoin		Isoflavone		Yield	M.p.	Lit.[61,64,70]
R_1	R_2	R_1	R_2	(%)		m.p.
4-OH	4'-OH	7-OH	4'-OH	84%	212-214^{0}C	213.5-214.5^{0}C
3-Me, 4-OH	4'-OH	7-OH, 8-Me	4'-OH	86%	218-220^{0}C	218-220^{0}C
4-OCH_3	4'-OH	7-OCH_3	4'-OH	80%	258-260^{0}C	257^{0}C
4-OH	4'- OCH_3	7-OH	4'- OCH_3	74%	228-229^{0}C	222-224^{0}C
4-OCH_3	4'-OCH_3	7-OCH_3	4'-OCH_3	76%	165-167^{0}C	162-164^{0}C

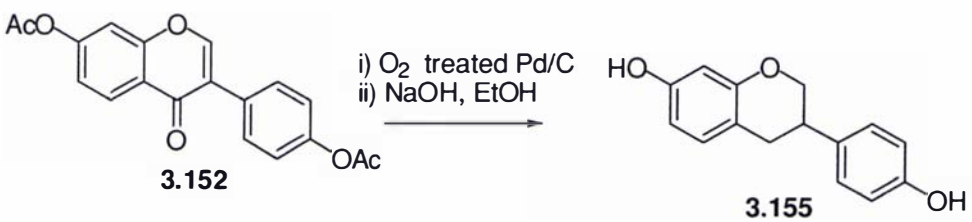
The valuable aspect of this reaction is that it is devoid of expensive chemicals and reagents as the appropriate deoxybenzoin often crystallised out from the reaction mixture and thus the expensive reagent $BF_3.OEt_2$ could be recycled. The cyclisation step requires the cheap and easily available $ZnBr_2$ as Lewis acid and the resulting work up was easy. In view of the experimental conditions employed, as well as the earlier work of Smith who demonstrated the mechanism of formylation of indole,[75] the probable path by which an isoflavone is formed when deoxybenzoin is submitted to the action of DMF and $MeSO_2Cl$ is shown in **Scheme 3.31**.


+ ZnBr2
3.146
3.147
3.94
3.148
3.149
-OMeSO2−
-H+
3.151
3.150
SO2MeOH
+ Me2NH2 + OMeSO2
3.95

Scheme 3.31

3.3.1.3 Reduction Of Isoflavones For The Syntheses Of Isoflavans

Several metabolites have been detected in urine from human subjects on soybean diets. The major metabolites of daidzein and formononetin are their reduction products equol (7,4'-dihydroxy isoflavane) **3.155** and methoxy equol (7-hydroxy, 4'-methoxy isoflavane) **3.129**. In an attempt to synthesise the glucuronides of equol and methoxy equol, a convenient method is required for their use as precursors in the synthesis of the isoflavane nucleus. The low solubility of isoflavones in suitable solvents limits reduction procedures. The best method to prepare an isoflavane from isoflavone appeared to be a modified Wessely-Prillinger method as reported by Lamberton *et al.*[76] Equol was synthesised by the reduction of the more soluble *O*,*O*-diacetyl daidzein **3.152** precursors followed by the hydrolysis of the resulting reduced product, in ethanolic NaOH. However the method of Wessely and Prillinger gave a satisfactory yield only if their special conditions for the preparation of the catalyst were applied.[76] The Pd/C catalyst was first suspended in glacial aretic acid and shaken in an atmosphere of oxygen for several days before use. Hydrogenation of daidzein over this catalyst is extremely rapid and conversion to (±) equol is usually complete in 15-30 minutes.[76]

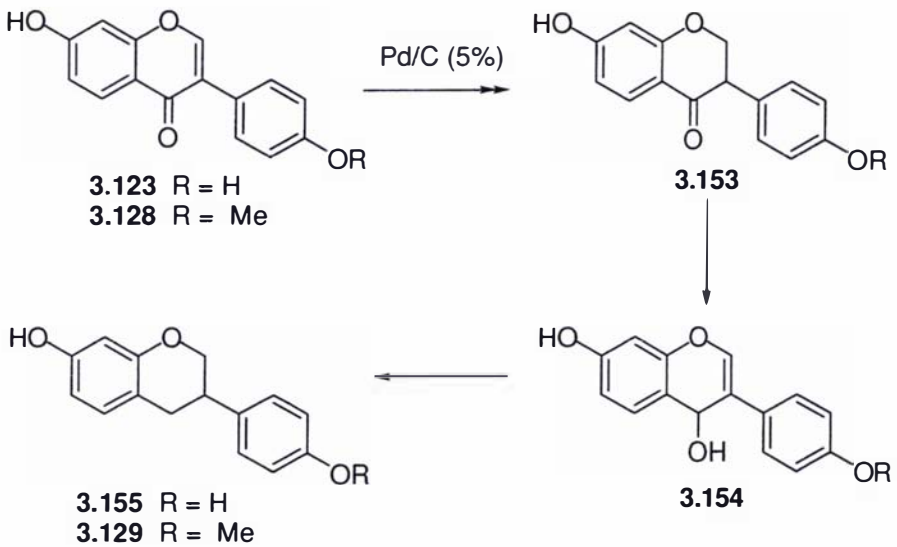


Scheme 3.32

The only disadvantage of this method is the large amount of Pd/C required for the complete reduction. Attempts to use less catalyst or to recover the catalyst and retreat with oxygen gave a mixture of reduced products.[76] To avoid this difficulty and to remove the extra step of synthesising *O*,*O*-diacetyl daidzein an alternative strategy has been designed.

Recently Chang *et al* [77] reported that their attempt to synthesise equol using the procedure of Adlercreutz *et al* [78] through the hydrogenation of daidzein in EtOH gave

4,7,4'-trihydroxy isoflavane as one of the products. They reported a modified procedure in which a solution of daidzein in EtOH or glacial HOAc was bubbled with H_2 for 15 min and then added to a pre reduced EtOH or glacial HOAc solution containing 5% Pd/C. They reported a 47% yield of equol when the reaction mixture was carried out at room temperature under a H_2 atmosphere. The attempts to synthesise reduced products using this methodology in the present work gave a complex mixture probably due to partial hydrogenation. Even though the reaction was continued for a long time no differences in the TLC was observed. The TLC analysis of the crude reaction mixture indicated unreacted starting material and 3 new products probably due to the presence of the partially reduced products **3.153**, **3.154**, and **3.155**.



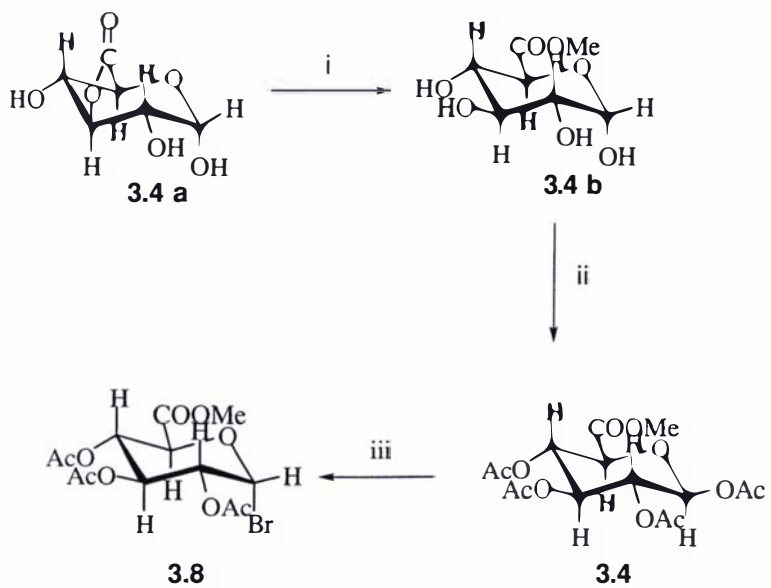
Scheme 3.33

Several attempts were made at achieving the reduction to isoflavanes before the correct reaction conditions were identified hence the reduction of daidzein and formononetin was successfully carried out according to the procedure of Luk *et al*[64] with little modification. Isoflavones were treated with 10% Pd/C in glacial HOAc at RT and atmospheric pressure under an atmosphere of H_2 for 16 h. The structures of the isoflavans (±) methoxy equol **3.129** and (±) equol **3.155** were confirmed by comparison of their NMR spectra with literature data. The ^{1}H NMR spectrum retained the familiar ABX and AA', XX' splitting pattern of the isoflavones, daidzein and formononetin. The protons of the methylene group in isoflavans **3.129** and **3.155** at C4 are not chemical shift equivalent. They couple with each other and also coupled with the vicinal protons

and hence appeared as two multiplets. The ^{13}C NMR spectrum of compound **3.129** and **3.155** gave a signal at ~71 ppm assigned to C-4 confirming that the C=O group was completely reduced and the usual C=O group at 175.6 ppm had disappeared.

3.3.2 Preparations Of Acyl Protected Methyl Glucopyranuronate As Glycosyl Donors For The Syntheses Of *O* -Glucuronides

A clear majority of all glucuronides have been synthesised via acyl protected sugar intermediates. The preparations of sugar intermediates (glycosyl donors) most often used for coupling reactions in this thesis are summarised below starting with commercially available D-glucurono-6,3-lactone ('glucurone') **3.4 a**. Tetraacetyl glucopyranuronate **3.4** was conveniently prepared from the glucuronolactone by base catalysed esterification in methanol to give the methyl glucuronide **3.4 b**. The acetylation reaction yields an α/β mixture from which the β-anomer **3.4** was easily separated by crystallisation. The tetraacetyl glucopyranuronate **3.4** on treatment with 45% HBr in HOAc gave the α-bromo sugar **3.8** in moderate yield according to the established literature procedure.[62]



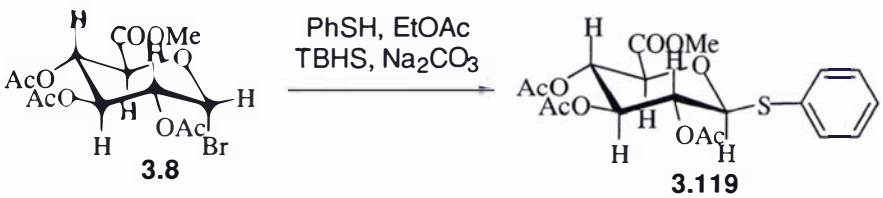
3.4 a **3.4 b** **3.8** **3.4**

Reagents and conditions : *(i) Na, MeOH, 0.5 h, (ii) CH_3COOH, $HClO_4$, 20-25 ^0C, 50%, (iii) HBr in CH_3COOH, (45%), Dark, RT, Overnight, 80%.*

Scheme 3.34

Although there is a new procedure reported[79] for the preparation of glycosyl halides using α -haloenamines under neutral conditions, the above classical method for making the bromo sugar **3.8** is still the easiest and cheapest procedure.

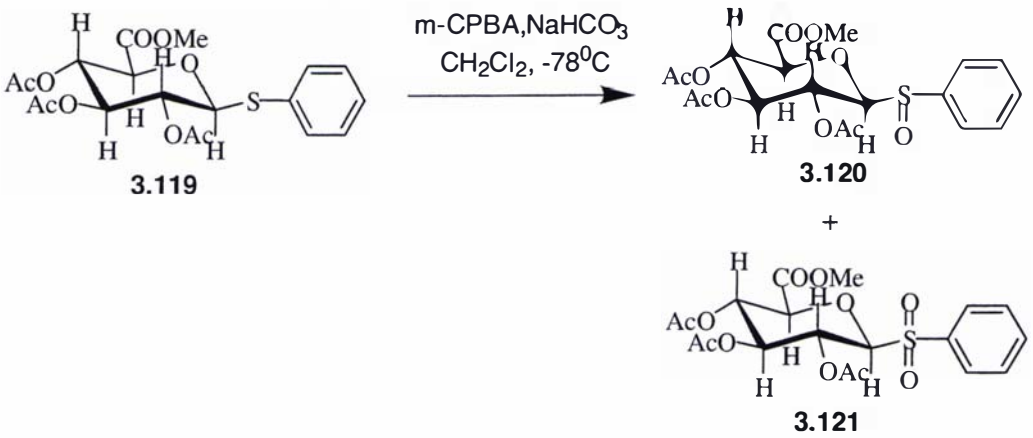
For the effective synthesis of analyte glucuronides new modified glycosyl donors are always in demand. Even though methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate is by far the most used glycosyl donor, this reagent is not stable and can be stored at 0^0 for only a few weeks before it turns black (although this can be recovered to some extent by recrystallised from EtOH). An attempt was made therefore to use the more stable glycosyl donor β-phenyl thioglucuronide **3.119** in glucuronidation reactions. The desired β-phenyl thioglucuronide **3.119** was easily prepared according to established literature procedures.[63] Hence the bromo sugar **3.8** was treated with thiophenol under phase transfer conditions to give the required β-phenyl thioglucuronide **3.119** in an excellent yield (95%).



3.8 **3.119**

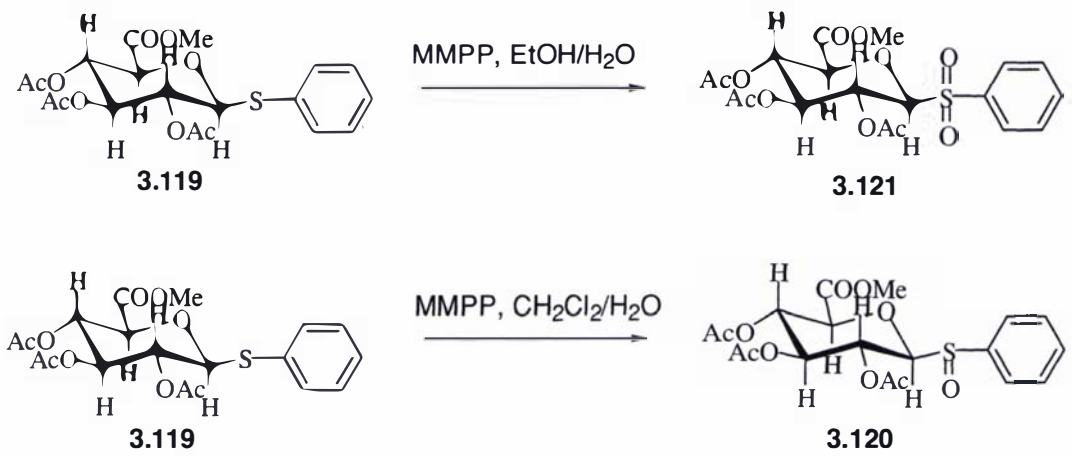
Scheme 3.35

Previous research also indicated that the anomeric sulfoxides such as **3.42** (**Section 3.1.4, Scheme 3.9**) were more reactive than the β-phenyl thioglucuronide **3.119** and therefore were particularly useful for reaction with deactivated phenols. Numerous attempts were made to obtain the sulfoxide in clean and controlled oxidation of the anomeric β-phenyl thioglucuronide **3.119**. Kahn's methodology[28] of m-CPBA/CH_2Cl_2 at -78^0C (**Scheme 3.36**) gave a very poor yield of the desired sulfoxide sugar **3.120** yielding mostly the unwanted over-oxidation product, the corresponding sulfone sugar **3.121**.



Scheme 3.36

More importantly this reaction worked on a small scale but attempts to perform this reaction on a larger scale resulted in an almost quantitative yield of the sulfone sugar **3.121**. Attempts to obtain the partial oxidation product (the sulfoxide sugar) under high dilution conditions also failed resulting largely in the unreacted thio sugar and small amounts of the sulfone sugar. The progress of the reaction was monitored by TLC analysis which showed that no sulfoxide sugar was formed at any stage of the reaction. An attempt was made to modify Kahn's methodology and hence m-CPBA and $NaHCO_3$ in CH_2Cl_2 were added into β-phenyl thioglucuronide **3.119** at -78^0C. Unfortunately again the reaction proceeded on a small scale (50 mg) but failed to give any sulfoxide sugar on a larger scale and gave either a poor yield of the sulfoxide or entirely the sulfone sugar. Attempts to increase the concentration of the oxidant and the reaction time as well as temperature also failed giving entirely the sulfone sugar. The controlled oxidation of β-phenyl thioglucuronide **3.119** was also attempted in the presence of the oxidant MMPP (magnesium salt of m-perpthalic acid)[80] in a combined solvent EtOH/H_2O and urea-H_2O_2 Complex[81] in EtOH. The reaction was monitored by TLC and again the product was only the sulfone sugar. Fortunately when this reaction was attempted in CH_2Cl_2/H_2O it resulted in a moderate yield of the sulfoxide sugar as the major product.

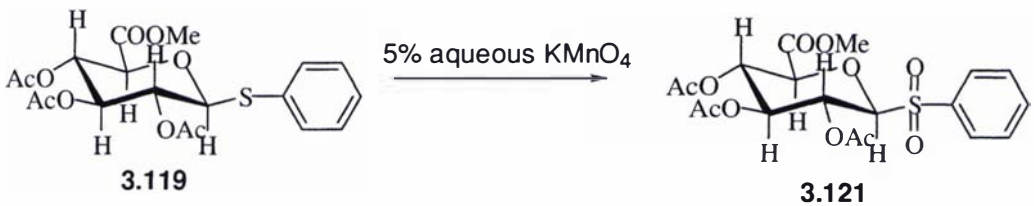


3.119 **3.121**

3.119 **3.120**

Scheme 3.37

The reason behind this success is probably due to the solubility of MMPP in both the organic and aqueous layers. MMPP being distributed in both phases is only partly available in the organic phase and hence acts as a mild oxidant and reacts with thiosugar **3.119** to give a moderate yield of the desired sulfoxide sugar **3.120**. This was further confirmed when the same reaction was repeated in the presence of a phase transfer catalyst (TBHS), the reaction gave an increased yield of the sulfone sugar at the expense of the sulfoxide. The anomeric sulfone sugar was also obtained using a literature method with the oxidant $KmnO_4$.[82]



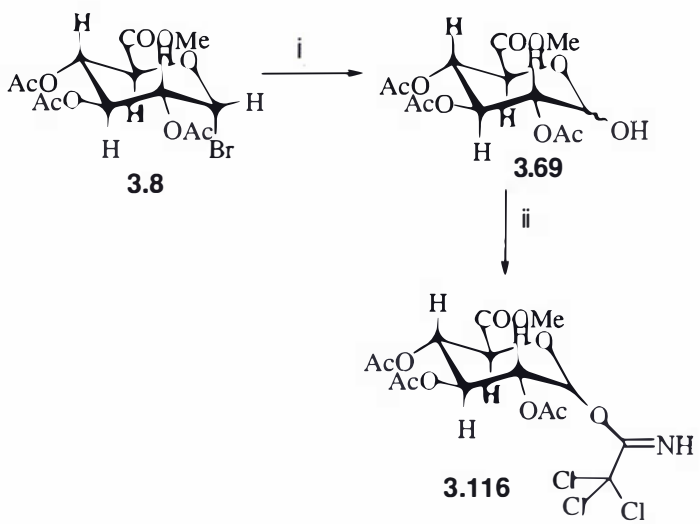
3.119 **3.121**

Scheme 3.38

The TLC and ^1H NMR analysis of the sulfone sugar using $KMnO_4$ method was identical with the sulfone sugar obtained using Kahn's methodology and the MMPP method.

With the limited success in the glycosylation reactions (**Section 3.3.3**) using the above glycosl donors attention was also focused on the synthesis of the hydroxy sugar **3.69** and the acetimidate sugar **3.116** as possible glycosyl donors. Both tetracetate **3.4**

and the bromo sugar **3.8** are suitable precursors for the 1-hydroxy sugar **3.69** which exists as an almost 5:1 (α:β) mixture (NMR in $CDCl_3$) after product formation. According to the literature procedure[35,36] the easiest method appeared to be deprotection at the anomeric sugar acetate of **3.4** by Bu_3SnOMe in THF under reflux to give crystalline **3.69** in good yields (78%). However the more circuitous route was chosen to avoid the use of the expensive and toxic reagent Bu_3SnOMe, and hence the hydroxy sugar **3.69** was conveniently prepared from the readily available bromo sugar **3.8.** To achieve this the bromo sugar **3.8** was catalytically hydrolysed using Ag_2CO_3 to obtain the hydroxy sugar in an excellent yield (>90%) (**scheme 3.39**)[36]. This hemiacetal itself was used as a glycosyl donor for various glucuronidation reactions or more conveniety it was converted into the more active glycosyl donor trichloroacetamidoyl pyranuronate sugar. The base catalysed reaction of hemiacetal **3.69** with trichloro acetonitrile could be tuned to give either the α or β imidate through the operation of what Schmidt has termed the 'kinetic' anomeric effect.[83] Hence the desired α-trichloro acetamidate was conveniety prepared according to a recently published literature procedure using the relatively mild base K_2CO_3 and Cl_3CCN in CH_2Cl_2 in an excellent yield (**Scheme 3.39**). The α-anomer was also obtained using the stronger and relatively expensive base DBU but the reaction mixture became black and required careful chromatography to isolate the product.



Reagents and conditions : *(i)Ag_2CO_3, Acetone, H_2O, RT, 6 h, 86%; (ii)Cl_3CCN, K_2CO_3, CH_2Cl_2, RT, 14 h, 85%.*

Scheme 3.39

The β-anomer could be obtained if a both milder or stronger base catalysed reaction of Cl_3CCN in CH_3CN with hydroxy sugar was performed at shorter reaction times. This tendency to rapidly form the β tricholoro acetimidate is thought be due to an enhanced nucleophilicity of the combined directive effects of the lone pair orbitals on the two oxygen atoms of the sugar in the β isomer (**3.116 b and 3.116 d**).

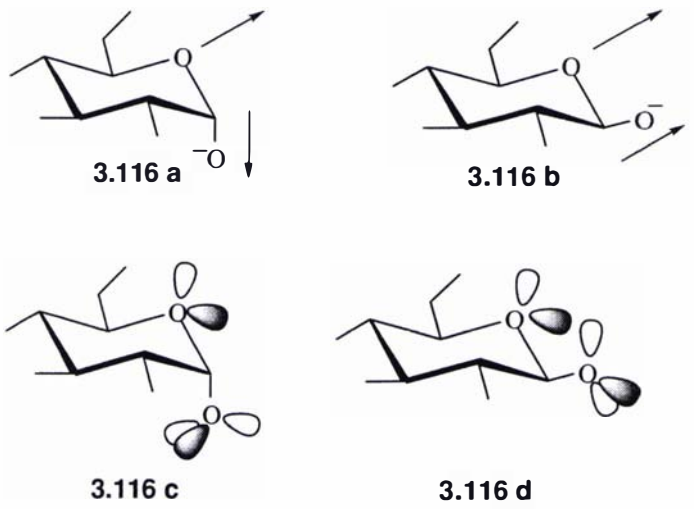


Figure 3.9

This kinetically effective stereoelectronic effect results from repulsion of the two lone electron pairs, by dipole effects, or both.

3.3.3 Investigation Into The Synthesis Of Isoflavone And Isoflavan Glucuronides By Coupling With Various Glycosyl Donors

In recent years, considerable attention has been focused on the medical problems associated with the metabolism and physiological role of isoflavone conjugates in humans and animals (**Section 2.1.8**). Despite this, a literature survey reveals no study into protein or enzyme conjugates and hence no immunoassay studies have been performed on isoflavone glucuronides.

Glucuronides have their own distinctive chemistry and are usually more difficult to prepare than the corresponding glucopyranosides. Schmidt and co-workers[84] have drawn up a glycosylation 'league table' of increasing ease of glycosyl donation which shows of all the common sugars the glycopyranuronates are the poorest glycosyl donors

and require the highest activation for a given aglycone. The reported methods for the synthesis of glucuronide conjugates in most cases involve the coupling of a protected glucuronic acid derivatives, activated at the anomeric center, with the aglycone followed by deprotection. No successful chemical method for the synthesis of isoflavone glucuronides so far has been reported. All previous syntheses of isoflavone glucuronides are limited to enzymatic pathways.[56]

3.3.3.1 Perester Coupling Method Using $BF_3.OEt_2$ As Catalyst

Aromatic phenols were used for model studies and hence attempts were made at the synthesis of various aromatic phenol glucuronides using the perester coupling method. Various simple phenols were successfully reacted at RT with the tetracetate sugar **3.4** in the presence of the Lewis acid catalyst $BF_3.OEt_2$ to give aryl-β-D-glucuronides. The glucuronides were obtained in moderate yields with 1 equivalent of the glycosyl donor. When *p*-cresol was reacted with 2.2 equivalent of the tetracetate sugar **3.4** as the glycosyl donor an improved yield was observed. Column chromatography was needed to purify the glucuronides and remove unreacted sugar. Hence later on reactions were performed using 2-3 equivalent of phenols and at the completion of the reaction, the crude reaction mixture was washed with aqueous KOH (2 M) to remove excess phenols. The yields were relatively low but this procedure avoided the chromatographic purification step. Unfortunately this acid catalysed coupling method which was often good for phenols proved to be unsuccessful for the isoflavones daidzein, formononetin and the isoflavan *m*-methoxy equol. When the tetracetate **3.4** was reacted with isoflavones or isoflavans in CH_2Cl_2 at RT or at higher temperatures in the presence of $BF_3.OEt_2$ no glucuronide was obtained. It was therefore decided to investigate the electronic demand on the glycosylation reaction and hence *m*-methoxy phenol, *m*-bromo phenol and *p*-hydroxy methyl benzoate were chosen as model compounds due to their presumed similarities of electronic demand with *m*-methoxy equol and formononetin. Since the reaction requires electron demand at the reaction centre, compounds with electron donating substituents *para* to the nucleophilic -OH group should accelerate the reaction. These observed variation in yield can be explained on the basis of Hammett σ values.[85] A positive value of (indicates an electron withdrawing group and a negative value an electron donating group. In agreement with this expectation *p*-cresol (σ*p*-Me = -0.14) gave 55% yield of the

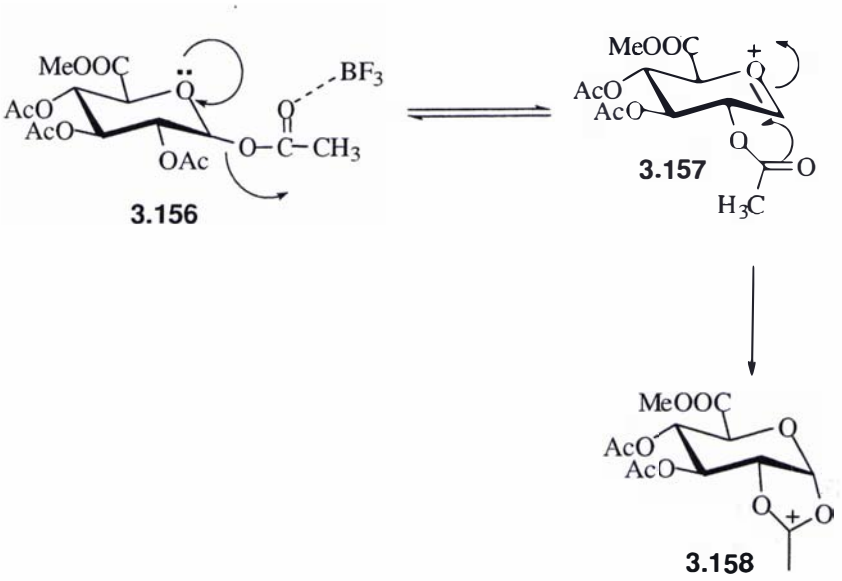
coupled product when a 1:1 molar ratio of glycosyl donor and aglycone was used. On the other hand *m*-cresol (σm-Me = -0.06) gave only 41% yield. When two *meta* methyl substutuents were employed (using 3,4 or 3,5 dimethyl phenol) there was little change in yield. No further detrimental effect on the tield was seen with a *p*-bromo substituent even though the Hammett σ value was now electron withdrawing (σp-Br = +0.26). On the other hand when the more electron withdrawing *meta* bromosubstituent (σm-Br = +0.37) was present the yield dropped to 12%. The fact that the presence of *p*-carbomethoxy (σp-COOMe = +0.44) and *m*-methoxy (σm-OMe = +0.10) substituents resulted in no yield at all suggests that these compounds are more deactivated than the Hammett σ values would suggest. This is presumably because in the presence of a Lewis acid catalyst such as $BF_3.OEt_2$ the oxygen atoms in the substituents are co-ordinated to the boron atom thus becoming much more electron withdrawing. Applying more forcing conditions by increasing the temperature, the concentration of reactants and increasing the amount of catalyst also failed to give any formononetin glucuronide. The glycosylation reaction was also studied in complexing solvents such as ethyl ether and THF but no coupling was observed. This was also the case for acetonitrile and DMF as solvent. The failure to effect this glycosylation can be attributed to the poor electronic demand among the isoflavones which act therefore as poor nucleophiles. In all successful aryl glucuronides esters the -2', 3'- and 4'- protons appeared further downfield (as in acetate sugar **3.4**) than the 1'- and 5'-protons due to the strongly electron withdrawing acetate groups. The table below summarises the results of successful and unsuccessful glycosylation reactions attempted.

Table 3.2 Preparation of phenolic glucuronide esters using $BF_3.OEt_2$ coupling method.

Phenols	Yield* (%)	M.p. ,^{0}C		References
		found	reported	
p-cresol	56%*	136	137-138	218
m-cresol	41%	102	113-115	218
p-bromo Phenol	45%	157-158	162-163	218
m-bromo phenol	12%	148-149	N	_
3,4-dimethyl phenol	46%	172	N	_
3,5-dimethyl pehenol	47%	172-175	N	_
p-hydroxy methyl benzoate	_	_	_	_
m-methoxy phenol	_	_	_	_

*The yields reported here are based on a 1:1 equivalence of aglycones and glycosyl donor N= new compound.

All of the glucuronides prepared were predominantly β-glucuronides as shown by the large coupling constants (J > 7 Hz). A plausible mechanism for this β-specificity of the successful coupling reactions can be imagined by the fact that the acetate group at position 2 can aid the departure of the anomeric acetate moiety to form an intermediate which can only form a β -adduct by attack of the oxygen atom of the phenols.

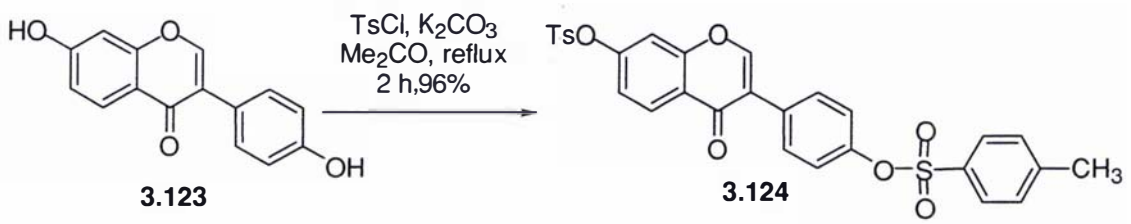


Scheme 3.40

The mechanism is not possible if the acetate group at position 2 is axial or if the sugar has an α-configuration, as only equatorial acetate groups at position 2 of sugars plays a very important assisting role during anomeric reactions.

3.3.3.2 Koenigs-Knorr Reactions For The Formation Of Glucuronides

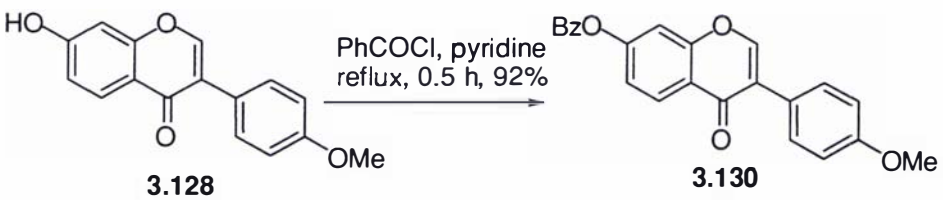
The classical Koenigs-Knorr method remains the most popular for the synthesis of a wide range of aryl glucuronides including steroid glucuronides (**Section 5.1.2**). It was decided to investigate the coupling of aglycone isoflavones and their metabolites with the bromo sugar **3.8** with various promoter and solvent systems. Initially one of the phenolic -OH of groups of daidzein was protected with tosyl chloride and K_2CO_3 to give4'-tosyl daizein (**Scheme 3.41**).



Scheme 3.41

It was expected that in daidzein **3.123** the 7-hydroxy group would be more acidic and hence deactivated than the 4'-OH substituent because of the electron withdrawing effect of the carbonyl group present in the chromene ring. This was confirmed by the ^{1}H NMR spectrum and Mass spectrum which indicated a selective protection of 4'-OH to give 4'-tosyl daidzein. However, initial Koenigs-Knorr attempts to glycosylate 4'-tosyl daidzein in the presence of the promoter $CdCO_3$ in toluene under reflux were not successful. Similar Koenigs-Knorr attempts in the presence of silver Salts especially Ag_2CO_3 and AgOTf also failed. The attempt to destroy any HBr formed during the glycosylation reaction by quenching with Et_3N also proved unsuccessful. When the glucuronidation reaction was performed on some of the simple phenols it gave the desired glucuronides in moderate yield (**Table 3.3**).

Farkas and co-workers[86] reported that formononetin benzoate can be coupled with acetylbromosugar **4.47** under Koenigs-Knorr conditions to obtain formononetin glycoside. Hence formononetin benzoate was also synthesised by reacting formononetin with benzoyl chloride and pyridine.



3.128 **3.130**

Scheme 3.42

However when the coupling reaction of formononetin benzoate with the bromosugar **3.8** was attempted with Ag_2CO_3/pyridine it gave largely dehydrohalogenated product.

Unfortunately none of the existing Koenigs-Knorr reactions gave the desired glucuronide for the isoflavones or any of their metabolites. The low organic solubility of the isoflavones and their metabolites compounded with the fact that they are poor nucleophiles was a major problem. A summary of all the attempted Koenigs-Knorr reaction is shown in **Table 3.3**.

Table 3.3 Attempted Koenigs-Knorr Glycosylation Reactions With Bromosugar (3.8)

Aglycone	Reaction Conditions	Yield (%)	Comments
4'-Tosyl Daidzein	$CdCO_3$, toluene, reflux	No Glucuronidation	Decomposition of bromosugar when refluxed.
Daidzein	$CdCO_3$, toluene, reflux	No Glucuronidation	Decomposition of bromosugar when refluxed.
Daidzein	$CdCO_3$, toluene, Et_3N, reflux	No Glucuronidation	Decomposition of bromosugar when refluxed.
7-Tosyl Daidzein	Pyridine, Ag_2CO_3, 0^0C	No Glucuronidation	TLC and ^1H NMR analysis indicated glycal formation
Formononetin	AgOTf, CH_2Cl_2, RT, Molecular sieves (4Å)	No Glucuronidation	Decomposition of bromosugar
Daidzein	Pyridine, Ag_2CO_3, 0^0C	No Glucuronidation	TLC and ^1H NMR analysis indicated glycal formation
Formononetin	Pyridine, Ag_2CO_3, 0^0C	No Glucuronidation	TLC and ^1H NMR analysis indicated glycal formation
Formononetin benzoate	Pyridine, Ag_2CO_3, drierite, 0^0C	No Glucuronidation	Glycal formation
Formononetin	AgOTf, Toluene, reflux	No Glucuronidation	Decomposition of bromosugar
Formononetin	AgO, isoquinoline, Ar, RT	No Glucuronidation	Hydrolysis of bromosugar
Formononetin	$CdCO_3$, Nitrobenzene, reflux	No Glucuronidation	Decomposition of bromosugar
Methoxy equol	$CdCO_3$, Toluene, reflux	No Glucuronidation	Decomposition of bromosugar seen

m-cresol	$CdCO_3$,toluene reflux, 3 h	54%	m.p.110^{0}C
m-bromo phenol	$CdCO_3$,toluene reflux, 5 h	4-5%	m.p.147-150^{0}C
3,4-dimethyl phenol	$CdCO_3$,toluene reflux, 3 h	44%	m.p.168-171^{0}C
3,5-dimethyl phenol	$CdCO_3$,toluene reflux, 3 h	46%	m.p.174-176^{0}C
m-methoxy phenol	$CdCO_3$,toluene reflux, 16 h	No glucuronidation	Decomposition of bromosugar
4-hydroxy methyl benzoate	$CdCO_3$,toluene reflux, 16 h	No glucuronidation	Decomposition of bromosugar

The Koenigs-Knorr reaction of formononetin was also attempted by refluxing at higher temperature in nitro benzene without any success. In all the Koenigs-Knorr attempts with the isoflavones and their metabolites as indicated above, decomposition of the bromosugar was shown on TLC or by ^{1}H NMR. Unreacted isoflavones and their metabolites were recovered unchanged in most examples. When the reaction was performed under alkaline conditions using pyridine, glycal was evident as a product. The ^{1}H NMR showed all the 3 x -OAc group were intact but a new singlet at 6.83 ppm due to the olefinic proton at C1 and at 4.84 ppm due to the proton at C5 was observed(the bromo sugar showed doublet for protons at C1 and C5).

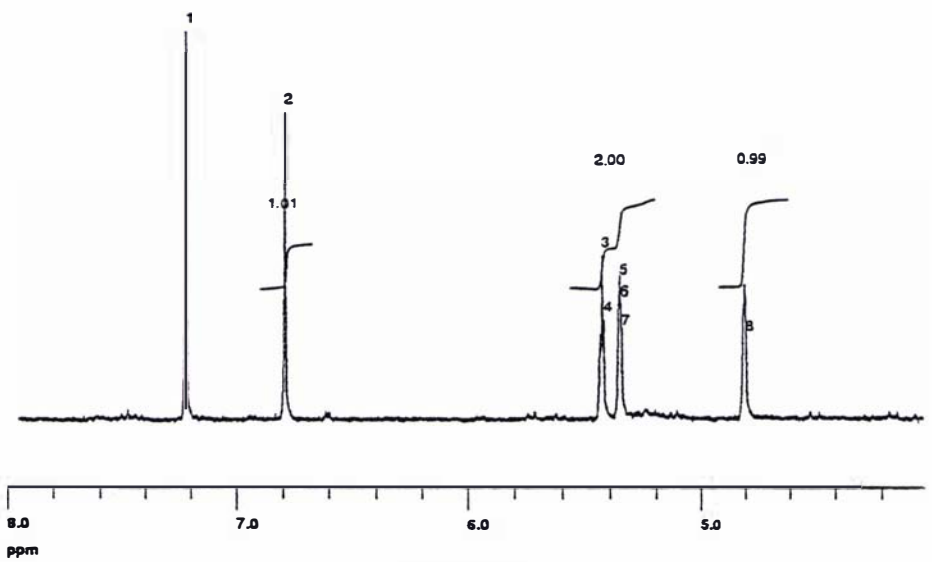
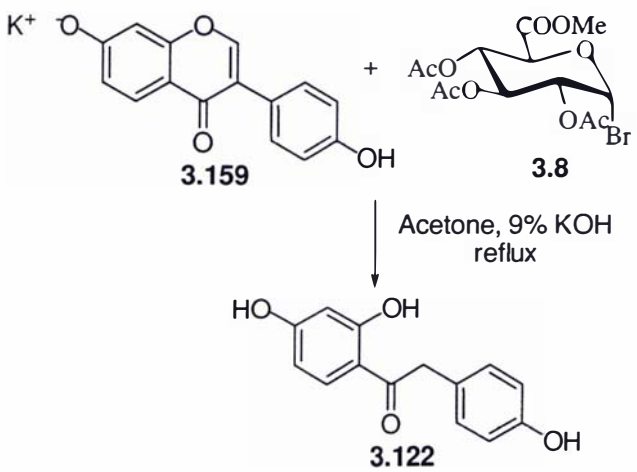


Figure 3.10

This result was also confirmed by ^{13}C NMR which indicated peaks at 139.2 ppm and 127.4 ppm due to the formation of the olefinic bond between C1 and C2.

3.3.3.3 Glycosylation Attempts Using Phenolate Anion Methods

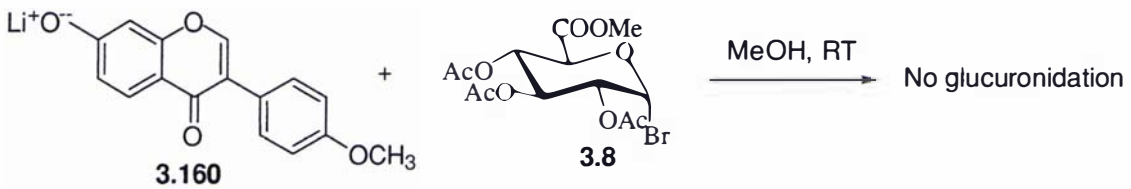
In our hands Zemplen's[87] base catalysed reaction (9% KOH) of the bromo sugar **3.8** with unprotected hydroxy isoflavones in acetone carried out in an attempt to produce daidzein and formononetin glucuronide caused mainly isoflavone C-ring cleavage and anomeric hydrolysis of bromosugar **3.8** but no glycosylation. The mass spectrum of the crude reaction mixture clearly showed a mass peak at 242, obtained due to C-ring cleavage of the chromene ring to give deoxy benzoin **3.122** as discussed in **Section 3.3.1.2**.



Scheme 3.43

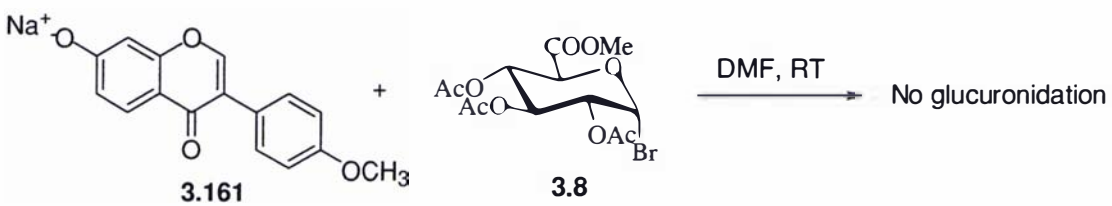
Berrang and co-workers[10] reported that their attempts to prepare morphine glucuronide **3.14** by Koenigs-Knorr or modified Koenigs-Knorr coupling of morphine and bromo sugar **3.8** were unsuccessful. However the reaction of the lithium phenolate derivative of morphine and bromo sugar **3.8** in methanol at RT gave a moderate yield of morphine glucuronide (**Section 3.1.3**). They also reported that the condensation of the potassium salt of morphine in EtOH at RT or under reflux yielded very little if any morphine glucuronide. Using this line of reasoning it was decided to employ this methodology with formononetin and hence the solid bromosugar **3.8** was added to a concentrated solution of slight excess of the lithium salt of formononetin at RT. The

reaction mixture was monitored by TLC which indicated that no glucuronidation was occuring even after storing for seven days at RT.



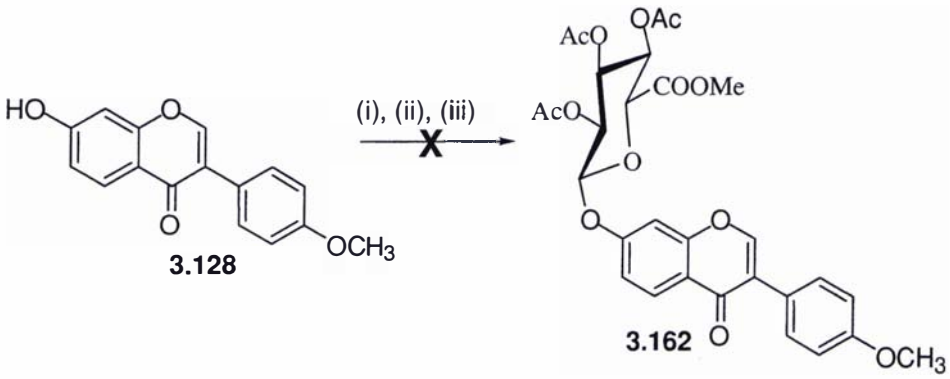
Scheme 3.44

The glcuronidation reaction was also attempted by treatment of formononetin with NaH to obtained the phenoxide anion in the absence of free base and then attack of this nucleophile on bromosuagr **3.8** in the polar solvent DMF. This attempt also proved unsuccessful giving 'glycoseen' (TLC) and unreacted formononetin.



Scheme 3.45

Recently Wahala *et al* [88] reported a glycosidation using a solid/liquid crown ether catalysed phase transfer system in acetonitrile for the synthesis of isoflavone glycosides. Our attempt to utilise this methodology for the coupling of isoflavone and the bromosugar **3.8** also resulted in failure.

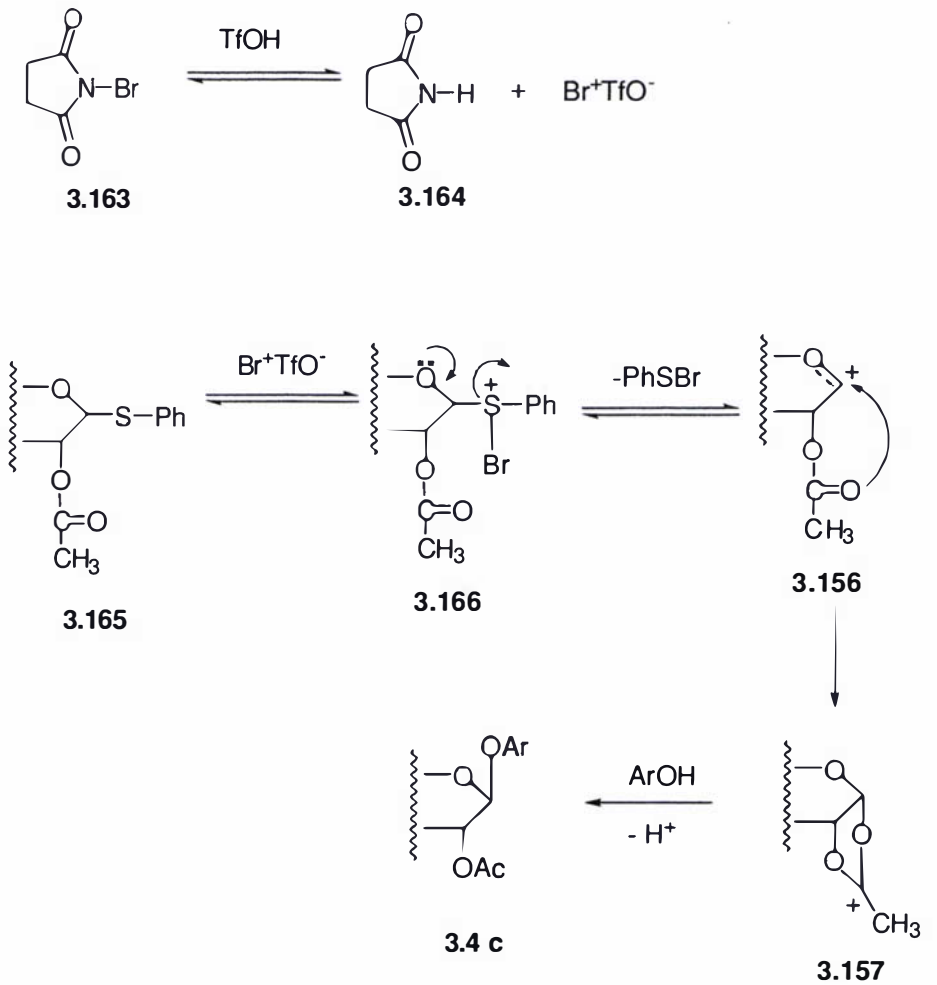


Scheme 3.46

Reagents and Conditions: *(i) t-BuOK, CH_3CN, (ii) 18-crown-6, (iii) Bromosugar (**3.8**), 25 ^0C, 5 h*

3.3.3.4 Thioderivatives As Glycosyl Donors In Glucuronidation Reactions

An attempt was also made to use a more stable glycosyl donor for preparing formononetin glucuronide. Hence the β-phenyl thio glucuronide which was easily prepared from methyl 1-bromo-1-deoxy-2,3,4 tri-*O*-acetyl-α-D-glucopyranuronate **3.8** was chosen. The possible mechanism of the glycosylation reaction using NBS (or NIS)/TfOH as a catalyst is shown in the **Scheme 3.47**.



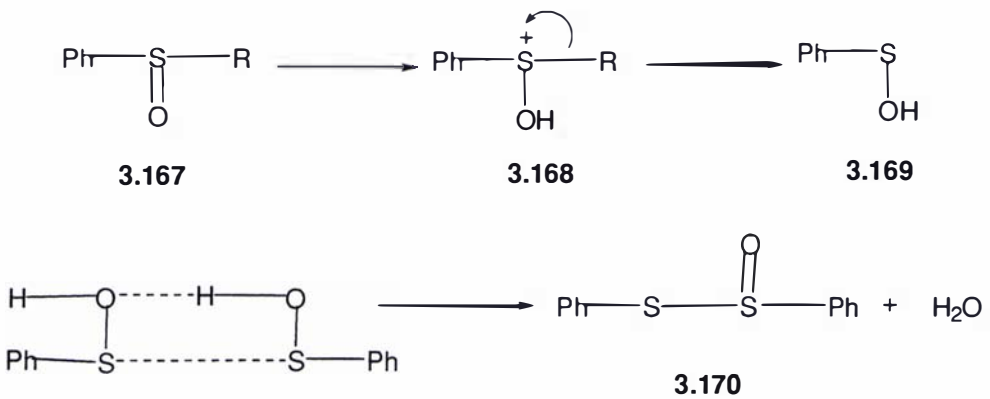
Scheme 3.47

NBS **3.163** reacts with triflic acid to give a more electrophilic bromonium triflate $Br^{+}TfO^{-}$.[89] The reactive intermediate $Br^{+}TfO^{-}$ then activates the thioglucuronide by forming a sulfonium ion **3.166**. The resulting sulfonium ion **3.166** is often fragmented to the oxonium ion **3.157** aided by resonance stabilisation from the oxygen atom. The oxonium ion **3.157** then reacts with the glycosyl donor with the aid of neighbouring group participation as in the Koenigs-Knorr reaction to give the β-glucuronide **3.4c**. In theory, NBS (or NIS) itself can activate thioglucuronides but the rate of the glycosylation reaction with NBS (or NIS) alone would be too slow. The use of NBS/TMSOTf as promoter should increase the reaction rate.

However the coupling reaction was again unavailing giving no product. The low solubility of the isoflavone again being a major problem. The failure of the

reaction was also thought to be due to the poor reactivity compared to the α-bromosugar **3.8** under the present conditions.

The most striking example of the effectiveness of the glycosylation reaction involves glycosylation of unreactive substrates using the more reactive sulfoxide sugars as glycosyl donors (**Scheme 3.9**). Generally, the less the electron density at the anomeric carbon, the faster the glycosylation reactions. The anomeric sulphoxide **3.120** has much less electron density at the anomeric carbon than does the anomeric sulfide **3.119** due to the electron withdrawing nature of the S=O group. Hence a glycosyl suphoxide donor is expected to be more reactive than the glycosyl sulphide analogues towards glycosylation reactions. However, the effectiveness of sulfoxide glycosylation reactions is not only dependent on the glycosyl donor used, but also to a large extent on the promoter system. The promoter system usually involves an activator Tf_2O[28,90], TMSOTf[91] or TfOH[92] and an acid scavenger triethyl phosphite (TEP)[31], 2, 6-di-tert-butylpyridine (DtBP) and 2, 6-di-tert-butyl-4-methylpyridine (DtBMP)[28,90]. Hence the synthesis of formononetin glucuronide using the sulfoxide sugar as a glycosyl donor and TfOH/TEP promoter system was also investigated but no success was achieved.

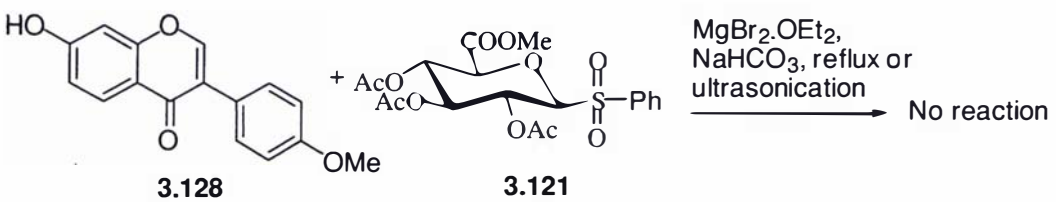


Scheme 3.48

One of the problems with the sulfoxide coupling reaction is that the acid activation of a sulfinylglycoside leads to the release of a highly reactive sulfenic acid species PhSOH **3.169** which undergoes a rapid bimolecular reaction promoted by hydrogen bonding affording the thiosulfinate **3.170** and water which can hydrolyze half of the oxonium ion. The reason that the sulfenic acid **3.169** forms the thiosulfinate **3.170** so easily is that intermolecular hydrogen bonding is particularly effective in

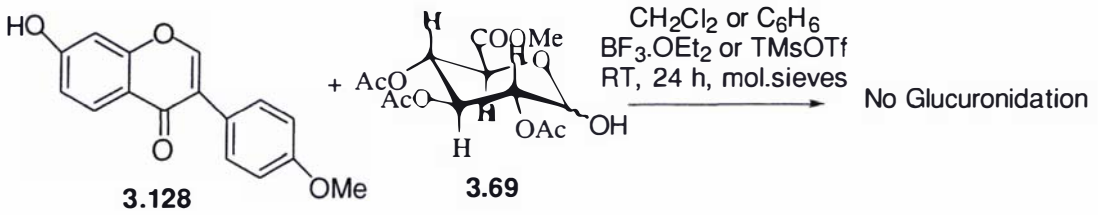
lowering the potential energy or enthalpy of the reaction. Since this reaction can compete efficiently with the glycosylation reaction for the less reactive glycosyl donors, the use of a very efficient acid scavenger which is able to react with the sulfenic acid is essential. Probably this is the reason why the coupling reaction of formononetin and sulfoxide **3.120** failed to give any glucuronide conjugate. The method worked for *p*-cresol but gave unacceptably low yield of glucuronide with formononetin (if any).

The anomeric sulphone sugar **3.121** is an even more reactive glycosyl donor than both the sulfide and sulfoxide in most glycosylations because of strong electron withdrawing effect at the anomeric carbon by the sulfonyl group (O=S=O). Furthermore the sulfone group is a well known leaving group in organic chemistry and can be prepared easily by oxidation of the corresponding sulfide. For these reasons even though a literature survey reveals no glycosylation method for phenols using this glycosyl donor an attempt was also made to use the sulfone sugar in the glycosylation reaction with formononetin (**Scheme 3.49**). However, this method also failed leaving the starting materials completely unreacted.



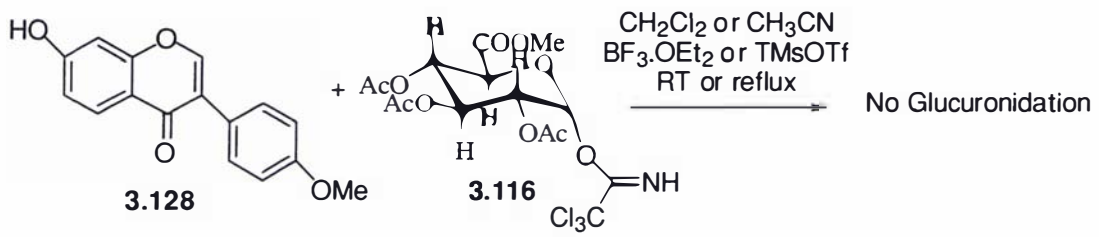
Scheme 3.49

The literature also reveals the use of the 1-hydroxy sugar **3.69** for glucuronidation using Mitsunobu coupling when Koenigs-Knorr reactions completely failed or gave very poor yields.[37] the more acidic *p*-Br and *p*-NO_2 phenol glucuronides were obtained using the 1-hydroxy sugar (**Section 3.1.6**) in a moderate yield.[1] Along this line formononetin was reacted with the 1-hydroxy sugar under Mitsunobu coupling conditions but again the reaction failed completely availing no glucuronide formation.



Scheme 3.50

With all our efforts at direct coupling with glucuronide derivatives having been in vain it was decided finally to try the recently discovered trichloroacetimidate glycosyl donor **3.116**. The donor properties of the trichloroacetimidate for many aglycones are reported to be superior to the donor properties of sugar derivatives used in the Koenigs-Knorr and Fischer-Helferich reactions.[83] Hence formononetin was reacted with the acetamidate sugar **3.116** in CH_2CI_2 in the presence of promoters $BF_3.OEt_2O$ and TMSOTf. The glycosylation reaction was also tried in CH_3CN but completely without any success. When possible unreacted formononetin was recovered by short column chromatography from these unsuccessful reactions.



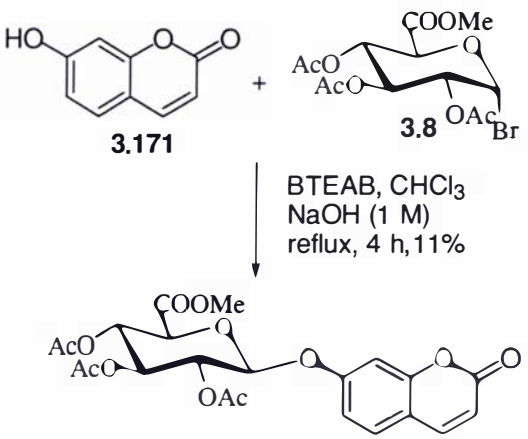
Scheme 3.51

3.3.4 Investigation Of The Bromosugar As Glycosyl Donor In The Synthesis Of Isoflavone Glucuronide Using A Phase Transfer Catalyst.

All the preceding reactions involve either an acid or Lewis acid as a reagent thus having the potential to form oxonium ions with the chromene ring oxygen atom in the isoflavones or isoflavanes. Hence the electron density on the nucleophilic oxygen (the 7-OH group) is much reduced resulting in very poorly nucleophilic aglycones. Successful synthesis of the glucuronide by direct reaction with glucuronide derivatives thus need basic or neutral reaction conditions. However even the lithium salt of formononetin was unreactive. On the other hand the use of phase transfer catalysts for

the synthesis of 2,3,4,6-tetra-*O*-benzyl-β-D-glucopyranosides and 2,3,4,6-tetra-*O*-acetyl-glycosides is well documented for aromatic phenols.[6,7] In the phase transfer catalysed procedure either CH_2Cl_2 or $CHCl_3$ was used as the organic solvent to contain the bromo sugar **3.8**. The isoflavone daidzein or formononetin, was dissolved in a slight excess of alkali thus generating the phenoxide anion as the nucleophile. Phase transfer catalysts such as n-tetrabutyl ammonium bromide (TBAB) or benzyl triethyl ammonium bromide (BTEAB) were used to transport the anionic aglycone nucleophile into the organic phase. It was hoped that the reaction product being lipophilic would distribute itself predominantly into the organic phase and consequently be protected from hydrolytic cleavage by the residual alkali.

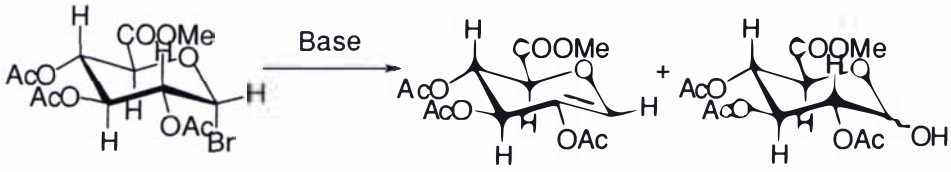
It has been reported that various attempts to synthesise aryl-β-D-glucuronides using phase transfer conditions[6] under similar conditions failed but recently Walsh et al reported the first successful synthesis of an *O*-glucuronide using a phase transfer catalyst.[85] A literature survey reveals no other sucessful *O*-glucuronidation reaction using phase transfer catalyst conditions. The aglycone in this reaction is apparently electronically analogous to an isoflavone. In this reaction 7-hydroxy coumarin was reacted with 1 M NaOH and coupled with methyl 2,3,4-tri-O-actetyl-1-bromo-1-α-D-glucopyranuronate in $CHCl_3$ using BTEAB (**Scheme 3..52**) to obtain 7-hydroxy coumarin glucuronide in poor yield (11%). There was no explanation given for the poor yield in the article.



3.171 **3.8** **3.172**

Scheme 3.52

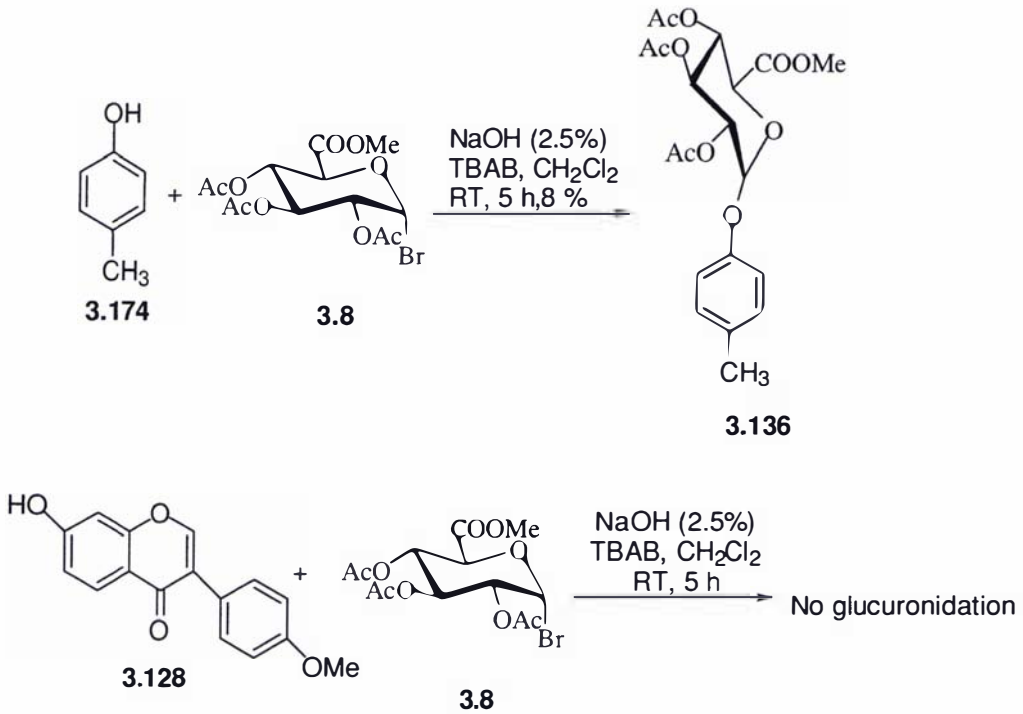
Unfortunately an adaptation of this procedure for the isoflavone daidzein, formononetin failed in the present work to give any glucuronide conjugate. The persistent by product observed from the reactions was the "glycoseen" or 2-acetoxy glycal **3.177** formed by HBr elimination of the bromosugar and not the desired glycosylated product. This is a facile reaction because of the *trans* arrangement of the β-hydrogen (on C3) and the 1-bromo substituent. This is consistent with the work reported by Dess *et al*[6] that β-elimination is a common side reaction in the gluco series. The TLC analysis of the crude reaction mixture also indicated hydrolysis of the bromosugar to form **3.69**.



3.8 **3.173** **3.69**

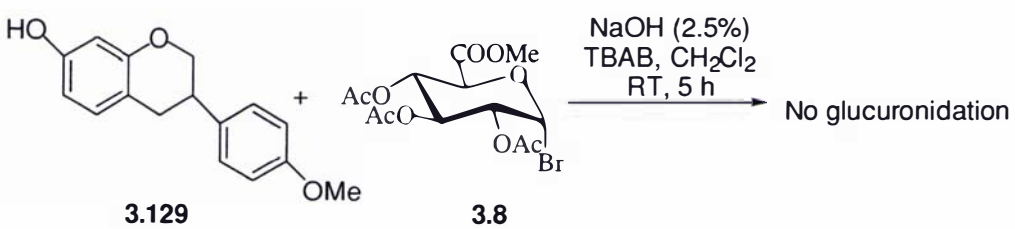
Scheme 3.53

The glucuronidation reaction was also attempted under the phase transfer conditions reported by Wahala and co-workers for isoflavone glycosides (**Chapter 4, Section 4.3.2**) but resulted in no glucuronidation and essentially the same side reactions. Numerous attempts to glycosylate the formononetin using phase transfer catalysts by varying reaction conditions such as more reaction time and reflux conditions also failed to give any glucuronide product. Even when the phase transfer reaction of the simple phenol *p*-cresol and methyl 2,3,4-tri-*O*-actetyl-1-bromo-1-α-D-glucopyranuronate was tried under essentially the same conditions an unacceptably low yield (8%) of glucuronide conjugate **3.136** was obtained.



Scheme 3.54

Large quantities of the protonated nucleophilic isoflavones were recovered unchanged from these reactions. This protonation was attributed to the HBr liberated during dehydrohalogenation of the bromosugar reprotonating the anionic form of the aglycon. Similarly all the attempts to obtain methoxy equol glucuronide using phase transfer catalysed conditions also resulted in failure (**Scheme 3.55**).



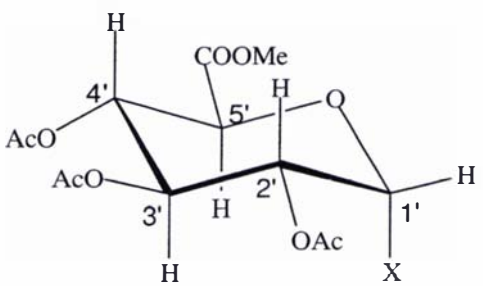
Scheme 3.55

The success of the phase transfer catalyst approach for promoting reactions between isoflavones and 2,3,4,6-tetra-*O*-acteyl-glycosides (**Chapter 4, Section 4.3.2**) and the failure of the same approach under essentially the same conditions to produce isoflavone glucuronides from isoflavones and methyl 2,3,4-tri-*O*-actetyl-1-bromo-1-α-D-glucopyranuronate implies that the procedure requires fully etherified glucopyranosyl

derivatives or at least a glucopyranosyl derivative with non-participating groups at C-2 and C-6.

3.3.5 General Characterisation Of Sugar Derivatives And Aromatic Glucuronides By NMR Spectra

The correct stereochemistry (α or β) of all the protons on the glucuronic acid ring (**Figure 3.11**), especially the 1'-proton is important. The stereochemistry of the conjugate glucuronide ester is also important as it would be essentially the same stereochemistry as the hydrolysed product, glucuronic acid. The success of the glycosylation reactions can also be sometimes determined by knowing the correct stereochemistry of the parent sugar derivative. For example the β-tetra acetate sugar **3.4** couples to the aglycone in the perester coupling method while the α-anomer does not react or gives a poor yield. More importantly the aryl glucuronide conjugates excreted from the body as xenobiotic molecules essentially possess the β-configuration. Hence for the purpose of synthesising standards it is usually desirable to synthesise the β-D-glucuronide ester. In this chapter, the basic structure of the glucuronide ester derivatives was confirmed on the basis of their ^{1}H NMR spectra. (see **Table 3.4**). The structure of the aryl glucuronide esters was then also confirmed by ^{13}C NMR spectra (see **Table 3.5**).



3.175

X = Leaving group or Ar

Figure 3.11 The structure of the glucuronide esters and the numbering system of the protons on the ring

The chemical shifts of the sugar portion of the different glucuronide derivatives were calculated from the ^{1}H NMR spectra and are collected in the **Tables 3.4 and 3.5**.

Table 3.4 The chemical shifts of the protons on glucuronide ester of the sugar derivatives

Sugar derivatives	Chemical shift of protons in δ (ppm)				Coupling constants of protons in J (Hz)		
	1'	2' 3' 4'		5'	$J_{1'2'}$	$J_{2'3'}$ $J_{3'4'}$	$J_{4'5'}$
Tetraacetate **3.4**	5.77	5.12-5.16		4.18	7.69	>9.2	9.16
Bromosugar **3.8**	6.65	4.85 5.24 5.62		4.58	4.03	>9.5	10.3
Thiosugar **3.119**	4.74	4.97-5.28		4.58	10.1	>9.2	9.7
Sulfoxide sugar **3.120**	4.54	4.91-5.33		4.04	9.7	>9.2	9.9
Sulfone sugar **3.121**	4.58	4.99-5.43		4.14	9.9	>9.2	9.9
Imidate sugar **3.116**	6.62	5.12-5.30	5.62	4.48	3.52	>9.5	10.3

Table 3.5 The chemical shifts of the protons with coupling constant and ^{13}C NMR value on C1 of aryl glucuronide esters

Aryl tri-O-acetyl glucuronide ester (Aglycone)	Chemical shift of protons in δ (ppm)			Coupling constants $J_{1'2'}$ (Hz)	^{13}C NMR $C_{1'}$ value ppm
	1'	2' 3' 4'	5'		
p-Cresol	5.05	5.11-5.35	4.15	7.25	99.5
m-Cresol	6.13	5.23-5.41	4.16	7.00	99.1
3,4-Dimethyl phenol	5.08	5.21-5.31	4.58	7.25	99.0
3,5-Dimethyl phenol	5.12	5.22-5.42	4.04	7.25	99.0
p-Bromophenol	4.58	5.24-5.36	4.14	7.03	99.0
m-Bromophenol	5.14	5.23-5.39	4.48	7.03	98.8

From **Table 3.4**, it is possible to assign unambiguously the 1'- and 5'-protons of all the glucuronides. Because the anomeric carbon (1'-C) is linked directly to two electron withdrawing atoms (an oxygen and a bromine atom for the α–bromosugar **3.8**, two oxygen atoms for the imidate sugar **3.116** and aryl glucuronide esters, an oxygen and a sulfur atom for the thioglucuronides **3.119**, sufoxide **3.120** and sulfone **3.121**, the anomeric proton (1'-H) is strongly deshielded. Thus it has a higher chemical shift value than that of the 5'-proton (5'-H) as carbon-5' is linked to only one electron withdrawing oxygen atom. The 1'- and 5'- protons can also be distinguished from the 2'-, 3'- and 4'-protons on the basis of their splitting patterns. The 1'- and 5'-protons both appear as doublets in the NMR spectrum due to splitting by the single neighbouring proton (2'-H for 1'-H, and 4'-H for 5'-H) while the 2'-, 3'- and 4'-protons appear as double doublets or triplets caused by splitting from two neighbouring protons (1'- and 3'-H for 2'-H, 2'- and 4'H for 3'-H, 3'- and 5'-H for 4'-H).

The anisotropy effect[93] explains the lower chemical shifts of the anomeric proton (1'-H) of the sulfoxide sugar **3.120** (4.54 ppm) and sulfone sugar **3.121** (4.58 ppm) compared with the thiosugar **3.119** (4.74 ppm) despite the evidently lower electron density at the sulfoxide sugar **3.120** and sulfone sugar **3.121** anomeric protons. The sulfoxide and sulfone anomeric protons are subject to the anisotropy effect of the S=O group which causes a upfield shift because the hydrogens are partially shielded. The same phenomenon was also reported in the literature.[94,95]

Since the 2'-, 3'- and 4'-carbon atoms are directly connected to the strong electron withdrawing acetate groups, the 2'-, 3'- and 4'- protons appear at lower field strength compared with the 1'- and 5'-protons in thiosugar derivatives. While in the tetracetate **3.4**, α-bromosugar **3.8** and imidate **3.116** sugar due to a strong electron withdrawing acetate group, bromine group and acetimidoyl group respectively the 1'-proton is substantially deshielded. Hence, the 1'-proton in these sugar derivatives has a much higher chemical shift value than 2'-, 3'- and 4'- protons. The conformation of the 1'- and 5'- protons of the glucuronide ester ring can be also confirmed by the coupling constants for all the neighbouring protons. The small coupling constant of the 1'- and 2'- protons for the bromosugar **3.8** ($J_{1'2'}$ = 4.03 Hz) and imidate sugar ($J_{1'2'}$ = 3.97 Hz) indicates that the 1'-proton is in a *cis*-axial-equatorial relationship (α-orienation) to the vicinal 2'-proton. The *trans*-diaxial relationship (β-orientation) of the anomeric protons

of the tetraacetate sugar **3.4**, thioglucuronides **3.119** to **3.121** and aryl glucuronide ester derivatives were demonstrated by the large coupling constant of the 1'- and 2'-protons ($J_{1'2'}$>9.7 Hz for thioglucuronides). However, the electronegative oxygen atoms of the tetraacaetatesugar and aryl glucuronide ester derivatives reduced the magnitude of the coupling constants ($J_{1'2'}$ = 7.69 Hz for tetraacetatesugar **3.4**, and $J_{1'2'}$>7.20 Hz for aryl glucuronide ester). The other protons (2'-, 3'- and 4'-H) of the glucuronic rings of the glucuronide esters appear to be all in the *trans*-diaxial relationship due to the large coupling constants for all of the neighbouring protons ($J_{2'3'}$, $J_{3'4'}$ and $J_{4'5'}$ 9.2 Hz).

Moreover examination of the acetylated D-glucuronide esters revealed different spectral patterns for the α and β anomers. Thus in the α-anomer the H-2, H-3 and H-4 protons appeared in the range of 1.0 ppm where as the β-anomer exhibited a quite different pattern and the H-2, H-3 and H-4 protons appeared within the narrow range of 0.5 ppm. Hence, this spectral pattern can also prove to be quite useful to assign the anomeric configuration of the glucuronide series.

The anomeric carbon atom resonance of the β-anomer is always observed at a lower field than the α-anomer (~6 ppm).[96] This was confirmed by the fact that the anomeric carbon atom C-1' in the α-tricholoacetimidate sugar appeared at 92.5 ppm where as in aryl glucuronide anomeric carbon atom C-1' appeared at ~99.0 ppm (see **Table 3.5**).

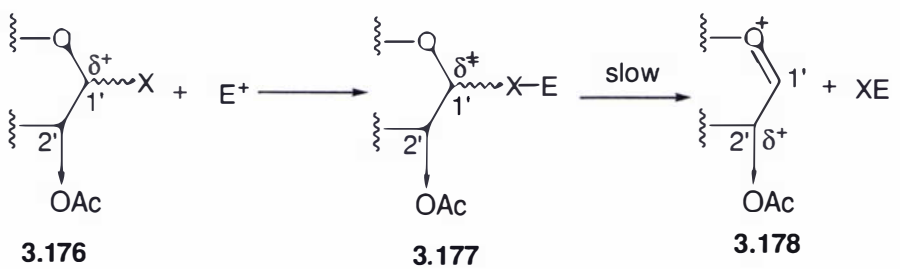
The protons H1' to H5' and aromatic protons in the aryl glucuronide ester derivatives were assigned unambiguously by ^{1}H-^{1}H 2D COSY spectra. For simple phenol glucuronide esters methyl (*p*-tolyl)-2', 3', 4'-tri-*O*-acetyl-glucopyranosiduronate **3.136** and methyl (*m*-tolyl)-2', 3', 4'-tri-*O*-acetyl-glucopyranosiduronate **3.137**, ^{1}H-^{1}H 2D COSY spectra are shown in the **Figure 3.14** and **Figure 3.16**. The five protons H1' to H5' were assigned based on the correlations of H1' (doublet, 5.05 ppm) and H5' with H2' and H4' (multiplet, 5.11-5.35 ppm) respectively. The correlation of the H2', H3' and H4' with each other was hidden due to all three protons appearing in the same region (5.11-5.35 ppm). The corelation of aromatic protons was also obvious in the spectrum. In addition HETCOR experiments were also carried out to support the assignment of the peaks for protons and carbons. The peaks for carbons were also easily recognisable as identified protons of the glucuronide esters were directly

correlated to respective carbons as shown in HETCOR spectrum **Figure 3.15** and **Figure 3.17**.

3.3.6 Summary

In summary, various aryl β-D glucuronide esters have been successfully synthesised as described in this chapter using the lewis acid catalyst $BF_3.OEt_2$ and Koenigs-Knorr conditions using $CdCO_3$. Even though some of the aryl glucuronide ester have been prepared before using other glycosylation reactions, it is the first time the newer synthetic route of $BF_3.OEt_2$ has been used for these phenols. The failure to effect glycosylation reactions with isoflavones and their metabolites with various glycosyl donor can be attributed to the following factors.

(i) The β –selective glycosylation reaction requires a neighbouring group (acetoxy or benzoxy group) at the C-2' position of the glucuronic acid ring to assist with the formation of the 1, 2-*trans*glucuronide. However, glycosyl donors possessing such groups with electron withdrawing properties at the C-2' position were found to be much less reactive.[18,97] Ester groups inductively disfavour activation and/or ejection of the leaving group at the anomeric carbon (1'-C) of the glucuronic acid ring. The main reason for deactivation of the sugar with respect to nucleophilic attack is due to the instability of the intermediate oxonium ion caused by a neighbouring positive charge resulting from an electron withdrawing group at the C-2' position (**Scheme 3.56**).[98]

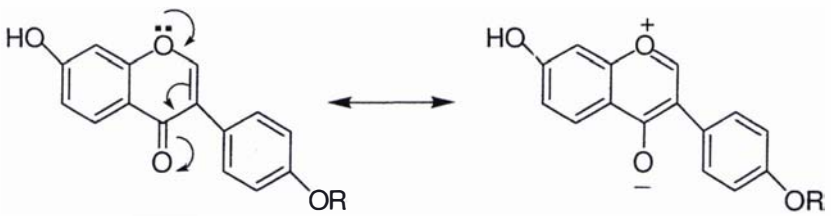


3.176 **3.177** **3.178**

Scheme 3.56

(ii) The presence of the chromene ring means that the isoflavones have a large degree of dipolar character with the oxygen atom of the chromene ring bearing a considerable amount of positive charge due to withdrawal of electrons by an adjacent

carbonyl group . This has a large deactivating effect on the 7-OH group causing it to function as poorer nucleophile.



3.179 **3.180**

Figure 3.12

(iii) The poor solubility of isoflavones and their metabolites in the non polar solvents (toluene, THF, CH_2Cl_2) required for the coupling reaction and the partial solubility in polar solvents at RT (MeOH, DMF) is also a major factor for the failure of this glycosylation reaction.

(iv) Acid and base sensitivity of the chromene ring of isoflavones also limits the applicability of various glycosylation methods by restricting the nature of the catalyst which can be used.

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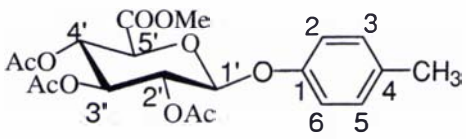
Figure 3.14 COSY Spectrum Of **3.136**



3.136

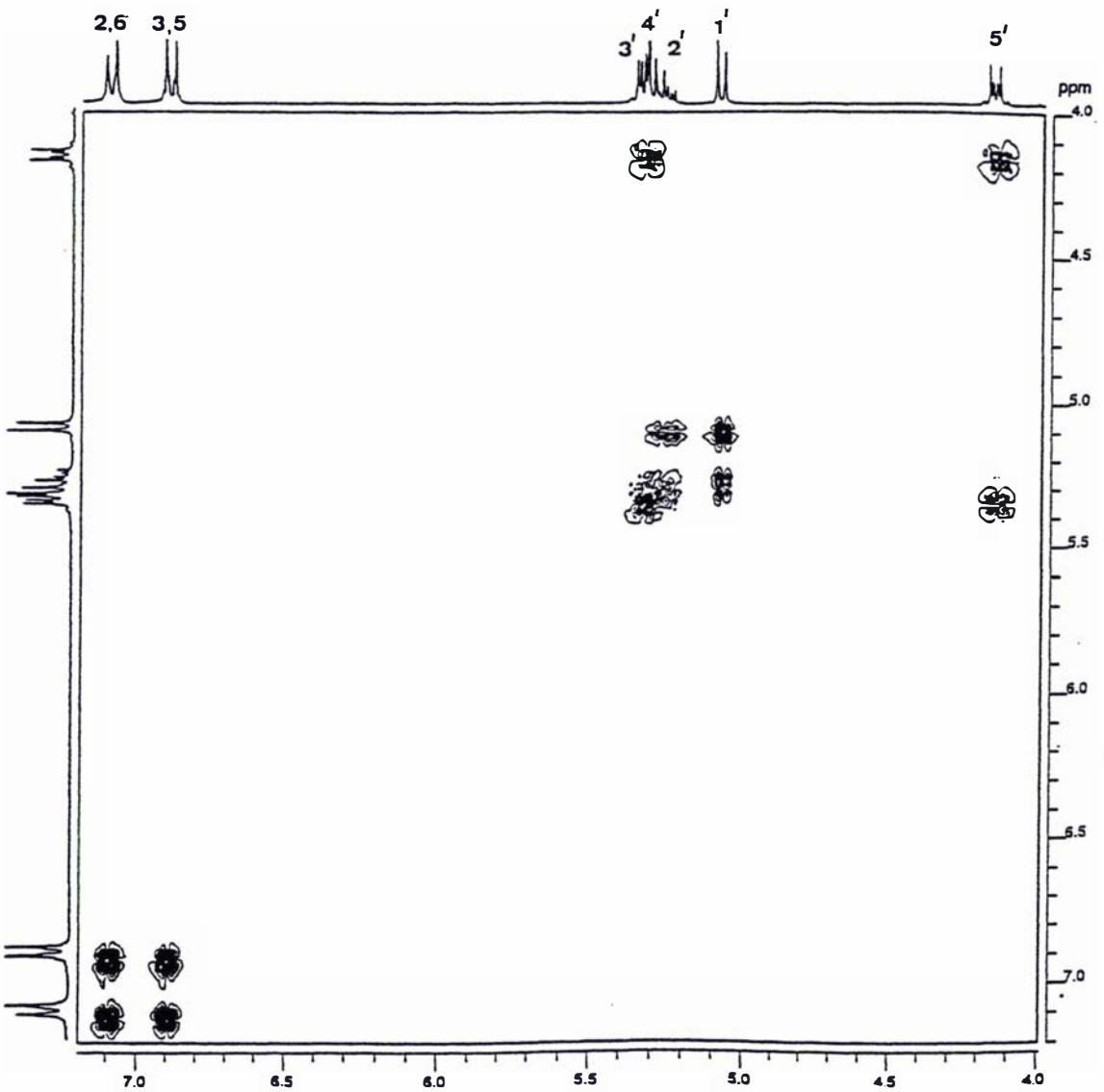
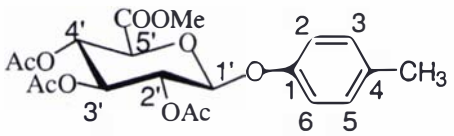


Figure 3.15 HETCOR Spectrum Of **3.136**



3.136

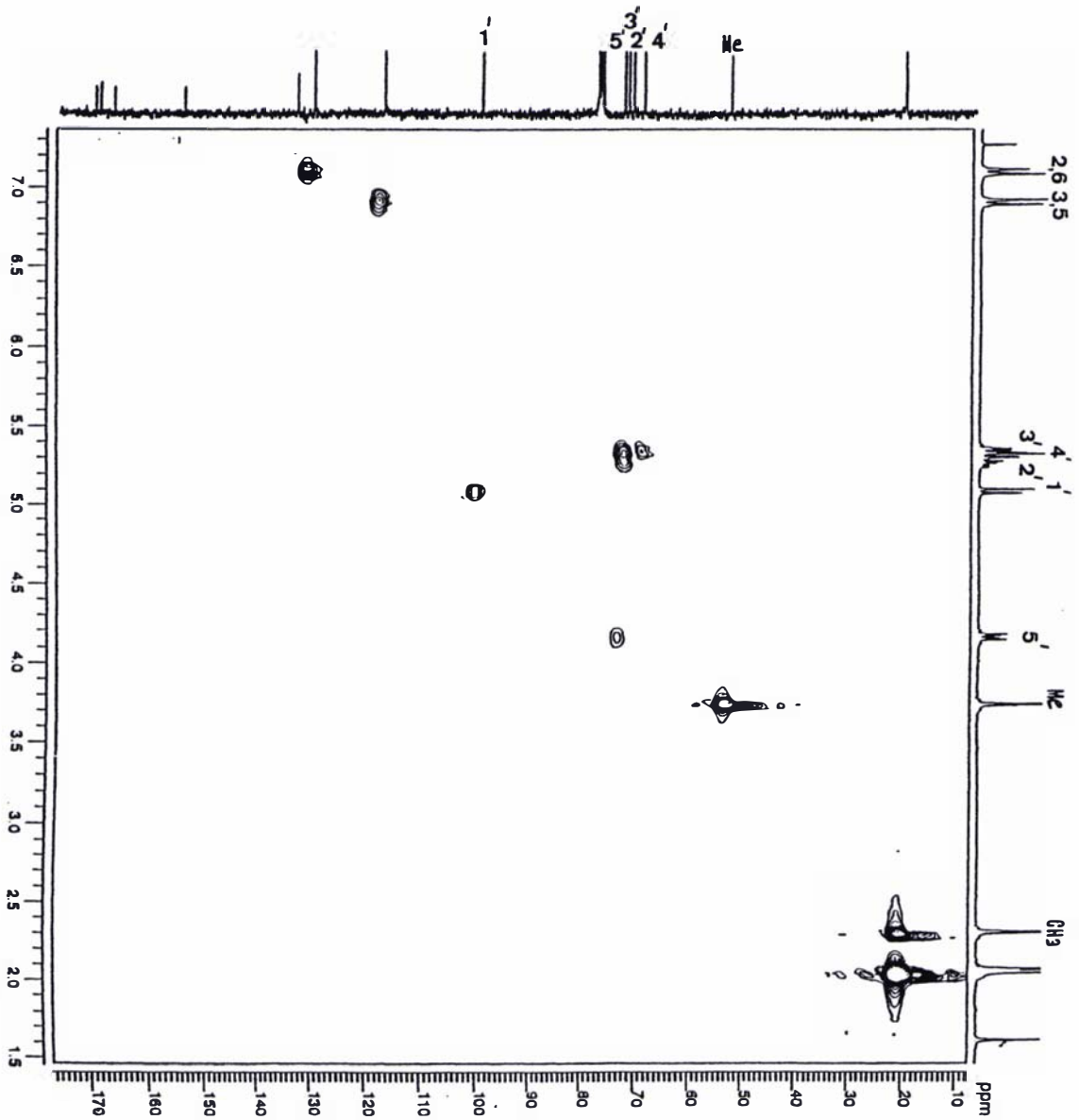
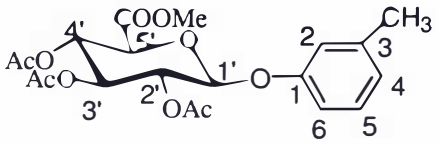


Figure 3.16 COSY Spectrum Of **3.137**



3.137

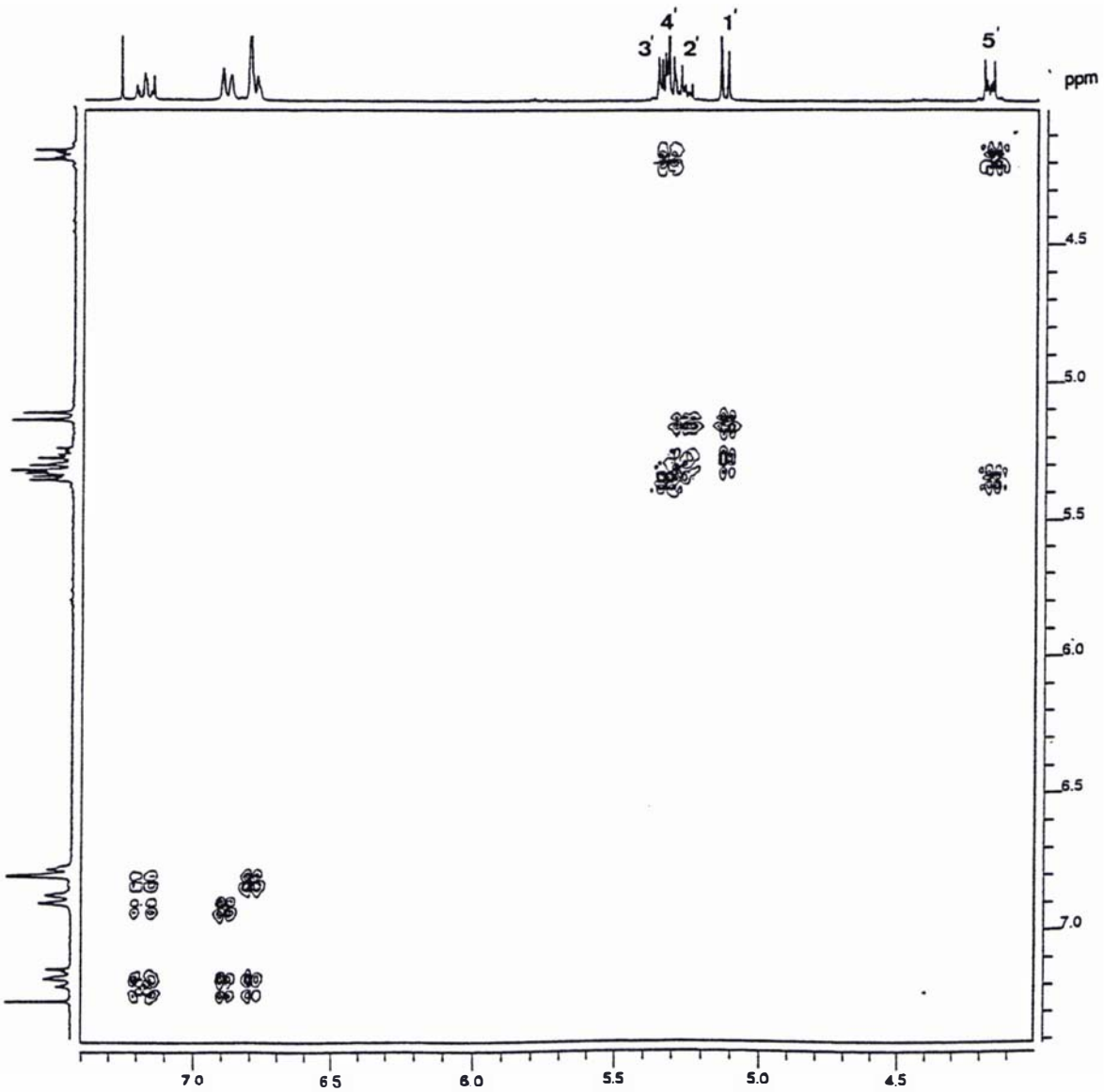
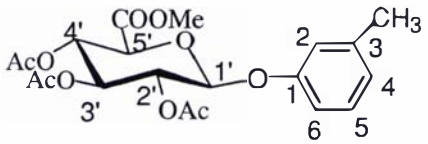
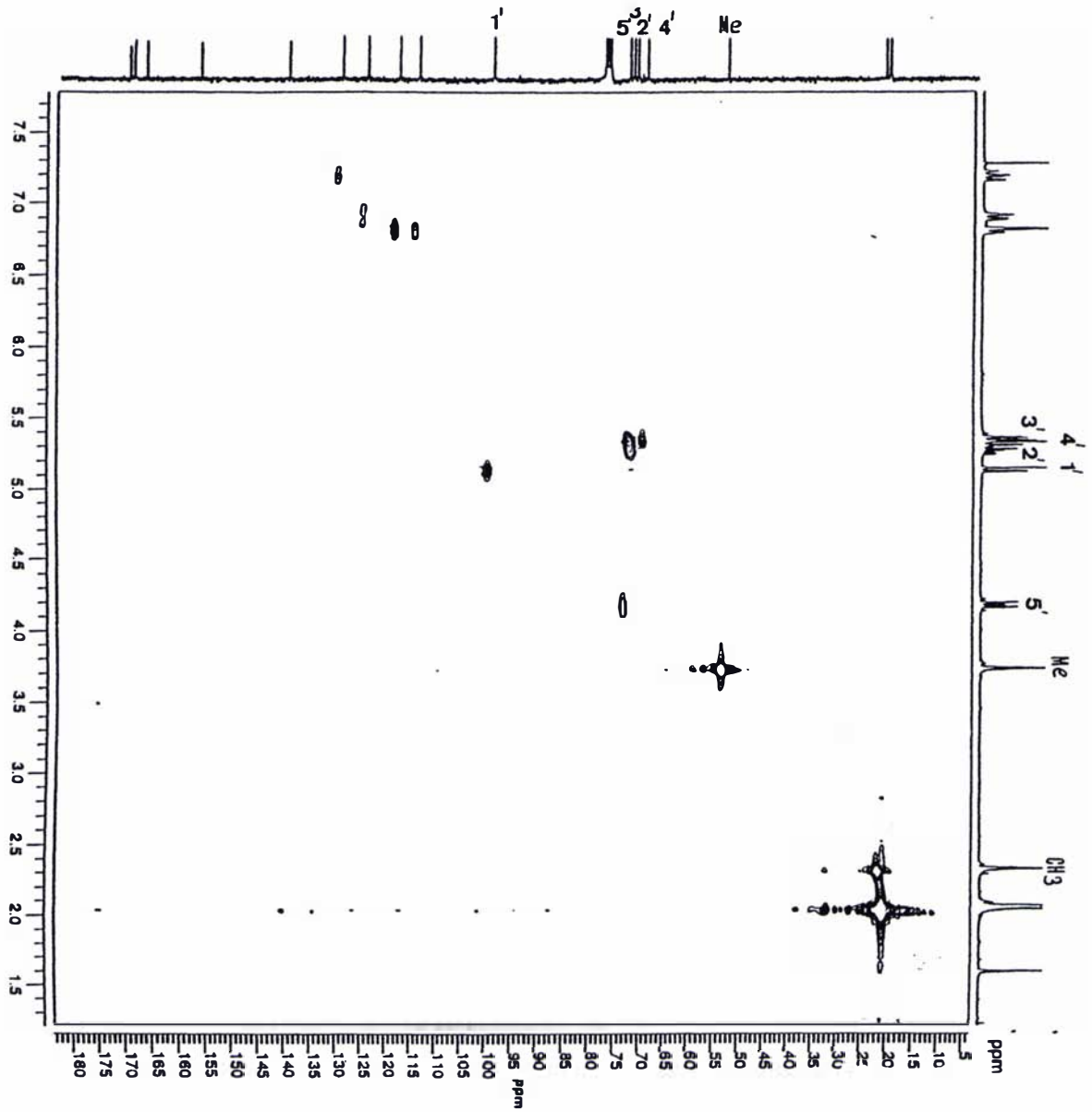


Figure 3.17 HETCOR Spectrum Of **3.137**



3.137



CHAPTER 4 SYNTHESIS OF β-D-GLUCURONIDES USING TEMPO MEDIATED SELECTIVE OXIDATION AND ENZYME CATALYSED GLUCURONIDATION

4.1 INTRODUCTION

Oxidations of alcohols to obtain aldehydes, ketones or carboxylic acids are fundamental transformations in synthetic organic chemistry. Many reagents are known for these conversions, the most well known being the mild non aqueous variants such as pyridinium chlorochromate (PCC)[1], pyridinium dichlorochromate (PDC)[2] and dipyridine chromium(VI) oxide[3] and the more aggressive chromium(VI) oxide oxidants.[4] In modern synthetic organic chemistry there is still a huge demand for mild and selective reagents for the oxidation of alcohols in the presence of other oxidisable groups. Several successful mild oxidation reagents such as Swern reagents[5], Dess-Martin periodinane[6] and tetra propyl ammonium perruthenate[7] have been developed to give high yields of carbonyl compounds from primary and secondary alcohols. However regio selective oxidation of one alcohol group in the presence of others is generally not feasible. In particular the selective oxidation of primary alcohols in the presence of secondary ones is a still difficult conversion in organic synthesis.[8]

The usefulness of NO_2 (N_2O_4) in preferential oxidation of primary alcohol groups in carbohydrates to obtain uronic acids was first realised by Maurer and Drefahl[9] and Yackel and Kenyon[10], more than a half a century ago in 1942. Later on Painter[11] reported a modified procedure for the oxidation of polysaccharides to polyuronic acids which involved the *in situ* generation of nitrogen oxides. Apart from the selective oxidation of the primary alcohol functions, substantial degradation of the polymer and nonselective oxidation products were obtained also.

4.1.1 Nitroxyl Radicals As Selective Oxidants

Nitroxyl radicals are compounds containing the N, N-disubstituted NO-group with one unpaired electron.[12] The substituents may be organic or inorganic.

Conjugated organic nitroxyl radicals, in which the unpaired electron is delocalised over the entire molecule have been known since the beginning of this century. A well known example of the later class is Fremy's radical which can be used as an oxidant for aromatic hydroxy compounds.[13] An example of this type of reagent is the diphenyl nitroxyl radical **4.1**.[14] Since conjugated nitroxyl radicals in contrast to non conjugated nitroxyl radicals, are not used for the oxidation of alcohols, they will not be discussed here. Non conjugated stable di-tert-alkyl organic nitroxyl radicals were first prepared by Lebedev and Kazarnorskii[15] in 1960 **4.2** and by Hoffman and Henderson[16] in 1961 **4.3**. The unpaired electron in these radicals is only delocalised over the nitrogen-oxygen bond, however their high stability is demonstrated by the fact that these were the first radicals that underwent chemical reaction involving no unpaired electron.

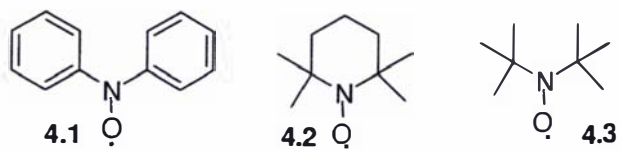


Figure 4.1

A large variety of radicals of this kind have been synthesised since, with important applications in biological fields.[17] In general, with only few exceptions these radicals are only stable when lacking α-hydrogens.[12] If one or more hydrogens are present in the α position the radical typically undergoes a disproportionation reaction leading to a hydroxyl amine **4.5** and nitrone **4.6** either or both of which may under go further reaction.[18]

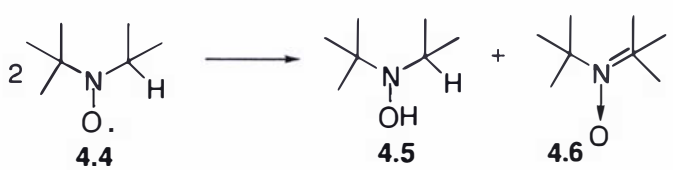
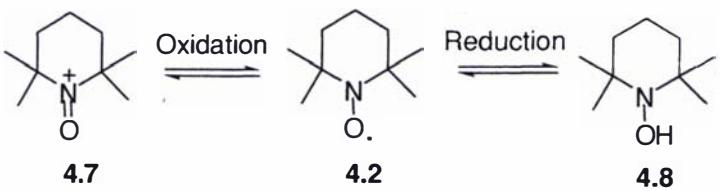


Figure 4.2

The first non conjugated nitroxyl radical to be synthesised was 2, 2, 6, 6-tetramethyl piperidin-1-oxyl **2**, more commonly known as TEMPO. Since then many

analogues have been prepared mainly from their common precursor 2, 2, 6, 6-tetramethyl-4-piperidone.[12]

It has been well established that stable organic nitroxyl radicals can be applied as mediators for the oxidation of primary and secondary alcohols. Several oxidants are able to oxidise the nitroxyl radical **4.2** to obtain the corresponding nitrosonium ion (oxyammonium salt) **4.7** which is the actual oxidant. During TEMPO oxidation the nitrosonium ion gets reduced to the corresponding hydroxyl amine **4.8** which thus needs re-oxidation for the cycle to continue.

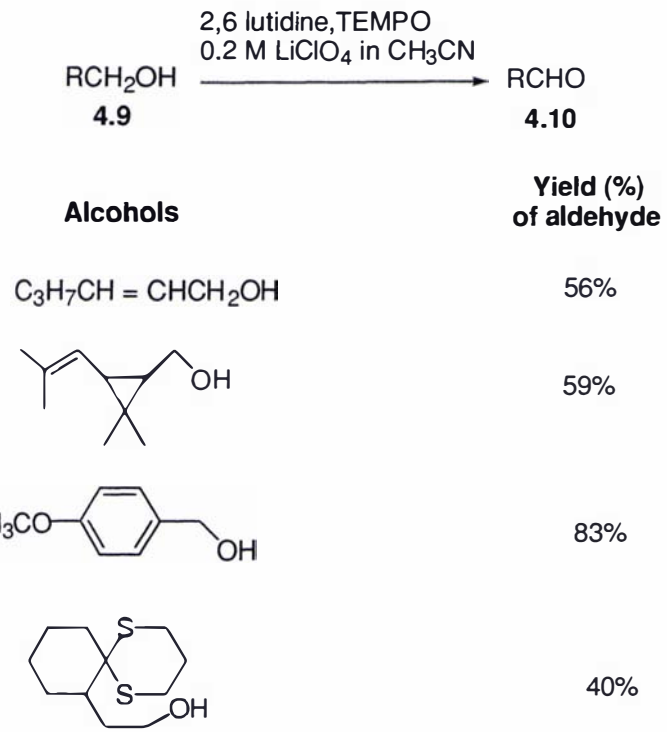


4.7 **4.2** **4.8**

Scheme 4.1

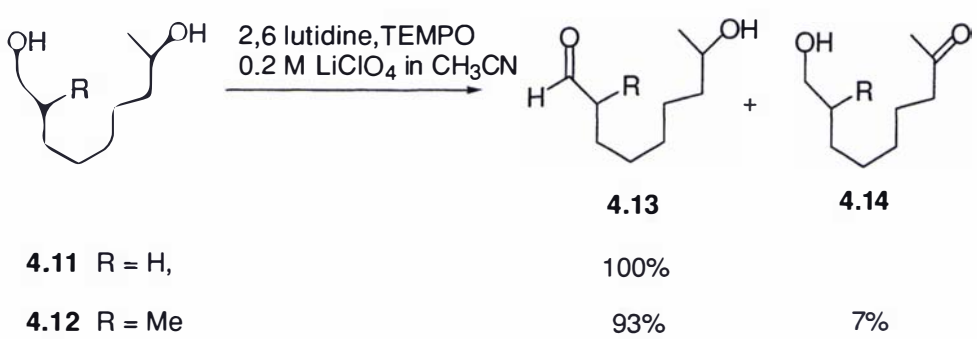
The nitrosonium ion can be used in stoichiometric amounts, however its high chemical reactivity generally hinders its isolation and purification. Fortunately radical **4.2** can also be used in catalytic amounts as a mediator which is oxidised *in situ* and regenerated by a second oxidant.[11]

A promising method for the selective oxidation of primary alcohols in the presence of secondary ones originates from the work by Semmelhack *et al*.[19] These authors used TEMPO as a mediator in the electro oxidation of alcohols to obtain aldehydes and ketones.



Scheme 4.2

The process occurs at low potential (ca. 0.4 V vs $Ag/AgNO_3$), with no tendency for over oxidation of aldehydes to acids. They also demonstrated a strong tendency for oxidation of primary alcohols over secondary ones using the same reagents and conditions.



Scheme 4.3

Only recently the use of organic nitroxyl radicals has been introduced into sugar chemistry for the selective oxidation of primary alcohols.[20,21,22]

4.1.1.1 The Mechanistic Aspects Of The Oxidation Of Alcohols

Although considerable effort has been made to elucidate the mechanism of the reaction between oxo ammonium salts and an alcoholic substrate, the details are still unclear. There are three different mechanistic pathways so far proposed in the literature i) a Cope like mechanism, ii) direct hydride abstraction and iii) a radical mechanism.

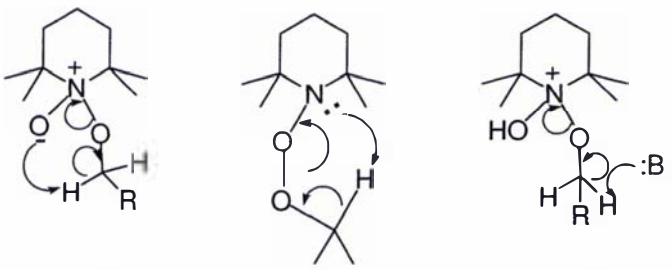
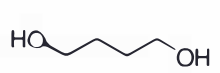
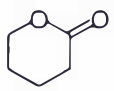
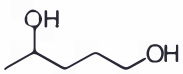
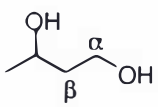
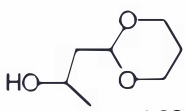
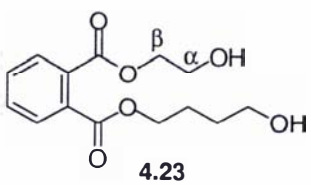
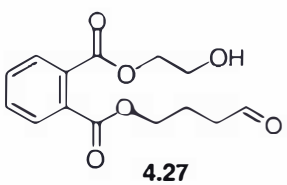


Figure 4.3

In an investigation towards understanding the mechanism Semmelhack and co-workers[19] favored the adduct **4.16** as a model for the transition state which undergoes a Cope like cyclic elimination. They excluded a radical mechanism as well as direct hydride abstraction. Although the site of the addition of the oxygen nucleophile from the alcohol to the N atom of TEMPO has not been established directly the Russian group has provided evidence for the addition of hydroxide (water) at the nitrogen of TEMPO through isotope exchange studies.[23] Overall the adduct **4.16** is nicely supported by the data but adduct **4.17** (by addition of the primary alcohol group to the oxygen of TEMPO) could not be ruled out. The high selectivity for less crowded hydroxyl groups can also be understood based on adducts **4.16** or **4.17**.

Ma and Bobbit[24] proposed adduct **4.18** which would undergo an acyclic concerted elimination, based on the fact that the acyclic form is probably less sterically confining in the transition state than the cyclic forms and indeed they found few steric effects in the TEMPO mediated oxidation of alcohols in contrast to Semmelhack *et al.*[19] Secondly they found that a β-oxygen functionality in the alcohol substantially inhibited the oxidation reaction. For example ethylene glycol, was completely unreactive while 2-phenoxy ethanol reacted slowly and incompletely. Endo and Yamaguchi *et al* [25] also investigated several diols in accord with previous studies and they pointed out that

alcohols having a β-oxygen are not oxidized by oxoammonium salts and those with a γ oxygen only do so slowly. Hence oxidation of diols with the oxoammonium chloride salt yielded lactones (**4.24** to **4.27**), most likely via an intermediate hemi acetal. Butane 1,3 diol **4.22** was oxidised to give a quantitative yield and the ester-alcohol **4.23** gave a new kind of selectivity for the oxidation of diols.

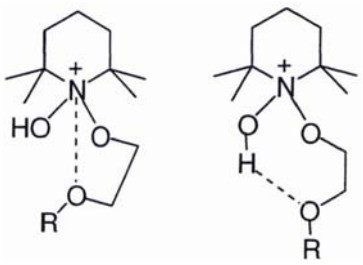
Alcohol	Product	Yield (%)	
		Chromatography	Isolated
 **4.19**	 **4.24**	100	81
 **4.20**	 **4.25**	61	40
 **4.21**	 **4.26**	83	
 **4.22**	 **4.28**	100	61
 **4.23**	 **4.27**	81	46

***Reagents and conditions**: Oxoammonium chloride (1.2 equiv.), CH_2Cl_2, Na_2CO_3, 0 ^{0}C-RT, Ar*

Scheme 4.4

Intramolecuar hydrogen bonding was ruled out as an explanation for this decreased reactivity. In a probable explanation Ma and Bobbit[24] suggested that a complex formed between the positive nitrogen and the β-oxygen as shown in **4.29** might be responsible for this decreased reactivity. Another perhaps more probable β-oxygen interaction was shown by Nooy *et al* [26] as illustrated in structure **4.30**. Since

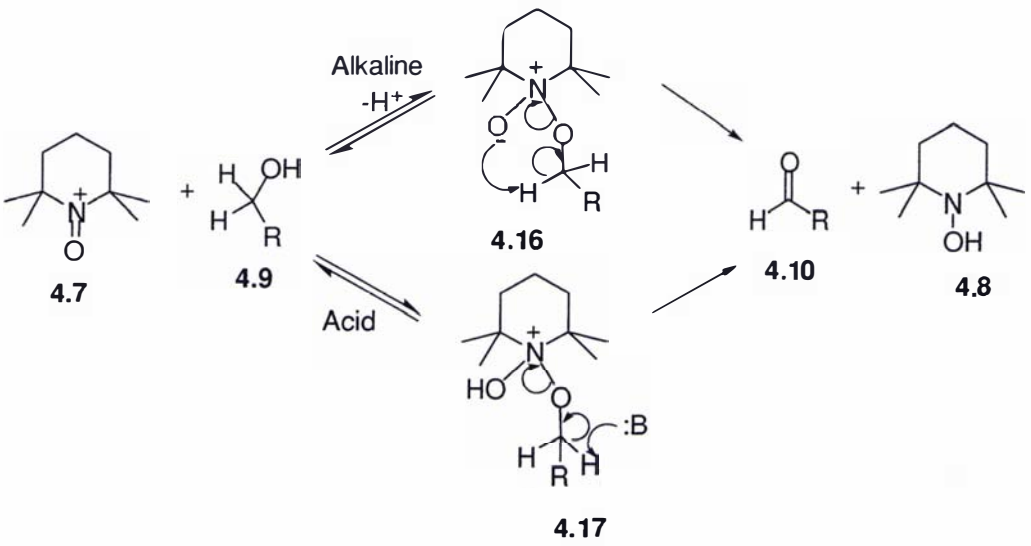
they worked under acidic conditions such a complex is conceivable, while in the cyclic transition state derived from **4.16** it is less likely due to the negative oxygen.



4.29 **4.30**

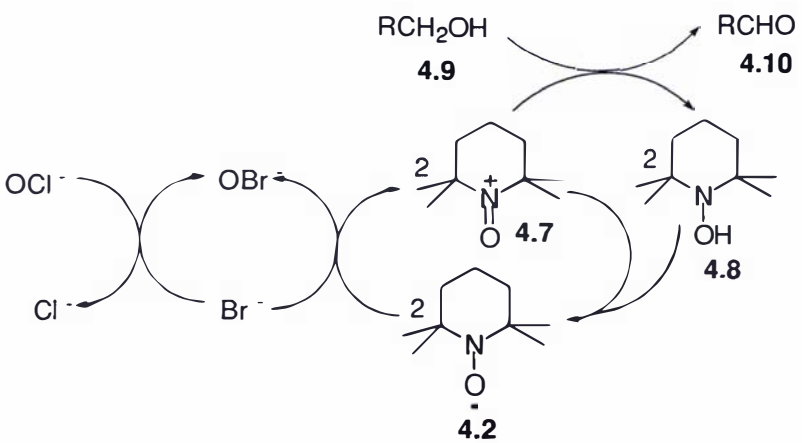
Figure 4.4

Nooy and van Bekkum *et al* [26] reported that under alkaline reaction conditions, primary alcohols were oxidised more rapidly than secondary ones. With primary/secondary polyol substrates, the selectivity for the primary hydroxyl depends on the steric demand of the secondary alcohols. For example, in contrast to several pyranosides, the acyclic substrates, 1,3 butanediol and mannitol could not be oxidised selectively. The reaction was followed with HPLC, and even in the initial stages, more than one product peak was found. In general, the observed regio-selectivity for different substrates under alkaline conditions depends on the accessibility of the alcohol, which would favour the more sterically confining transition state **4.16.** A study of the literature on nitroxyl-mediated oxidation of the alcohols reveals that this sterically directed selectivity only occurs under basic reaction conditions,[19,20,21] while under acidic reaction conditions[24,27] this selectivity disappears and secondary alcohols may be oxidised more rapidly. These observations suggest two different reaction path ways; the one under basic conditions based on a cyclic transition state similar to **4.16** and one under acid reaction conditions based on an acyclic reaction mechanism utilising a transition state similar to **4.17** as in the following **Scheme 4.5**. Obviously base will facilitate the proton abstraction step present in both mechanisms which is also thought to be the rate limiting step in both pathways.



Scheme 4.5

It appeared that the reaction is much faster at pH values higher than 9.[26] This is profitable because nonselective oxidation due to hypohalite is slower at higher pH values. TEMPO is known to be a stable radical which could be oxidised by several reagents to give a nitrosonium ion. The later is a strong oxidant and shows selectivity towards primary hydroxyl groups over secondary ones. Generally hypobromite has been used as the regenerating oxidant which in turn is regenerated by hypochlorite (**Scheme 4.6**).



Scheme 4.6

From the kinetic experiments carried out on methyl glucopyranuronate (MGP) as the substrate Nooy *et al*[283] discovered that [OCl^-] had no influence on the observed

reaction rate. The fact that the concentration of the primary oxidant had no influence on the reaction rate implies that the regeneration of the nitrosonium ion, which is thought to proceed according to reactions i, ii, and iv is more rapid than the oxidation of substrate (reaction iii). The amount of oxidant nitrosonium ion produced was considered constant during the reaction after approximately 30% oxidation.

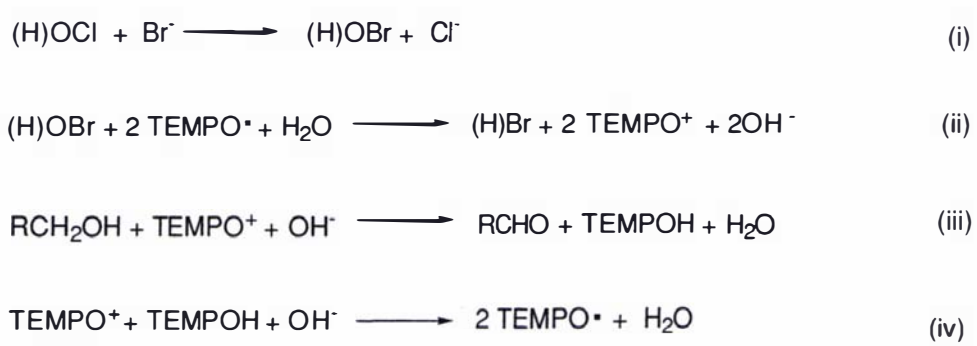


Figure 4.5

Initially it was expected that the aldehyde intermediate was oxidised by hypobromite to obtain the acid however from the oxidation of butanal with and without TEMPO, Nooy *et al* [26] discovered that the oxidation with TEMPO added was much more rapid It was thus concluded that the aldehyde intermediate is oxidised in the same way as the alcohol and the aldehyde is not oxidised by the presence of other oxidants.

4.1.1.2 Factors Influencing The Reactivity Of TEMPO Mediated Oxidations.

1 Aqueous medium

In organic solvents, without water or with only low concentrations of water the reaction stops at the aldehyde stage, which indicates that water is necessary for the TEMPO mediated oxidation of alcohols to carboxylic acids.

2 Steric factors

A study of the literature on nitroxyl mediated oxidation of alcohols reveals that this sterically directed selectivity only occurs under alkaline reaction condition while under acid reaction conditions the selectivity disappears and secondary alcohols may be

oxidised more rapidly than primary alcohols.[26] The marked difference in reactivity was also observed for sterically hindered alcohols under alkaline reaction conditions. For example 3-methyl cyclohexanol was oxidised by TEMPO with the same reaction rate as for cyclohexanol, whereas the more hindered alcohol 2-methyl cyclohexanol was oxidised more slowly.[26]

3. Influence of TEMPO

The presence of TEMPO has much influence on the reaction rate for the oxidation of alcohols. For example simple primary alcohols such as n-butanol were oxidised more rapidly and quantitatively to the corresponding carboxylates than cyclohexanols but without TEMPO added to the reaction mixture, no reaction was observed over 8 h.[26] Secondary alcohols tested were oxidised faster with TEMPO added to the reaction mixture than with only hypobromite.

4. Influence of ring size

It was found that primary alcohols in pyranosides were oxidised more selectively than those in furanosides. However this difference becomes smaller with more sterically hindered secondary alcohols.[20]

5. Anomeric configuration

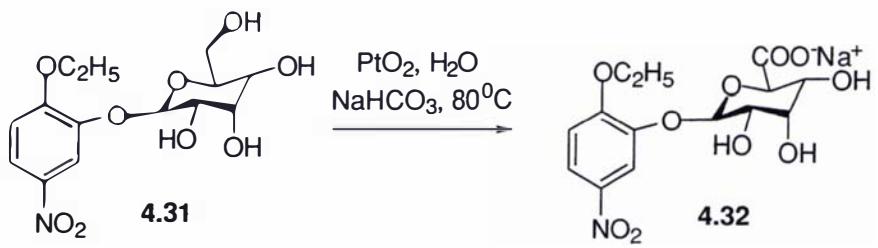
In the oxidation of various pyranosides it appeared that the substituent on the anomeric center did not have much influence on the reaction rate.[26] However the anomeric configuration at C-4, had a substantial influence on the reaction rate. For example methyl α-D glucopyranoside and octyl α-D glucopyranoside were observed to have the same rate constant (7.8 units), while methyl β-D glucopyranoside had a rate constant of 12 units.

4.1.2 Selective Oxidations In Carbohydrate Chemistry

Selective oxidation of primary hydroxyl groups is known to be achieved with difficulty using Pt/O_2 (PtO_2) but the yield can be low.[28,29] Smaller substrates (monomers, oligomers) can be selectively oxidised with Pt/O_2. However, this reaction

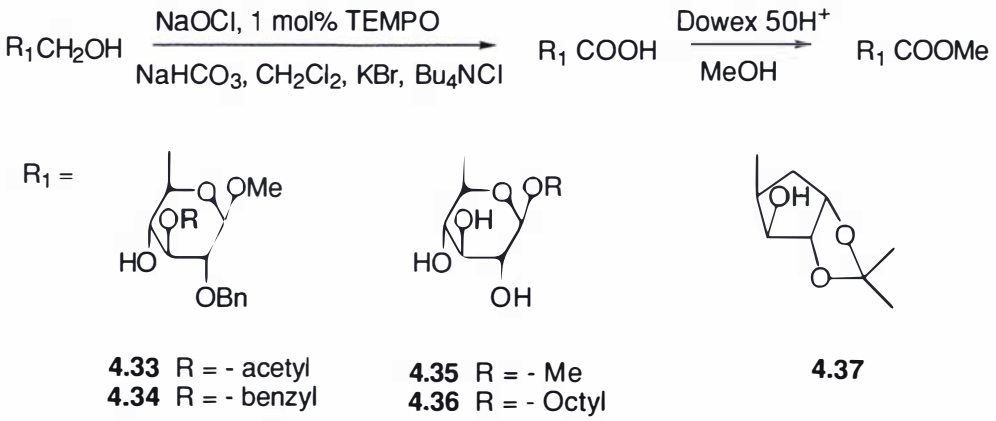
proceeds only sluggishly and with a low degree of oxidation when it is applied to polysaccharides because of the heterogeneous character of the catalyst.

Kiss *et al* [29] reported the preparation of 2-ethoxy 5-nitro phenol glucuronide using PtO_2 as the oxidising agent for the selective oxidation of the primary hydroxyl group of its glucoside moiety. No yield or spectroscopic data were recorded however.



Scheme 4.7

The TEMPO mediated oxidation of various carbohydrates in water at pH 10-11 with hypobromite, formed by reaction of hypochorite and bromide, as the regenerating oxidant was first investigated by Davis *et al.*.[21] Under the applied conditions primary alcohols were oxidised more rapidly than secondary ones and only carboxylates were found as the reaction products. The variety of partially protected monosacharide derivatives were selectively oxidised to carboxylic acids using sodium hypochlorite in the presence of catalytic amounts of TEMPO.[19] The oxidation leaves benzyl ethers, (in compound **4.34**), acetyl groups (in compound **4.33**) and acetonides (in compound **4.37**) intact. The less protected methyl (**4.35**) and octyl glucosides (**4.36**), where three secondary hydroxyl groups were competing with the primary hydroxyl group still led to the carboxylates as the main products. The selectivity for the primary hydroxyl groups in these sugars thus seems to be greater than expected from the published examples shown in **Scheme 4.8**.



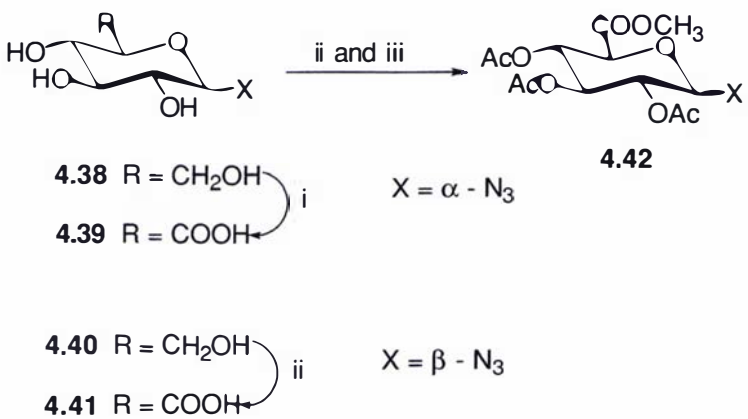
4.33 R = - acetyl
4.34 R = - benzyl

4.35 R = - Me
4.36 R = - Octyl

4.37

Scheme 4.8

Employing this oxidation procedure in sodium bicarbonate with sodium hypochlorite and catalytic amounts of TEMPO on both α and β–D-glucopyranosyl azide the corresponding glucuronic acid derivatives could be obtained in a smooth reaction and very good yields.[30] A considerable amount of inorganic salt was present in the crude mixture after the oxidation and hence the water soluble acids **4.39** and **4.41** were not isolated.

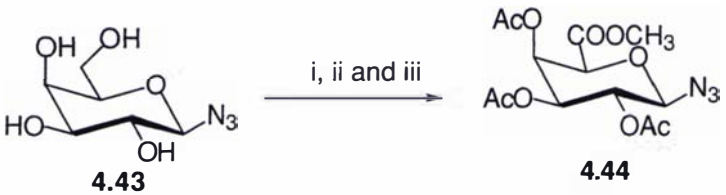


4.42

Reagents and conditions : *(i) TEMPO, NaOCl, $NaHCO_3$, (ii) CH_3I, (iii) Ac_2O, DMAP*

Scheme 4.9

By similar oxidation of β-D-galactopyranosyl azide the crystalline glucopyranuronate could be prepared in good yield following chromatographic purification.

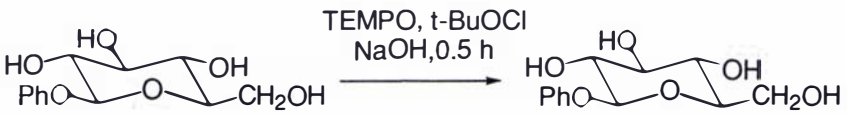


4.43 **4.44**

Reagents and conditions : *(i) TEMPO, NaOCl, $NaHCO_3$, (ii) CH_3I, (iii) Ac_2O, DMAP*

Scheme 4.10

Very recently Herbert *et al* [31] developed a high yielding route to various labelled β-D-glucuronides involving as a pivotal step, the oxidation of βD-glucosides by TEMPO and tert-butyl hypochlorite. The route is readily applicable to the preparation of glucuronides from other glucose samples labelled with stable or radio active isotopes. With water insoluble alcohols a two phase system is recommended but the TEMPO oxidation of octyl-β-D-glucoside under two phase conditions failed where as TEMPO/t-BuOCl method gave a quantitative yield.



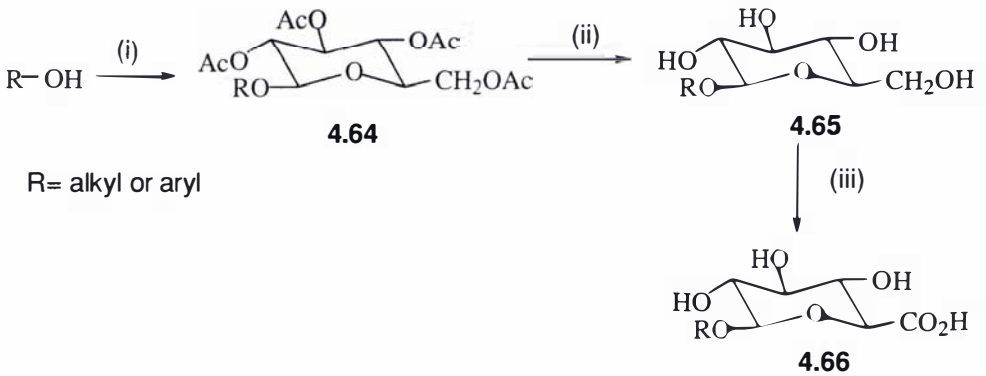
4.45 **4.46**

Scheme 4.11

4.1.3 Scope And Aims Of This Investigation

The direct synthesis of β-D-glucuronides of the deactivated phenols such as used in this work combined with the solubility problems in suitable solvents has presented a formidable challenge. As described in **Chapter 3** a variety of attempts have been made to synthesise isoflavone β-D-glucuronides by direct coupling of the isoflavone with various acyl protected glucuronides as glycosyl donors under different reaction conditions and all failed. The most disappointing aspect was the inability of the isoflavone to form glucuronides with more reactive glycosyl donors such as the sulfoxide glucuronide and the acetimidate glucuronide. Since an isoflavone β-D-glucuronide was not obtained using any of these various glycosylation methods it was decided to investigate the synthesis of the β-D-glucuronide using an alternative route.

It has been well presented that glycosides are more reactive than glucuronides[32] and less susceptible to form by products such as the β-eliminated products. Hence it was decided to investigate the synthesis of 1-*O*-β-D-glucuronide using the general sequence shown in the **Scheme 4.12**.



Scheme 4.12

Reagents and conditions : *(i) Glucuronidation, (ii) Hydrolysis, (iii) TEMPO, NaOCl, NaOH (0.5 M)*

In order to gain a greater understanding of the possible generality of this reaction and to find a procedure for formation of the glucuronides of simple phenols such as *p*-cresol, sterically hindered phenols such as 2,6-dimethyl phenol, steroids such as estrone (aryl -OH) and testosterone (alkyl -OH) and formononetin the corresponding glucosides were synthesised and the selective oxidation of the primary –OH group in the sugar moiety was investigated.

4.2 EXPERIMENTAL

4.2.1 General Experimental Details

See **Section 1.2.1** for more **General Experimental Details.**

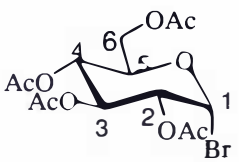
The molecular sieves (4 A^0) were activated at 450^{0}C and stored in an oven at 100°C before use in the reactions. Thin layer chromatography (TLC) plates for all molecules were visualised by UV lamp (254 nm) and spraying with 10% concentrated H_2SO_4 in EtOH and heating at 100^{0}C for 2 minutes.

Reverse phase Waters™Sep-Pak C_{18} cartridges were supplied by Millipore Corporation (Milford, United States) and were used for the purification of all glucosides and glucuronides. Amberlite XAD-2 resin (SERVA, 0.3-1 mm) column chromatography was also used for further purification of formononetin glucosides and steroid glucuronides (when required). The chromatographic analysis for estrone glucuronide was performed on SMART™ fast protein liquid chromatography (FPLC) system at RT using a μRPC C2/C18 PC3.2/3 column. LiChroprep RP-18 (silica derivatised with C-18 hydro carbon) (Merck, 40-63 μm) was generously provided by Dr David Stevenson, Industrial Research Limited, Wellington and was used for the purification of glucuronides prepared by the enzymatic route.

400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra were obtained using Bruker spectrometer. Spectral assignments were made and product stereochemistries were elucidated using the double resonance techniques COSY, HETCOR, DEPT and HMBC.

4.2.2 Preparation Of Glycoside Ester Derivatives

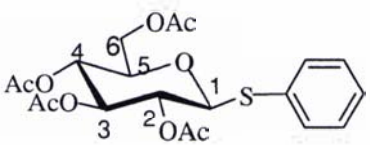
4.2.2.1 1-Bromo-1-deoxy-2, 3, 4, 6–tetra-*O*-acetyl-α-D-glucopyranose (4.47)



4.47

This compound was prepared according to the method described for the synthesis of methyl 1-bromo-1-deoxy-2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate **3.8** as white crystals using commercially available β-D-glucose pentaacetate **4.67** as precursor. Mp, 89-90^{0}C, (Lit. mp, 86-88^{0}C)[33]. ^{1}H NMR δ/ppm 6.61 (1H, d, J = 3.96 Hz, H1), 5.56 (1H, t, H4), 5.16 (1H, t, H3), 4.85 (1H, dd, J = 4.17, 3.96 Hz, H2), 4.22-4.41 (2H, m, H6), 4.13 (1H, d, J = 10.76 Hz, H5), 2.12 (3H, s, -OC(O) CH_3), 2.11 (3H, s, -OC(O) CH_3), 2.06 (3H, s, -OC(O) CH_3), 2.05 (3H, s, -OC(O) CH_3).

4.2.2.2 1-Thiophenyl-1-deoxy-2, 3 ,4, 6-tetra-*O* -acetyl-β-D-glucopyranose (4.48)

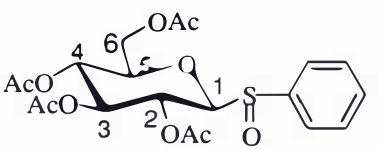


4.48

1-Bromo-1-deoxy-2,3,4,6-tri-*O* -acetyl-α-D-glucopyranose **4.47** (3.0 g, 7.29 mmol) and tetrabutyl ammonium hydrogen sulfate (Bu_4NHSO_4, 2.6 g) were dissolved in EtOAc (30 ml) and the solution was stirred vigorously at RT. After the addition of Na_2CO_3 (1 M, 3 ml) and thiophenol (PhSH, 1.5 ml, excess), the solution was continuously stirred for a further 0.5 hrs until the TLC showed the completion of the reaction (Hexane/ EtOAc 1:1, R_f = 0.62). The reaction mixture was diluted with EtOAc (50 ml) and the organic phase was washed with Na_2CO_3 (1 M, 25 ml x 3), water (30 ml x 2), brine (30 ml) and dried over $MgSO_4$ overnight. The EtOAc was removed under reduced pressure to afford an off white solid. The crude solid was recrystallised twice from EtOH to give

the pure product, 1-thiophenyl -1-deoxy-2, 3, 4, 6-tetra-*O* -acetyl-β-D-glucose **4.48** as white crystals (2.60 g, 81% yield); mp, 115^{0}C (sharp); Found: MH$^+$ 441.1219; $C_{20}H_{25}O_9S$; requires 441.1217. ^{1}H NMR (400 MHz) δ/ppm 7.29-7.50 (5H, m, phenyl), 4.91-5.09 (3H, m, H2-H4), 4.72 (1H, d, *J* = 10.1 Hz H1), 4.16-4.19 (2H, m, H6), 3.71-3.74 (1H, m, H5), 2.10 (3H, s, -OC(O) CH_3), 2.09 (3H, s, -OC(O) CH_3), 2.01 (3H, s, -OC(O) CH_3), 1.98 (3H, s, -OC(O) CH_3).

4.2.2.3 1-Phenylsulfenyl-1-deoxy-2,3,4,6-tetra-*O* -acetyl-β-D-glucopyranose (4.49)

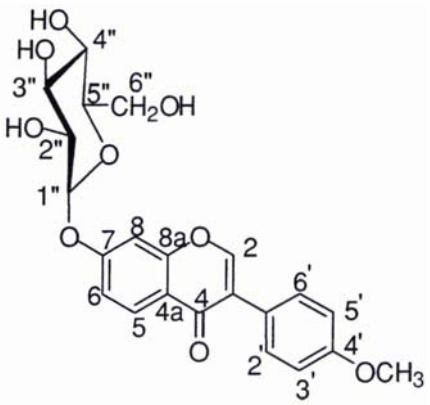


4.49

1-Thiophenyl -1-deoxy-2, 3 ,4, 6-tetra-O -acetyl-β-D-glucopyranose (2.5 g, 5.67 mmol) was dissolved in CH_2Cl_2 (80 ml) and was cooled to -70^{0}C in dry ice/acetone. A solution of *m*-chloroperbenzoic acid (m-CPBA) (2.25 g, 50-60%, 6.52-7.82 mmol) in distilled water (30.0 ml) was added slowly at -70^{0}C. The reaction mixture was stirred for 1 hour until TLC showed completion of the reaction (Hex/ EtOAc 1:1, R_f = 0.33). The reaction mixture was poured into saturated aqueous $NaHCO_3$ (1000 ml), the organic layer was washed with saturated aqueous $NaHCO_3$ (200 ml x 3), water (50 ml x 2), brine (50 ml) and dried over Na_2SO_4 for several hours. The solvent was removed under reduced pressure to afford a white solid, which was purified by short column chromatography on neutral alumina using Hex/EtOAc (1:1) as eluting solvent Hex/EtOAc (1:1) to give the desired 1-phenylsulfenyl-1-deoxy-2,3,4-tri-O -acetyl-β-D-glucopyranuronate as a white crystalline solid (2.101 g, 81% yield). mp, 133-135^{0}C. Found: MH$^+$ 457.1148; $C_{20}H_{25}O_{10}S$; requires 457.1168. ^{1}H NMR δ/ppm 7.31-7.54 (5H, m, phenyl), 4.92-5.07 (3H, m, H2-H4), 4.42 (1H, d, *J* = 10.7 Hz, H1), 4.16-4.19 (2H, m, H6), 3.64-3.72 (1H, m, H5), 2.10 (3H, s, -OC(O) CH_3), 2.09 (3H, s, -OC(O) CH_3), 2.00 (3H, s, -OC(O) CH_3), 1.98 (3H, s, -OC(O) CH_3).

4.2.3 Synthesis Of Formononetin Glucoside

4.2.3.1 Formononetin glucoside (4.50) using phase transfer catalysis[34]



4.50

A solution of formononetin (0.5 g, 1.86 mmol) in 2.5% aqueous NaOH (3.57 ml) was stirred at RT for 15-20 mins. To this solution was added a CH_2Cl_2 (12 ml) solution of TBAB (0.75 g, 0.45 mmol) and 1–bromo-2, 3, 4, 6-tetra-*O*-acetyl-α-D-glucopyranuronate **4.47** (1.15 g, 2.80 mmol). The two phase reaction mixture was stirred vigorously for 5 h before the reaction mixture was neutralised with 0.1M H_2SO_4 (0.1M). The reaction mixture was extracted with EtOAc (4 x 50 ml), washed with brine (50 ml) and then dried over Na_2SO_4. The solvent was removed under reduced pressure to give a crude yellow gummy mass. The crude sample was dissolved in a solution of NaOMe (1.0 g) in MeOH (200 ml) and allowed to stand at 5^0C for 15 h. The reaction mixture was neutralised using aqueous HCl (0.1 M). The solution was filtered and the solvent was removed under reduced pressure to give an off white solid. Initial purification was carried out by dissolving the solid in DMF:H_2O (1:2.6, 100 ml) and subjected to polystyrene resin XAD-2 column chromatography. The column was eluted with water, MeOH:H_2O (1:1, 100 ml), MeOH:H_2O (2:1, 200 ml), MeOH:H_2O (3:1, 200 ml) and finally with pure MeOH. A by product (16 mg) obtained by C-ring cleavage of formononetin was eluted first followed by the desired title compound as a white solid and finally unreacted formononetin (0.260 g). The desired product **4.50** was further purified onto Sep-Pak C_{18} column by eluting with aq. MeOH (1:1) and pure MeOH. The solvent was removed under reduced pressure from the desired fractions to give **4.50** as pure white solid (0.255 g, 32%, R_f = 0.66, 3:1 EtOAc/EtOH). ^{1}H NMR (DMSO-d_6)

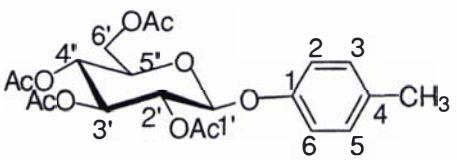
δ/ppm 8.48 (1H, s, H2), 8.34 (1H, d, J = 9.0 Hz, H5), 7.79 (2H, d, = 8.79 Hz, H2', H6'), 7.44 (1H, d, J = 2.2 Hz, H8), 7.41 (1H, dd, J = 8.7, 2.2 Hz, H6)), 7.22 (2H, d, J = 2.2, 8.79 Hz, H3', H5'), 5.41 (1H, d, 7.58 Hz, H1"), 4.06 (3H, s, -OCH_3), 3.53-3.95 (6H, m, , H2", H3", H4", H5", H6"). ^{13}C NMR δ/ppm 174.6 (C4), 162.5 (C7), 160.0 (C4'), 158.0 (C8a), 153.5 (C2), 130.7 (C2', C6'), 27.6 (C5), 125.0 (C3), 124.6 (C4a), 116.2 (C6), 114.1 (C3', C5'), 104.2 (C8), 101.4 (C1"), 78.1 (C5"), 77.6 (C3'), 74.2 (C2'), 70.9 (C4"), 62.1 (C6"), 55.4 (-OCH_3). The ^{1}H NMR and ^{13}C NMR agreed with those reported in the literature.

4.2.3.2 Formononetin glucoside (4.47) using phenolate anion (NaH) method

To a solution of formononetin (0.1 g, 0.37 mmol) in dry and freshly distilled DMF (1 ml) was added a solution of NaH (0.01 g, washed with n-pentane) in dry distilled DMF (0.5 ml). The solution was stirred at RT for 10 mins under an atmosphere of Ar. To this solution was added 1-bromo- 2, 3, 4, 6-tetra-*O* -acetyl-α-D-glucopyranuronate **4.47** (0.152 g, 0.37 mmol). The reaction mixture was stirred vigorously for 2 h before addition of another lot of 1–bromo- 2, 3, 4, 6-tetra-*O* -acetyl-α-D-glucopyranuronate **4.47** (0.075 g, 0.19 mmol). The reaction mixture was stirred for a further 1 h. The solvent was removed under reduced pressure to yield the yellow crude formononetin-*O* -β-D-(tetra-*O* -acetyl glycopyranosides). The crude product was dissolved in a solution of NaOMe (0.54 g) in MeOH (100 ml) and allowed to stand at 5^{0}C for 15 h. The reaction mixture was neutralised using aqueous HCl (0.1 M). The solution was filtered and the solvent was removed under reduced pressure to give an off white solid. The purification was achieved by dissolving it in DMF:H_2O (1:2.5, 25 ml) and then subjecting to polystyrene resin XAD-2 column, eluting it with H_2O (50 ml), MeOH:H_2O (1:1, 50 ml), MeOH:H_2O (2:1, 50 ml), MeOH:H_2O (3:1, 150 ml) and MeOH (50 ml). The solvent was removed from the appropriate fractions to give the desired formononetin glucoside (33 mg, 21%, R_f = 0.64, 3:1 EtOAc/EtOH) and unreacted formononetin (28 mg). The spectral data were identical in all respect with the product obtained by phase transfer catalyst method.

4.2.4 Synthesis Of Glycoside Ester Derivatives Using Various Glycosyl Donors

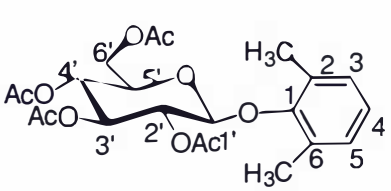
4.2.4.1 *p*-Tolyl–2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranoside (4.51)



4.51

To a solution of *p*-cresol (0.2 g, 1.84 mmol) in CH_2Cl_2 (5 ml) and molecular sieves ($4A^0$, activated) was added β-D-glucose pentaacetate **4.67** (0.360 g, 0.92 mmol) and $BF_3.OEt_2$ (0.13 ml, 0.92 mmol). The resulting reaction mixture was protected from moisture and stirred at 25-30^{0}C overnight (16 h). The reaction mixture was diluted with CH_2Cl_2 (40 ml) and washed with aqueous KOH (2N, 25 ml x 4), water (25 ml x 2), brine (25 ml) and dried over $MgSO_4/Na_2SO_4$. The solvent was removed under reduced pressure to afford a crude off white solid. The crude product was then recrystallised using absolute EtOH to give colourless crystals of the desired *p*-tolyl–2', 3' ,4', 6'-tetra-*O*-acetyl-β-D-glycopyranoside **4.51** (214 mg, 53%, R_f= 0.70, 1:2 Hex/ EtOAc). Mp, 110-111^{0}C. ^{1}H NMR (400 MHz) δ/ppm 7.11 (2H, d, *J* = 8.57 Hz, phenyl H2, H6), 6.90 (2H, d, *J* = 8.57 Hz, phenyl H3, H5), 5.15-5.31 (3H, m, H2'-H4'), 5.03 (1H, d, *J* = 7.26 Hz, H1'), 4.18 (1H, dd, *J* = 2.3, 11.9 Hz H6'), 4.27 (1H, dd, *J* = 5.04, 11.9 Hz H6'), 3.79-3.90 (1H, m, H5'), 2.31 (3H, s, -CH_3 Ar) 2.09 (3H, s, -OC(O) CH_3), 2.07 (3H, s, -OC(O) CH_3), 2.06 (3H, s, -OC(O) CH_3), 2.04 (3H, s, -OC(O) CH_3). ^{13}C NMR (100 MHz) δ/ppm 170.4, 170.1, 169.2, 169.1, (4 x –*O*C (O) CH_3), 154.6, 132.7, 129.8, 116.9 (4 x aryl C), 99.4 (C1'), 72.4(C3'), 71.9 (C5'), 71.1 (C2'), 68.2 (C4'), 61.9 (C6'), 20.6, 20.5 (-OC(O) *C* H_3, -CH_3 Ar).

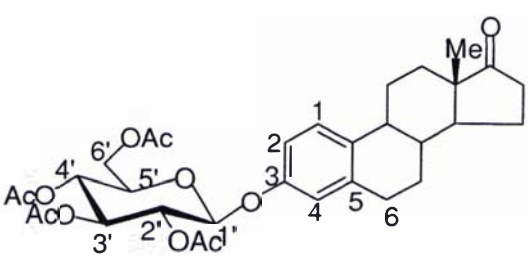
4.2.4.2 2,6-Dimethyl-2',3',4',6'-tetra-*O*-acetyl-β-D-glucopyranoside (4.53)



4.53

In a typical reaction tetra-*O*-acetyl sulfoxide sugar **4.49** (2.57 g, 5.62 mmol) in CH_2Cl_2 (15 ml) was added to Tf_2O (1.61 g) in CH_2Cl_2 (21 ml) at -78^0C followed by an acid scavanger triethyl phosphite (0.717 g, 4.30 mmol) in CH_2Cl_2 (3.5 ml) and 2, 6-dimethyl phenol (0.350 g, 2.86 mmol) in CH_2Cl_2 (10 ml). The resulting reaction mixture was protected from moisture and stirred under Ar. The reaction mixture was slowly warmed to 0^0C to observe a purple color which became intense as the reaction proceeded. The reaction mixture was poured into aqueous $NaHCO_3$ (250 ml), washed with 0.2 N KOH and dried over Na_2SO_4. The solvent was removed under reduced pressure to afford a crude yellow solid. The crude product was then purified by column chromatography eluting with Hex/EtOAc (2:1) and then Hex/EtOAc (1:1) to give a pale yellow solid. The pale yellow solid was again purified through quick column chromatography eluting with Hex/EtOAc (1:1) before recrystallisation in boiling propan-2-ol to give a white solid of the desired 2, 6-dimethyl-2', 3', 4', 6'-tetra-*O*-acetyl-β-D-glycopyranoside **4.53** (446 mg, 34%, R_f= 0.48, 1:1 Hex/ EtOAc). Mp, 115-116^{0}C,. Found: ($M+Cs^+$) 585.0736; $C_{22}H_{28}O_{10}Cs$ requires 585.0732. ^{1}H NMR δ/ppm 7.00-7.27 (3H, m, phenyl H3-H5), 5.18-5.5.36 (3H, m, H2'-H4'), 4.84 (1H, d, *J* = 7.69 Hz, Hl'), 4.23 (1H, dd, *J* = 4.8, 11.5 Hz H6'), 4.27 (1H, dd, *J* = 2.41, 11.5 Hz H6'), 3.56-3.60 (1H, m, H5'), 2.28 (3H, s, -CH_3 Ar), 2.19 (3H, s, -OC(O) CH_3), 2.13 (3H, s, -OC(O) CH_3), 2.05 (3H, s, -OC(O) CH_3), 2.04 (3H, s, -OC(O) CH_3). ^{13}C NMR δ/ppm 170.4, 170.2, 169.2, 169.1, (4 x –O*C* (O) CH_3), 152.6, 132.5, 128.8, 124.9 (4 x aryl C), 101.4 (C1'), 73.0(C3'), 71.8 (C5'), 71.6 (C2'), 68.5 (C4'), 61.7 (C6'), 20.6, 16.9 (-OC(O) *C* H_3, -CH_3 Ar).

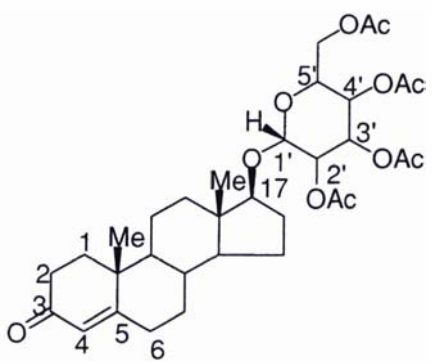
4.2.4.3 17-Oxo-1,3,5(10)–estratrien–3–yl-2,3,4,6–tetra–*O*–acetyl-β -D-glucopyranoside (4.55)



4.55

The coupling reaction was performed by a procedure similar to that used in the preparation of **4.51** using the Lewis acid $BF_3.OEt_2$. The title compound was obtained as white crystals (0.58 g, 88%, R_f= 0.42, 1:1 Hex/ EtOAc) from estrone (0.3 g, 1.10 mmol), β-D-glucose penta acetate (0.85 g, 2.20 mmol) and $BF_3.OEt_2$ (0.31 ml, 2.20 mmol). For compound **4.55** mp, 212-213^{0}C. Found: MH^+, 601.2648; $C_{32}H_{41}O_{11}$ requires 601.2589, Found: (M+Na) 623.2468; $C_{32}H_{41}O_{11}Na$ requires 623.2387. ^1H NMR δ/ppm (400 MHz) 7.21 (1H, d, J = 8.35 Hz, phenyl H2), 6.74-6.80 (2H, m, phenyl H1, H4), 5.14-5.30 (3H, m, H2'-H4'), 5.05 (1H, d, J = 7.25 Hz, H1'), 4.30 (1H, dd, J = 4.8, 16.7 Hz H6'), 4.17 (1H, dd, J = 2.41, 16.7 Hz H6'), 3.81-3.86 (1H, m, H5'), 2.068 (3H, s, -OC(O) CH_3), 2.035 (3H, s, -OC(O) CH_3, 2.030 (3H, s, -OC(O) CH_3), 2.016 (3H, s, -OC(O) CH_3), 0.92 (3H, s, 18-CH_3) ^{13}C NMR (100 MHz) δ/ppm 220.5 (-C(O)R), 170.4, 170.0, 169.2, 169.1 (4 x –*O*C (O) CH_3), 154.7, 137.9, 134.6, 126.3, 117.0, 114.2 (6 x aryl C), 99.0 (C1'), 72.7(C3'), 71.9 (C5'), 71.1 (C2'), 68.3(C4'), 62.0(C6'), 21.6, 2 x 20.8, 20.6 (-OC(O) *C* H_3), 13.9 (-CH_3(Ar)).

4.2.4.4 Androst-4-en–3-one 17-β-D-yl-2', 3', 4', 6'-tetra-*O*–acetyl glucopyranoside (4.57)



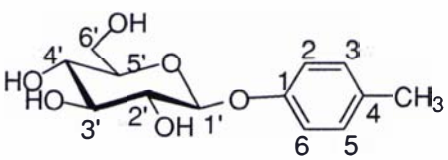
4.57

The coupling reaction of testosterone **4.71** with 1-bromo-1-deoxy-2, 3, 4, 6–tetra-*O*-acetyl-α-D-glucopyranose **4.47** was carried out as follows: To a solution of testosterone **4.71** (500 mg, 1.73 mmol) in anhydrous toluene (100 ml) was added dried $CdCO_3$ (1.10 g, 6.37 mmol) and the suspension was concentrated to approximately 70 ml by distillation to remove residual moisture. After distillation, the solution of methyl 1-bromo-1-deoxy-2, 3, 4-tri-*O* -acetyl-α-D-glucopyranuronate **4.47** (1.71 g, 4.16 mmol)

in anhydrous toluene (30 ml) was added slowly using a pressure equalising funnel, keeping the addition rate the same as the rate of distillation. Finally, the whole reaction mixture was refluxed for 20 h. The extent of the reaction was monitored by TLC and the colour of the reaction mixture. The reaction mixture slowly gained a bluish grey colour as the reaction proceeded. The reaction mixture was then filtered through a celite pad and washed with CH_2Cl_2. The combined filtrate was washed with water (30 ml) and dried over anhydrous K_2CO_3 for several hours. The organic phase was filtered and concentrated at 25-40^{0}C under vacuum to reduce the volume (30 ml), and the resultant syrup was purified by flash chromatography column by eluting first with toluene and then with 20% acetone in hexane to get unreacted testosterone and the desired product (R_f = 0.29; acetone:Hex, 3:7). The product was further purified by dissolving it in acetone (10 ml) and poured in to H_2O (40 ml) (acetone/ H_2O ratio 1:4) to obtain a white amorphous solid. The solid was washed with water and then recrystallised with absolute EtOH to give androst-4-en–3-one 17-β-D -yl-2', 3', 4', 6'-tetra-*O*–acetyl glucopyranoside **4.57** (698 mg, 65 %) as white crystals, mp, 208-209^{0}C, Found: MH^+, 619.3118; $C_{33}H_{47}O_{11}$ requires 619.3121. ^{1}H NMR δ/ppm 5.73 (1H, s, H4), 4.94-5.26 (3H, m, H2'-H4'), 4.53 (1H, d, J = 7.90 Hz, H1'), 4.12-4.30 (2H, m, H6'), 3.63-3.70 (1H, m, H5'), 3.55 (1H, t, H17), 2.09 (3H, s, –OC(O)CH_3), 2.06 (3H, s, –OC(O)CH_3), 2.03 (3H, s, –OC(O)CH_3), 2.02 (3H, s, -CH_3), 0.75 (3H, s, -CH_3).

4.2.5 Synthesis Of Glucosides By Hydrolysis Of Glycoside Ester Derivatives

4.2.5.1 *p*-Tolyl-β-D-glucopyranoside (4.52)

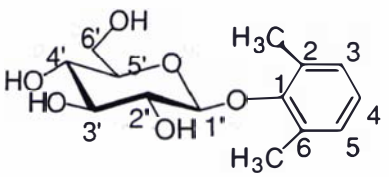


4.52

Compound **4.51** (0.250 g, 0.570 mmol) was dissolved in MeOH (10 ml) and aqueous Na_2CO_3 (2M, 3.5 ml) was added. The reaction mixture was stirred at RT for 5 h. The TLC (EtOAc/EtOH, 3:1) showed no starting material was left. The reaction mixture was neutralised or brought to pH ~6 by titrating with HCl (1 M) and the solvent was

removed under reduced pressure. The compound was then purified by reversed phase chromatography on a Waters C_{18} Sep-Pak column by eluting with water and then 50% aqueous MeOH. Appropriate fractions were pooled and the solvent was removed under reduced pressure to give a white solid (88 mg, 57%). Found: ($M+Na^+$), 293.1001; $C_{13}H_{18}O_6Na$ requires 293.1000. ^1H NMR (400 MHz) (D_2O) δ/ppm 7.44 (2H, d, J = 8.57 Hz, H2, H6), 7.35 (2H, d, J = 8.57 Hz, H3, H5), 4.25 (1H, d, 9.8 Hz, H1'), 4.08 (1H, d, 3.92 Hz, H6'a), 3.80-3.86 (5H, m, H2'-H5', H6'b), 2.63 (3H, s, $-CH_3$ Ar). ^{13}C NMR (100 MHz) δ/ppm 155.8 (C1), 131.7 (C4), 129.7 (C2, C6), 116.6 (C3, C5), 101.4 (C1'), 76.9 (C3'), 76.8 (C5'), 73.8 (C2'), 70.3 (C4'), 61.4 (C6'), 19.7 ($-CH_3$(Ar)). Found: M^+, 270.1079; $C_{13}H_{18}O_6$ requires 270.1089.

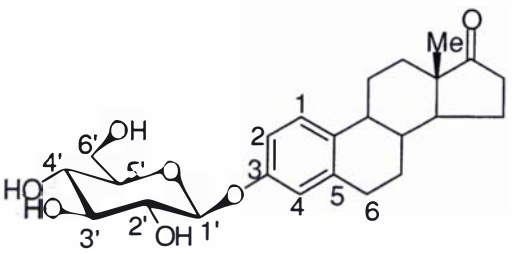
4.2.5.2 2, 6-Dimethyl phenol-β-D-glucopyranoside (4.54)



4.54

The hydrolysis reaction was performed by a procedure similar to that used in the preparation of **4.52.** Compound **4.54** (107 mg, 68%) was obtained from compound **4.53** (0.250 g, 0.553 mmol). Found: ($M+Na^+$) 307.1157; $C_{14}H_{20}O_6Na$ requires 307.1156. ^1H NMR (400 MHz) (D_2O) 6.96-7.22 (3H, m, phenyl), 4.58 (1H, d, 7.88 Hz, H1'), 3.67 (1H, d, J = 3.88 Hz, H6'a), 3.34-3.64 (5H, m, H2'-H4', H6'b), 3.18-3.21 (1H, m, H2'), 2.27 (6H, s, 2 X $-CH_3$Ar).. ^{13}C NMR (100 MHz) δ/ppm 152.8 (C1), 132.3 (C3, C5), 129.5 (C2, C6), 125.7 (C4), 103.5 (C1'), 96.3 (C5'), 76.8 (C3'), 74.5 (C2'), 70.0 (C4'), 61.0 (C6), 16.7 (2 x $-CH_3$ Ar).

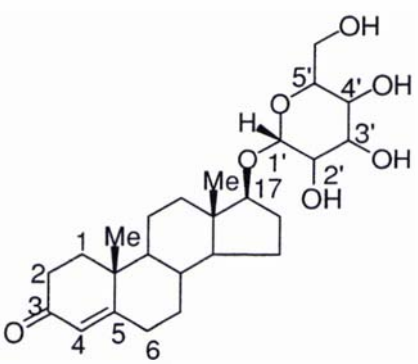
4.2.5.3 Estrone-β-D-glucoside (17-Oxo-1,3,5(10)–estratrien–3-yl-β-D-glucopyranoside) (4.56)



4.56

The hydrolysis reaction was performed by a procedure similar to that used in the preparation of **4.52**. Compound **4.56** (96.4 mg, 67%) was obtained from compound **4.55** (200 mg, 0.332 mmol). Found: $(M+Na^+)$ 307.1157; $C_{14}H_{20}O_6Na$ requires 307.1156. ^1H NMR (400 MHz) (DMSO-d_6) δ/ppm 6.67-7.14 (3H, m, phenyl), 5.12 (1H, d, J = 7.88 Hz, H1'), 4.89-5.01 (1H, m, H6'a), 4.71 (1H, d, J = 7.45 Hz, H6'b), 3.06-3.18 (4H, m, H2'-H5'), 0.79 (3H, s, 18-CH_3). ^{13}C NMR (100 MHz) δ/ppm 222.4 (-C(O)R), 155.9, 137.7, 133.8, 126.1, 116.7, 114.2 (6 x aryl C), 101.4 (C1'), 77.0(C3'), 76.9 (C5'), 73.9 (C2'), 70.4 (C4'), 61.5 (C6'), 50.6, 44.4, 38.9, 35.7, 31.8, 29.6, 26.7, 26.0, 21.5 (6 x -CH_2, 3 x --CH, estrone) 13.3 (-CH_3 Ar).

4.2.5.4 Testosterone-β-D-glucoside (Androst-4-en-3-one-17-β-D-yl-glucopyranoside) (4.58)



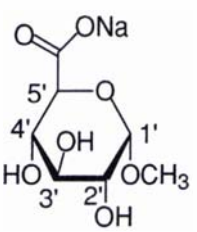
4.58

The hydrolysis reaction was performed by a procedure similar to that used in the preparation of **4.52**. Compound **4.58** (70.0 mg, 65%) was obtained from compound **4.57** (0.148 g, 0.239 mmol). Found: MH^+, 451.2681; $C_{25}H_{39}O_7$ requires 451.2695. Found: (M+ Na^+), 473.2491 $C_{25}H_{38}O_7Na$ requires 473.2515. ^1H NMR δ/ppm 5.64 (1H, s, H4), 4.35 (1H, d, J = 6.78 Hz, H1'), 3.85 (1H, d, J = 4.23 Hz, H6'a), 3.65 (1H, t, H17), 3.56 (1H, dd, J = 5.32, 4.23 Hz, H6'b), 3.24-3.38 (3H, m, H3'-H5'), 3.16 (1H, t, H2'), 1.26 (3H, s, $-CH_3$), 0.92 (3H, s, $-CH_3$). ^{13}C NMR δ/ppm 202.8 (-C(O)R), 175.6 (C5), 124.6 (C4), 105.1 (C1'), 63.2 (C3'), 90.0, 78.5, 72.1, 63.2, 55.9, 52.1, 44.6, 40.4, 37.2, 35.1, 34.3, 33.2, 30.2, 24.6, 22.2, 37.1 (C6')18.1 (C18), 12.4 (C19).

4.2.6 General Procedure For TEMPO Mediated Oxidation Reaction[35]

The β-D-glucoside (1 equiv.) was dissolved in distilled H_2O (~ 10 ml). TEMPO (0.005 equiv.) and NaBr (0.15 equiv.) were dissolved in this solution, which was cooled to 0^0C and a cold solution of 12-15% hypochlorite in H_2O previously brought to pH ~ 10 by adding 4 M HCl was added. The pH was controlled at ~10-10.5 by drop wise addition of NaOH or KOH (0.5 M) with a syringe. The reaction went to completion after about 0.5 h (TLC), when the pH was generally stable indicating no more NaOH was needed. The reaction was quenched by addition of EtOH (~ 5 ml) and the mixture was neutralised by adding 1 M HCl. The organic solvent was removed under reduced pressure and the remaining solution was then freeze-dried. The crude product was purified using either XAD-2 column or Waters™ Sep-Pak column chromatography by eluting with water, aqueous MeOH (50%) and MeOH. Appropriate fractions were pooled and the solvent was removed under reduced pressure. The reaction mixture was then freeze-dried to give almost pure sodium salts of the glucuronides.

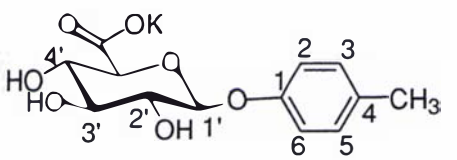
4.2.6.1 Methyl-α-D-glucuronide (4.59)



4.59

The general procedure was followed using methyl-α-D-glucoside (0.476 g, 2.456 mmol) and the crude product **4.59** was obtained in >90% yield. ^{1}H NMR (D_2O) δ/ 4.89 (1H, d, 4.89 Hz, H1'), 3.96 (1H, d, 9.9 Hz, H5'), 3.56-3.72 (3H, m, H2'-H4'), 3.45 (3H, m, -OCH_3). ^{13}C NMR δ/ppm 176.8 (-C=O), 99.6 (C1'), 73.2 (C3'), 72.3 (C5'), 72.4 (C4'), 71.3 (C2'), 55.6 (-OCH_3). The ^{1}H NMR and the ^{13}C NMR assignments agreed with those obtained by Nooy and Besemer.[35]

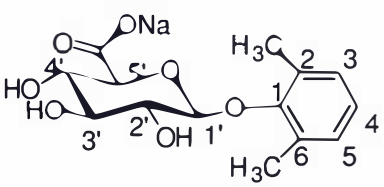
4.2.6.2 *p*-Tolyl-β-D-glucuronide (4.60)



4.60

The general procedure was followed using p-cresol-β-D-glucoside **4.52** (200 mg, 0.740 mmol) using KOH (0.5 M) and the product **4.60** was isolated in pure form (176 mg, 74%). Found: M^+, 323.0520; $C_{13}H_{16}O_7K$ requires 323.0533. ^{1}H NMR (D_2O) (400 MHz) δ/ppm 7.14 (2H, d, J = 8.7 Hz, H2, H6), 6.98 (2H, d, J = 8.7 Hz, H3, H5), 4.96 (1H, d, 9.2 Hz, H1'), 3.77 (1H, d, 3.98 Hz, H5'), 3.53-3.57 (3H, m, H2'-H4'), 2.13 (3H, s, -CH_3 Ar). ^{13}C NMR δ/ppm 175.9 (-C=O), 154.9 (C1), 133.7 (C4), 130.7 (C2, C6), 117.1 (C3, C5), 100.9 (C1'), 76.5 (C3'), 75.8 (C5'), 73.2 (C4'), 72.2 (C2'), 20.1 (-CH_3Ar).

4.2.6.3 2,6-Dimethyl phenol-β-D-glucuronide (4.61)

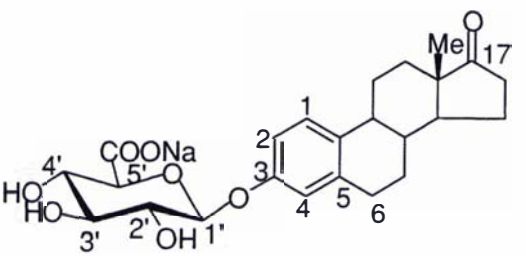


4.61

The general procedure was followed using 2,6-dimethyl phenol-β-D-glucoside **4.54** (200 mg, 0.740 mmol) and the product **4.61** was isolated in pure form (176 mg, 74%). Found: MH^+, 299.1116; $C_{14}H_{19}O_7$ requires 299.1130, M^+, 321.0520; $C_{13}H_{16}O_7Na$

requires 321.0533. ^{1}H NMR (D_2O) δ/ppm 7.00-7.22 (3H, m, H3-H5), 4.77 (1H, d, 7.69 Hz, H1'), 4.58 (1H, d, 7.69 Hz, H1'), 3.32-3.81 (3H, m, H2'-H4'), 2.27 (6H, s, 2 X -CH_3 Ar). ^{13}C NMR δ/ppm 172.7 (-C=O), 152.4 (C1), 132.0 (C2, C6), 129.3 (C3, C5), 125.6 (C4), 103.3 (C1'), 75.5 (C3'), 75.1 (C5'), 73.7 (C4'), 71.7 (C2'), 16.6 (-2 X -CH_3 Ar).

4.2.6.4 Estrone-β-D-glucuronide (4.62)

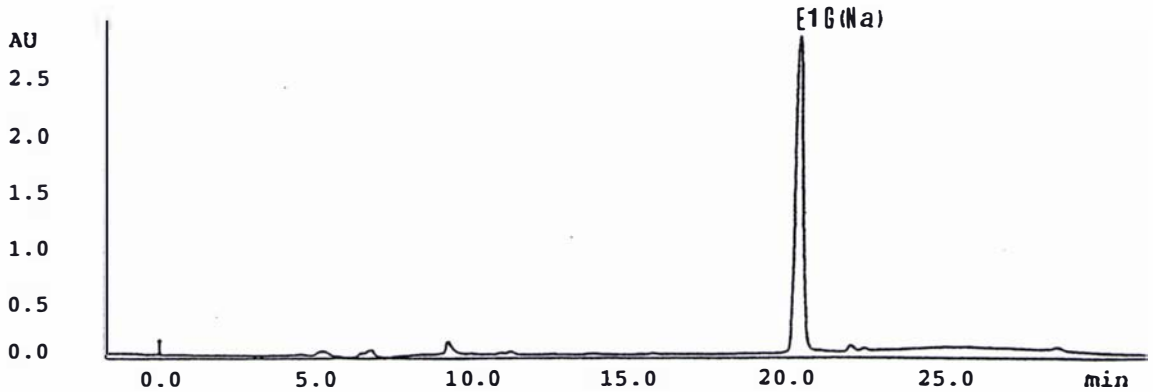


4.62

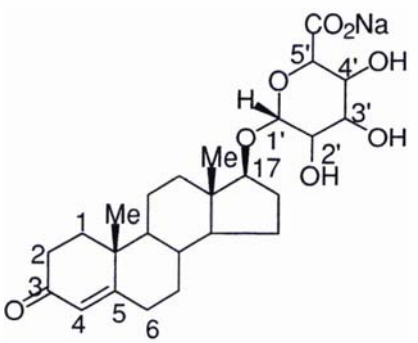
The general procedure was followed using estrone-β-D-glucoside **4.56** (216 mg, 0.499 mmol) and the product **4.62** was isolated in pure form (145 mg, 62%). %). Found: M^+, 432.2152; $C_{24}H_{32}O_7$ requires 432.2148. ^{1}H NMR δ/ppm (MeOH-d_4) 6.72-7.20 (3H, m, phenyl), 5.04 (1H, d, J = 7.33 Hz, H1'), 4.03 (1H, d, J = 9.53 Hz, H5'), 3.46-3.74 (3H, m, H2'-H4'), 0.89 (3H, s, 18-CH_3). In all other respects the NMR data were identical with previously obtained spectra for E1G.

The purity of the product was checked on the Smart® FPLC system by dissolving the product E1G **5.1** (0.5 mg) in CH_3CN/H_2O (3:7) and loading 0.05 ml of the resulting solution onto a reverse phase C2/C18 column (solvent A: H_2O/CH_3CN/HCOOH/NaCl, 90:10:0.05/1.0) at a flow rate of 1 ml/min and using a linear gradient from 30 to 70% solvent over 60 mins. A wavelength of 220 nm was used for the detection. The resulting chromatogram is shown in **Figure 4.6**.

Figure 4.6 FPLC analysis of E1G (Na) prepared using TEMPO method showing that the product was pure.



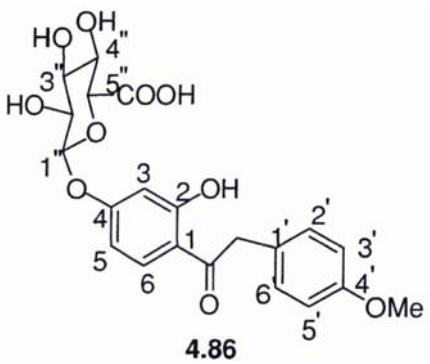
4.2.6.5 Testosterone-β-D-glucuronide (4.63)



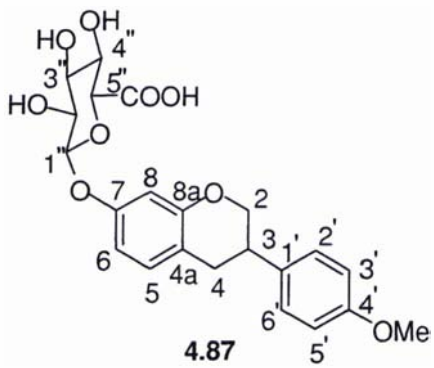
4.63

The general procedure was followed using testosterone-β-D-glucoside **4.58** (114 mg, 0.252 mmol) and the product **4.63** was isolated in pure form (59 mg, 48%). ^{1}H NMR (400 MHz) (D_2O) δ/ppm 5.98 (1H, s, H4), 4.70 (1H, d, J = 7.69 Hz, H1'), 4.01 (1H, t, H17), 3.83-3.87 (1H, m, H5'), 3.92 (1H, t, H3'), 3.75 (1H, d, H4'), 3.43-3.50 (1H, m, H2'), 1.19 (3H, s, 19-CH_3), 0.76 (3H, s, 18-CH_3). ^{13}C NMR (100 MHz) δ/ppm 205.1 (-C(O)R), 178.9 (-*C* O_2CH_3), 175.8 (C5), 122.2 (C4), 102.6 (C1'), 89.1 (C17), 76.6 (C5'), 75.7 (C3'), 73.5 (C2'), 72.2 (C4'), 16.9 (-C18), 11.2 (-C19).

4.2.7 Enzymatic Synthesis Of 2-Hydroxy, 4'-Methoxy Deoxybenzoin Glucuronide (4.86) And (±) Methoxy Equol Glucuronide (4.87)[36]



4.86



4.87

These reactions were carried out under contract by Dr David Stevenson of IRL, wellington: DTT (35 mg, 0.22 mmol), UDPGA (400 mg, 0.64 mmol), BSA (3.2 g, 3% w/v) and ovine liver microsomes (10 mg) were mixed to get a homogeneous solution. Tris-HCl buffer pH ~8 (105 ml, 200 mmol), glucuronic acid (100 mmol) and $CaCl_2$ (6 mmol) were added to the above homogeneous solution. The reaction mixture was stirred at 30^0C to get homogeneous solution and formononetin (100 mg, 0.37 mmol) or methoxy equol (100 mg, 0.39 mmol) in CH_3CN (2 ml) was added and the reaction mixture was stirred vigorously. The reaction mixture was then allowed to stirr gently at 30^0C overnight. The reaction mixture was then diluted with H_2O (100 ml) and freeze dried. The crude solid thus obtained was then extracted with MeOH (2 x 50 ml), filtered and the solvent was removed under reduced pressure to get a crude yellow solid. The crude yellow solid was then subjected to RP 18 flash silica column chromatography for purification.

4.2.7.1 Purification Of 2-Hydroxy, 4'-Methoxy Deoxybenzoin (4.86) And (±) Methoxy Equol Glucuronide (4.87) Conjugates

The crude yellow solid obtained from the enzymatic synthesis was dissolved in 1M KCl (100 ml) and loaded onto a LiChroprep RP-18 (40-63 μm) column from Merck. The column was washed with distilled H_2O (200 ml) to remove salts followed by a 0-80% gradient of CH_3CN in H_2O over 2 h at a flow rate 4 ml/ min. The fractions were collected at 3 min intervals (~12 ml) and each fraction was analysed by TLC. The TLC analysis indicated that the fractions contained unreacted starting material, impurity and the product contaminated with impurity with increasing elution time. The fractions

containing product and impurity were pooled together and further purified using HPLC on a RP 18 column using gradients of 20-55% of CH_3CN in H_2O over 1 h at a flow rate of 2 ml/ min. The fractions were pooled together from the trailing edge of the product peak and analysed by TLC which showed it to contain pure glucuronide. The solvent was removed under reduced pressure to give a small amount of pure product **4.86** (4 mg) and **4.87** (2 mg) for spectroscopic analysis.

a) (±) Methoxy equol-β-D-glucuronide (4.87)

Found: MH^+, 433.1494; $C_{22}H_{25}O_9$ requires 433.1498. ^1H NMR (400 MHz) (MeOH-d_4) δ/ppm 7.18 (2H, d, *J* = 8.69 Hz, H2', H6'), 6.97 (1H, d, *J* = 8.32 Hz, H5), 6.86 (2H, d, *J* = 8.69 Hz, H3', H5'), 6.74 (1H, d, *J* = 8.41 Hz, H6), 6.52 (1H, s, H8), 4.92 (1H, H1''), 4.19-4.23 (1H, m, H4), 3.94-3.97 (1H, m, H4), 3.75-3.86 (1H, m, H5''), 3.74 (3H, s, -OCH_3), 3.55-3.59 (1H, m, H4''), 3.44-3.46 (2H, m, H2'', H3''), 2.96-3.10 (1H, m, H3), 2.88-2.92 (2H, m, H2). ^{13}C NMR δ/ppm (100 MHz) 159.1 (C4'), 157.3, (C8a), 155.2 (C7), 133.9 (C1'), 130.2 (C5), 128.4 (C2', C6'), 116.7 (C4a), 114.1 (C3', C5'), 109.4 (C6), 104.8 (C8), 101.6 (C1''), 76.6 (C5''), 75.7 (C3''), 73.7 (C2''), 72.3 (C4), 71.1 (C4''), 54.6 (-OCH_3), 38.2 (C3), 32.0 (C2).

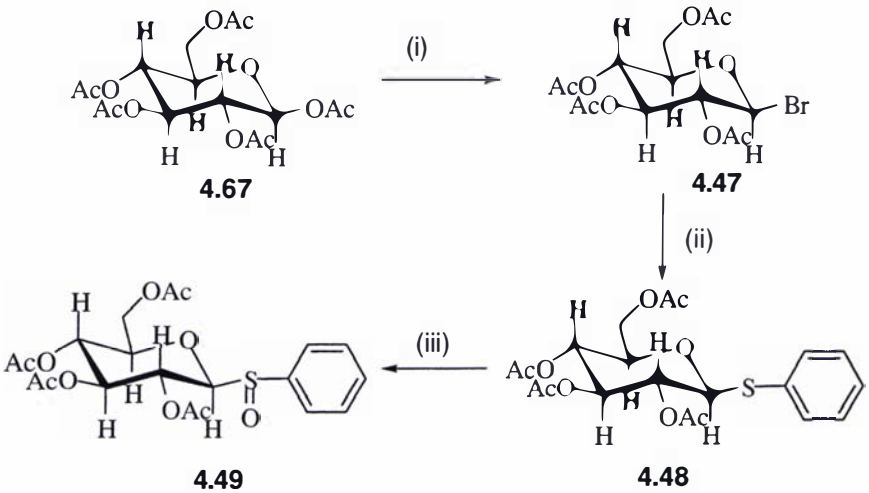
b) 2-Hydroxy-4'-methoxy deoxybenzoin-4-β-D-glucuronide (4.86)

Found: MH^+, 435.1291; $C_{21}H_{23}O_{10}$ requires 435.1498. ^1H NMR (400 MHz) (MeOH-d_4) δ/ppm 8.0 (1H, d, *J* = 9.01 Hz, H6), 7.26 (2H, d, *J* = 8.73 Hz, H2', H6'), 6.76 (2H, d, *J* = 8.73 Hz, H3', H5'), 6.66 (1H, dd, *J* = 8.62, 2.71 Hz, H5), 6.42 (1H, d, *J* = 2.42 Hz, H3), 5.06 (1H, d, *J* = 7.62 Hz, H1''), 4.35 (2H, s, -CH_2), 4.05 (1H, d, *J* = 7.86 Hz, H5''), 3.74 (3H, s, -OCH_3), 3.59-3.63 (1H, m, H4''), 3.45-3.48 (2H, m, H2'', H3''). ^{13}C NMR (100 MHz) δ/ppm 203.8 (-C(O)R), 174.2 (C2), 165.1(C4), 159.1 (C4'), 133.1 (C6), 130.4 (C2', C6'), 127.1 (C1'), 114.7 (C1), 114.0 (C3', C5'), 108.3 (C5), 104.2 (C3), 100.2 (C1''), 76.3 (C3'', C5''), 73.4 (C2''), 72.0 (C4''), 54.6 (-OCH_3), 44.0 (-CH_2).

4.3 RESULTS AND DISCUSSION

4.3.1 Synthesis Of Acyl Glycoside Esters As Fundamental Glycosyl Donor Intermediates

A survey of the literature reveals numerous procedures[37] for the synthesis of aryl and alkyl glycosides using various acyl protected glycoside ester intermediates. For a facile way of preparing these glycoside ester intermediates it was decided to employ the same methodology used for the synthesis of acyl protected glucopyranuronate intermediates (**Chapter 3**). Hence commercially available β-D-glucose pentaacetate **4.67** was reacted with 35% HBr in HOAc to obtain the α-tetraacetyl bromo sugar **4.47** in moderate yield. The α-tetraacetyl bromosugar was then reacted with thiophenol in the presence of the phase transfer catalyst TBHS to synthesise the β-phenyl thioglycoside. With the thioglycoside in hand it was also decided to synthesise the stable and potentially more reactive sulfoxide glycoside ester **4.49**. Surprisingly Kahn's procedure which failed to give a clean controlled oxidation product of sulfoxide glucuronide from the corresponding β-phenyl thioglucuronide (**Chapter 3, Section 3.3.2**) worked well for the controlled oxidation of the β-phenyl thioglycoside **4.48**. Hence β-phenyl thioglycoside **4.48** was reacted with m-CPBA/ CH_2Cl_2 at -78^0C and the sulfoxide glycoside **4.49** was obtained in an excellent yield without any unwanted oxidation product (the sulfone glycoside). The reaction was scaled up to (1.0 g) without any reduction in the yield.



4.67 4.47 4.48 4.49

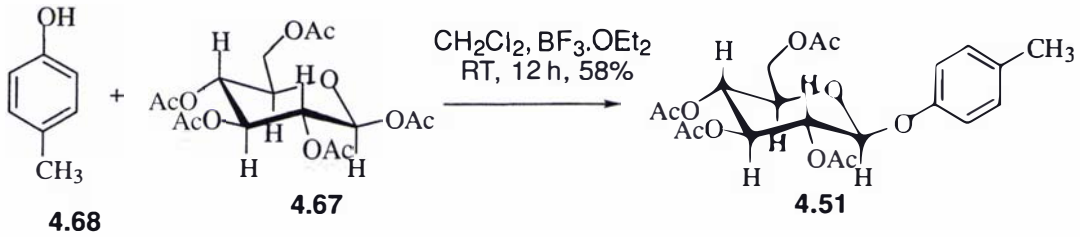
Reagents and conditions : *(i) HBr in CH_3COOH, (45%), dark, RT, Overnight, 80% (ii)TBHS, EtOAc, RT, Na_2CO_3, PhSH, 0.5 h, 81%, (iii) m-CPBA, CH_2Cl_2,- 70 ^{0}C, 1 h, 81%.*

Scheme 4.13

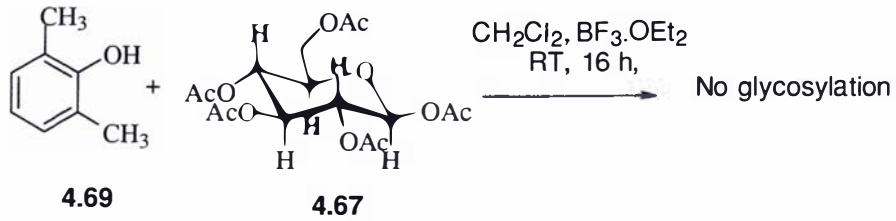
4.3.2 Investigation Into The Synthesis Of *O*-Glucosides

With the background in synthetic organic methodology of *O*-glycosylation methods (developed in **Chapter 3**) to attach sugars to a variety of aglycons it was decided to investigate the synthesis of various glycosides and produce the glucosides by hydrolysis. It was also hoped that the synthesis of isoflavone β-D-tetraacetyl glycoside would be obtained using one of the many glycosylation methods.

p-Cresol was directly reacted with β-D-glucose pentaacetate **4.67** in the presence of the Lewis acid catalyst $BF_3.OEt_2$ at RT to obtain *p*-cresol glycoside **4.51** in good yield (**Scheme 4.14**). Similar attempts to synthesise the β-glycoside of the sterically hindered 2,6-dimethyl phenol using the perester coupling failed to give any product. A variety of reaction parameters were changed but the 2,6-dimethyl phenol glycoside was not obtained.

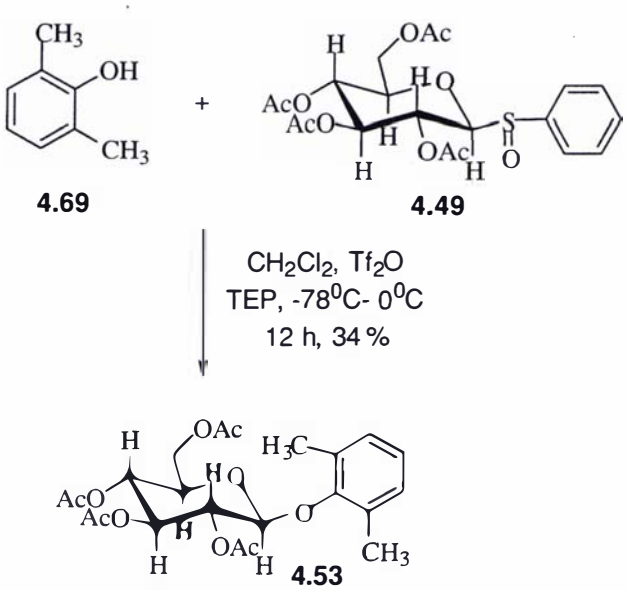


Scheme 4.14



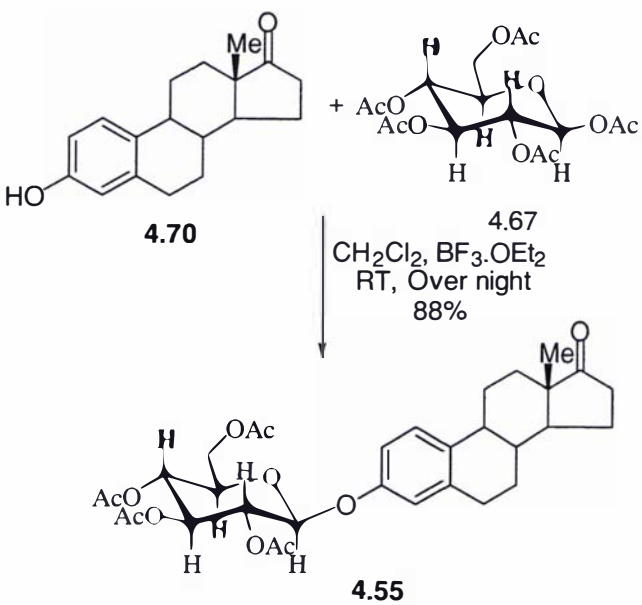
Scheme 4.15

The success reported by Kahn and co-workers[46] in the glycosylation of unreactive substrates such as the sterically hindered 2,6-dimethyl phenol with tetra-*O*-benzyl sulfoxide (**Section *3.1.4.2*, Scheme 3.9**), prompted an investigation of their methodology. Hence the tetra-*O*-acteyl sulfoxide sugar and Tf_2O in CH_2Cl_2 were treated with 2,6-dimethyl phenol in the presence of the acid scavenger TEP to give the desired 2,6-dimethyl phenol glycoside **4.53** in a reasonable yield. The yield was poorer then expected due to the side product thiosugar **4.48** obtained due to the reduction of the glycosyl donor **4.49** by the acid scavanger TEP which is known to be an effective deoxygenating agent.[47]



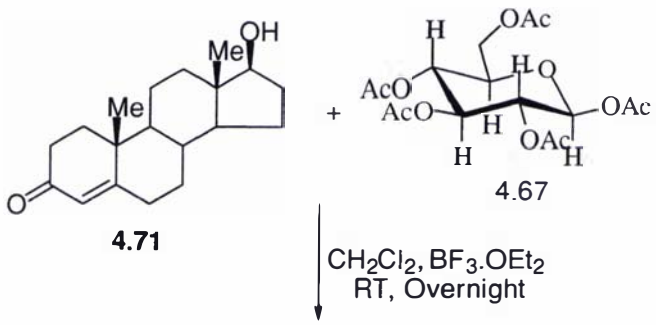
Scheme 4.16

It was also possible to synthesise estrone β-D-tetraacetyl glycoside ester using perester coupling method with the Lewis acid $BF_3.OEt_2$ as the catalyst. Estrone was directly reacted with β -D-glucose pentaacetate in the presence of activated molecular sieves (4Å) and a catalytic amount of $BF_3.OEt_2$ in CH_2Cl_2. Estrone glycoside **4.55** was obtained in an excellent yield after purification and recrystallisation.



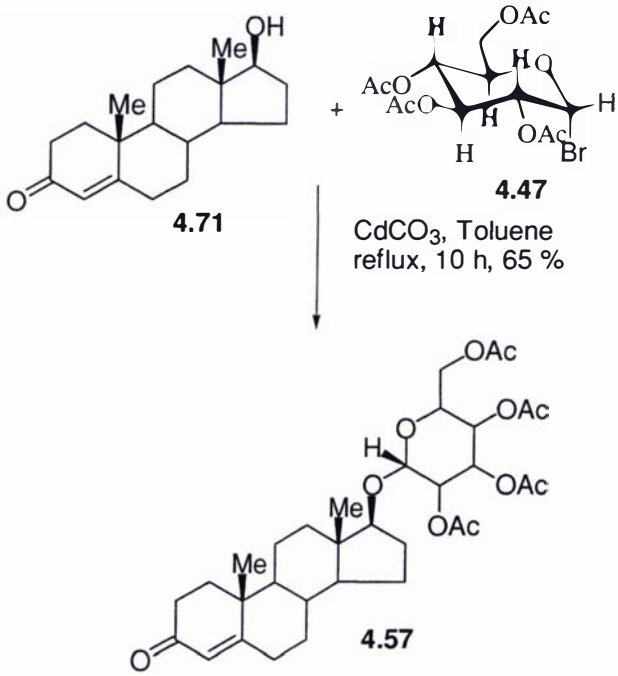
Scheme 4.17

Similar attempts were also made to couple the aliphatic hydroxyl group of testosterone **4.71** using the perester coupling method to obtain testosterone β-D-tetra-*O*-acetyl glycoside ester. Unfortunately the crude reaction mixture turned black after stirring under Ar at RT for 2 h. The TLC analysis of the crude reaction mixture indicated no glycosylation but decomposition of both the testosterone and β-D-glucose pentaacetate.



Scheme 4.18

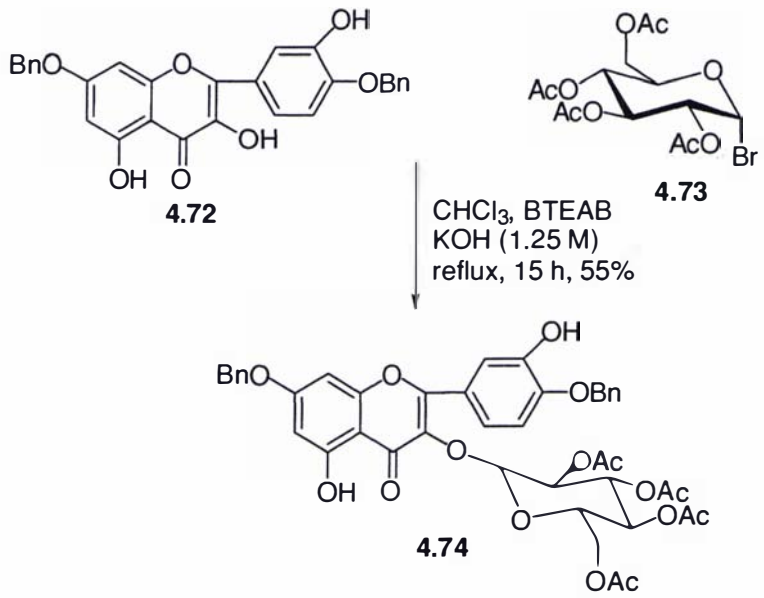
Hence efforts were concentrated on synthesising testosterone β-D-tetra-O-acetyl glycoside ester by the standard classical Koenigs-Knorr procedure. Testosterone was reacted with the α-tetraacetyl bromosugar **4.67** under reflux in toluene using $CdCO_3$ as the promoter. The reaction was refluxed under anhydrous conditions for several hours after removing any of the water present by azeotropic distillation. An interesting feature of the reaction was the development of a bluish gray color of the crude reaction mixture from pale yellow as the reaction proceeded. These aspects will be discussed further in **Chapter 5**. The desired testosterone β-D-tetra-*O*-acetyl glycoside ester was isolated after purification in acetone/H_2O and crystallisation in a good yield.



Scheme 4.19

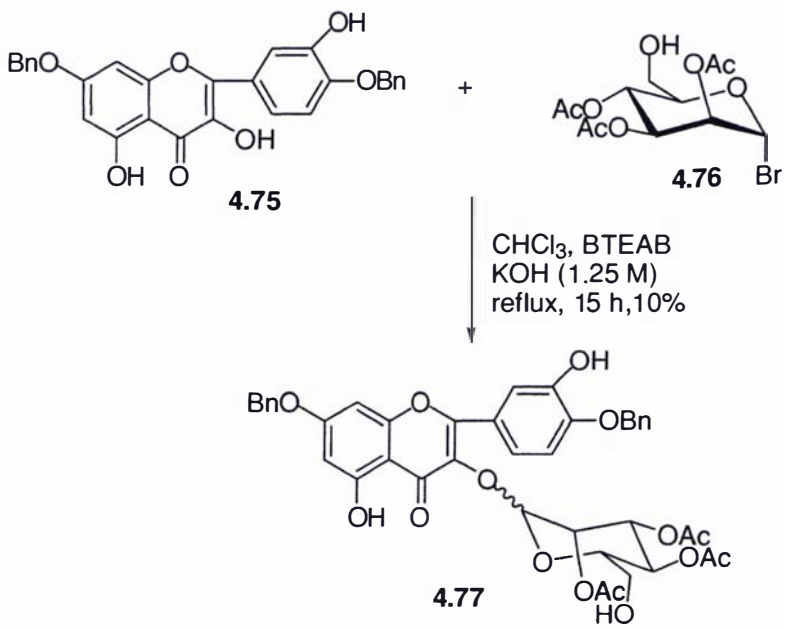
Attempts were also made to synthesise formononetin β-D-tetra-*O*-acetyl glycoside ester using i) the perester coupling method with β-D-glucose penataacetate, ii) the Koenigs-Knorr reaction using $CdCO_3$ as the promoter with α-tetraacetyl bromosugar 4.47 and iii) using the reaction conditions successfully applied for sterically hindered phenols using the β-D-tetraactate sulfoxide sugar **4.49**. All of these attempts failed to give any of the desired formononetin β-D-tetra-*O*-acetyl glycoside ester. The insolubility of formononetin in suitable solvents and the electronic effects as discussed in **Chapter 3** were thought to be a major hurdle against success of the glycosylation reaction.

While a literature survey revealed very few syntheses of aryl glycosides using phase transfer catalysis of phenols[39] with an α-tetraacetyl bromosugar **4.47**, a report on the application of phase transfer catalysis to the syntheses of 4',7-di-*O*-benzylquercetin (**Scheme 4.20**) (an aglycon similar to isoflavones) using α-tetraacetyl bromosugar **4.73** has been reported earlier.[38]



Scheme 4.20

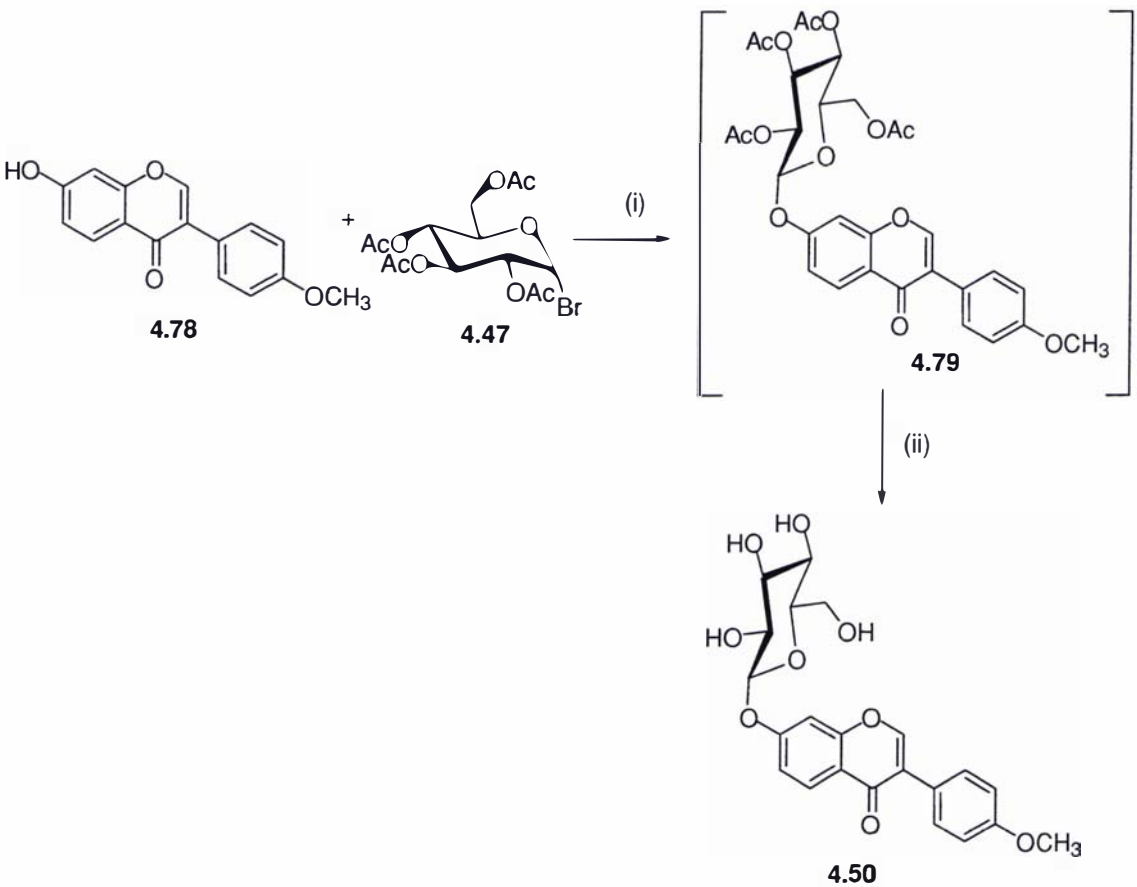
In contrast to this result, when 2,3,4-tri-*O*-acetyl-a-L-rhamnopyranosyl bromide **4.76** was used as alkylating agent glycosylation took place only in poor yield, and with a lack of stereospecificity.



Scheme 4.21

During the course of this research into the synthesis of glucuronides using this methodology Wahala and co-workers[34] reported a promising method of the synthesising of various isoflavone glycosides using phase transfer conditions.

These results encouraged an attempt at using this reaction for the synthesis of formononetin tetra-*O*-acetyl glycoside. It was decided to use established literature procedures and hence in the presence of the phase transfer catalyst tetrabutyl ammonium bromide (TBAB) the formononetin anion was coupled with α-tetraacetyl bromosugar **4.47** (**Scheme 4.22**).



Scheme 4.22

Reagents and conditions : *(i) NaOH (2.5%), TBAB, CH_2Cl_2, RT, 5 h, (ii) NaOMe in MeOH, 4 ^0C, 12 h, 32 %.*

The crude reaction mixture was monitored by TLC analysis which indicated the presence of unreacted formononetin, the desired product formononetin tetra-*O*-acetyl glycoside and other sugar impurities. The product from this organic-aqueous two phase reaction formononetin–*O*-tetra-acetyl-β-D-glycopyranoside was isolated by extracting with EtOAc and taken to the next step without purification. The yield of the product obtained was poor and repetitive efforts to obtain a better yield failed. Various attempts to improve the situation such as increased reaction time and increased concentration of

phase transfer catalyst in the reaction also failed. It was presumed that the poor yield was due to the formation of by products such as compounds **4.80** obtained by HBr elimination and **4.81** obtained due to anomeric hydrolysis of the α-acetyl bromosugar **4.47**.[39] Moreover unreacted formononetin was also recovered in substantial quantities.

4.80 **4.81**

Figure 4.7

Synthesis of formononetin–*O*-tetra-acetyl-β-D-glycopyranoside by generating the phenolate anion of formononetin by reaction with NaH in DMF and then solid addition of α-tetraacetylbromoglucose was also investigated. The reaction mixture was stirred for 5-6 hrs at RT under an atmosphere of Ar but the yield of the desired product formononetin–*O*-tetra-acetyl-β-D-glycopyranoside was poorer than the general phase transfer catalyst method.

Selectivity towards the 1, 2-*trans* or β-glycoside suggests either an SN2 mechanism reaction of the α-acetyl bromosugar **4.73** *via* a cyclic acyl oxonium ion with attack by the phenolate thus being limited to the β-position.

4.3.2.1 Hydrolysis Of Ester-Protected Glycosides And Purification Of Glucosides

Deprotection (deacetylation) of the sugar moiety was carried out using the standard procedure of treatment with NaOMe/MeOH to give the corresponding free glucosides. However it was found that glycoside esters can be also conveniently hydrolysed using the milder base Na_2CO_3 in MeOH at RT without raising any problem of partial deprotection. In fact it was found in the case of formononetin tetra-*O*-acetyl-β-D-glucopyranoside that it is safer to treat it with Na_2CO_3 in aqueous MeOH at RT then to treat it with the stronger base NaOMe This procedure lessens the risk of generating any 4,5-didehydro glycoside, a known elimination by-product from such hydrolysis and more importantly avoids any irreversible ring opening reaction due to the presence of the base sensitive chromene ring (**Chapter 3, Scheme 3.30**).

The sodium salts of various glycosides usually require many repeated crystallisations from methanol in order to obtain a pure product. This results in a significant loss of valuable product. Hence purification of the sodium salt of *p*-cresol, 2, 6 dimethyl phenol, estrone and testosterone glycosides was conveniently achieved easily by neutralising the corresponding salt with HCl (1M) or by bringing the pH of the reaction mixture to pH~6. The compounds were then purified by reversed phase chromatography on a Waters™ C_{18} Sep-Pak column by eluting with water to remove water soluble inorganic salts and then with 50% aqueous methanol to recover the desired glucoside in almost pure form. However the purification of formononetin β-D-glucoside required special care because of the acid and base sensitive chromene ring. The sodium salt solution obtained after alkaline hydrolysis of formononetin-(tetra-*O*-acetyl-β-D-glycopyranoside) was carefully neutralised with Amberlite resin 120 (H). the pH of the crude reaction mixture being brought to pH ~7. The glycosidic linkage is stable at pH 7 for long periods of time.

The literature method for general purification of isoflavone-β-D-glucoside involves a two step purification initially by reversed phase preparative TLC and then on a hypersil ODS 5 μ semipreparative column. A two step convenient purification procedure was also employed initially using non ionic neutral resin XAD 2 chromatography as described by Mattox *et al* [40] and Nuzawa *et al* [41] with formononetin glucoside being eluted with a 50-70% aqueous methanol solution. The methanol was removed and the product was further purified by adsorption onto a C_{18} Sep-Pak column, eluting with water and then with 50% aqueous methanol and MeOH. The aqueous solution retains the low molecular weight reagents and sodium salts.

4.3.3 TEMPO Mediated Oxidation Of 1-β-D-Glucosides

Recent success reported by several research groups in the selective oxidation of primary hydroxyl groups in the presence of catalytic amounts of TEMPO in sugar chemistry prompted an investigation of the applicability of this method to the glucosides prepared in this work. While there have been few reports of the use of TEMPO in the selective oxidation of pyranosides, a literature survey revealed only one method of use of TEMPO mediated selective oxidation on various phenyl β-D-glucosides.

The simple phenol *p*-cresol glucuronide was conveniently obtained using t-BuOCl/TEMPO in aqueous solution as reported by Melvin *et al* [31] in good yield. The advantage of this method was the absence of any inorganic salts and hence resulted in easy isolation and purification of the glucuronide. The disadvantage was that t-BuOCl being extremely unstable at RT must be freshly prepared and can be stored at 0^0C for only a few days. It was decided to investigate an alternative TEMPO/NaOCl method reported by Nooy *et al* [35] with some modifications to account for the various glycosides at hand. Initially methyl-β-D-glucoside along with TEMPO and NaBr were dissolved in H_2O and kept at 0^0C. The NaOCl solution (15%, initially brought to pH 10 by addition of 4 M HCl) was added and the pH of the reaction mixture was controlled to 10.5 using a pH-stat by controlled addition of 0.5 M NaOH. The reaction was virtually over in 45 minutes giving a quantitative yield of the desired methyl-β-D-glucuronide. The attempt to remove inorganic salt were not highly successful but TLC and ^{1}H NMR indicated a positive reaction. Due to the ease of this homogeneous reaction other β-D-glucuronides were also successfully prepared by controlling the pH of the reaction mixture to ~10.5 by addition of NaOH (0.5 M) with a syringe. The following table indicates the successful synthesis of 1-*O*-β-D-glucuronides using TEMPO mediated oxidation. It is worth noticing that the reduction in yield of estrone glucuronide was observed when the estrone glucoside was reacted in a mixture of organic/aqueous solvent. Slight decomposition was observed and some estrone was recovered by extracting the crude reaction mixture with $CHCl_3$.

Table 4.1 TEMPO mediated selective oxidation of primary hydroxyl group of various glucosides

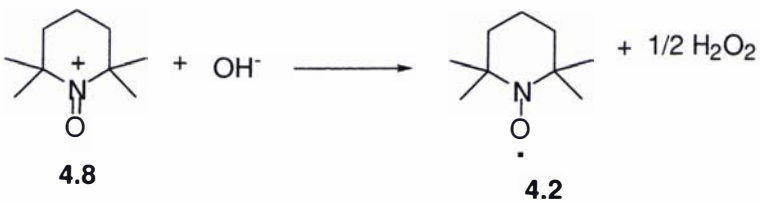
Glucoside	Glucuronide	Solvent	Yield(%)
1-*O*-Methyl glucoside	1-*O*-Methyl glucuronide	Water	>90%
p-Cresol glucoside	*p*-Cresol glucuronide	Water	74%
2,6- Dimethyl phenol glucoside	2,6- Dimethyl phenol glucuronide	water	56%

Estrone glucoside	Estrone glucuronide	water	62%
Estrone glucoside	Estrone glucuronide	Water, DMF	45%
Estrone glucoside	Estrone glucuronide	Water, 1,4-Dioxan	43%
Testosterone glucoside	Testosterone glucuronide	Water	48%
Formononetin Glucoside	Formononetin Glucuronide	Water	NO glucuronide
Formononetin Glucoside	Formononetin Glucuronide	Water, 1,4-Dioxan	NO glucuronide
Formononetin Glucoside	Formononetin Glucuronide	Water, DMF	NO glucuronide

A literature survey revealed that one of the problems with TEMPO mediated oxidation is the isolation of water soluble acid unstable glucuronides from substantial amounts of inorganic salts.[21] Most of the time the carboxylic acid was methylated before the work up to aid extraction and purification. However the simple expedient of using a Waters™ Sep-Pak C_{18} column proved to be fortuitous as the desired carboxylic acid was obtained in an almost pure form. The crude product from the TEMPO reaction was dissolved in a small quantity of distilled water, stirred on a vortex mixer and filtered through a Sep-Pak column. The product carboxylic acids as their sodium salts stayed adsorbed in the Sep-Pak column while the water soluble inorganic salts eluted through. The product was obtained by eluting with MeOH:H_2O (2:1) and contained little or no side products after this work up. The filtered aqueous washings were analysed by TLC and NMR and were found to contain tiny amounts of the carboxylic acid sodium salt and a mixture of inorganic salts. All yields reported are isolated yields and the products were fully characterised spectroscopically (except for the sodium salt of methyl glucuronide which proved difficult to purify due to its high solubility in H_2O).

The initial results obtained with TEMPO mediated oxidation were very encouraging and suggested that a proper selection of the reaction conditions would

result in obtaining the target formononetin β–D-glucuronide. However, various attempts to synthesise formononetin β–D-glucuronide using the same reaction conditions failed. The insolubility of formononetin in aqueous solution was again a major problem to the success of the reaction. It was also presumed that, perhaps due to the solubility problem the oxidation reaction of the oxoammonium ion with alcohols was slow allowing available oxoammonium salt to react with hydroxyl ion (**Scheme 4.23**) rather than the alcohol.



4.8 **4.2**

Scheme 4.23

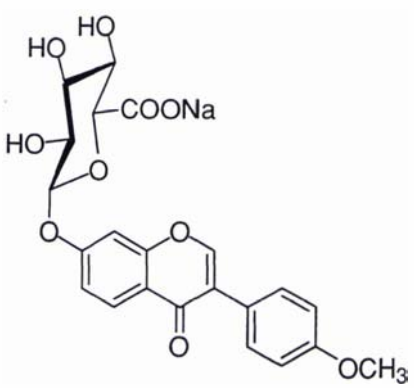
For these reasons even though the literature indicated that in an organic solvent without water or with a low concentration of water the reaction inhibits the formation of the carboxylic acid, the reaction was repeated with stoichiometric amounts of TEMPO in the more polar solvents DMF, 1,4-dioxan and acetonitrile with H_2O (thus providing homogeneous reaction conditions). Unfortunately the TLC and mass spectra indicated no formation of aldehyde or acid but only more decomposed product. This was confirmed when estrone glucoside was reacted under similar conditions in DMF/H_2O and 1,4-dioxan/H_2O since it gave a reduced yield of the final product estrone glucuronide compared with the reaction in aqueous medium only. Due to instability problems with the chromene ring of isoflavone the TEMPO mediated reaction was also performed at buffered condition using $NaHCO_3$. Under this condition the starting material formononetin β -D-glucoside remained unreacted. Various attempts were made by changing the conditions but all attempts were unavailing. The table below summarises the attempts to selectively oxidise the primary hydroxyl group of formnononetin-β-D-glucoside (ononin).

Table 4.2 Attempted TEMPO mediated selective oxidation of formononetin glucoside (ononin)

Equiv of TEMPO	Reaction conditions	Comment	Ref.
0.005	H_2O, NaBr, NaOCl (15%), 0^0C, 0.5 h	Initial drop in pH was fast which changed slowly after initial NaOH consumption and then the change in pH was very slow. TLC indicated unreacted ononin **4.50** and decomposed product due to mass 242.	35
0.01	H_2O, NaBr, NaOCl (15%), 0^0C, Overnight	TLC was monitored frequently which indicated decomposition with no difference in TLC ovenight.	35
0.01	H_2O, NaBr, NaOCl (15%), 0-20^0C, 0.5 h-overnight	Observation was same as before with very little difference overnight.	35
1	H_2O, NaBr, NaOCl (15%), 0-20^0C, 0.5 h-overnight	Observation was same as before with very little difference overnight.	25, 35
0.01	1,4-dioxan, H_2O, NaBr, NaOCl (15%), 0^0C, 0.5 h	Drop in pH was seen immediately, 0.1ml NaOH was consumed, TLC analysis indicated unreacted ononin, formononetin and other decomposed products. Mass spectrum indicted weak product peak among other impurities.	_
0.1	DMF, H_2O, NaBr, NaOCl (15%), 0^0C, 3-5 mins.	Drop in pH was fast, initially NaOH was consumed, solution turned pale yellow color, TLC showed unreacted ononin and decomposition. Weak product peak seen in mass spectrum.	_
0.1	DMF, H_2O, KBr, NaOCl (15%), 0-20^0C, 2 h	Solution became intense yellow, initially NaOH was consumed, unreacted ononin and decomposed products seen.	_

0.1	DMF, H_2O, KBr, NaOCl (15%), 0^0C, Air was bubbled in to the reaction mixture, 1h.	Initial drop in pH was fast, initial NaOH consumption was fast, solution turned pale yellow color, TLC showed unreacted ononin and decomposition product due to mass 242. Weak product peak seen in mass spectrum.	42
0.2	CH_3CN, KBr, NaOCl (15%), 0-5^0C, 0.5-2 h	TLC analysis showed unreacted ononin, decomposed product formononetin and other base line impurities.	_
0.2, 1.0	CH_2Cl_2, aq. Na_2CO_3, 0^0C, NaOCl (15%), brine, 3 h	TLC analysis showed unreacted starting material, excess hypochorite (3 mol equi.) gave slight decomposition and mass spectrum indicated no peak due to desired product.	21, 25
0.01	CH_2Cl_2, KBr, 0^0C, NaOCl (15%), mixture stirred on vertex, 1 h	TLC analysis showed unreacted starting material and base line impurity	43, 44

As indicated in the above table various parameters and reaction conditions were changed without any success. When TEMPO mediated oxidation was tried in organic/aqueous medium and the products were purified using a Sep-Pak column the mass spectrum indicated a weak peak due to $(M-H)^+$ or MH^+ at 465 or 467 respectively for the product **4.88** on several occasions.



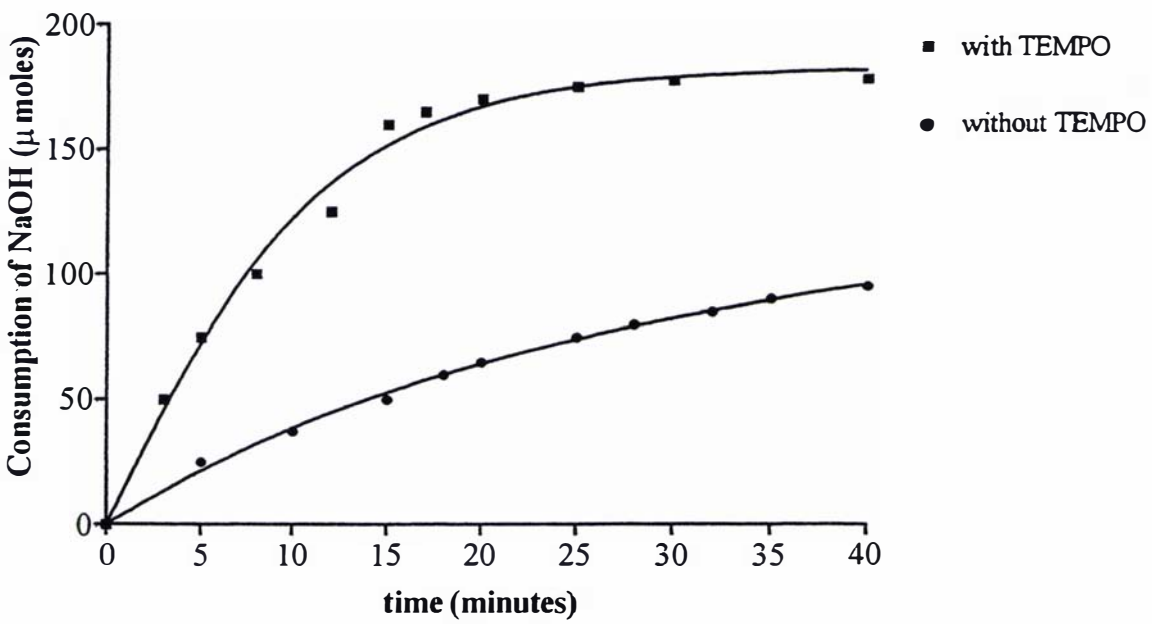
4.88

Figure 4.8

The product peak was accompanied with a strong peak due to decomposition products. The ^{1}H NMR and ^{13}C NMR indicated only a complex mixture and no peaks due to desired product seen.

To understand the role of TEMPO in the selective oxidation reaction formononetin glucoside was reacted with and without the presence of TEMPO under standard conditions of general procedure. Surprisingly there was very little difference in the consumption of NaOH (0.5 M) with or without the presence of TEMPO. This shows that the base consumed formononetin glucoside reaction predominates over the TEMPO mediated oxidation of formononetin glucoside and explains why the reaction failed in alkaline conditions. When the consumption of NaOH was studied in the TEMPO mediated oxidation of 2,6-dimethyl glucoside a marked difference in the consumption of NaOH was seen (**Figure 4.8**). The consumption of NaOH was rapid with TEMPO present but was slow in its absence. This explains why the TEMPO mediated oxidation was successful.

Figure 4.9 Attempted oxidation of 2,6-dimethyl phenol glucoside with and without TEMPO added to the mixture at pH 10



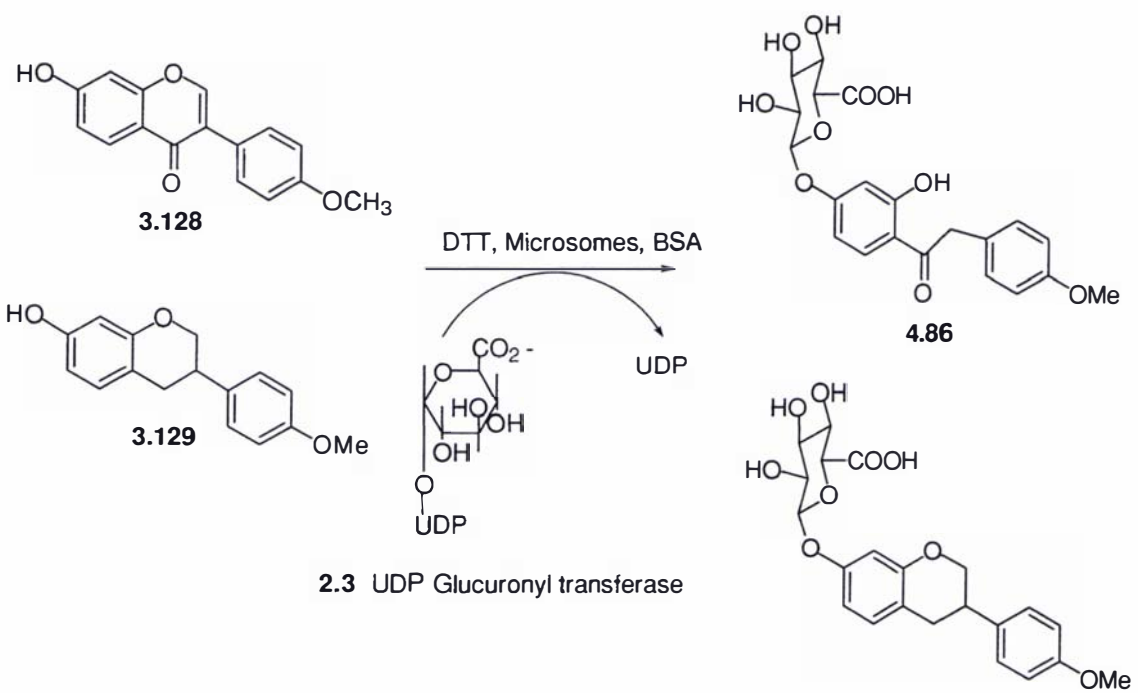
While alkaline conditions are preferred (to neutralise the acid produced during the reaction) the decomposition was an obvious result under alkaline conditions for

formononetin glucoside. The reaction gave no oxidation product under neutral or slightly acidic conditions either.

Its been also reported that carbohydrates can be oxidised selectively in the presence of catalytic amounts of PtO_2, but the method not only failed to oxidise formononetin glucoside but also the simple phenolic glucoside, *p*-cresol-β-D-glucoside. These results were in agreement with a recent report by Herbert et al who showed that their attempts to oxidise phenyl-β-D-glucoside with PtO_2 failed.[31] Earlier Marsh[45] also reported that methyl-α and β-glucuronide and methyl-α and β-galactopyranuronide were prepared by oxidation with catalytic amounts of PtO_2, but attempts to oxidise phenyl-α- and β-D-glucosides failed.

4.3.4 Enzymatic Synthesis Of Formononetin And Methoxy Equol Glucuronides

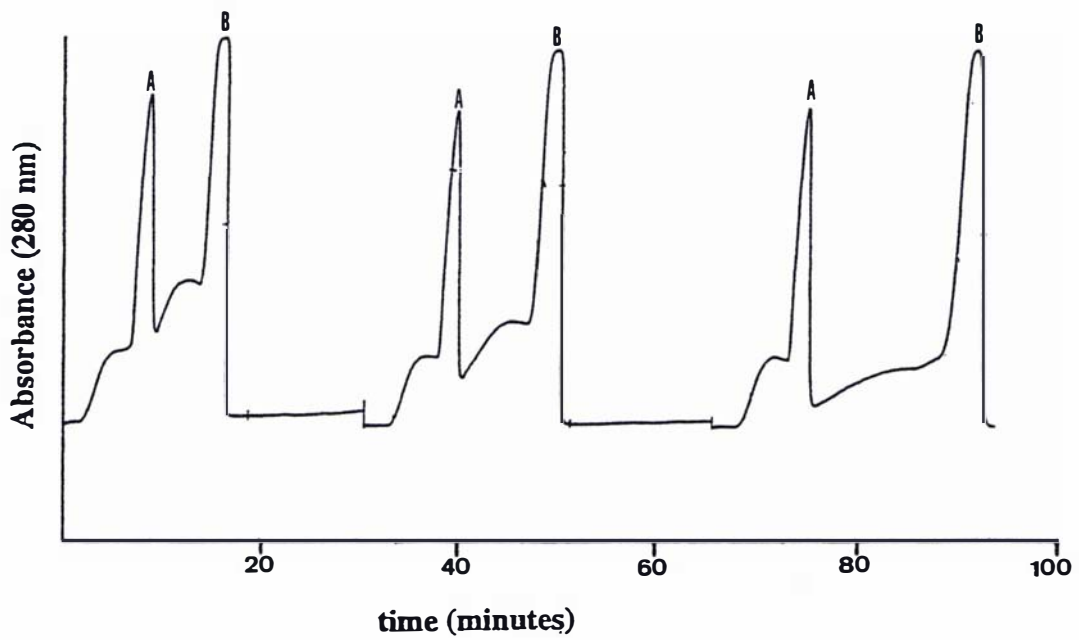
With all previous attempts to synthesise isoflavone and isoflavan glucuronide using various chemical methods such as coupling with various glycosyl donors and TEMPO mediated selective oxidation of formononetin glucoside having failed attention was focused onto the synthesis of these compounds using UDP-glucuronyl transferase (**Scheme 4.24**).



Scheme 4.24

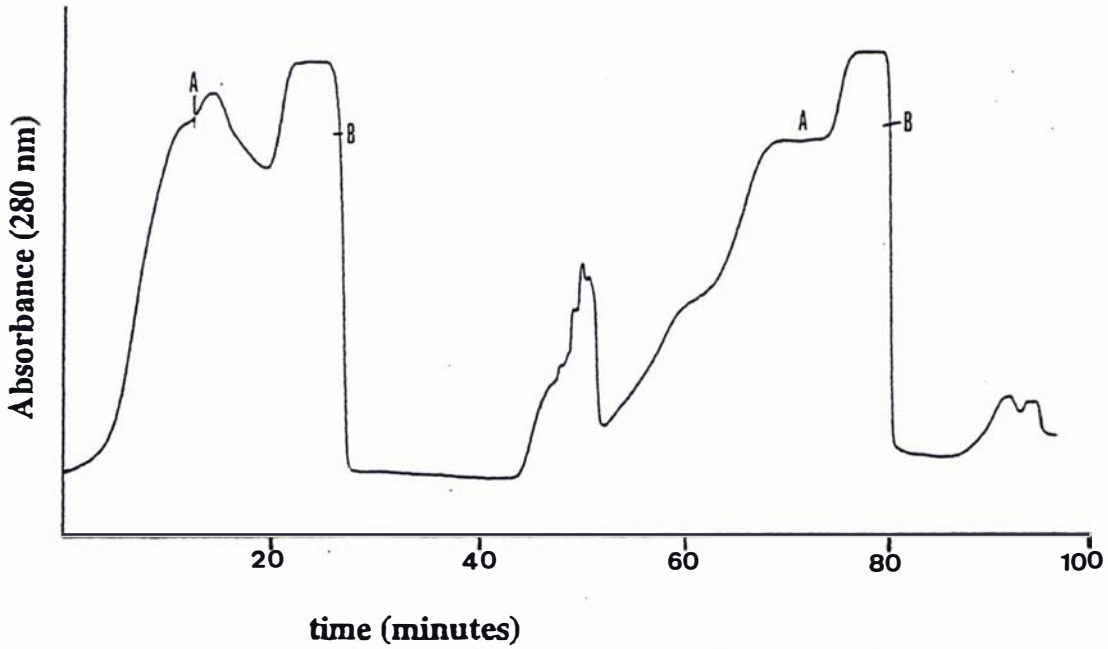
The crude yellow extract obtained from the enzymatic reaction of formononetin and (±) methoxy equol provided by Dr David stevenson showed on TLC unreacted starting material and other unknown impurities. Initial attempts to purify the crude reaction mixture and hence isolate the glucuronide in pure form failed. Thus the crude reaction mixture was purified using a LiChroprep RP-18 (40-63 μm) column from Merck. The eluted fractions from the column were collected and monitored on silica TLC plates using EtOAc/MeOH/AcOH (67:30:3) as solvent system. Visualisation of the TLC plates was by UV or spraying with concentrated H_2SO_4. The unreacted starting material appeared at an R_f value of 0.75 whereas the product appeared at R_f = 0.47. The chromatogram in **Figure 4.10** reveals the peaks obtained during the purification of methoxy equol glucuronide; the desired product and the starting material.

Figure 4.10 LiChroprep RP-18 elution profile for the mixture obtained by enzymatic reaction of (±) methoxy equol



The fractions from the trailing edge of the product peak B in **Figure 4.10** were pooled together and the spectroscopic and mass spectral data (**Section 4.2.7**) obtained to confirm the product as (±) methoxy equol glucuronide **4.87**. The same purification procedure was also applied to the crude reaction mixture obtained from the enzymatic reaction of formononetin which showed the chromatogram as shown in **Figure 4.11**.

Figure 4.11 LiChroprep RP-18 elution profile for the mixture obtained by enzymatic reaction of formononetin



The spectroscopic and mass spectrum data (**Section 4.2.7**) suggested that the pure product obtained was in fact 2,4-dihydroxy, 4'-methoxy deoxybenzoin glucuronide **4.86**. The failure to affect the glucuronidation of formononetin but rather the production of 4-hydroxy, 4'-methoxy deoxybenzoin glucuronide **4.86** instead shows that cleavage of the chrome ring has occurred (**Chapter 3, Section 3.3.1.2**). Even though the actual reason for the formation of 2,4-dihydroxy, 4'-methoxy deoxybenzoin is unclear it is believed that the decomposition occurred either during the enzymatic conversion or the isolation procedure.

4.3.5 General Characterisation Of Glycoside Esters, Glucosides And Glucuronides By NMR Spectra

For all the prepared tetra acetyl glycoside ester derivatives, the 2'-, 3'- and 4'-protons are substantially deshielded compared with the 1'- and 5'-protons due to the fact that they are directly connected to the strong electron withdrawing acetate groups. However, in all the prepared glucosides since the 2'-, 3'- and 4'-protons are now directly linked to hydroxyl groups after hydrolysis of the acetyl groups, their chemical shift values moved upfield and hence they had lower values than 1'- and 5'-protons. A similar shift was also seen in the selective oxidation products (the glucuronides). The diastereotopic methylene protons at C6' in the glycoside ester derivatives and glucosides

showed an *AB* or *AX* pattern as they are not chemical shift equivalent (presumably due to restricted rotation) and hence splits by vicinal protons giving two doublets or a doublet and a multiplet were observed. The *trans*-diaxial relationship (β-orientation) of the sugar ring with the aryl or steroid moeties were confirmed by the large coupling coupling constants (> 7 Hz). The β-orientation was also confirmed by the ^{13} C NMR which showed anomeric carbon (C1') peak in the range of 99 to 105 for all the prepared glycosides, glucosides and glucuronides. In all glycoside ester derivatives, H1' to H5'-protons of the sugar moiety were assigned unambigiously by ^{1}H-^{1}H 2D-COSYspectra (**Figure 4.13** for example). The correlation of H1' (5.06 ppm) with H2', H2' with H3', H3' with H4', with H5', H5' with one of the protons at C6' (H6') and the correlation of the two protons at C6' (H6') with each other were all recognisable in the spectrum. Similarly the ^{1}H-^{1}H 2D-COSYspectra of all the glucoside derivatives were used to recognise the H1' to H5'-protons of the sugar moiety (**Figure 4.14** for example). In addition HETCOR and DEPT (**Figure 4.15**) for example experiments were also carried out to support the assignments of peaks

Similarly for the enzymatically prepared 2,4-dihydroxy, 4'-methoxy deoxybenzoin glucuronide (**4.86**) the aromatic protons (H3, H5 and H6 in the A ring and H2', H6', H3', H5'- in ring B), the $-CH_2$ and the $-OCH_3$ group and the H1" to H5"-protons of the glucuronic acid moiety were all assigned unambigiously by ^{1}H-^{1}H 2D-COSYspectra (**Figure 4.16**). In addition HETCOR (**Figure 4.18**) experiment was also carried out to support the assignment of the peaks.

Whereas for enzymatically prepared methoxy equol glucuronide (**4.87**) the aromatic protons (H3, H5 and H6- in the A ring and H2', H6', H3', H5'- in ring B), the 2 x $-CH_2$ group in ring C, the $-OCH_3$ group and the H1" to H5" of the glucuronic acid moiety were all assigned unambiguously by ^{1}H-^{1}H 2D-COSYspectra (**Figure 4.17**). In addition the DEPT experiment (**Figure 4.19**) were also carried out to support the assignment of the peaks.

The presence of the sugar ring in the correct orientation and the β-orientation of the glucuronide moiety with respect to the aryl moiety, was confirmed by the large coupling constant J =7.62 Hz in the ^{1}H NMR. This was also confirmed by ^{13}C NMR as

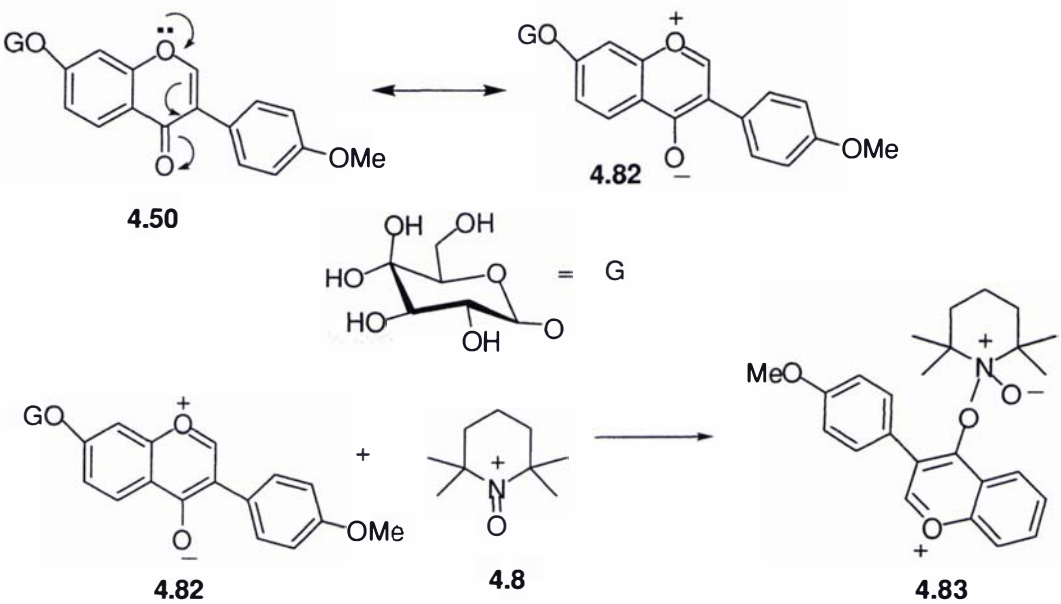
the anomeric carbon (C1") showed a peak at 101.6 ppm and 100.2 for 2,4-dihydroxy, 4'-methoxy deoxybenzoin glucuronide and methoxy equol glucuronide respectively.

In the 2D COSY spectrum for 2,4-dihydroxy, 4'-methoxy deoxybenzoin glucuronide **4.86** (**Figure 4.16**), two pairs of correlated aromatic protons (H2', H6' and H3', H5') appeared at 7.26 and 6.76 ppm respectively as doublets, while the aromatic proton H6 (8.0 ppm, d) correlated to H5 (6.66 ppm, dd). The H3-proton appeared at 6.80 ppm as a singlet. The correlation of H1" (5.06 ppm) with H2" (3.61 ppm), H2" with H3" (3.45-3.48 ppm), H3" with H4" (3.59-3.63 ppm), and H4" with H5" (4.05 ppm) were all clearly recognisable in the spectrum. The correlation of the H2" and H3" protons was hidden due to both protons appearing at the same position (3.61 ppm). The -CH_2 group at 4.35 ppm and the -OCH_3 group at 3.74 ppm appeared as a singlets.

The presence of the glucuronic acid moiety of 2,4-dihydroxy, 4'-methoxy deoxybenzoin glucuronide (**4.86**) at C-4 was also supported by long range coupling experiment HMBC (**Figure 4.20**). The 2D HMBC spectrum correlates chemical shifts of heteronucleus (generally ^{13}C) and protons (^1H) *via* the long-range heteronuclear coupling $^nJ_{CH}$ where n>1. In 2D HMBC spectrum each cross peaks means that the corresponding ^1H and ^{13}C are two ($^2J_{CH}$) or three bonds ($^3J_{CH}$) away. Residual direct connectivities are usually present as large doublets due to $^1J_{CH}$. The present long range HMBC experiment (**Figure 4.20**) was optimised for J_{CH} =7 Hz. The $^2J_{CH}$ values for aromatic rings are <7 Hz and hence the $^2J_{CH}$ values were not seen. The $^3J_{CH}$ values on aromatic ring were clearly recognisable based on the following correlations: H6 correlates to C3 (104.2 ppm), C4 (165.1 ppm) and –COR (203.8 ppm), (H2', H6') correlates to CH_2 (44.0 ppm), (C2', C6') (130.4 ppm), C4' (159.1 ppm) and (C3', C5') (114.0 ppm), H5 correlates to C3 (104.2 ppm) and C1 (114.7 ppm), H3 correlates to C5 (108.3 ppm), C1 (114.7) and C4 (165.1 ppm), where as -CH_2 correlates to, C1' (127.1 ppm), (C2', C6') (130.4 ppm), (C3', C5') (114.0 ppm) and –COR (203.8 ppm) and OCH_3 correlates to C4' (159.1 ppm). In the glucuronide moiety the sugar protons showed only ($^3J_{CH\ eq}$) and ($^3J_{CH\ ax}$) were not seen due to small coupling constant (J<7 Hz) for ($^3J_{CH\ ax}$). Hence H1" correlates to C4 (165.1), and H2", H3" correlates to C4" (72.0), (C3", C5") (76.3). The fact that H1" correlates to C4 proved that sugar moiety is attached at C4 and not at C2.

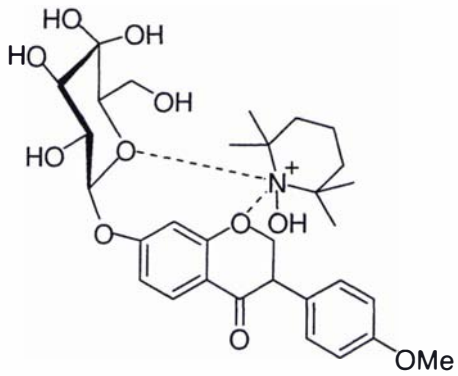
4.3.6 Summary

While selective oxidation of the various glucosides has been successfully achieved on phenol glucosides and steroid glucosides the method was unsuccessful for formononetin glucoside. The literature reveals no previous attempts of synthesis of steroid glucuronides using this new methodology. The relative insolubility of formononetin and the instability of the chromene ring under alkaline conditions were compounding problems in this case. The failure to oxidise the primary -OH group of the formononetin glucoside in the presence of TEMPO was also thought to be due to interferences by the electron withdrawing aryl ether ring *via* possible formation of a TEMPO-formononetin complex due to the presence of the large degree of negative charge resident on the carbonyl oxygen of resonance structure **4.82**.

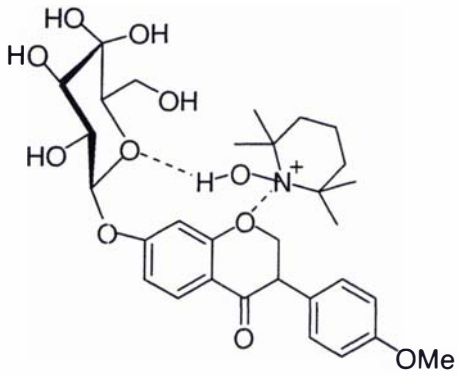


Scheme 4.25

Two other possibilities for the formation of formononetin glucoside-TEMPO complexes also exist where the positive charge on TEMPO binds to the negative charge on the oxygen atom of the glucoside ring and the negative oxygen of the chromene ring such as **4.84** and **4.85** as shown in the **Figure 4.12**.



4.84



4.85

Figure 4.12

The TEMPO oxidation method proved very valuable for the synthesis of the sterically hindered phenol 2,6-dimethyl phenol glucuronide in good yield. This is the first time 2,6-dimethyl phenol glucuonide has been successfully synthesised. Hence this method provides access for the synthesis of glucuronides which are otherwise difficult to achieve using direct coupling methodologies.

Unfortunately even with the enzymatic method synthesis of the phytoestrogen metabolite formononetin glucuronide failed giving insteadthe deoxybenzoin derivative. Although (±) methoxy equol glucuronide was synthesised successfully by this methodology the time available did not allow larger amounts of pure material to be isolated. As a result insufficient quantities of methoxy equol glucuronide were available to allow syntheses of immunogens and conjugates for homogeneous enzyme immunoassays. Thus, to explore the possibility that home multi purpose monitor for a range of biomarkers can be developed attention was focussed on other steroid glucuronides. Future efforts will however produce sufficient amounts of (±) methoxy equol glucuronide for further research into establishing an immuno assay.

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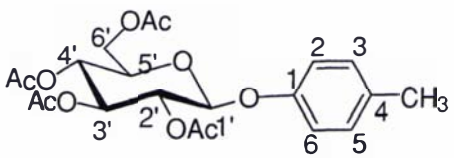
Figure 4.13 COSY Spectrum Of **4.51**



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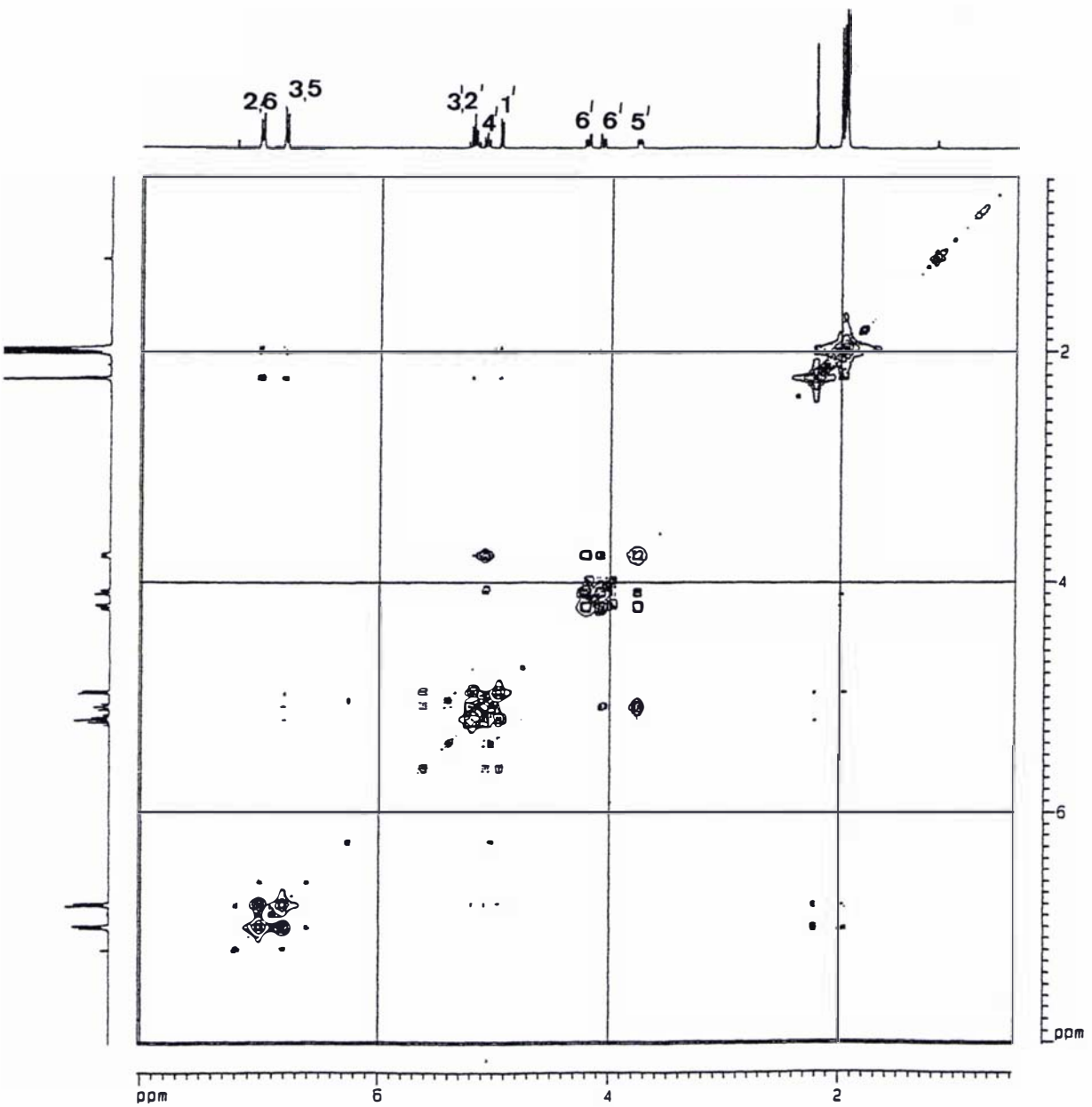

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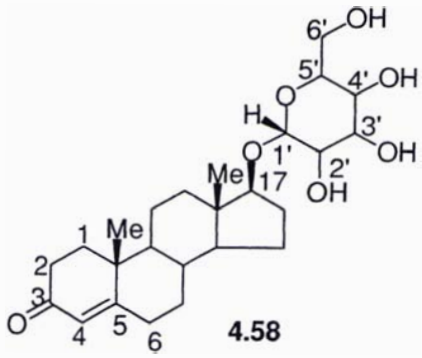

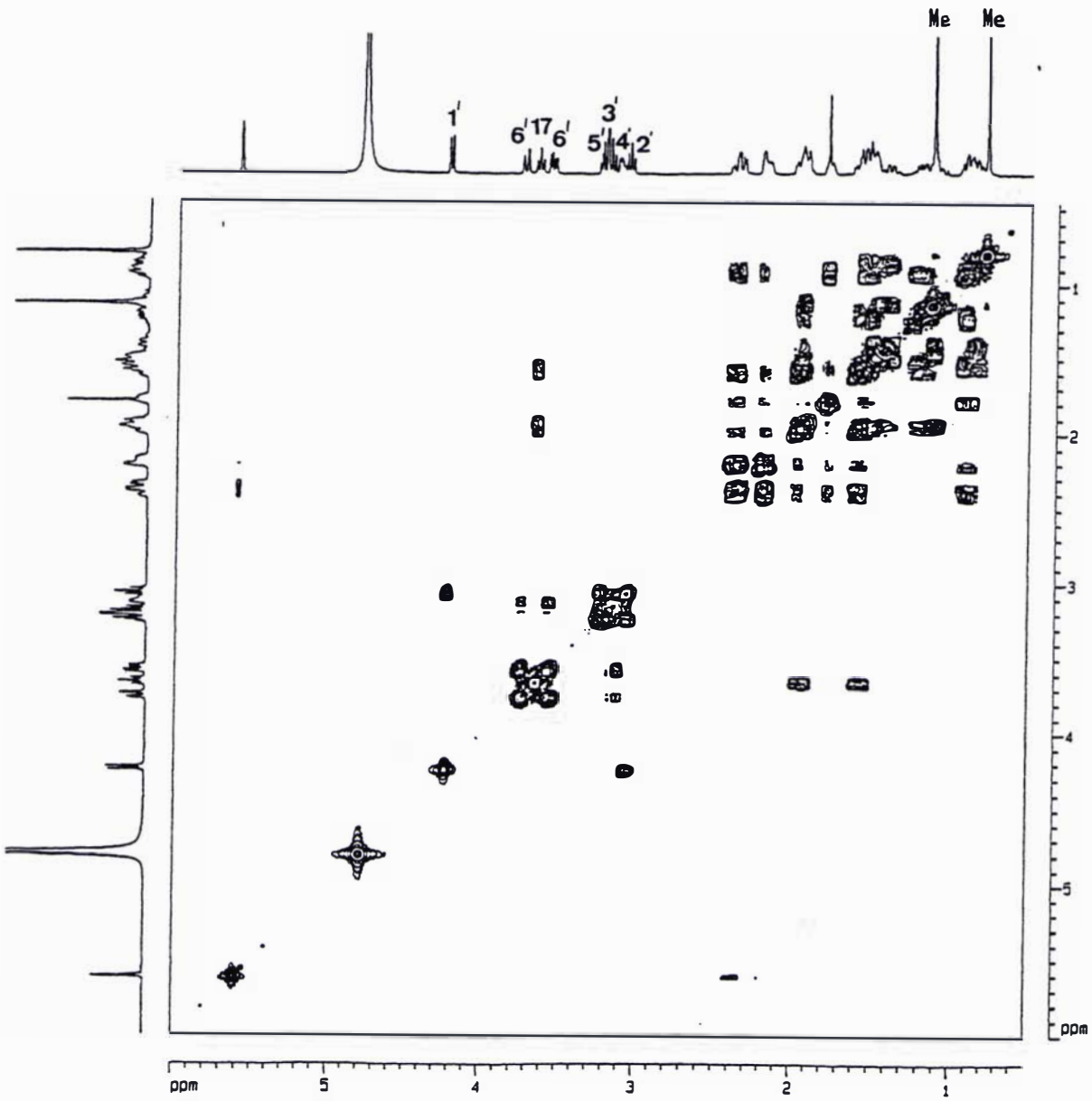

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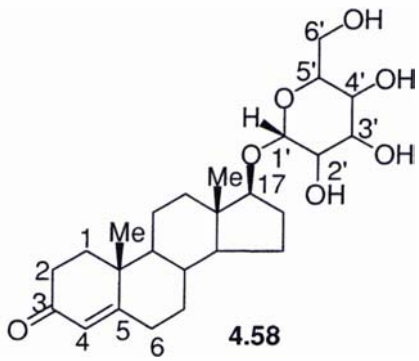


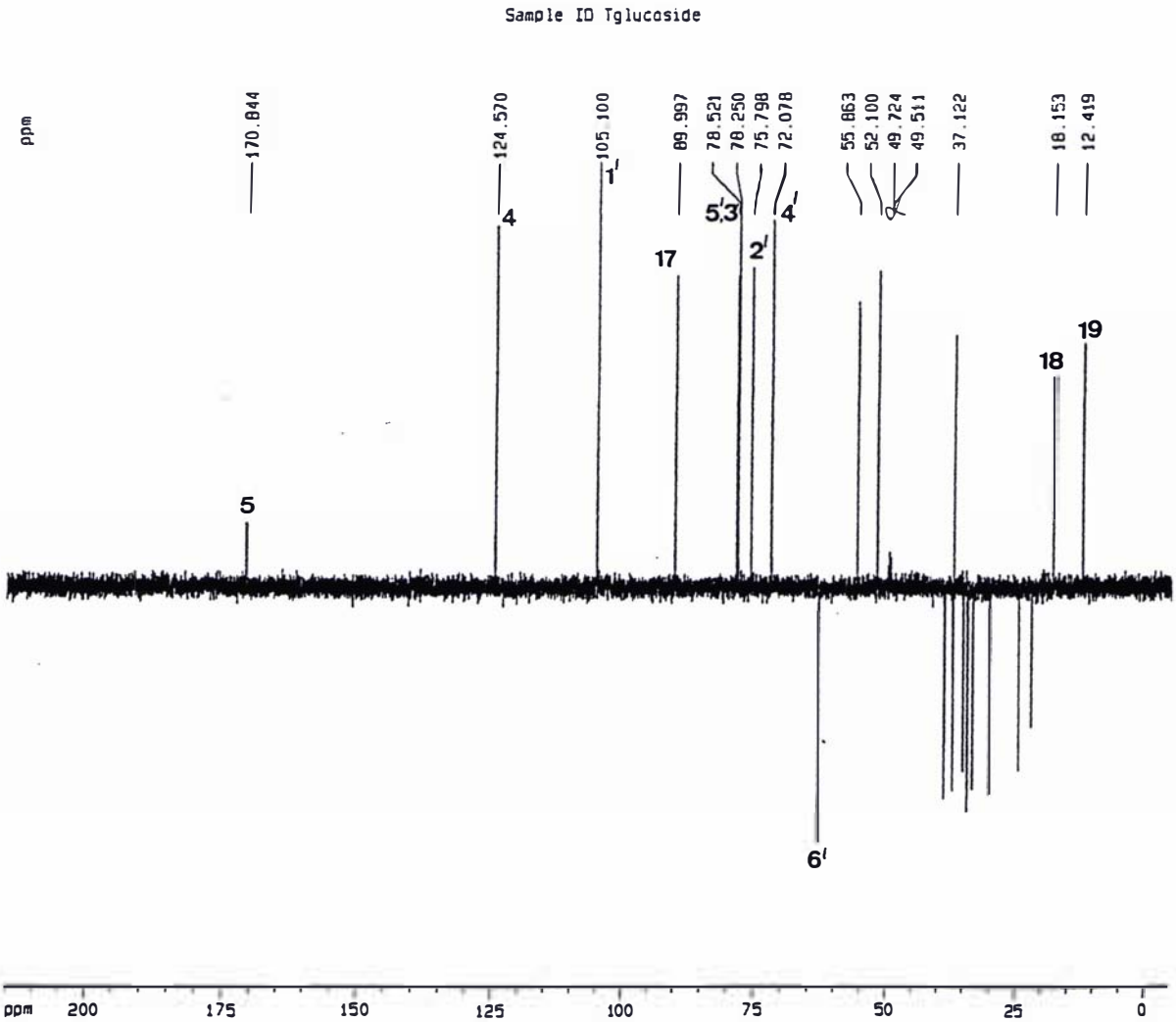
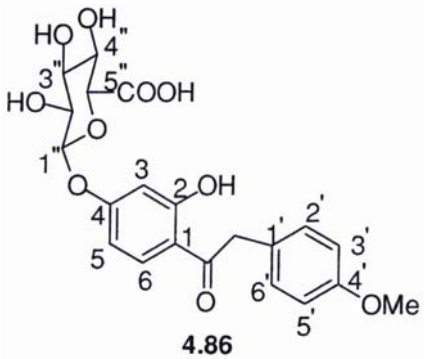


Figure 4.16 COSY Spectrum Of **4.86**



4.86

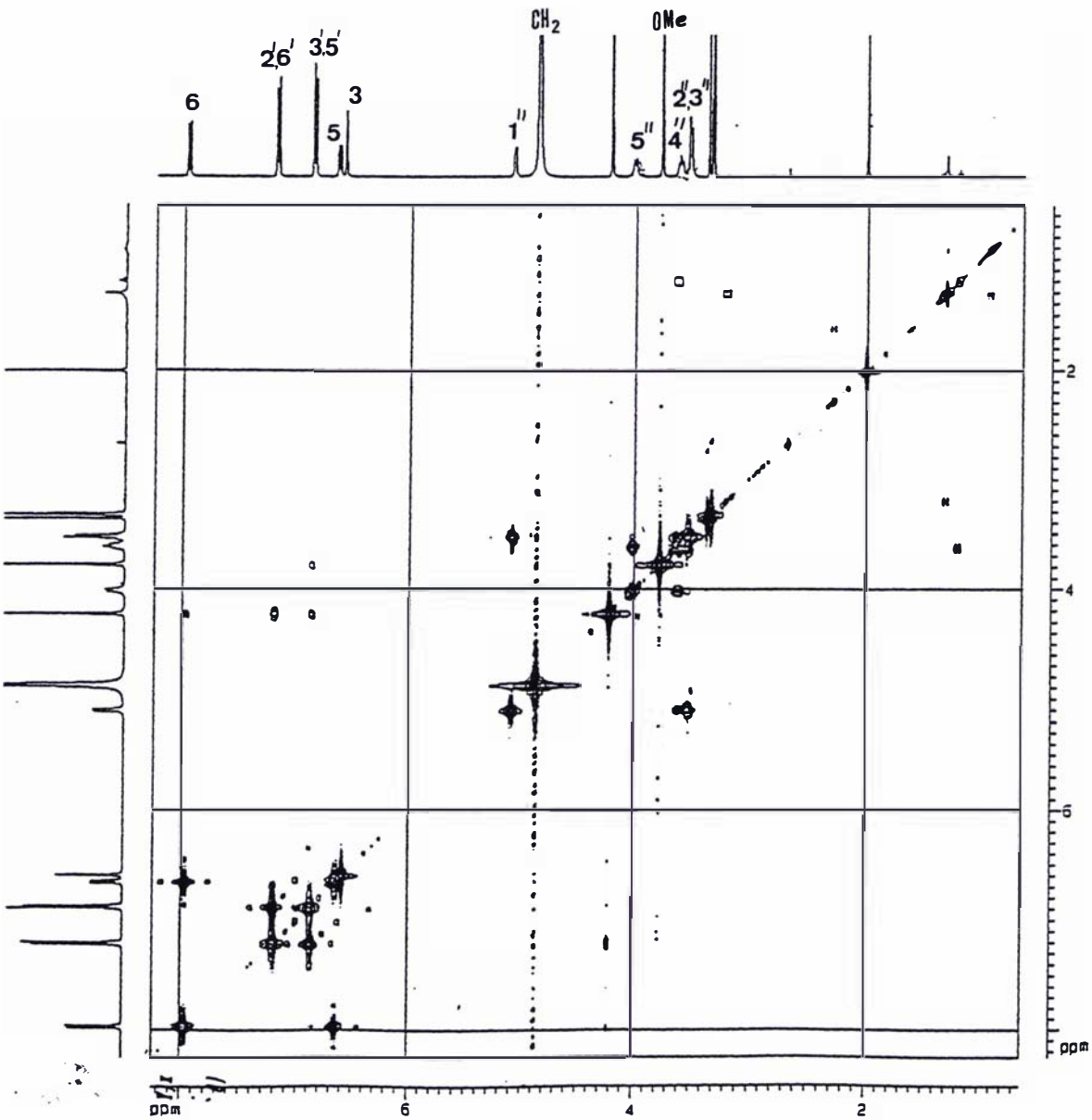
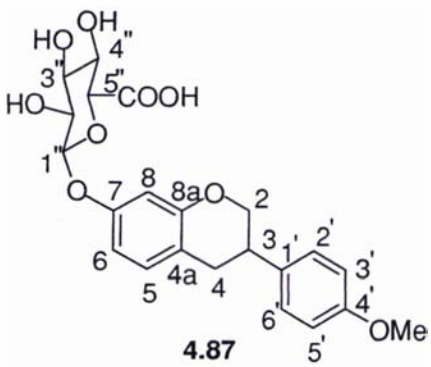


Figure 4.17 COSY Spectrum Of **4.87**



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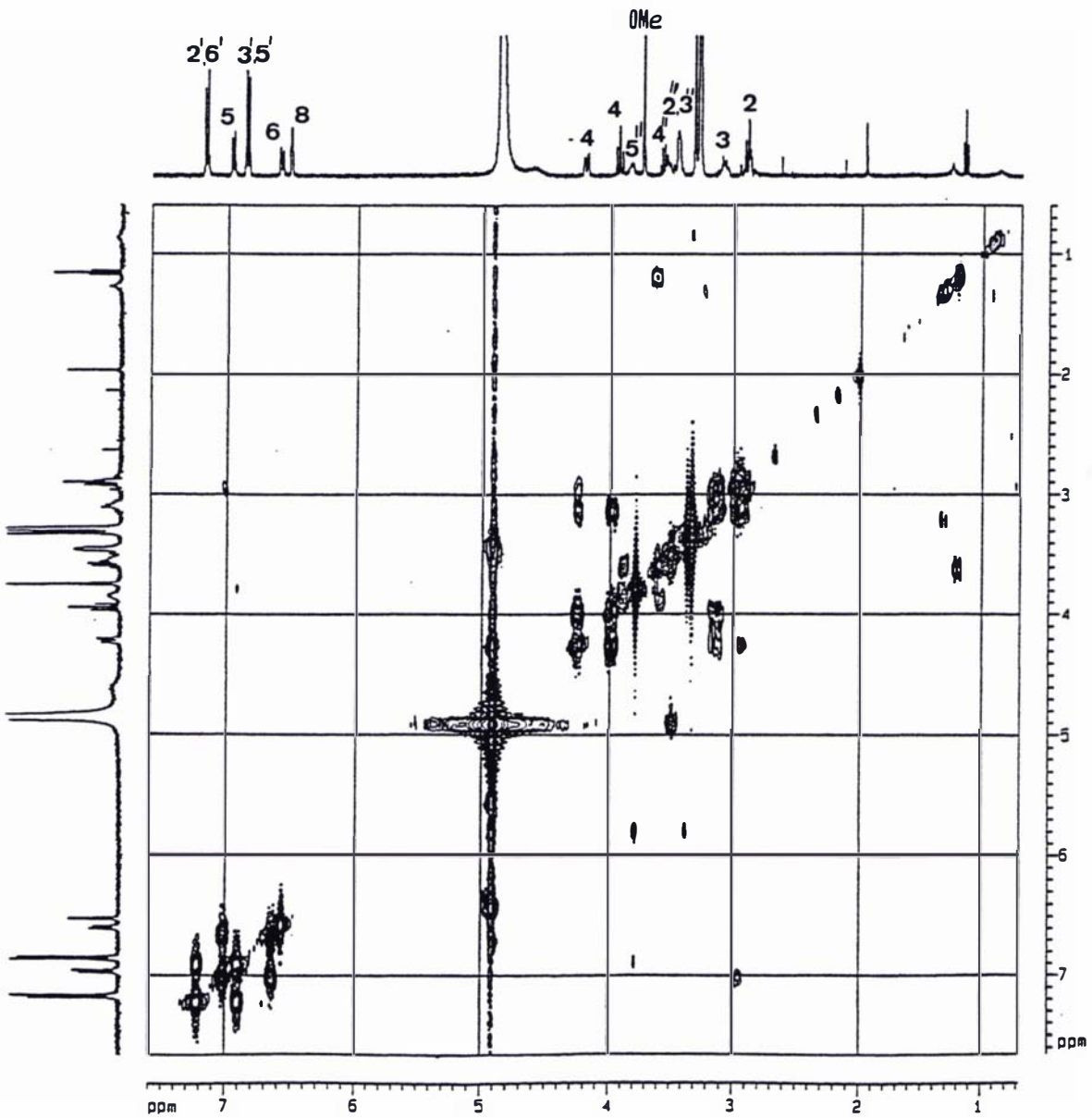

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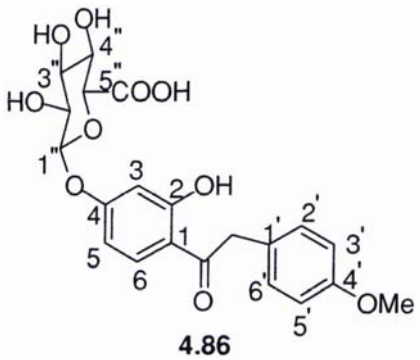

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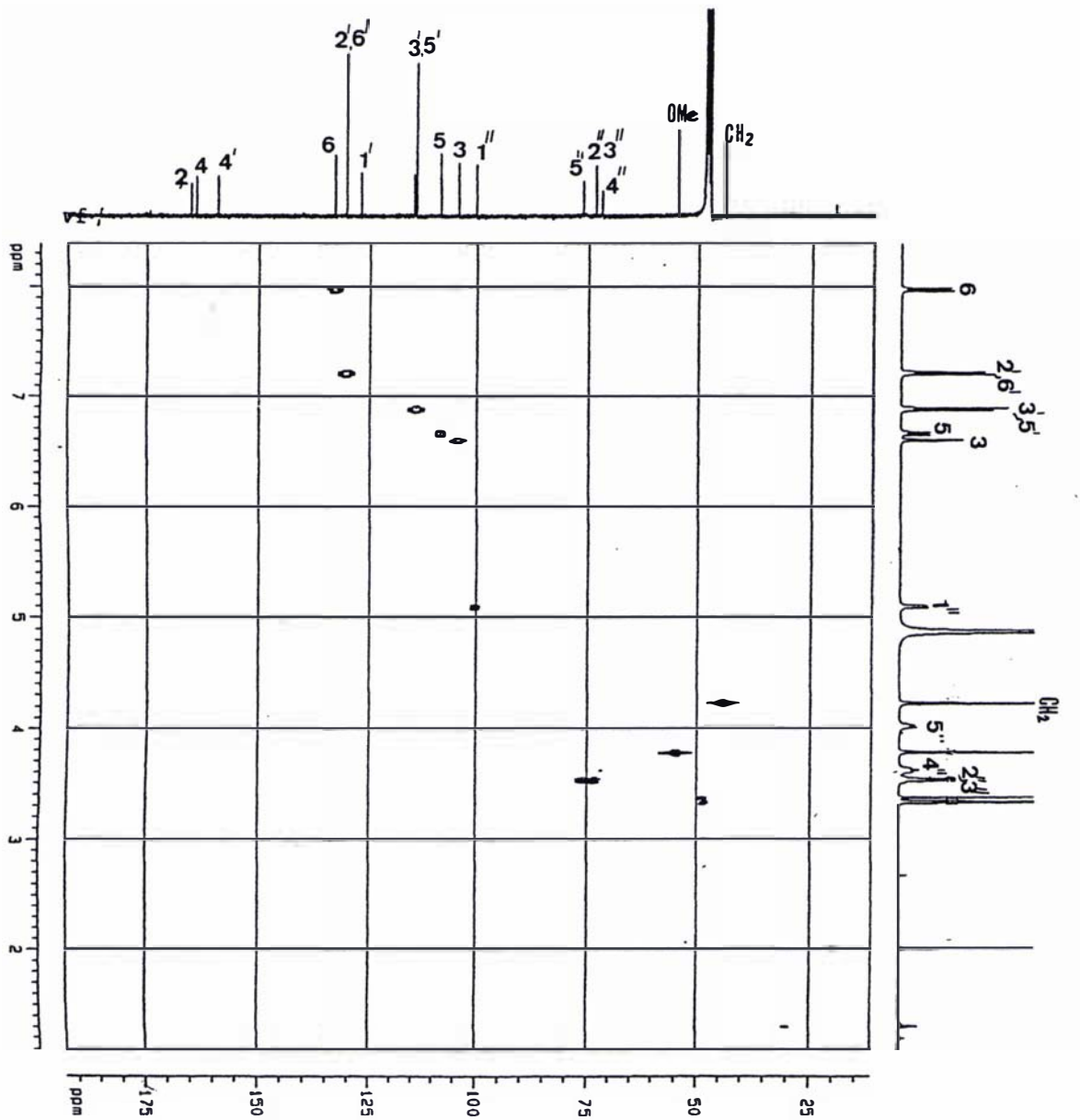

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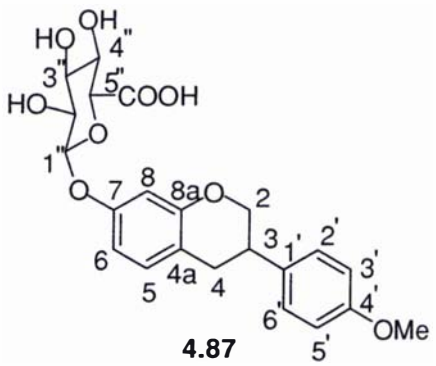
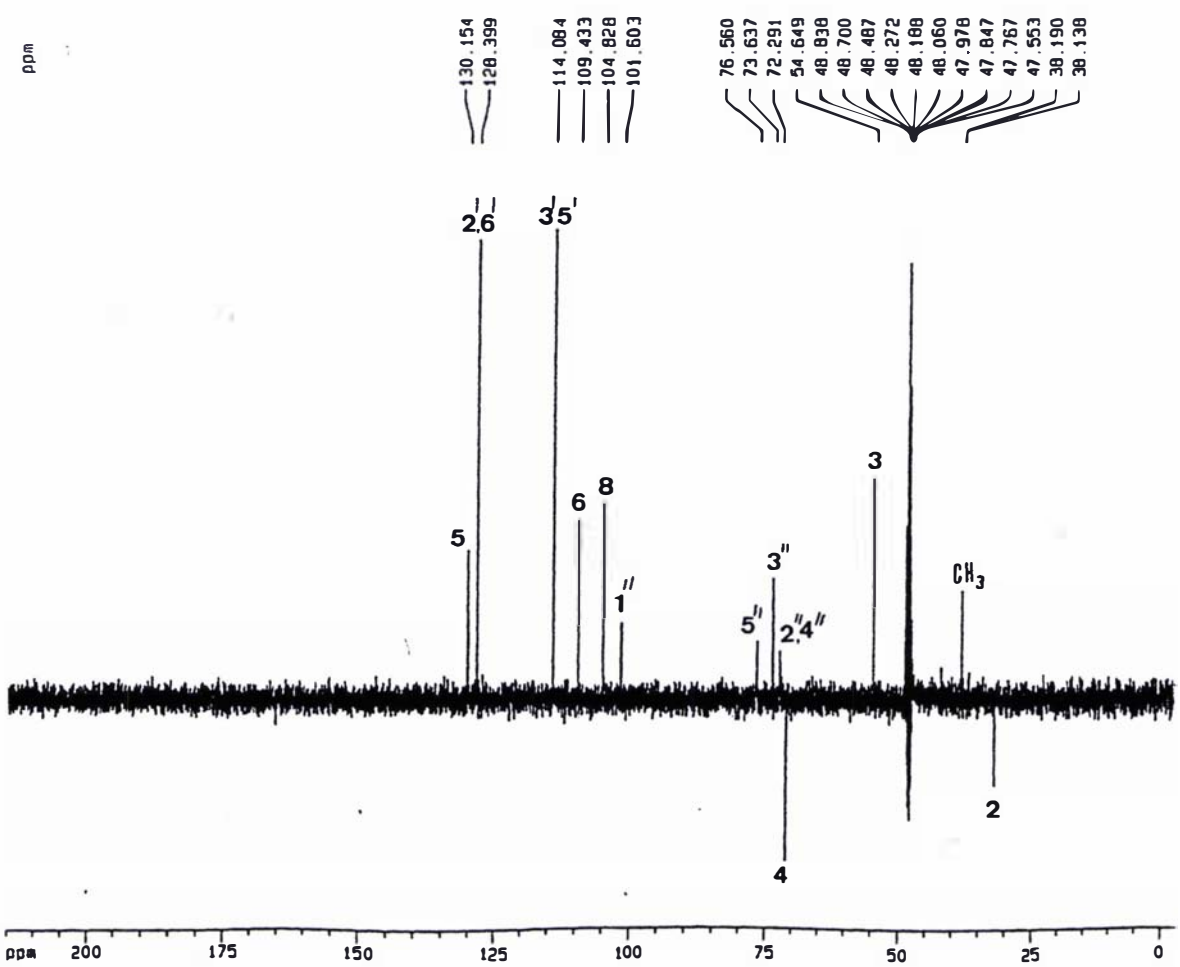


4.87



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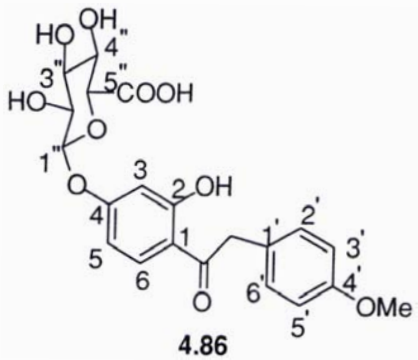

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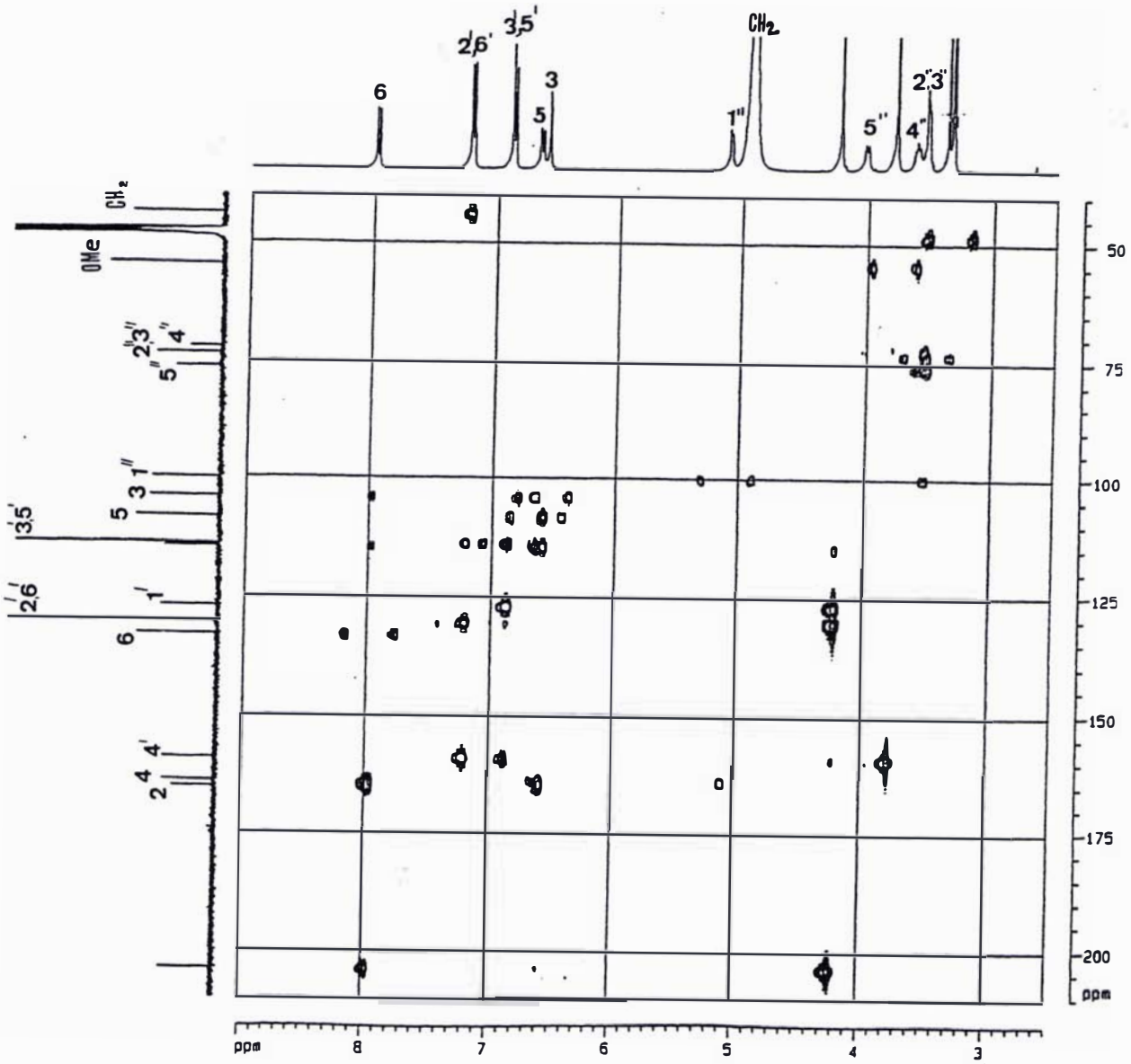


CHAPTER 5 SYNTHESIS AND CHARACTERISATION OF STEROID GLUCURONIDES AS HAPTENS FOR USE IN A HOME MULTI-PURPOSE MONITOR

5.1 INTRODUCTION

As the first point listed in **Section 2.1.10** ((i) synthesis of the analyte glucuronide) has not been achieved for formononetin glucuronide and so far only in small amounts for (±) methoxy equol glucuronide, the requirements for a multi-purpose monitor (**Section 2.1.10**, points i - v) were tested with the more familiar and more easily obtainable steroid glucuronides. Even without the target phytoestrogen glucuronide there is still a considerable interest in extending the range of applications of the monitor to include other steroids such as testosterone and cortisol (or its metabolites). As a first step in the raising of an appropriate antiserum in sheep or rabbits for the development of new enzyme-steroid glucuronide immunoassays studies a ready supply of steroid glucuronides is needed as standards and starting materials. In general the desired steroid glucuronides can be obtained through three main sources: (i) commercial purchase (up to NZ$ 10,000 per gram for E1G and NZ$ 42,000 per gram for TG); (ii) extraction from urine; or (iii) by direct chemical synthesis. As it is very expensive to purchase the amount of steroid glucuronides needed for assay development and the extraction method is difficult and time consuming, the chemical synthetic approach was taken for the preparation of steroid and other glucuronides in this thesis. The most commonly used method for the synthesis of steroid glucuronides involves the *O*-glycosylation reaction of a glycosyl donor with an appropriate steroid aglycone under Koenigs-Knorr conditions.[1,2,3] From a synthetic point of view, the efficiency of the *O*-glycosylation reaction generally involves a high chemical yield, regioselectivity and stereo selectivity. High stereoselectivity is easily achieved by selective protection of the hydroxyl groups of the glycosyl acceptor. Therefore research in this area has focused on obtaining a high chemical yield and high stereo selectivity for *O*-glycosylation reactions.

For the synthesis of steroid glucuronides, the correct stereochemistry at the anomeric carbon atom of the glucuronide ring is crucial. Since the naturally occuring metabolites of steroids all have the β-orientation at the anomeric carbon atom, any immunoassay system must be designed so that the anti-steroid glucuronide antibodies, which are raised against synthetic steroid glucuronide protein conjugates, specifically recognise the β-form of the steroid glucuronide metabolites. Clearly if linkages of the glucuronide ring in the synthetic materials were of α-orientation, the antibodies raised by injection of steroid glucuronide protein conjugates into sheep would have the wrong stereospecificity. All antibodies so far raised against authentic samples of steroid glucuronides have high specificities and bind only weakly to the natural materials. Hence an appropriate methodology for the synthesis and characterisation of the β-glucuronides is an essential pre-requisite for success in this project. Therefore in the first part of this chapter the chemical synthesis of selected steroid glucuronides as haptens will focus on linkage of the glucuronic acid moiety with the aryl hydroxyl groups of steroids to give β-linked steroid glucuronides.

5.1.1 Classification Of Steroid Glucuronides

A variety of steroid glucuronides have been isolated from natural sources, and their structures may be classified chemically into four different classes depending on the nature of the acetal linkage,[3] (i) aryl glucuronides which involve linkage to the aglycone moiety with a phenolic OH group such as **5.1,** (ii) alkyl glucuronides which involve linkage to an alcoholic OH group such as **5.2**, (iii) acyl glucuronides involving linkage to a carboxylic acid OH group such as **5.3** and (iv) "hemiacetal" glucuronides where the linkage to the glucuronide is *via* a hemiacetal OH group such as **5.4**.

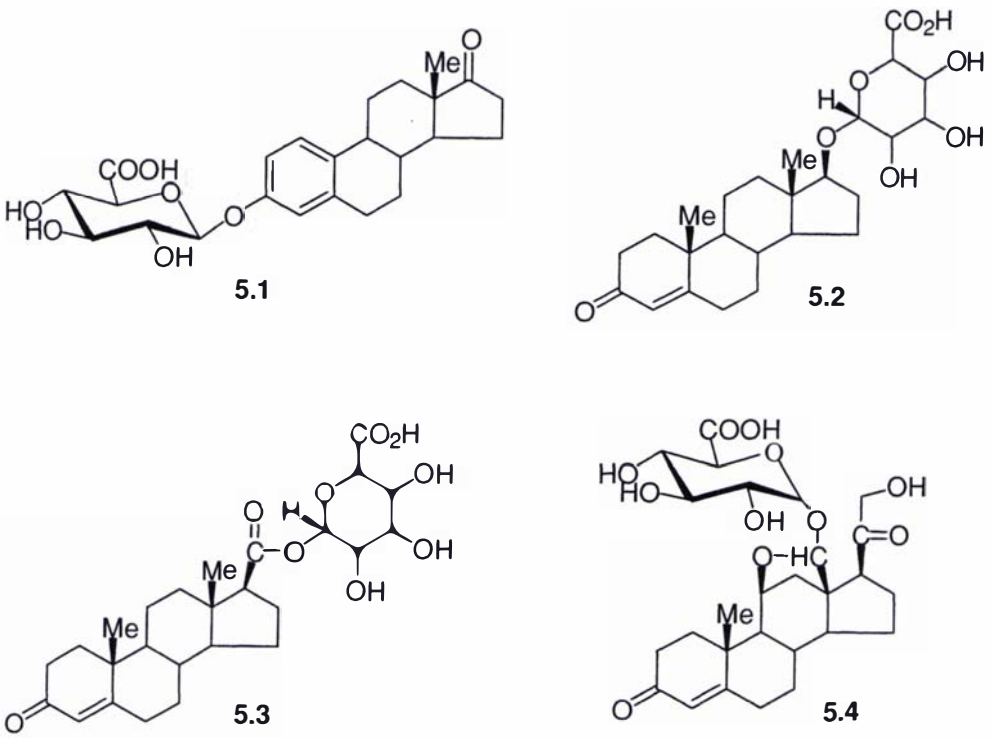


Figure 5.1

The structures of these various steroid glucuronides are shown in **Figure 5.1**. Aryl and alkyl glucuronides are probably the most commonly occurring in nature and only these two types of steroid glucronides are pertinent to this chapter.

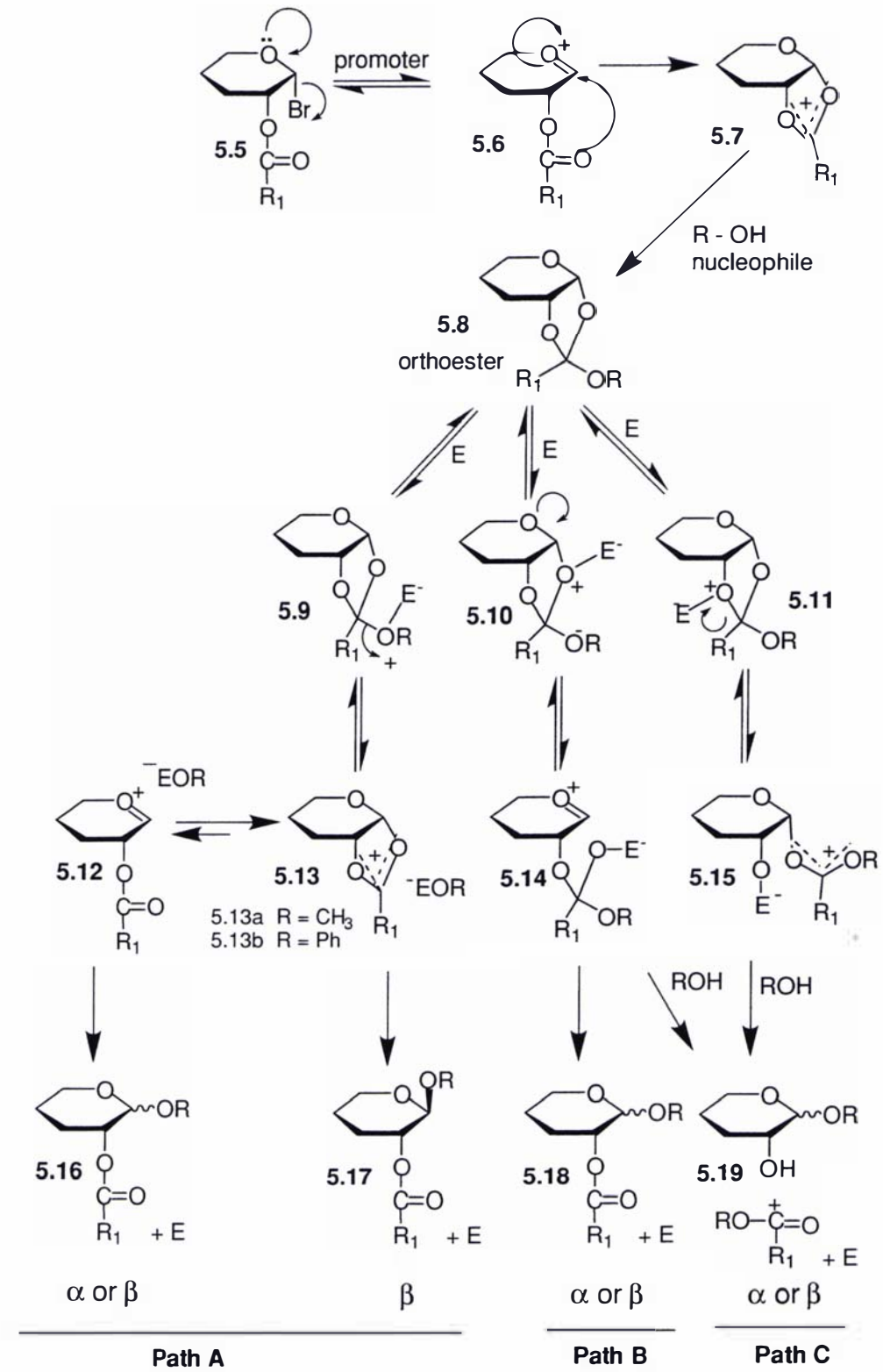
5.1.2 Koenigs-Knorr Reactions For The Synthesis Of Steroid glucuronides

The Koenigs-Knorr reaction has been described as the coupling of a halo sugar (i.e., the glycosyl halide) with an alcoholic or phenolic group under anhydrous conditions, in the presence of an appropriate promoter system. In relation to the anomeric stereo chemistry of the glycosylation reaction, there are three basic methods which have been developed: (i) the neighbouring group assisted methods for the construction of a 1,2 *trans* glycoside such as a β-gluco or α -manno type glycoside; (ii) the anomerisation method[4] for the synthesis of an α-gluco or α-manno type glycosides and (iii) the heterogenic catalyst method[5] for preparation of a β -mannoglycoside. Clearly only method (i) is related to, and most used, in the synthesis of 1,2-*trans* -glycosides such as steroid β-D-glucuronides. The reaction has been known as Koenigs-Knorr reaction since that time.

The classical Koenigs-Knorr reactions used heavy metal salts (mainly silver and mercury salts) as activating reagents and as acid scavangers.[2] Water was generally removed by drierite and molecular sieves during the glycosylation reactions.

The proposed mechanism of the Koenigs-Knorr reaction (**Scheme 5.1**) shows that the chemical yield and stereochemistry are dependent on the following parameters: (i) the structure of the glycosyl halides; (ii) the structure of the alcohol or phenol compounds (glycosyl acceptor); (iii) the promoter used; and (iv) the solvent used. The generally acceptable mechanism proposed by Garegg et al[6] to account for glycoside formation in most Koenigs-Knorr reactions is shown in **Scheme 5.1**.

Initially, the glycosyl halide, which possesses a participating acetoxy group at C-2, undergoes heterolysis of the carbon-halogen bond assisted by the promoter (halophilic reagents or halide ion acceptors) to form a shielded carbocation **5.6**, which subsequently forms a 1,2-dioxolenium ion **5.7** by intermolecular reaction of the neighbouring 2-acetoxy carbonyl oxygen atom with the electron deficient anomeric carbon atom. Subsequent nucleophilic reaction of an alcoholic or phenolic hydroxyl group with the 1, 2-dioxolenium ion results in formation of the orthoester **5.8**.



Scheme 5.1

There are three possible reaction pathways for acid catalysed glycoside formation deriving from the ortho ester **5.8**. For most Koenigs-Knorr reactions path A is the main pathway because the cyclic ion pair intermediate **5.13** is much more stable

than the acyclic ion intermediates **5.14** and **5.15** of pathways **B** and **C**. The cyclic ion pair **5.13** subsequently forms the β-glucuronide **5.17** by internal rearrangement, or via the acyclic ion **5.12**, which is in equilibrium with **5.13**, leading to a mixture of the α and β-glycosides **5.16**. It was found that the stereochemistry of Koenigs-Knorr reactions was affected by the structure of the participating alcohols.[6] For alcohols which had either decreased electron density at the alkoxyl oxygen due to electron withdrawing substituents or electron delocalisation such as in phenols, or bulky substituents on the adjacent carbon atoms, the Koenigs-Knorr reaction was more likely to produce a high proportion of α-glycosides. This can be explained on the basis of **Scheme 5.1** as follows: when the oxygen electron density of the counterion (EOR-) in the cyclic ion pair intemediate **5.13** is decreased, or if **R** is made more bulky, the rate of the β-glycoside yielding reaction (**5.13** to **5.17**) is decreased. Thus reaction by way of the more reactive but lower concentration, of the acyclic ion **5.12** then becomes more important, hence leading to a higher proportion of α-glycosides.

From the mechanistic studies of Koenigs-Knorr reactions (**Scheme 5.1**), it can be seen that the orthoester **5.8**, which is the most important intermediate, results from the nucleophilic reaction of an alcohol or phenol with the 1,2- dioxolenium ion **5.7**. Thus for phenolate oxygen atoms with low electron density (weak base) or for sterically hindered alcohol compounds there will be a decrease in the yield of Koenigs-Knorr reactions or no Koenigs-Knorr reaction, because these alcohol compounds function as poor nucleophiles. The higher yield reported[6,7] for glycosylations with benzoylated bromo-sugars, as compared with acetylated bromo-sugars, can be accounted for on the basis that the side reaction products, 2-OH glycosides **5.19**, which are an important cause of low yields in normal glycosylation reactions, are avoided. The reason could be due to the better resonance stabilisation[8] of the benzoylated cyclic ion **5.13b** than for the acetylated cyclic ion **5.13a**, which is also more stable than the related acyclic ion pairs **5.14** and **5.15** in paths B and C. Hence the pathway **5.13** to **5.17** predominates.

The type of acid used, or generated from the promoter system, is also an important factor which influences the rearrangement rate of the cyclic ion pair **5.13** to β-glycosides **5.17**. It seems that strongly acidic species in Koenigs-Knorr reactions give a high β/α ratio in the product, probably as a result of their effects on the oxygen electron density of the counterion (EOR^-), which strongly stabilises the shielded cyclic

ion pair **5.13**. The influence of the solvent on the product composition is also noteworthy. Because of the higher ionic character of the ion pair **5.12** compared with species **5.13**, and thus the higher demand for solvation by a polar solvent, polar solvents tend to give higher proportions of α-glycosides *via* pathway **A**. However, for some special solvents such as nitriles using glycosyl donor imidate sugars (**Section 5.1.3**), highly selective β-glycoside synthesis can be achieved by α-nitrilium-nitrile-conjugates as intermediates in glycosylation reactions.

So, for the synthesis of 1,2-*trans* -glycosides under classic Koenigs-Knorr conditions, the general choice of reaction condition should be: strongly basic (high electron density on oxygen) and less sterically hindered alcohols or phenols as glycosyl acceptors; (ii) benzoylated or acetylated halosugars as glycosyl donors; and (iii) non polar solvents, or some special solvents under acid conditions.

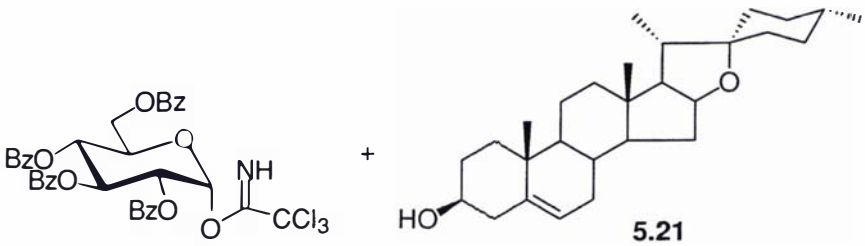
5.1.3 Trichloroacetimidate Coupling Methods

Schmidt's pioneering studies on glycosylation using trichloroacetimidates[9] have led to an increasing number of applications to glucuronidation. The thermally and chemically stable trichloroacetimidate glycosyl donor was easily synthesised from the corresponding 1-hydroxy sugar by treatment of trichloroacetonitrile with different bases such as K_2CO_3[10], Cs_2CO_3[9], and NaH or 1,8-diaza bicyclo (5,4,0) undec-7-ene (DBU).[11] Both α- and β-*O*-activated anomers can be isolated in pure form and in high yields under kinetic or thermodynamic control. Crystalline trichloroacetamidate glucuronides can be stored at -20^0C under dessication for several weeks with little decomposition. However glycoside acetimidates are thermally more stable towards storage and handling and can be kept easily at 0^0C over longer periods of time.

The catalyst used is almost invariably $BF_3.OEt_2$ or occasionally TMSOTf, however chloral has been used also as a non-acidic catalyst for glycosylations.[9] The value of the method is seen clearly in the reaction of acetimidate sugar **3.116** with *p*-nitrophenol (which is deactivated towards nucleophilic reaction) using $BF_3.OEt_2$ catalysis, affording the β-conjugate in 85% yield. For *O*-alkyl glucuronides the method may not be quite so reliable as the monoacetate of androstane-3α,17β-diol gave the 3-glucuronide ester in only 40% yield, slightly superior to the Koenigs-Knorr method but

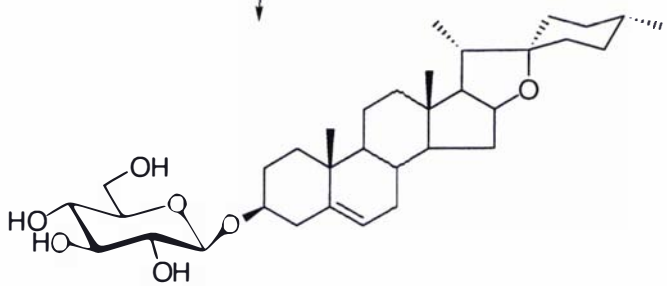
the 17-glucuronide ester was obtained in only 8% yield. Both products were stereo specifically β.

Trichloroacetimidate glycosylation has been found to have wide applications in the synthesis of natural products. Most recently, one of the most efficient synthesis of trichloro acetimidate glycosylation is reported[12] to give pharmacologically active trillin (diosegenyl-β-D-glucopyranose) in quantitative yield (**Scheme 5.2**). All the previous protocols of thermal glycosylations, 1,2 anhydro sugar and glycal methodology involve expensive and toxic promoters and moderate yields. Benzoyl protected stable imidates were readily prepared from the corresponding 1-hydroxy sugars and coupled with various steroidal glycosyl acceptors using catalytic amounts of TMSOTf as the promoter in CH_3CN. This gave the corresponding β-coupling products (saponins) in excellent yield. Moreover it was found that the reaction was virtually complete within 5 minutes and no special care was needed regarding for example, the amount of TMSOTf used, the addition sequence, the addition speed or the reaction temperature. The only two important factors are the benzoyl protection and the TMSOTf promotion. The glucuronide moiety was also introduced into sapogenins in excellent yields by the present procedure. Moreover these sapogenins can be selectively oxidised afterwards to give glucuronic acids.



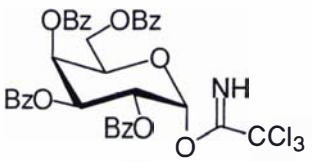
5.20 + 5.21

(i) TMsOTf (0.05 eq),CH_3CN
(ii) NaOMe

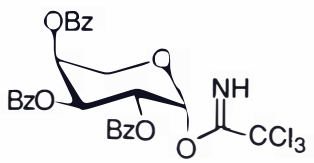


5.22 Trillin (100%)

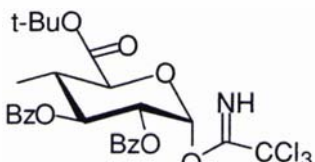
Selected donors



5.23

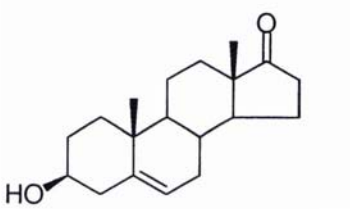


5.24

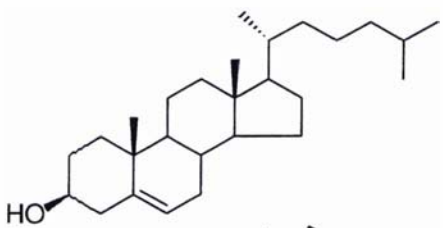


5.25

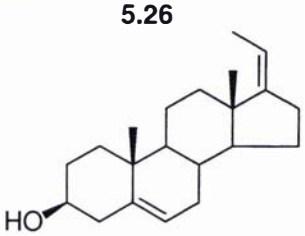
Selected acceptors



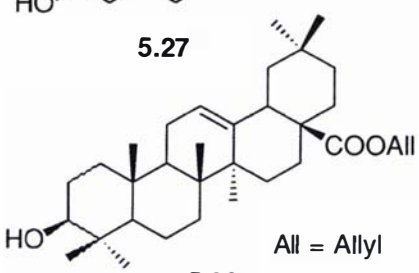
5.26



5.27



5.28



All = Allyl

5.29

Scheme 5.2

5.1.4 β-Selective Glycosylation Reactions Of The steroids Estrone And Testosterone

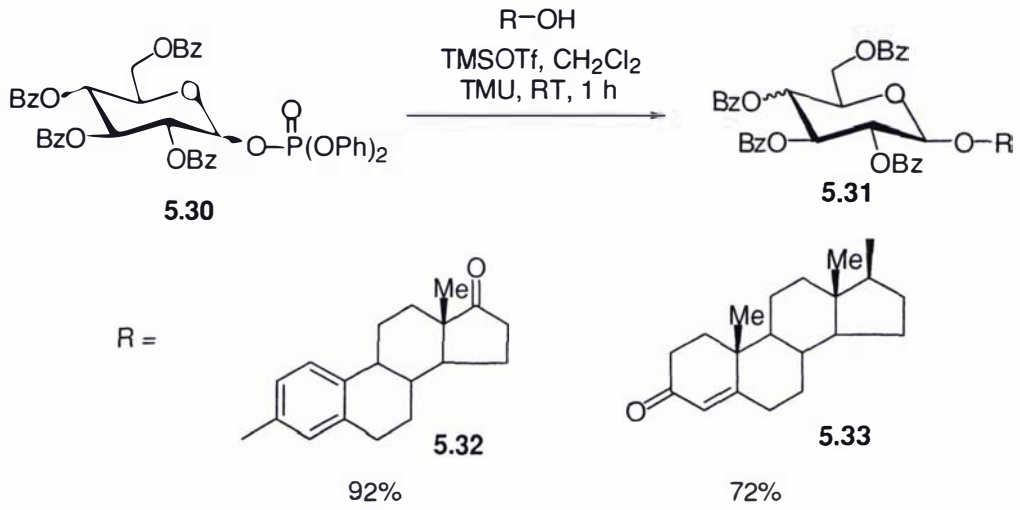
Estrone glucuronide can be prepared simply by direct glucuronidation of estrone **5.53** with various glycosyl donors followed by subsequent hydrolysis to remove the protecting groups. Previously, standard Koenigs-Knorr reactions using Ag_2CO_3 to obtain methyl [17-oxo-estra-1,3,5(10)-trien-3-yl-2,3,4-tri-*O*-acetyl-β-D-glucopyranoside] uronate **5.50** (henceforth abbreviated to estrone–3-β-D-glucuronide triacetate methyl ester) have not exceeded yields of approximately 10%. In 1971 Conrow and Bernstein[2] published a report on an investigation of the catalytic effect of various metals mainly as their carbonates or oxides on the glucuronidation of estrone. They were able to synthesise estrone–3-β-D-glucuronide triacetate methyl ester with methyl 2,3, 4-tri-*O*-acetyl-1-bromo-α-D-glucopyranuronate (bromo sugar) **3.8** in refluxing toluene using HgO as a catalyst, to give an improved yield of 25%. The disadvantage of this method was that the product was contaminated with organo mercury complexes which were difficult to remove. Later on they reported a much improved Koenigs-Knorr synthesis of estrone–3-β-D-glucuronide triacetate methyl ester by using $CdCO_3$ as acid scavanger which avoided the problem of low yield.

There has been also previously two methods reported[13,14] for the synthesis of methyl 2,3,4-tri-O-acetyl-β-D-glucopyranosiduronate 17-β-3-oxo-androst-4-en-17-yl **5.56** (referred to as testosterone 17-β-glucuronide triacetate methyl ester) by classical Koenigs-Knorr coupling of testosterone with bromo sugar **3.8** in the presence of the promoter Ag_2CO_3. The product **5.51** formed in the reaction was separated from the excess reactants by counter current distribution and purified by fractional re-crystallisation before subsequent hydrolysis to give testosterone glucuronide (TG). Recently a synthesis of d_3-testosterone glucuronide has been reported.[15] Hence d_3-tesosterone 17-β-glucuronide triacetate methyl ester was prepared using the promoter $CdCO_3$ in 60% yield. The product was isolated using column chromatography and then subsequently hydrolysed to give the desired d_3-testosterone glucuronide as fine crystals.

Newly developed phosphorus containing glycosyl donor groups have been shown by Hashimoto *et al* [16] to be very promising alternative promoters for Koenigs-Knorr reactions. The glycosyl phosphate **5.30** can be conveniently prepared from the

corresponding bromo sugars by silver carbonate catalysed hydrolysis of the glycosyl halides followed by rapid phosphorylation with dicholorophosphate and DMAP, or simply by reacting the corresponding bromo sugar with dibenzyl phosphate in a catalytic two phase system using TBHS as a catalyst.

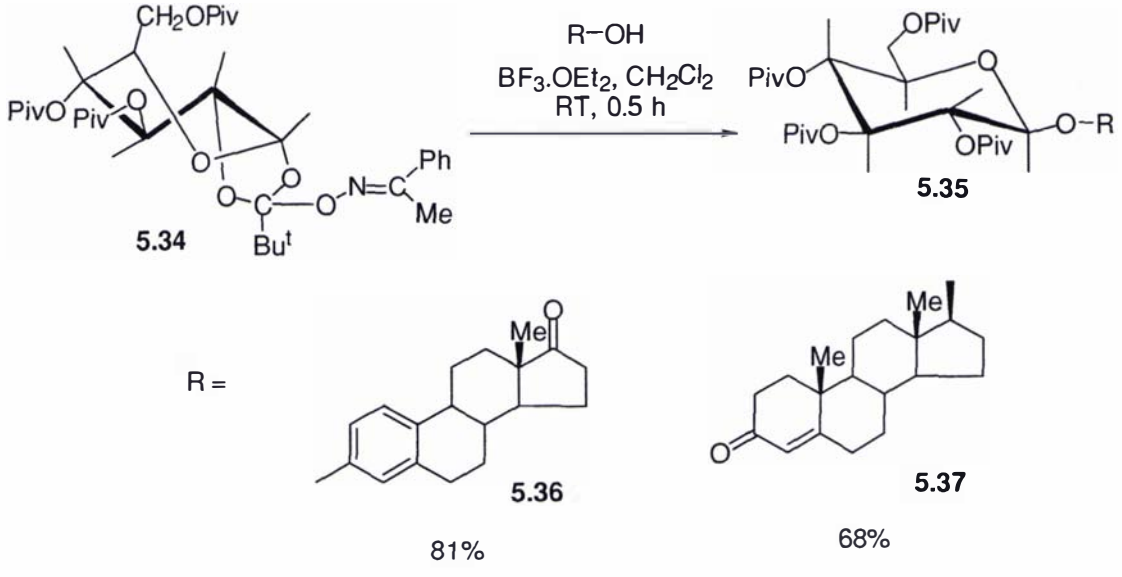
Hashimoto *et al* [16] successfully used the glycopyranosyl phosphate **5.30** as a glycosyl donor in the presence of TMSOTf for extremely rapid entry into glycosylation of estrone and testosterone respectively with high β-selectivity. The reaction afforded excellent yields as well. The glycosylation reactions also showed that the anomeric configuration of the glycosyl donor was not crucial to either the stereochemical outcome or the final yield of the glycosylation reactions. Besides the use of glycosyl bromide and phosphorus derivatives as glycosyl donors there have been some other types of glycosyl donors used in Koenigs-Knorr reactions of steroids. Among the known 1, 2-*trans* glycosylations the traditional orthoester methods suffer from the disadvantage that besides the desired glycoside from the direct glycosylation of the starting ortho ester, a second isomeric glycoside is also obtained by rearrangement (reesterification) of the starting orthoester to a new orthoester followed by glycosylation.



Scheme 5.3

To rectify this re-esterification problem a new type of oximate ortho ester has been developed by Kunz *et al* [17] and employed in the effective 1,2-*trans*-glycosylation of some complex alcohols and phenols including estrone and testosterone. The

advantage this method offers is that it does not give rise to the unwanted side products which are normally produced in orthoester glycosylations and also allows the stereo selective β-glycosylation to take place of both alcoholic (testosterone) and phenolic hydroxyl groups (estrone) under identical conditions.



Scheme 5.4

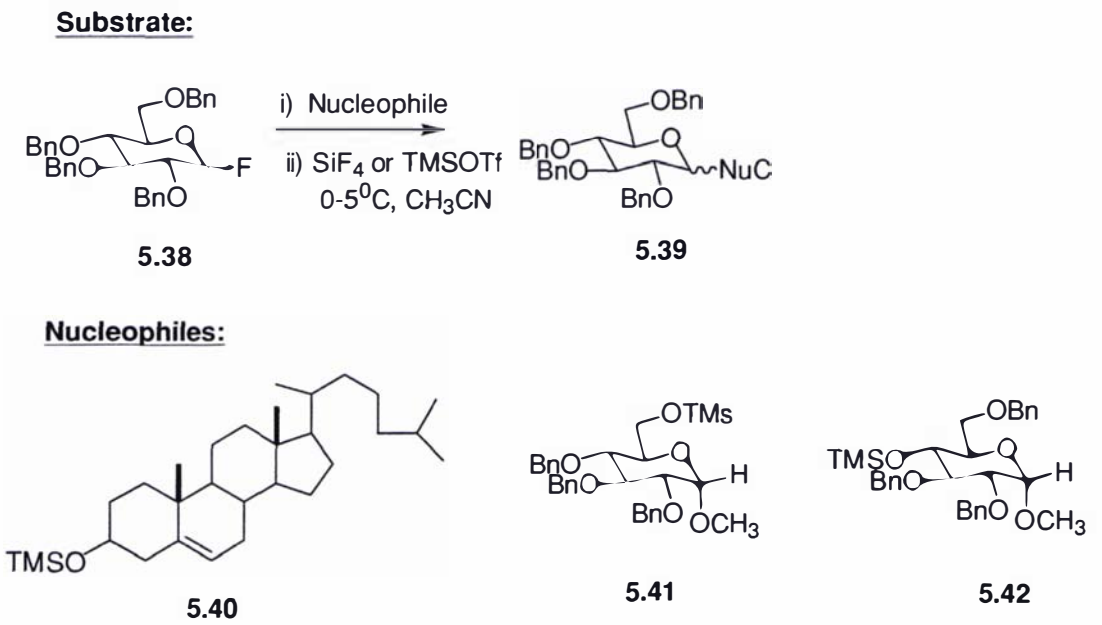
During the course of writing this thesis Werschkun and co-workers[18] reported the powerful Lewis acid $BF_3.OEt_2$ as a catalyst in the synthesis of estrone–3-β-D-glucuronide triacetate methyl ester by coupling of estrone to the tetracetate sugar **3.4** in CH_2Cl_2 at RT. The reaction was stereochemically reliable giving only β-anomers of the conjugates.

5.1.4.1 Influence Of Nitriles As Solvents On The Glycosylation Reactions

The stereochemical outcome of the glycosylation reaction in the neighbouring group participation is strongly influenced by the solvent. In many cases either the α or the β isomer can be obtained selectively by varying the solvent conditions. In general the percentage of the β-glycoside or glucuronide produced increases with the solvent polarity.

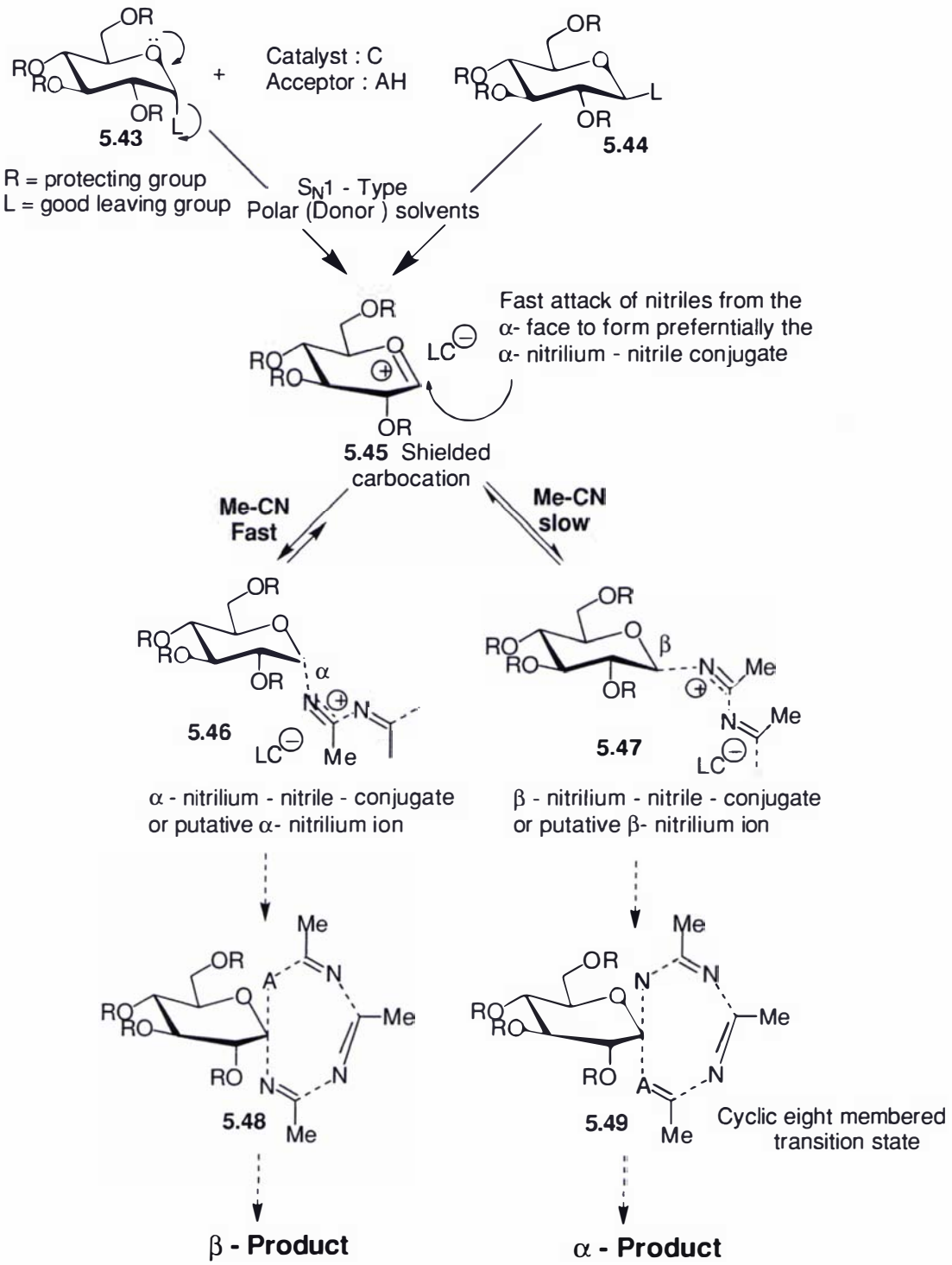
Particularly noteworthy is the glycosylation reactions of various methyl silyl ethers with substrates having a nonparticipating benzyloxy group at C-2.[19] The

glycosylation reaction in the presence of the catalyst tetrafluorosilane or TMSOTf in acetonitrile gave the β-glycoside with moderate to high stereoselectivity whereas the reaction in ether afforded the α-anomers predominantly.



Scheme 5.5

Besides the general polarity of the solvents, which affects the stabilities of different ion pair intermediates (such as ion pair **5.12** and **5.13** in **Scheme 5.1**) by solvation, some nitriles react with different glycosyl donors and function as important reaction intermediates to control the stereochemical outcome of the glycosylation reactions. A literature survey reveals that a number of activators have been used for β-glycosylation using nitrile as a solvent. Activators include fluoride (F^-)[19], methyl thio (-SMe), diphenyl phosphate[-OP(O)$(Oph)_2$], p,p-diphenyl-N-(p-toluenesulfonyl) phosphine imidate [-OP(=NTs)$(Ph)_2$][16] and trichloroacetimidate [-OC(=NH)CCl_3][12] as leaving groups at the anomeric carbon. The explanation of these highly β-selective glycosylation reactions using nitrile as solvents is outlined in **Scheme 5.6**.



Scheme 5.6

The shielded carbocation **5.45** as the reaction intermediate was generated from different glycosyl donors, which need excellent leaving groups, under S_N1-type conditions. At very low temperatures, fast attack of solvent nitriles from the α-face of the equatorial ion **5.45** provided the α-nitrilium-nitrile-conjugate **5.46** which led to the

β-coupling products (kinetic control). However at relatively high temperatures, formation of the themodynamically more stable β-nitrilium-nitrile conjugate is favoured thus furnishing the α-products. A cyclic eight membered transition state, leading to intramolecular glycoside-bond formation as shown in **Scheme 5.5** may be hypothesized to explain the high reactivity and selectivity. Hence, highly selective β-glycosylation reactions in solvents such as in nitriles require excellent leaving groups at the anomeric carbon of the glycosyl donors and performance of glycosylation reaction at low temperatures.

5.1.5 RIA Method For Measurement Of Testosterone Glucuronide In Urine

Given that only small amounts of (±) methoxy equol glucuronide (**Section 4.2.7**) are currently available another target molecule was needed to test the hypothesis that any hapten glucuronide can be used in place of E1G or PdG in the Ovarian monitor assay, thus producing a multi-purpose monitor home test. It is necessary to demonstrate that another analyte glucuronide can be used to prepare good anti-hapten glucuronide antibodies and highly inhibitable lysozyme-hapten glucuronide conjugates. Although it will be possible in the future to obtain sufficient quantities of (±) methoxy equol glucuronide for this purpose the time constraints of this thesis dictated that another choice be made.

In males testosterone is secreted by the leydig cells of the testes. Females produce about 5% to 10% as much testosterone as do males of which 50% is derived from peripheral conversion of androstenedione, 25% from the ovaries, and 25% from adrenal glands (except at the midpoint of the menstrual cycle, where the ovarian contribution increases by 10-15%). The first simple and systematic assay for determination of testosterone glucuronide (TG) in urine was described by Hennam and colleagues.[23] They reported an RIA using Kellie's antibody[24,25] against TG and showed that the steroid conjugate can be estimated directly without prior hydrolysis or extraction. This method has distinct advantages over procedures based upon the principle of isotope dilution, gas liquid chromatography and competitive protein binding using plasma proteins.[23] For example TG is measured as such and not as testosterone released by various hydrolytic procedures. Furthermore the method is quick and reliable, the results on 12 urine samples being obtained on the same day. The

individual steps are simple to perform and the capacity to analyse a large number of samples is high being up to 100 per run. Another advantage is that day to day running costs are low and the only expensive equipment required was a liquid scintillation counter.

Along this line Tresguerres *et al* [26] also developed an RIA for the determination of TG in crude urine and investigated the role of two protein-TG complexes in raising antibodies: BSA-TG and human plasma Cohn's fraction IV (CF)-TG. The results presented demonstrated that Cohn's fraction IV used as a carrier protein with TG as hapten gives high titres of antibodies when injected in to rabbits compared with the BSA-TG complex. The specificity of the antiserum was sufficiently high. This is probably due to the fact that CF consists partly of carbohydrate moieties. However the author did mention that higher titres of antibodies would have been obtained with the BSA-TG complex after a long period of immunization of the animals. They reported that due to technical reasons the rabbits had to be killed after 13 weeks of treatment.

5.1.6 Aims of the Chapter

The aims of this chapter were therefore two fold.

(i) To investigate other coupling methods of attaching the glucuronide moiety to estrone by a β-linkage and to validate this with clinical data using the Ovarian Monitor.

(ii) To investigate the synthesis of testosterone glucuronide (TG) and prepare its immunogen and conjugates for a development of a HEIA as the basis of a multi-purpose home monitor for the measurement of TG in urine.

5.2 EXPERIMENTAL

5.2.1 General Experimental Details

See **Section 1.2.1** for more **General Experimental Details**.

Thin layer chromatography (TLC) plates for all molecules were visualised by UV lamp (254 nm) and spraying with 10% concentrated H_2SO_4 in EtOH and heating at 100^0C for 2 minutes.

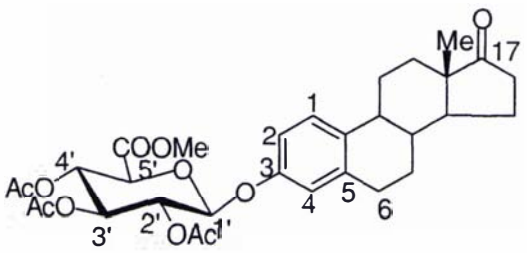
All the chromatographic analytical procedures for steroid glucuronides were performed on a SMART™ fast protein liquid chromatography (FPLC) system at RT using a μRPC C2/C18 PC3.2/3 column. The Ovarian Monitor equipment including meter (**Figure 2.4**), empty and pre-coated ovarian monitor assay tubes and multi-assay heating block were supplied by St Michael Natural Family Planning Services Pty, 2/380 Riversdale Road, East Hawthorn, Victoria, 3123, Australia.

Hen egg white lysozyme (grade VI, 3 times recrystallised, dialysed and lyophilised) and the lyophilised Micrococcus lysodeikticus were obtained from the Sigma Chemical Company (St. Louis, MO, USA). Purification procedures were performed on a prepacked Pharmacia CM HR 16/50 (500 x 16 mm I.D.) columns packed with Pharmacia S-Sepharose (fast flow) resin and a butyl Sepharose hydrophobic interaction column HR 5/5 (150 x 16 mm I.D.).

400 MHz ^{1}H NMR and 100 MHz ^{13}C NMR spectra were obtained using a Bruker spectrometer. Spectral assignments were made using the double resonance techniques COSY and HETCOR.

5.2.2 Synthesis Of Estrone Glucuronide

5.2.2.1 Methyl 17–oxo-1,3,5 (10)–estratrien–3–yl-2',3',4'-tri-*O*–acetyl-β -D–gluco-pyranosiduronate (5.50) using Koenigs-Knorr method[20]

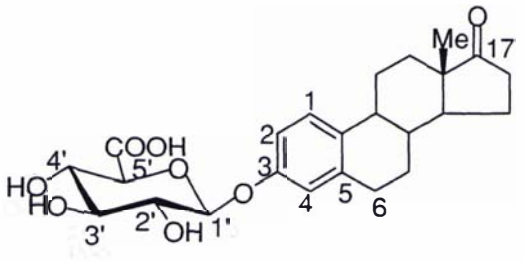


5.50

The coupling reaction of estrone **5.53** with methyl 1-bromo-1-deoxy-2, 3, 4-tri-O-acetyl-α-D-glucopyranuronate **3.8** was carried out as follows: Dried $CdCO_3$ (1.07 g, 6.23 mmol) was added to a solution of estrone **5.53** (682 mg, 2.52 mmol) in anhydrous toluene (100 ml) and the suspension was concentrated to approximately 60 ml by azeotropic distillation to remove residual moisture. After distillation, the solution of methyl 1-bromo-1-deoxy-2, 3, 4-tri-*O*-acetyl-α-D-glucopyranuronate **3.8** (2.91 g, 7.33 mmol) in anhydrous toluene (50 ml) was added dropwise by means of a pressure equalising funnel, keeping the addition rate the same as the rate of distillation. Finally, the whole reaction mixture was refluxed for 5 hours. The precipitate was then removed by filtration on celite and washed with CH_2Cl_2. The filtrate and washings were combined and the solvent was removed under reduced pressure. The syrup was purified by dissolving it in acetone (65 ml) and pouring it into water (250 ml) (acetone/water ratio 1:3.85) to precipitate the crude product. The solid was washed with water and recrystallised with CH_2Cl_2/EtOH (1:1, 60 ml) to give the desired methyl 17-oxo-1, 3, 5(10)-estratrien-3-yl-2, 3, 4-tri-*O*-acetyl-β-D-glucopyranosiduronate **5.50** (775 mg, 55%, R_f = 0.71; 1:1 Hex/ EtOAc) as a pure white solid, mp, 229-230^{0}C, (Lit. mp, 222-230^{0}C)[2]; ^{1}H NMR δ/ppm 6.73-7.23 (3H, m, phenyl), 5.23-5.35 (3H, m, H2'-H4'), 5.11 (1H, d, J = 7.33 Hz, H1'), 4.15-4.18 (1H, m, H5'), 3.74 (3H, S, -CO_2CH_3), 2.054 (3H, s, -OC(O) CH_3, 2.049 (3H, s, -OC(O) CH_3), 2.043 (3H, s, -OC(O) CH_3), 0.90 (3H, s, 18-CH_3) ^{13}C NMR δ/ppm 220.5 (-C(O)R), 169.9, 169.1, 169.0 (3 x –*O*C (O) CH_3), 166.7 (-*C* O_2CH_3), 154.5, 138.0, 134.8, 126.0, 117.0, 114.3 (6 x aryl C), 99.1 (C1'), 72.6(C3'),

71.9 (C5'), 71.0 (C2'), 69.2(C4'), 53.0 (-CO_2C H_3), 21.6, 20.7, 20.6 (-OC(O) C H_3), 13.9 (-CH_3(Ar)).

5.2.2.2 17-oxo-1,3,5 (10)–estratrien–3-yl-β-D-glucopyranosiduronicacid E1G (H) (5.1)



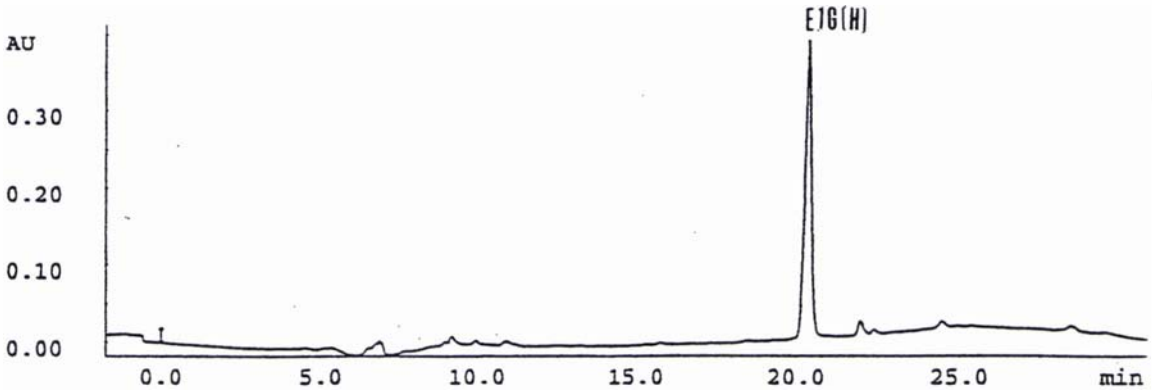
5.1

Methyl 17-oxo-1, 3, 5(10)-estratrien-3-yl-2, 3, 4-tri-*O*-acetyl-β-D-glucopyranosiduronate **5.50** (252 mg, 0.43 mmol) was dissolved in MeOH (50 ml) and aqueous NaOH (5ml, 2M) was added slowly. The reaction mixture was stirred at RT overnight after which TLC showed that the reaction was complete. The reaction mixture was titrated to pH= 8 with dilute HCl (1M) and allowed to stand at 5^0C overnight to give white crystals. The white crystals were dissolved in (MeOH:H_2O, 3:2, 35 ml) and titrated to pH = 2.5 with dilute HCl (1M). The methanol was removed under reduced pressure and the glucuronide solution was passed through an Amberlite® XAD-2 resin column, and the product was eluted with 50% aqueous MeOH. The solvent was removed to give a white solid which was recrystallised from aqueous MeOH (1:1) to give desired 17-oxo-1, 3, 5(10)-estratrien-3-yl-β-D-glucopyranosiduronic acid (E1G) as fine crystals (125 mg, 65%, R_f = 0.56, 3:1:1; EtOAc/EtOH/HOAc), mp, 166-168^{0}C, (Lit. mp, 165-168^{0}C)[20]; Found: M^-, 445.1862; $C_{24}H_{29}O_8$ requires 445.1855. ^{1}H NMR δ/ppm (acetone-d_6) 6.78-7.23 (3H, m, phenyl), 5.06 (1H, d, J = 7.33 Hz, H1'), 4.06 (1H, d, J = 9.53 Hz, H5'), 3.47-3.74 (3H, m, H2'-H4'), 0.89 (3H, s, 18-CH_3). ^{13}C NMR δ/ppm 220.5 (-C(O)R), 154.5, 138.0, 134.8, 126.0, 117.0, 114.3 (6 x aryl C), 99.1 (C1'), 72.6(C3'), 71.9 (C5'), 71.0 (C2'), 69.2(C4'), 13.9 (-CH_3(Ar)).

The purity of the product was checked on a Smart® FPLC system by dissolving the product E1G **5.1** (0.5 mg) in CH_3CN/H_2O (3:7) and loading 0.05 ml of the resulting solution onto a reverse phase column C2/C18 (solvent A: H_2O/CH_3CN/HCOOH/NaCl,

90:10:0.05/1.0) at a flow rate of 1 ml/min and using a linear gradient from 30 to 70% solvent over 60 mins. A wavelength of 220 nm was used for the detection. The resulting chromatogram is shown in **Figure 5.2.**

Figure 5.2 FPLC analysis of E1G(H) prepared by Koenigs-Knorr coupling method



Only one major peak was observed with a retention time similar to that of an authentic sample of E1G.

5.2.2.3 Methyl 17–oxo-1,3,5 (10)–estratrien–3–yl-2',3',4'-tri-*O*–acetyl-β -D–gluco-pyranosiduronate (5.50) using acetimidate coupling method

A typical procedure was as follows: To estrone **5.53** (58 mg, 0.21 mmol) in dichloromethane (2.5 ml) and molecular sieves (activated, $4A^0$) was added methyl 2,3,4-tri-*O* -acetyl-1-O-(trichloroacetimidoyl)-α -D-glucopyrano-uronate (152 mg, 0.31 mmol) and $BF_3.OEt_2$ (0.12 ml, 50%, 0.42 mmol). The reaction mixture was stirred at 0^0C and allowed to rise to RT over 4 h. The TLC was used to monitor the reaction. The reaction mixture was then diluted with CH_2Cl_2 (25 ml) washed with aqueous KOH (2M, 25 ml), water (25 ml) and brine (25 ml) and dried over $MgSO_4$ for several hours. The solvent was removed under reduced pressure and the resulting solid was purified on a silica pad by eluting with Hex:EtOAc (1:1) to give unreacted estrone (12 mg) and the product. The crude product was then recrystallised in CH_2Cl_2:MeOH(1:1) to give fine crystals of methyl 17–oxo-1,3,5(10)–estratrien–3–yl-2',3',4'-tri-*O*–acetyl-β D–glucopyranosiduronate **5.50** (46 mg, 62%, R_f = 0.71; EtOAc:Hex, 1:1), mp, 226-

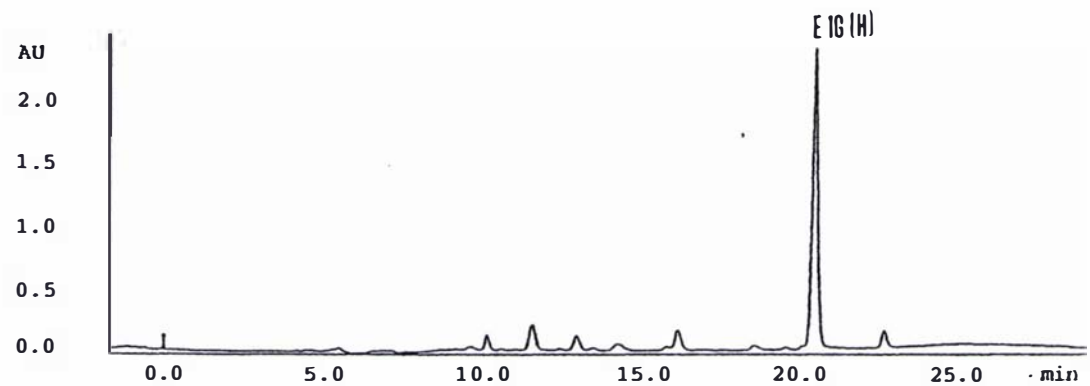
228^{0}C, (Lit. mp, 222-230^{0}C).[20] The NMR spectra were identical to the spectra reported above for **5.2.2.1**.

5.2.2.4 17-oxo-1,3,5 (10)–estratrien–3-yl-β-D-glucopyranosiduronicacid E1G (H) (5.1)

The hydrolysis reaction was performed by a procedure similar to that used in the preparation of **5.1** using the Koenigs-Knorr method. E1G(H) **5.1** (22 mg, 63%) was obtained from compound **5.50** (46 mg, 0.078 mmol) as fine crystals; mp, 167-170^{0}C, (Lit. mp, 165-168^{0}C); Found: M-, 445.1862; $C_{24}H_{29}O_8$ requires 445.1855.

The purity of the product was checked by FPLC using the Smart® system and the same conditions as in **Figure 5.2** to give essentially the same chromatogram.

Figure 5.3 FPLC analysis of E1G(H) prepared by acetimidate coupling method



5.2.2.5 Methyl 17–oxo-1,3,5 (10)–estratrien–3–yl-2',3',4'-tri-*O*–acetyl-β -D–gluco-pyranosiduronate (5.50) using the perester coupling method

A typical experimental procedure for the coupling reaction of estrone **5.53** with methyl tetra-*O*-acetyl glucopyranuronate **3.4** was as follows: To a solution of estrone (0.1 g, 0.37 mmol) in CH_2Cl_2 (6.0 ml) and molecular sieves (4A^0, activated) was added methyl tetra-*O*-acetyl glucopyranuronate (0.35 g, 0.93 mmol) and $BF_3.OEt_2$ (0.312 ml, 50%, 1.10 mmol). The resulting reaction mixture was protected from moisture and stirred at 25-30^{0}C overnight (16 hrs). The reaction mixture was then diluted with CH_2Cl_2 (50 ml)

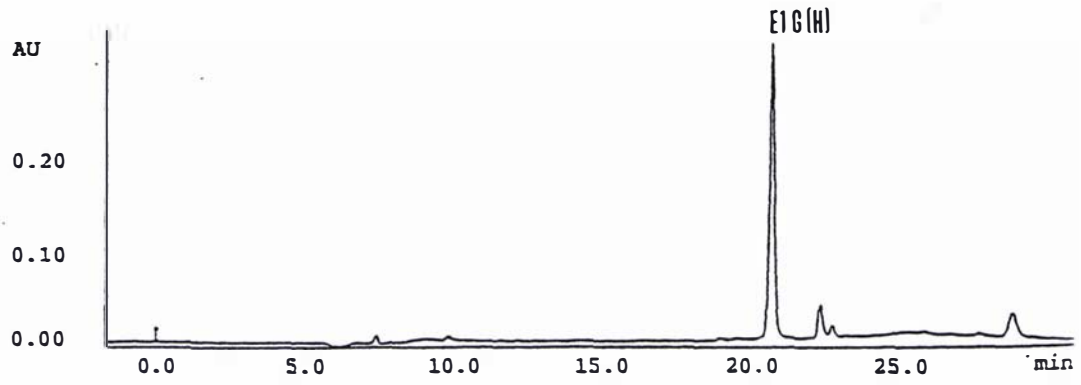
and washed with aqueous KOH (2M, 25 ml), water, (25 ml), brine (25 ml) and dried over $MgSO_4/Na_2SO_4$. The solvent was removed under reduced pressure to afford a crude white solid. The solid was purified by dissolving it in acetone (25 ml) and pouring into water (100 ml) (acetone/H_2O ratio 1:4) to give the crude product. The solid was washed with water and recrystallised with CH_2Cl_2/MeOH (1:1, 20 ml) or acetone to give the desired methyl 17–oxo-1, 3, 5 (10)–estratrien–3–yl-2', 3', 4'-tri-*O*–acetyl-β -D–glucopyranosid-uronate **5.50** (154 mg, 71%, R_f = 0.56; EtOAc:Hex, 1:1) as a pure white solid, mp, 228-230^{0}C, (Lit. mp, 222-230^{0}C)[20]. The ^{1}H NMR and ^{13}C NMR spectra were identical to those recorded earlier in **5.2.2.1**.

5.2.2.6 17-oxo-1,3,5 (10)-estratrien–3-yl-β-D-glucopyranosiduronicacid E1G (H) (5.1)

The hydrolysis reaction was performed by a procedure similar to that used in the preparation of **5.1** using the Koenigs-Knorr method. E1G(H) **5.1** (56 mg, 63%) was obtained from compound **5.50** (115 mg, 0.195 mmol) as fine crystals; Mp, 167-170^{0}C, (Lit. mp, 165-168^{0}C)[20] Found: MH^+, 445.1862; $C_{24}H_{29}O_8$ requires 445.1855.

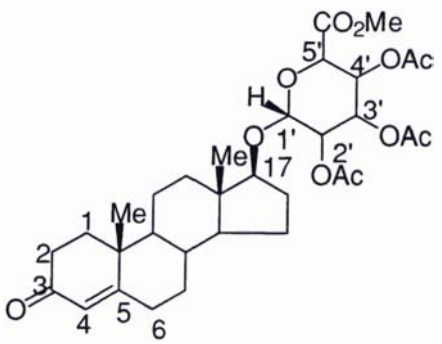
The purity of the product was checked by FPLC using the Smart® system and the same conditions as in figure **5.2** to give essentially a similar chromatogram.

Figure 5.4 FPLC analysis of E1G(H) prepared by the perester coupling method



5.2.3 Synthesis Of Testosterone Glucuronide

5.2.3.1 Methyl-2',3',4'–tri-*O*-acetyl-β -D-glucopyranosiduronate,(17β)–3-oxo-androst-4–en–17-yl (5.51)



5.51

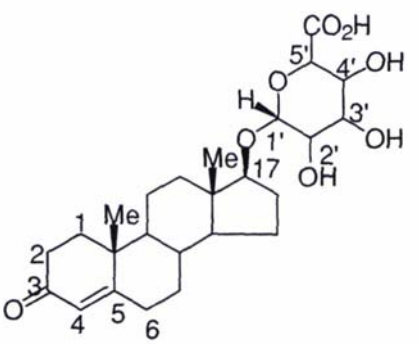
The coupling reaction of testosterone **5.54** with methyl 1-bromo-1-deoxy-2, 3, 4-tri-*O*-acetyl-α-D-glucopyranuronate **3.8** was carried out as follows: To a solution of testosterone **5.54** (500 mg, 1.73 mmol) in anhydrous toluene (100 ml) was added dried $CdCO_3$ (1.02 g, 5.91 mmol) and the suspension was concentrated to approximately 70 ml by distillation to remove residual moisture. After distillation, the solution of methyl 1-bromo-1-deoxy-2, 3, 4-tri-*O* -acetyl-α-D-glucopyranuronate **3.8** (1.99 g, 5.02 mmol) in anhydrous toluene (30 ml) was added slowly using a pressure equalising funnel, keeping the addition rate the same as the rate of distillation. Finally, the whole reaction mixture was refluxed for 30 hours. The extent of the reaction was monitored by TLC and by the colour of the reaction mixture. The reaction mixture slowly gained a pale bottle green colour, which became intense as the reaction proceeded. The reaction mixture was then filtered through a celite pad and washed with CH_2Cl_2. The combined filtrate was washed with water (30 ml) and dried over anhydrous K_2CO_3 for several hours. The organic phase was filtered and concentrated at 25-40^{0}C under vacuum to reduce the volume (30 ml), and the resultant syrup was purified by flash chromatography by eluting first with toluene and then with 20% acetone in hexane to get unreacted testosterone and the desired product (R_f = 0.32; acetone:Hex, 3:7). The product was further purified by dissolving it in acetone (10 ml) and pouring it in to H_2O (40 ml) (acetone/ H_2O ratio 1:4) to obtain a white amorphous solid. The solid was washed with water and then recrystallised with boiling propan-2-ol to give methyl-

2',3',4'–tri-*O*-acetyl-β -D-glucopyranosiduronate,(17β)–3-oxoandrost-4–en–17-yl **5.51** (572 mg, 54%) as white needles, mp, 183-184^{0}C, (Lit. mp, 186.8-188.8^{0}C)[13] Found: MH$^+$, 605.2961; $C_{32}H_{45}O_{11}$ requires 605.2966. ^{1}H NMR δ/ppm 5.73 (1H, s, H4), 5.19-5.25 (2H, m, H3'-H4'), 5.01(1H, t, H2'), 4.58 (1H, d, *J* = 7.70 Hz, H1'), 4.00 (1H, d, *J* = 9.43 Hz, H5'), 3.75 (3H, s, -CO_2CH_3), 3.58 (1H, t, H17), 2.056 (3H, s, –OC(O)CH_3), 2.026 (3H, s, –OC(O)CH_3), 2.020 (3H, s, –OC(O)CH_3), 1.19 (3H, s, -CH_3), 0.76 (3H, s, -CH_3). ^{13}C NMR δ/ppm 196.6 (-C(O)R), 170.7, 170.0, 169.1 (3 x –O*C* (O) CH_3), 168.8 (-*C* O_2CH_3), 167.0 (C5), 123.8 (C4), 101.3 (C1'), 72.5(C3'), 72.0 (C5'), 71.4 (C2'), 69.4(C4'), 53.8 (-CO_2CH_3), 20.7, 20.6, 20.5 (-OC(O) *C* H_3), 17.4 (-C18), 11.6 (-C19).

The title compound **5.51** was also prepared using freshly prepared Ag_2CO_3 as a different promoter for the coupling reaction as described below.

To a solution of testosterone **5.54** (100 mg, 0.35 mmol), powdered dry drierite and Ag_2CO_3 (0.18 g, 0.65 mmol) in anhydrous benzene (20 ml) was added methyl 1-bromo-1-deoxy-2, 3, 4-tri-O-acetyl-α-D-glucopyranuronate **3.8** (200 mg, 1.25 mmol) in anhydrous benzene (10) over 0.5 h. The reaction mixture was refluxed for 20 hrs under an atmosphere of Ar. The reaction mixture was then filtered on celite® and the residue was washed with benzene. The solvent was removed under reduced pressure and the crude dark yellow solid was purified by column chromatography with an eluting solvent of Hex:EtOAc (1:2) before recrystallisation in propan-2-ol to give **5.2** (47 mg, 22 %, R_f = 0.32) as off white crystals, mp, 182-186^{0}C, (Lit. mp, 186.8-188.8^{0}C).[13]

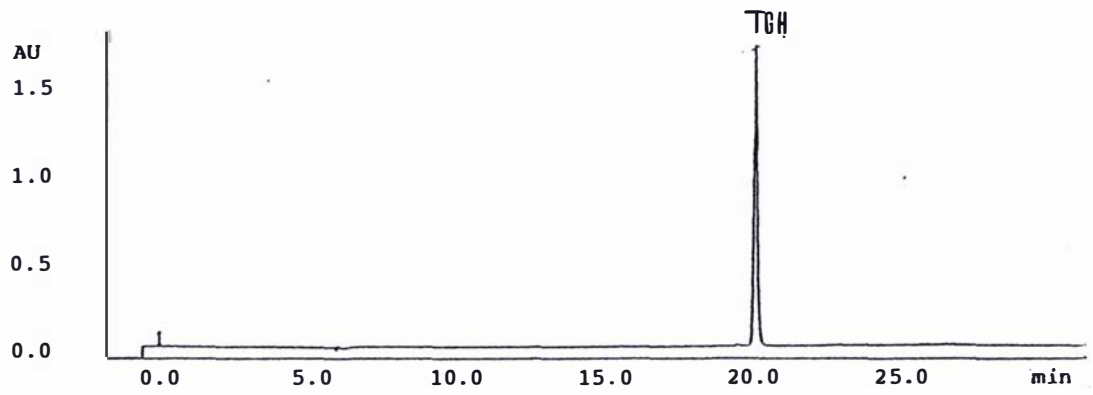
5.2.3.2 β -D-glucopyranosiduronic acid, (17β)-3–oxoandrost-4-en-17-yl (5.2)



5.2

A solution of methyl-2', 3', 4'–tri-*O* - acetyl-β-D - glucopyranosiduronate, (17β) –3-oxoandrost –4–en–17-yl **5.51** (105 mg, 0.17 mmol)) in methanol (25 ml) was stirred with NaOH (0.5 M, 2.5 ml) at RT for 4 hours. The hydrolysis was monitored by TLC until no starting material was detected (R_f = 0.56; Hex:EtOAc, 1:1). The solution was then titrated to pH 8.5 with HCl (1M) and the solution was concentrated to ~7-8 ml under reduced pressure. The solution was allowed to stand for several hours at RT to give the white crystalline sodium salt. The crystals were filtered and washed with cold methanol (3 ml). The crystals were dissolved in MeOH:H_2O (3:2, 10 ml) and titrated with HCl (0.5 M) to pH 2.9. The solvent was removed under reduced pressure and the product was purified by dissolving the solid in water (2 ml) and loading on a Waters Sep-pak® Plus C_{18} cartridge for absorption and eluting it with water (0.5 ml), and MeOH (5 ml). The solvent was removed under reduced pressure to give a white solid which on recrystalisation from aqueous MeOH (1:1) gave the desired product β-D-glucopyranosiduronic acid, (17β)-3-oxoandrost-4-en-17-yl **5.2** (48 mg, 60%, as fine crystals, mp, 174-175^{0}C, (Lit. mp, 182-183.5^{0}C).[13] Found: MH^+, 465.2490; $C_{25}H_{37}O_8$ requires 465.2488. ^{1}H NMR δ/ppm (acetone-d_6) 5.68 (1H, s, H4), 4.40 (1H, d, *J* = 7.69 Hz, H1'), 3.71 (1H, t, H17), 3.55 (1H, d, *J* = 9.23 Hz, H5'), 3.32-3.40 (2H, m, H3', H4'), 3.62 (1H, t, H3'), 3.14 (1H, t, H2'), 1.09 (3H, s, 19-CH_3), 0.76 (3H, s, 18-CH_3). ^{13}C NMR δ/ppm 205.1 (-C(O)R), 178.9 (-*C* O_2CH_3), 175.8 (C5), 122.2 (C4), 102.6 (C1'), 89.1 (C17), 76.6 (C5'), 75.7 (C3'), 73.5 (C2'), 72.2 (C4'), 16.9 (-C18), 11.2 (-C19). The purity of the product was checked on Smart® FPLC system by dissolving the product TG **5.2** (0.2 mg) in CH_3CN/H_2O (2:3) and loading 0.05 ml of the resulting solution onto a reverse phase C2/C18 column (solvent A: H_2O/CH_3CN/HCOOH/NaCl, 90:10:0.05/1.0) at a flow rate of 1 ml/min and using a linear gradient from 30 to 70% solvent over 60 mins. A wavelength of 237 nm was used for the detection. The resulting chromatogram is shown in **Figure 5.2**.

Figure 5.5 FPLC analysis of TG (H) prepared by Koenigs-Knorr coupling method



5.2.4 Preparation Of Testosterone Glucuronide-Lysozme Conjugate

Testosterone glucuronide **5.2** (7.5 mg, 0.016 mmol) was dissolved in dry DMF (100 μL) to give a clear solution. Standard solutions of DCC and NHS in dry DMF (0.6 M each) were freshly made up. To the TG solution was added this freshly made up solutions of NHS (35 μL) and DCC (35 μL). The reaction was allowed to stand at RT with occasional gentle stirring for 1 h. During this time the earlier clear solution gave white crystals due to the formation of the active ester (the insoluble product is seen due to the formation of dicyclohexyl urea). After that, the peptide bond between TG and HEW lysozyme was formed by adding the active ester solution dropwise to a stirred solution of purified HEW lysozyme (150 mg, 10 μmol) dissolved in 1% aqueous $NaHCO_3$ (4.0 ml) at 0^0C. The reaction mixture was gently stirred for 15 minutes and then allowed to stand over night at 5^0C. The crude reaction mixture was then dialysed to remove the dicyclohexyl urea and $NaHCO_3$ using (7 x 0.5 L) Milli-Q water. The crude reaction mixture was then freeze dried and stored at -10^0C.

5.2.5 Purification Of Testosterone Glucuronide-Lysozyme Conjugate

The success of the conjugation experiments and purity of the derivatives was determined by analytical chromatography on a Mono-S cation-exchange column in 7 M urea buffers (pH 6.0) as described below. Testosterone glucuronide-lysozyme conjugate was purified in a two step procedure from the conjugation reaction mixture

by S-Sepharose fast flow ion-exchange chromatography in NaH_2PO_4 buffers at pH 6.0 followed by butyl Sepharose hydrophobic interaction chromatography in pH 6.6 buffers as described below.

5.2.5.1 Mono-S Cation Exchange Chromatogrpahy

Mono-S cation exchange chromatography was routinely used to analyse the initial HEWL-TG conjugation reaction mixtures as well as to assess the efficiency of any conjugate purification procedures. The analysis on the conjugation reaction mixture was performed on the dialysed sample. The thick milky precipitate which was associated with all conjugations was removed by centrifuging the dialysed reaction mixture for 15 minutes at speed 5 on a Gallenkamp Junior bench centrifuge. The supernatant was prepared for loading by preparing a 6:1 dilution of an aliquot of the sample with the equilibrating buffer (50 mM phosphate/7 M urea, pH 6.0).

After loading of the sample onto the column by injection, elution was effected using a linear sodium chloride gradient by using increasing quantities of the elution buffer (50 mM phosphate/7 M urea/1 M sodium chloride, pH 6.0). The standard chromatographic conditions were: flow rate 0.5 ml/min; Chart speed 0.2 cm/min; programme - 0-15 minutes, 0.1 M NaCl; 15-55 minutes, 0-0.3 M NaCl; 55-65 minutes, 1 M NaCl).

5.2.5.2 S-Sepharose Fast Flow Chromatohraphy (Ion Exchange Column-With 7 M Urea Buffers)

The supernatant was prepared for loading onto a S-Sepharose fast flow cation exchange column by the addition of urea (0.126 g per 300 μL of sample), and then sodium dihydrogen phosphate buffer (pH 6.0) was added to the new increased volume to give a 50 mM phosphate/7 M urea solution. After filtering the sample was loaded *via* a peristaltic pump onto the column which was pre-equilibrated with 50 mM phosphate buffer/7 M urea (pH 6.0) and the elution effected using a long, linear sodium chloride gradient (0-0.21 M NaCl in 690 minutes).

The peak fractions from the S-Sepharose fast flow column were pooled and dialysed against water (3 changes x 20 L) and then concentrated to a final volume of 10-20 mL.

5.2.5.3 Butyl Sepharose Chromatography (Hydrophobic Interaction Column-With Ammonium Sulphate Buffers)

The final step in the purification of conjugate T1 involved a single passage through a hydrophobic interaction column. Ammonium sulphate and sodium dihydrogen phosphate were added to the concentrated samples from the S-Sepharose fast flow cation exchange column to give a 1.26 M ammonium sulphate/50 mM phosphate solution which was adjusted to pH 6.6 and filtered. The filtered sample was then loaded via a peristaltic pump onto a Butyl Sepharose column (150 mm x 16 mm i.d.) which was pre-equilibrated with 1.26 M ammonium sulphate/50 mM phosphate buffer (pH 6.6). Elution of the sample from the column was effected using a linear negative ammonium sulphate gradient (1.26-0 M ammonium sulphate in 20 minutes).

5.2.6 Production Of Antisera With A Testosterone Glucuronide-BSA Conjugate As Immunogen

Testosterone glucuronide-bovine serum albumin conjugates were prepared using the same methodology as described earlier in the conjugation of TG to HEW lysozyme and used to generate antisera in sheep. Antisera were raised in sheep by Debbie Chesterfield, of the Agriculture services, Massey University. The primary imunisation was given by subcutaneous injection under the skin behind the neck with 5 mg of TG-BSA conjugate dissolved in 1.0 ml saline mixed with 1.0 ml Freund's complete adjuvant, in a total volume of 2 ml. The solution was injected into 2-3 sites of the neck and was dispersed under the skin by gentle rubbbing. Three weeks later a booster injection of a further 1.5 mg of TG-BSA immunogen in 1.0 ml saline mixed with 1.0 ml Freud's incomplete adjuvant was carried out in the same manner. The sheep were blood sampled 68 days following the booster shot using (10 ml) vacutainers, by venepuncture of the jugular vein. The blood samples were left to clot and spun down for 0.5 h at 4000 rpm on a SS34 centrifuge head to obtain the serum. The serum was stored at -10^{0}C.

5.2.7 Testosterone Glucuronide Lysozyme Conjugate Assay

To avoid cross contamination, separate syringes were used for the lysozyme, lysozyme conjugates, antiserum and substrate in the preparation of the reagents for the assay.

5.2.7.1 Buffers

All tris-maleate buffers used in the assay were made by appropriate dilution of a 1 M stock buffer (pH 7.0) with water. The stock buffer was prepared by mixing maleic acid (7.25 g), tris (19.80 g), sodium chloride (12.75 g), tween 80 (20.0 mL) (prediluted 1/100 with water) and concentrated hydrochloric acid (2.8 mL) in water. The pH of the buffer was adjusted to 7.0 with concentrated hydrochloric acid and then made to a final volume of 375 mL.

5.2.7.2 Substrate

The bacterial suspension of *Micrococcus lysodeikticus (M. lysodeikticus)* was prepared fresh. Lyophilised *M. lysodeikticus* (30 mg) was triturated with 4 mL of 75 mM tris-maleate buffer (pH 7.0) in a manual two piece glass homogeniser. When a visually uniform suspension was obtained, it was transferred to a small glass vial and sonicated using a Bandelin Sonorex RK100 sonicator for five minutes to ensure homogeneity. The sonicated suspension was kept on ice when not using it.

5.2.7.3 The Standard Activity Assay Protocol

The assay was initiated by the addition of a 10 µL aliquot of the bacterial suspension via a stepper syringe. The reaction mixture was quickly vortexed (2-3 seconds) and the assay tube placed in the Ovarian Monitor. The first transmission value was recorded upon shutting the lid, followed by the second transmission value five minutes later. The rate was recorded as change in transmission (ΔT) (650 nm). All assays were performed in duplicate or triplicate.

5.2.7.4 Inhibition Studies

For the inhibition studies, lysozyme conjugate T1 (12 µL) was chosen to give a change in transmission (ΔT) of approximately 350 over 5 minutes

The undiluted antiserum (1-60 µL) and conjugate (12 µL) were added to opposite corners at the bottom of an empty Ovarian Monitor assay tube, the total volume in the assay tube was made up to 50 µL with 75 mM tris-maleate buffer (pH 7.0), and the resulting mixture shaken for a count of three. A further 238 µL of 40 mM tris-maleate buffer was then added and the assay mixture was shaken on a vortex mixture and allowed to incubate for 5 minutes at 40°C using a custom made heating block. The assay was initiated with an aliquot of *M. lysodeikticus* (10 µL).

5.2.7.5 Standard Curve

The following general protocol was used in the production of the standard curve. Testosterone glucuronide (6.7 mg, see **Section 5.2.3**) was dissolved in tris maleate buffer (5.19 ml, 40 mM) to prepare various dilution series of standards. Testosterone glucuronide standard (50 µL) and undiluted antiserum (40 µL) were dispensed into diagonally opposed corners of an empty assay tube, and the immune reaction was initiated by vortexing the assay tube for a count of three. The undiluted conjugate stock solution (12 µL) was added and the contents of the assay tube vortexed again. Assay buffer was then added to bring the total volume in the assay tube to 340 µL, and the resulting reaction mixture re mixed and left to incubate for 5 minutes at 40°C. The assay was then initiated with an aliquot of *M. lysodeikticus* suspension (10 µL).

5.3 RESULTS AND DISCUSSION

In recent years considerable attention has been directed to the biological and medical importance of steroidal estrogens and investigation into their glucuronic acid conjugates as significant metabolites has been pursued. With regard to the important role conjugates of steroidal estrogens play as starting materials for the preparation of protein-or enzyme- conjugates in immuno assay, the synthesis of estrone and testosterone glucuronide has attracted considerable attention. In connection with our investigation of the biological function of steroid conjugates, a more convenient method for obtaining these compounds was required.

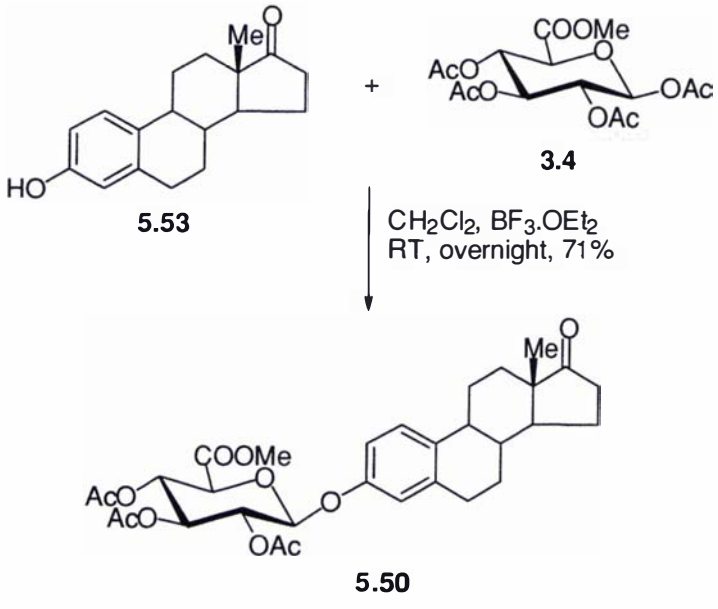
Moreover the present unavailability of the target phytoestrogen glucuronides necessitated a switch to testosterone glucuronide as an alternative biomarker which might be measured at home using the monitor. This assay serves also to validate the concept of a multi-purpose home monitor for a wide variety of analyte glucuronides of clinical interest.

The findings of the synthesis of various aryl glucuronides by tetraacetyl glucopyranuronate and phenols using $BF_3.OEt_2$ as a catalyst (**Chapter 3, Section 3.1.2**) encouraged us to investigate this reaction on steroids. The acetimidate method (**Section 5.1.3**) utilising $BF_3.OEt_2$ was also used. Also the synthesis of steroidal glucuronides was adopted from the literature with the synthesis of estrone and testosterone glucuronides using the classical Koenigs-Knorr reaction conditions. This chapter is also concerned with the production of antibodies *via* preparations of conjugates of testosterone glucuronide with HEW (hen egg white lysozyme) and BSA (bovine serum albumin) and the establishment of a homogeneous enzyme immuno assay for TG.

5.3.1 Investigation Of The Perester Coupling Reaction In The Synthesis Of Steroid Glucuronides

β-glycosylation is one of the key reaction step in the synthesis of the different steroid glucuronides. During the course of this work some interesting aspects of the glucuronidation reactions were noted. Starting from D-glucuronolactone, peracetylated glucuronic methyl ester **3.4** could be prepared as a crystalline β-anomer in one step as

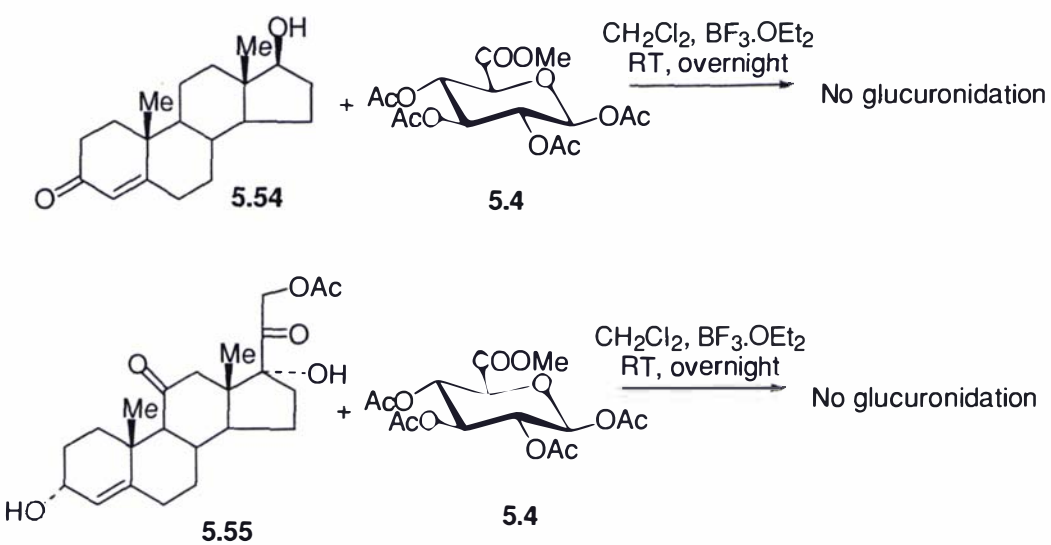
described earlier (**Chapter 3 Section 3.3.2**). Estrone was successfully reacted with peracetylated glucuronic acid methyl ester **3.4** utilising $BF_3.OEt_2$ methodology. The reaction proceeded satisfactorily at RT and heating was unnecessary. The reaction was stereochemically reliable giving only the β-anomers of the conjugates in excellent yields as judged from the NMR spectral data.



Scheme 5.7

Convincing yields of such glucuronic acid conjugates were achieved, and the inconvenient use of the glycosyl halides and catalysis with some heavy metal salts was avoided. In order to optimise the coupling reaction the effects of solvent, temperature and reaction time on the yield of the reaction was studied. It was found that optimal yields were obtained in CH_2Cl_2 at RT after overnight stirring. The reaction was very slow in toluene and resulted in a very poor yield after overnight stirring at RT, probably due to the insolubility of estrone in toluene at RT. In complexing solvents such as acetonitrile and DMF no glucuronidation was observed. The reaction was also slow in CH_2Cl_2 at lower temperature due to the fact that estrone was sparingly soluble in CH_2Cl_2 at lower temperatures as well. While the reaction was not studied at higher temperatures it was believed that increasing the temperature would affect the anomeric configuration and might give an α:β mixture of conjugates.[21]

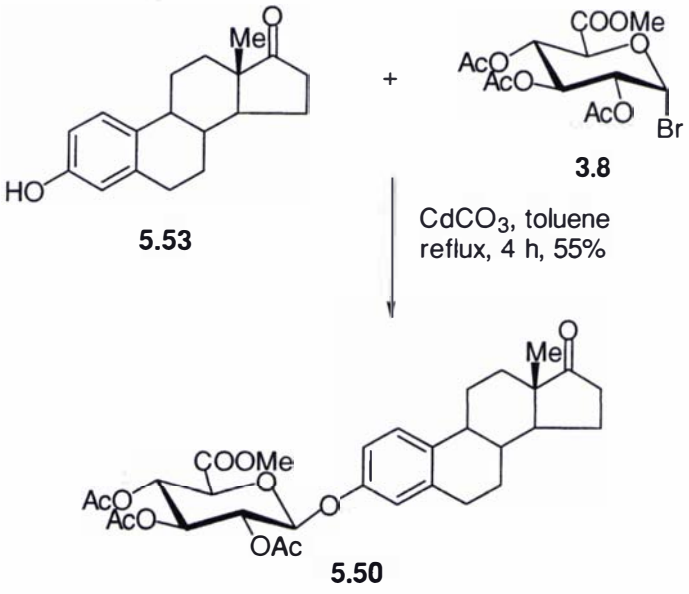
When the coupling reaction of testosterone was attempted with peracetylated glucuronic methyl ester **3.4** under similar conditions no glucuronidation was seen and the reaction mixture turned black. This was surprising from a reactivity point of view as for the phenol compounds (glycosyl acceptors) the lone pair of electrons of the nucleophilic oxygen atom are delocalised at the aromatic ring through the conjugation system. Hence the lower electron density at the phenolic hydroxyl group causes it to function as a much poorer nucleophile than the does the alcoholic hydroxyl group. Although the reason for the failure to synthesise conjugate **5.2** is not clear it was thought to be due to the complex formation between the α,β unsaturated C=O group of testosterone and the acid catalyst $BF_3.OEt_2$ leading to decomposition. This was also the case when 3α,17,21-trihydroxy-5-β-pregnan-20-one-21-acetate (THE-21-acetate)[22] was reacted with peracetylated glucuronic methyl ester **3.4** under similar conditions. No glucuronidation was seen and extensive decomposition occurred.



Scheme 5.8

5.3.2 Classical Koenigs-Knorr Reaction For The Synthesis Of Steroid Glucuronides

The glycosyl donor α-bromosugar **3.8** used in the synthesis of the steroid glucuronides was easily prepared and showed good chemical reactivity and β-selectivity in the glycosylation reaction as reported in the literature.

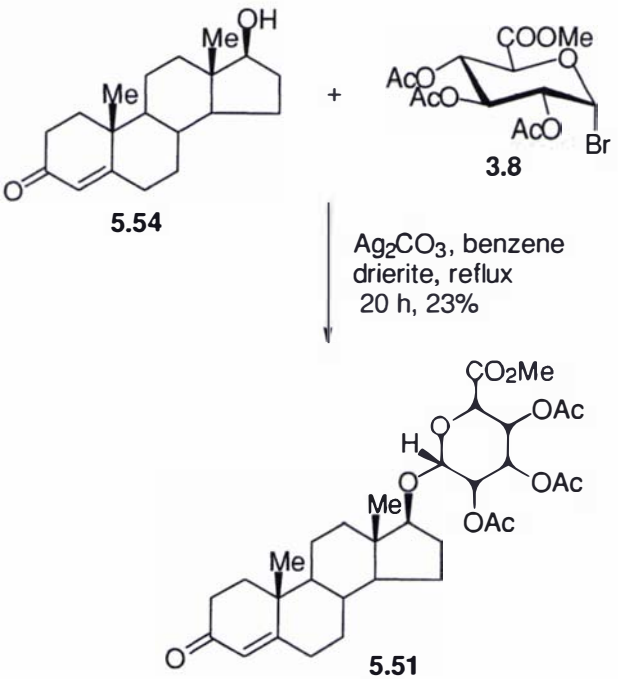


Scheme 5.9

Efforts in this direction showed that the continuous distillation of toluene from the mixture was effective in bringing the reaction to completion when more than 2 equivalent of bromosugar were added dropwise, over 1 h to a mixture of estrone and $CdCO_3$ in distilling toluene as described by Wu Y[20] followed by an additional 4-5 h reaction time. The initiation of the reaction was manifested by a development of color on the surface of the cadmium carbonate (colorless to pale yellow and finally pink). The product estrone 3-β-D-glucuronide triacetate methyl ester after isolation and purification was subsequently hydrolysed to give estrone 3-β-D-glucuronide.

The synthesis of testosterone glucuronide triacetate methyl ester was investigated first in the presence of Ag_2CO_3 using essentially the same literature procedure as reported[13] except, that column chromatography was used to isolate the product from the excess reactants. The literature procedure used a tedious counter current distribution method for this purpose. Hence the synthesis of testosterone glucuronide triacetate methyl ester was carried out with powdered drierite previously dried at 250^{0}C and freshly prepared Ag_2CO_3 in freshly distilled anhydrous benzene for the coupling of testosterone and the bromosugar. The reaction mixture was stirred at reflux for 40 h during which benzene aliquots were removed periodically. The TLC of the crude reaction mixture showed unreacted testosterone, byproducts due to decomposition of the bromosugar along with the desired product testosterone

glucuronide triacetate methyl ester. The product was purified using column chromatography to give pure testosterone glucuronide triacetate methyl ester in poor yield.

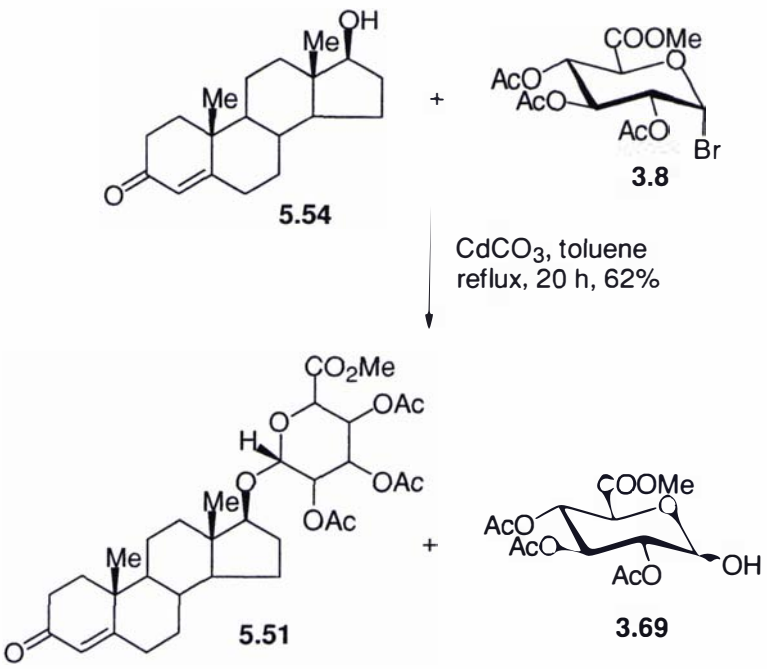


Scheme 5.10

More recently Sanaullah and Bowers[15] employed an improved procedure for the synthesis of d_3-testosterone glucuronide triacetate methyl ester using $CdCO_3$ as the promoter. They added the bromosugar **3.8** dissolved in anhydrous toluene at reflux to the solution of d_3-testosterone and $CdCO_3$ in anhydrous toluene over 1 h. The reaction mixture was then allowed to reflux for 2 days. The crude reaction mixture was then filtered and purified through flash silica gel column chromatography to give d_3-testosterone glucuronide triacetate methyl ester.

Efforts in this direction were made using this general method of the modified Koenigs-Knorr method and hence anhydrous conditions maintained during the addition of α-bromosugar by distillation as described in the literature.[13] A limited investigation of the reaction variables showed that complete reaction of testosterone was achieved when 2.5 equivalents of the bromosugar were added dropwise, over 2 h to a mixture of testosterone and $CdCO_3$ in distilling toluene followed by an additional 20 h reaction time. In fact, initially the reaction mixture was allowed to reflux for 2 days as reported

in the literature but since complete reaction of testosterone was achieved and no observed difference in TLC was seen, the reaction mixture was stopped after 20 h reflux. An interesting feature of the reaction was the that the initiation of the reaction was manifested by a development of color on the surface of the cadmium carbonate (colorless to pale yellow and finally bottle green). At this stage the TLC analysis of the organic soluble components of the mixture showed predominantly the product and anomeric hydrolysis byproduct of the bromosugar.



Scheme 5.11

Since the latter compound is soluble in water initially the product was purified according to the method of Conrow and Bernstein[2] by dissolving the crude mixture in acetone and pouring the solution into water to final concentration of 25%. The desired glucuronide was then further purified using flash silica gel column chromatography to give the pure white crystalline material.

5.3.2.1 Ag_2CO_3 And $CdCO_3$ As Promoters For The β-Glycosylations Of Steroid Glucuronide.

Both Ag_2CO_3 and $CdCO_3$ are widely used as catalysts and as acid scavengers in Koenigs-Knorr reactions for the synthesis of steroid glucuronides. Ag_2CO_3 is a traditional catalyst and has been used for many years. In 1971, $CdCO_3$ was introduced

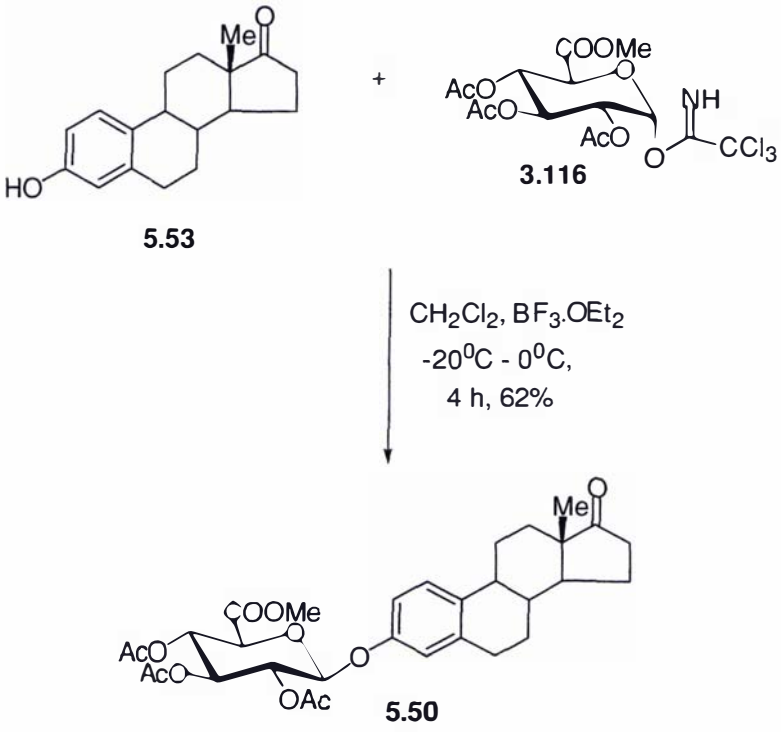
by Bernstein and co-workers[2] as a new catalyst for the Koenigs-Knorr synthesis of aryl glucuronides but there was no indication of its use in the Koenigs-Knorr synthesis of alkyl glucuronides. Until a few years ago testosterone glucuronide was synthesised only *via* Ag_2CO_3 as the catalyst in the Koenigs-Knorr reaction. Only recently Sanaullah and Bowers[312] used $CdCO_3$ for the successful synthesis of d_3-testosterone glucuronide.

The beneficial role of $CdCO_3$ over Ag_2CO_3 is well known in estrone glucuronide.[2] In the present work, comparing Ag_2CO_3 and $CdCO_3$ in Koenigs-Knorr reactions of testosterone glucuronide, the use of $CdCO_3$ also appeared to have some advantages over Ag_2CO_3. For example in the present work testosterone glucuronide triacetate methyl ester **5.56** was prepared by reaction of testosterone with bromo sugar **3.8** catalysed by Ag_2CO_3 in benzene in 23% yield, compared with 54% yield when using $CdCO_3$ (see experimental). When $CdCO_3$ was used in glucuronidation reactions, the commercial reagent could be used directly without any purification. All the compounds and the solvent (toluene) could be easily and thoroughly dried by azeotropic distillation to remove moisture before starting the reaction. It is thus easier to control the reaction under scrupulously anhydrous conditions. During this work, $CdCO_3$ has been used as a catalyst in the Koenigs-Knorr reaction and stored in a desiccator over long periods of time. The steroid glucuronides **5.50 and 5.56** synthesised in this chapter were made by using this catalyst directly without any purification and progress of the reaction was monitored by the development of colour on the surface of $CdCO_3$.

5.3.3 Synthesis Of Estrone Glucuronide Using The Trichloro Acetimidate Method

During the course of writing this thesis Werschkun and co-workers[30] have reported a similar synthesis of the 3 β-D-glucuronide of 17 β-estradiol and estrone using the peracetylated glucuronic acid methyl ester **3.4** (1:1.2 eq.) in 42% yield and the 3 β-D-glucuronide of 17 α-ethnylestradiol using the acetimidate sugar **3.116** in 61% yield in the same paper. Surprisingly how ever they did not mentioned the attempt to use the acetimidate sugar **3.116** for the synthesis of estrone triacetate 3-β-D-glucuronide ester **5.50**.

The methyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranuronate **3.116** was easily prepared in three steps from the tetracetate derivative **3.4** (**Scheme 3.39**). The relatively mild catalysis required and very high β-stereoselectivity makes the tricholoroacetimidate sugar **3.116** an attractive intermediate. The reaction conditions successfully used in the synthesis of the 7-hydroxy coumarin glucuronide using BF_3OEt_2 catalysis[10] were used with little modification. Hence the acetimidate sugar **3.116** was activated and dried before its coupling with estrone **5.51** by stirring at RT with molecular sieves (4 Å) in CH_2Cl_2.



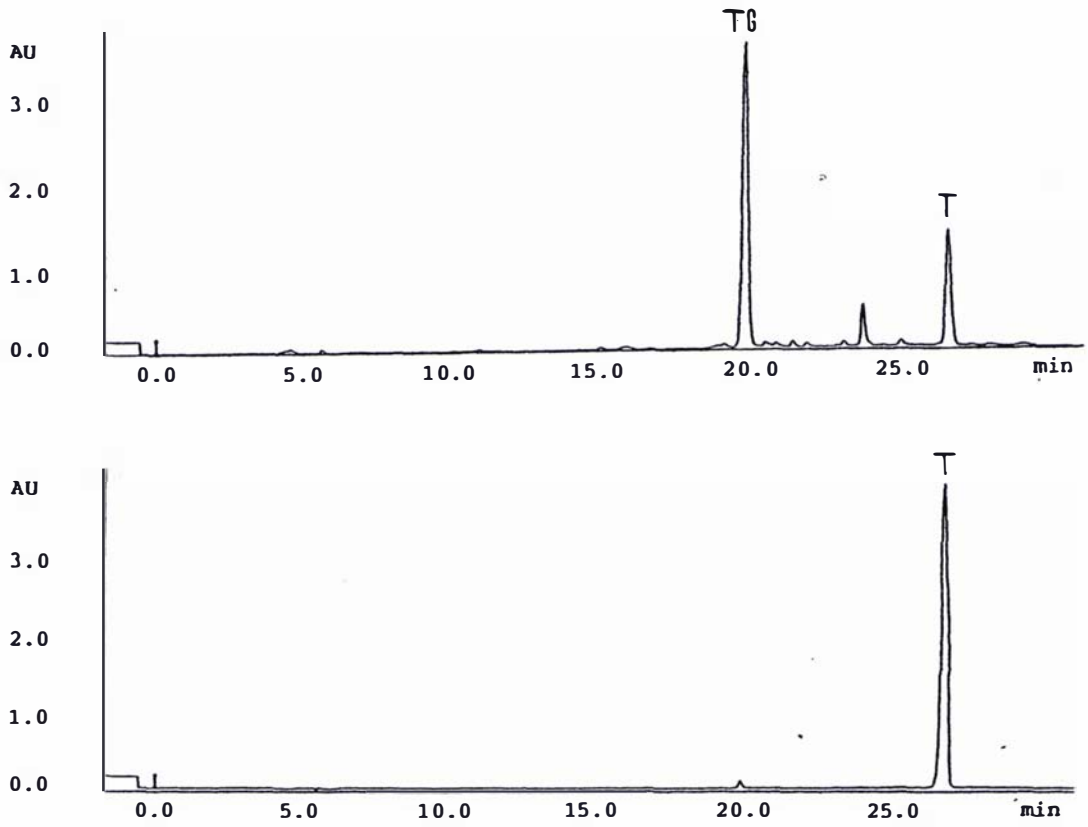
Scheme 5.12

This slightly improved yield over the classical Koenigs-Knorr reaction using $CdCO_3$ as the catalyst can be explained on the basis of the superior donor properties of the acetimidate sugar towards the aglycone.[9] Because of its high stability in dry solvents the reaction is not hampered by the facile formation of elimination by-products in dry solvents. In the presence of an equatorial acetate group at position 2, the reaction is thought to be proceed *via* an oxonium ion intermediate **5.7** as in **Scheme 5.1** by neighbouring group participation and lead to the β-adduct.

5.3.3.1 Purification Of Steroid Glucuronides In Their Free Acid Forms

It was especially notable that estrone glucuronide and testosterone glucuronides which were synthesised in this chapter were transformed to the free acid form by titrating the sodium salt solution, obtained after alkaline hydrolysis of the protected estrone and testosterone glucuronide esters to pH 2.5 and 2.9 respectively. In practice this was the most difficult and irreproducible step in the synthesis. The free acid forms of the steroid glucuronides showed some differences in stability in acidic media. It was found that testosterone glucuronide at pH 2.5 was more acid sensitive and showed some decomposition unlike estrone glucuronide which was found to be quite stable at this pH value at least for short periods of time. Repetitive efforts to obtain testosterone glucuronide in the free acid form at pH 2.5 failed. The TLC of the solution and the melting point of the white solid obtained after removal of the solvent under reduced pressure showed a mixture of compounds including testosterone and the parent compound testosterone glucuronide sodium salt. When the purity was checked on a Smart® FPLC system it clearly showed decomposition as the chromatogram indicated the presence of testosterone (**Figure 5.6**). The mixture (0.5 mg) was analysed in CH_3CN/H_2O (3:7) and loading 0.05 ml of the resulting solution onto a reverse phase column C2/C18 (solvent A: $H_2O/CH_3CN/HCOOH/NaCl$, 90:10:0.05/1.0) at a flow rate of 1 ml/min and using a linear gradient from 30 to 70% solvent over 60 mins. A wavelength of 237 nm was used for the detection.

Figure 5.6 FPLC analysis of testosterone glucuronide after treatment at pH 2.5 showing decomposition (top) and testosterone reference (bottom)

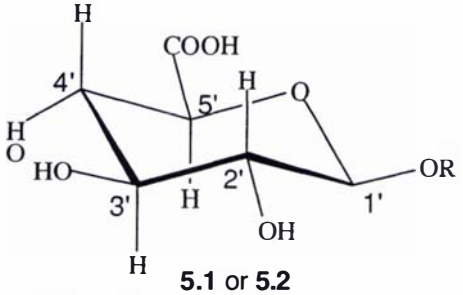


This was thought to be due to partial hydrolysis of the glycosidic bonds of the steroid glucuronides, which being acetal linkages are always acid sensitive. A different procedure was developed therefore in order to obtain testosterone glucuronide free acid in pure form. The testosterone glucuronide salt after hydrolysis of the ester was acidified to pH~2.9 and then the solvent was removed under reduced pressure. The white solid thus obtained was dissolved in a minimum amount of H_2O (63 mg in 2 ml H_2O) and passed through a Sep-Pak C_{18} column. The Sep-Pak column was then eluted with MeOH:H_2O(1:1) to give nice white needle shaped crystals. This procedure not only afforded the free acid, which is the necessary form of the steroid glucuronides for the later amidation reactions, but it also provided a useful method for the purification of steroid glucuronides. Since the free acids of steroid glucuronides are normally insoluble in water, unlike the sodium salts they can be separated comparatively easily from aqueous solution by absorption on the C_{18} column. Washing with water removes

the low molecular weight reagents and sodium salts in the washings leaving the pure steroid glucuronide product on the column. Purifications of the sodium salt form of the steroid glucuronides on the other hand usually requires many repeated recrystallisations from MeOH in order to obtain a pure product, which results in significant losses of valuable products.

5.3.4 General Characterisation Of Estrone And Testosterone Glucuronide

It is important to be certain of the correct stereochemistry of the glucuronides after synthesis. This is reflected in the protons on the glucuronic acid ring especially the proton at the hemiacetal carbon atom (1'). All naturally occurring steroid glucuronides found in urine (which are formed enzymatically and stereospecifically from glucuronyl transferase) have the β-configuration. For the naturally occurring steroid glucuronides to be recognised specifically by antibodies it is essential that the synthetic steroid glucuronide used to generate the anti-steroid antibodies has the same stereochemistry. In this chapter the basic structure of the glucuronic acid ring of the estrone glucuronide and testosterone glucuronide was confirmed by ^{1}H NMR and ^{13}C NMR techniques.



5.1 or **5.2**
R = Estrone or Testosterone

Figure 5.7

For estrone glucuronide the aromatic protons (1-, 2- and 4-H in the A ring of estrone), the 18-CH_3 group and the 1'- to 5'-protons of the glucuronic acid moiety were assigned unambiguously by ^{1}H-^{1}H 2D-COSYspectra (**Figure 5.14**). For testosterone glucuronide the H-4 proton in the A ring and the H-17 proton in the D ring of testosterone), the -$COOCH_3$ group and the 1'- to 5'-protons of the glucuronic acid moiety were assigned unambiguously by ^{1}H-^{1}H 2D-COSYspectra (**Figure 5.15**). In

addition a HETCOR experiment (**Figure 5.16**) was also carried out to support the assignment of the peaks.

The presence of the sugar ring in the correct orientation and the *trans*-diaxial relationship (β-orientation) of the glucuronide moieties with respect to the steroid skeleton, were confirmed by the large coupling constants ($J_{1'2'}$,=7.32 Hz for E1G and $J_{1'2'}$,=7.69 Hz for TG whereas $J_{1'2'}$,=7.33 Hz for the E1G acetyl ester and $J_{1'2'}$,=7.70 Hz for the TG acetyl ester) for the two readily identified protons in the sugar moiety at C-1' and C-5'. The anomeric carbon (C-1') of the glucuronide ring is directly linked to two electron withdrawing oxygen atoms while the C-5' carbon is directly linked to only one electron withdrawing oxygen atom. As a result, the strongly deshielded anomeric proton (H-1') has a higher chemical shift value that that of the 5'-proton H-5'. For example for E1G the H-1' chemical shift was 5.06 ppm compared to 4.06 ppm for H-5' whereas for TG the H-1' chemical shift was 4.70 ppm compared to 3.85 ppm for H-5'. Since the 2', 3' and 4'-carbon atoms in the steroid glucuronide triacetate methyl ester are directly linked to strong electron withdrawing acetate groups, the 2', 3' and 4'-protons have higher chemical shift values than the 1' and 5'-protons. However, in the free steroid glucuronides, since the 2', 3' and 4'-protons are now linked to hydroxyl groups, the chemical shift values of the steroid glucuronide are moved up field and have lower values than the 1' and 5'-protons.

In the 2D COSY spectrum of estrone 3-glucuronide **5.1** (**Figure 5.14**), two correlated aromatic protons (1-H and 2-H) appeared at 7.20 and 6.85 ppm respectively, while the aromatic 4-proton occurred at 6.80 ppm as a singlet. The correlation of 1'-H (5.06 ppm) with 2'-H (3.61 ppm), 2'-H with 3'-H (3.61 ppm), 3'-H with 4'-H (3.71 ppm), and 4'-H with 5'-H (4.06 ppm) were all clearly recognisable in the spectrum. The correlation of the 2'-H and 3'-H protons was hidden due to both protons appearing at the same position (3.61 ppm). The 18-CH_3 group appeared as a singlet at 0.89 ppm.

In the 2D cosy spectrum of TG **5.2** (**Figure 5.15**), the five protons (1'-H to 5'-H) of the glucuronic acid ring were established again on the basis of the following correlations: 1'-H (doublet, 4.40 ppm) with 2'-H (triplet, 3.14 ppm); 2'-H with 3'-H (multiplet, 3.32-3.40 ppm); 3'-H with 4'-H (multiplet, 3.32-3.40 ppm); 4'-H with 5'-H

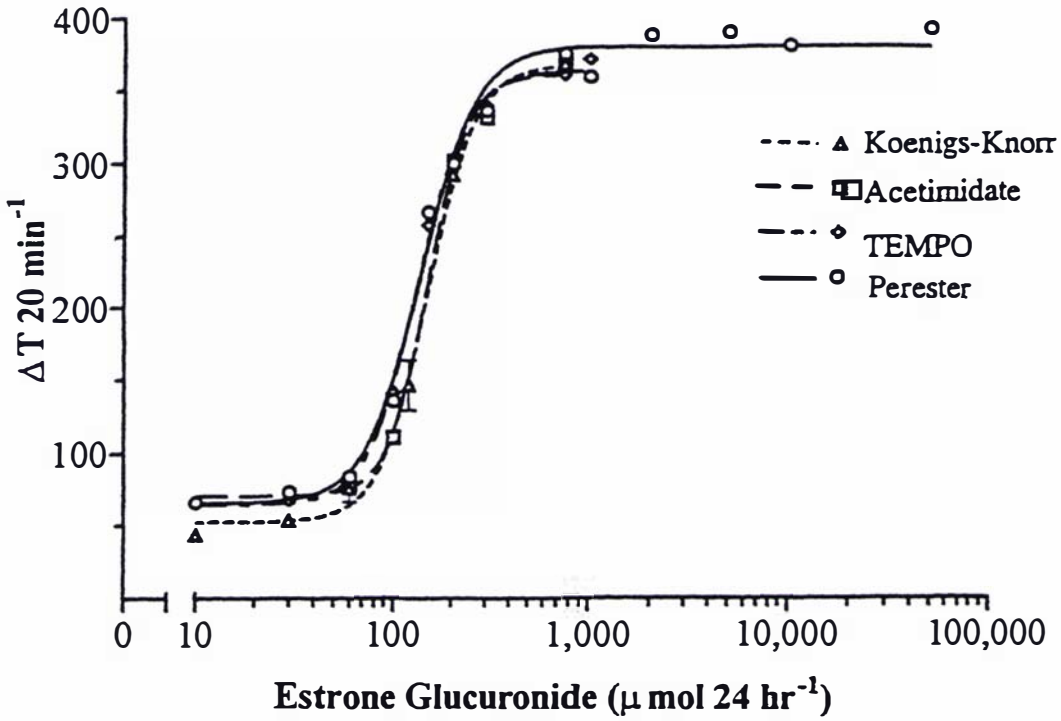
(doublet, 3.55 ppm). In addition the H-4 proton signal appeared as singlet at 5.68 ppm, while H-17 appeared as triplet at 3.71 due to its splitting by H-16.

Thus, the spectroscopic data clearly confirm the expected structures of E1G (**5.1**) for the 4 synthetic procedures and for the synthesis of TG (**5.2**).

5.3.5 Assays For Estrone Glucuronide Using Pre-Coated Estrone Glucuronide Assay Tubes With Freeze-Dried Components

The final proof of the structures of the different E1G(H) samples prepared using different synthetic routes was carried out by constructing standard curves with the different E1G(H) prepared by the Koenigs-Knorr reaction.[30] These was carried out using the freeze dried assay tubes from Melbourne which contained the ingredients for the assay (**Section 5.2.6**) to generate the standard curves. Single assays were performed according to the Ovarian Monitor instruction manual.[31] In standard curves prepared using these tubes, the 50 μL urine sample was replaced with 50 μL of the appropriate estrone glucuronide standard solution. A custom made heating block was utilised to incubate the samples at 40°C between transmission. The first transmission value was recorded 10 seconds after addition of an appropriate estrone glucuronide standard solution, followed by second transmission value 20 minutes later and the rate was measured as ΔT/ 20 minutes. The ΔT/ 20 minutes data obtained for estrone glucuronides prepared using various coupling methods were used to construct the standard curves. The standard curves were fitted by the sigmoidal dose response curve with a variable slope using the data analysis and graphic program PRISM. The results are shown in **Figure 5.8**.

Figure 5.8 Standard curves for E1G prepared using various synthetic routes



Clearly, within experimental error the standard curves are the same. The key parameters obtained from the standard curves are summarised in the **Table 5.1**.

Table 5.1 Standard curve fit for various estrone glucuronide samples

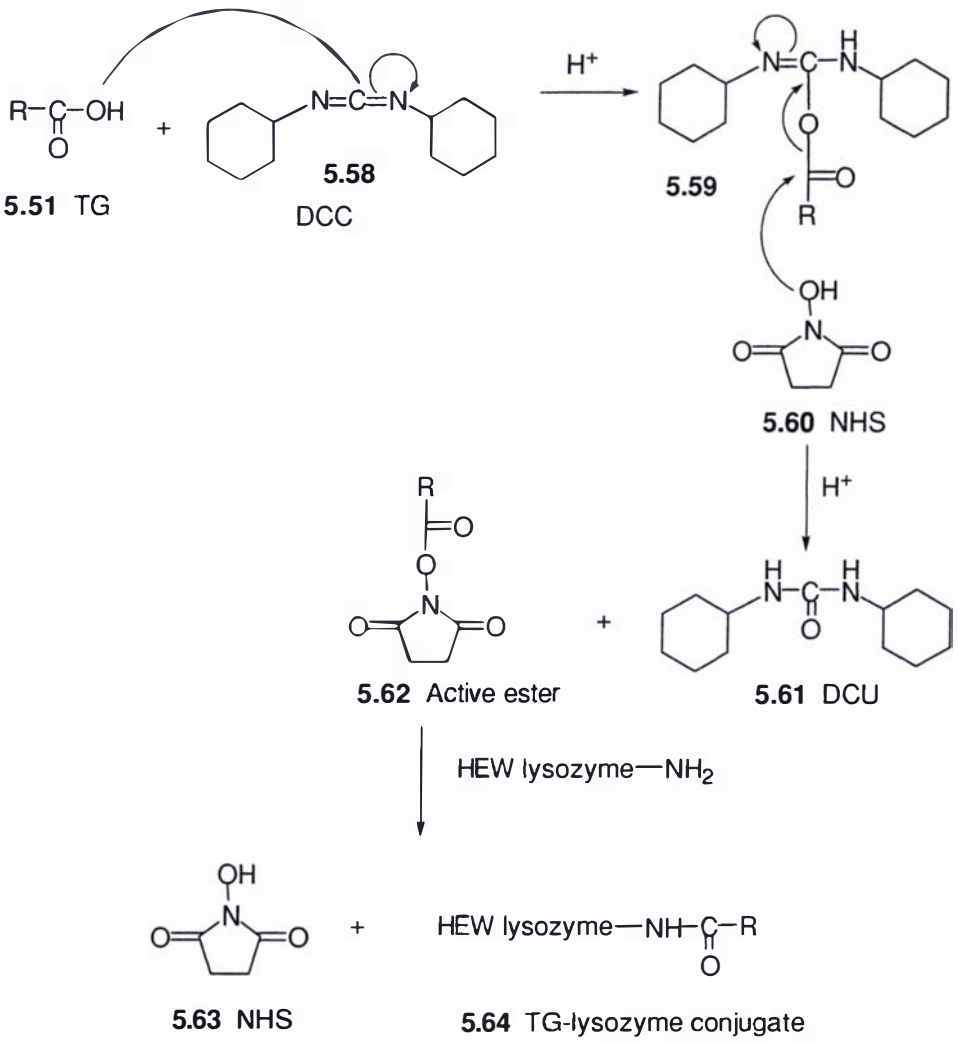
Labels	Koenigs-Knorr method	Perester method	Acetimidate method	TEMPO method
BOTTOM	50.39	65.68	70.73	64.35
TOP	368.7	380.8	360.8	363.6
HILLSLOPE	3.276	3.083	4.244	3.466
EC50	144.5	137.3	149.3	131.3
R^2	0.9964	0.9924	0.9950	0.9972

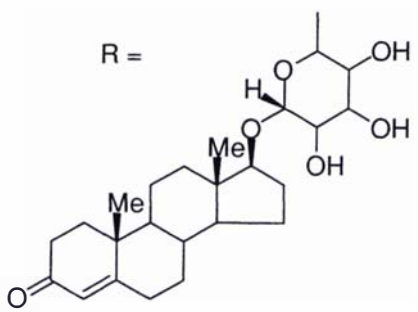
Within experimental error these are identical and give the same sensitivity at the mid-point (EC50) of the standard curve. The standard curve fits were excellent as shown by the R^2 values and the Hill slopes indicate a steep slope for the standard curves

making them ideal for the measurement of physiological levels of E1G. This suggests that the estrone glucuronide standards prepared from using the various coupling methods in this chapter were identical within experimental error over the critical concentration range with the Melbourne samples (Koenigs-Knorr method) used to generate the standard curves supplied with the pre-coated assay tubes. This result can be considered as conclusive proof that the estrone glucuronide synthesised using the various coupling methods were of the correct stereochemistry and were essentially pure.

5.3.6 Preparation Of Testosterone Glucuronide-Lysozyme Conjugates

Testosterone glucuronide conjugates of hen egg white lysozyme were prepared by the active ester coupling method at a 1.5:1 molar ratio of testosterone glucuronide to lysozyme as shown in **Scheme 5.13**.





Scheme 5.13

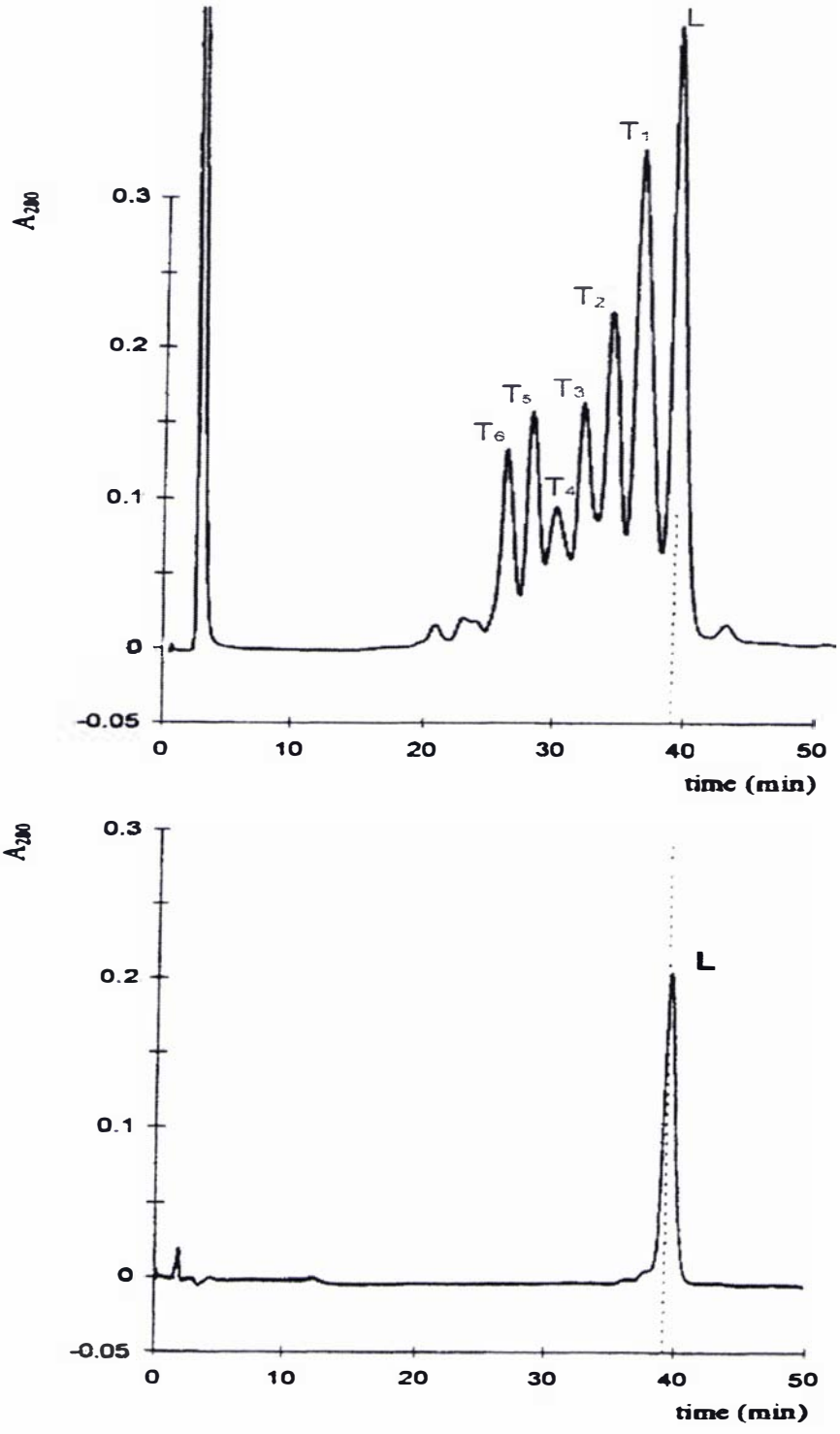
The preparation of the active ester reagent using the combined NHS/DCC method (**Scheme 5.13**) was preferred over the direct use of DCC (or EDC) for the coupling of testosterone glucuronide to lysozyme. While DCC is a good leaving group, the TG-DCC complex is insoluble in water and the formation of the carbodiimide

reagent is optimal at low pH (pH 4-5) where the amino groups are rather unreactive, being protonated. Thus the substitution of the DCC derivative for a more soluble compound which retains the good leaving group properties of DCC was achieved through addition of NHS to **5.59** which led to the formation of the active ester of testosterone glucuronide **5.62**. The active ester is quite stable and its hydrolysis rate is slow in aqueous medium compared with the rate of reaction with amino groups at the reaction pH ~9.[27] Another advantage of the active ester method is that DCC is insoluble in water and thus any unreacted DCC does not react with lysozyme to any great extent to generate cross linking between lysozyme molecules as is the case with protein conjugates utilising water soluble carbodiimides.

5.3.7 Purification Of Testosterone Glucuronide-Lysozyme Conjugates

Analytical cation-exchange chromatography[29] in 6M urea buffers showed that the coupling reaction resulted in conjugation of 73% of the initial amount of lysozyme with TG based on the relative peak areas. The largest peak consisted of unreacted lysozyme (L), while the six peaks (T1-T6) which eluted earlier consisted of different TG-lysozyme conjugates (see **Figure 5.9**).

Figure 5.9 Mono-S analysis of TG-lysozyme conjugation mixture (top) and lysozyme reference (bottom)

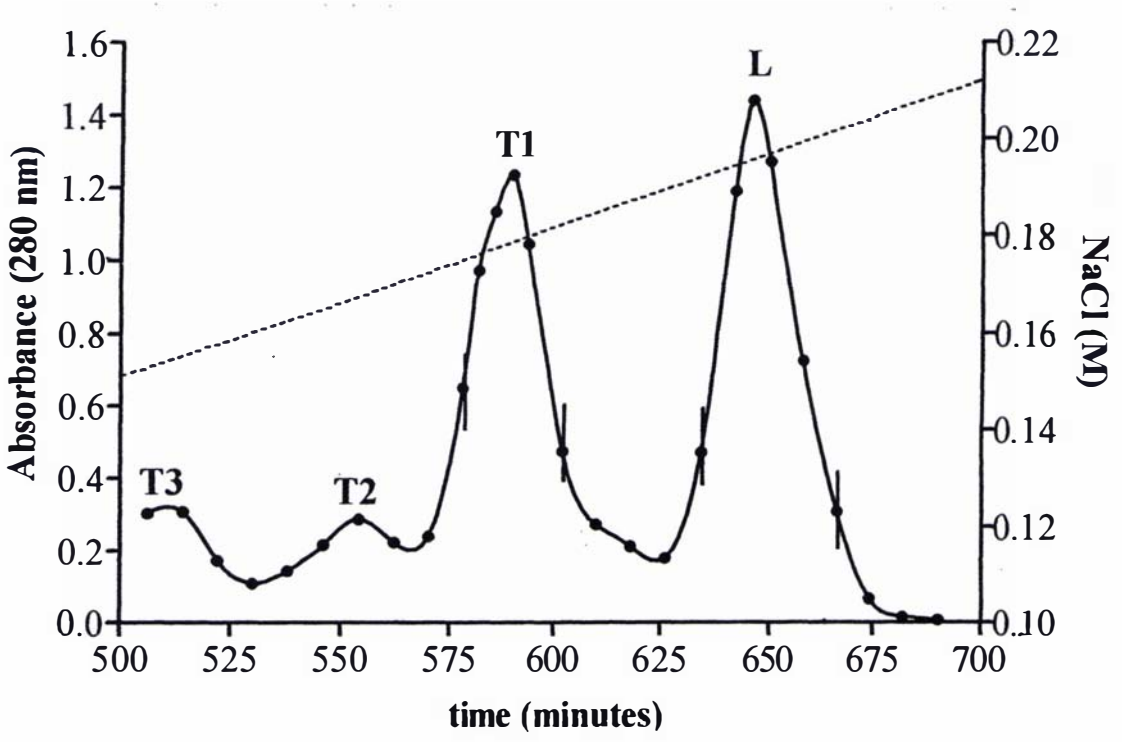


The peak areas were determined from the peak height multiplied by the width at half-height. The largest peak (27.3 % of the total) consisted of unreacted lysozyme (L) as shown by comparison with the elution profile of a pure sample of lysozyme (**Figure**

5.6). The six peaks (T1-T6) which eluted earlier are the different TG-lysozyme fractions. The conjugates are numbered relative to their elution position for lysozyme, T1 being closest to the lysozyme and T6 being the first conjugate to elute from the column (as for the E1G and PdG conjugates).[29] The overall yields of the three largest conjugate fractions, T1, T2 and T3 were 21.5%, 14.7% and 10.8% respectively, which together comprised 64.6% of the total conjugate yield. The remaining 35.4% of the conjugate yield consisted of fractions T4 (6.3% overall yield), T5 (10.5% overall yield) and T6 (8.9% overall yield) which are presumably the more highly substituted conjugates as is the case with E1G and PdG.[28] The major difference between the conjugates obtained by acylation with E1G and PdG is that peak T1 is the major conjugate fraction followed by T2 whereas for E1G peaks E3 and E1 are the predominant fractions (in that order) and for PdG, P1 is the major fraction followed by P3 with very little P2 being evident. The relative retention times for T1, E1 and P1 and T2, E2 and P2 are identical. Previous work by Smales *et al*[28] has shown that E1 and P1 are both conjugate mixtures where the steroid glucuronide is acylated on lysine 97 and 116 whereas E3 and P3 are both conjugated on lysine 33. Hence it is likely that T1 is conjugated with testosterone glucuronide on lysine 97, 116 or both and T2 is a disubstituted conjugate where both lysine 97 and 116 are acylated. Confirmation of this must await further work on tryptic digestion of the conjugates,[30] however, this information is not required for this thesis. These results show that the direct substitution of TG for E1G and PdG is possible and that exactly the same procedures can be used to prepare the TG conjugates. The coupling chemistry is determined by the carboxyl group of the glucuronide moiety. Although there are differences in the relative amounts of the various conjugate families for different hapten glucuronides as discussed above the structure of the hapten can clearly be varied over a wide range as required for a multi-purpose monitor using the lysozyme HEIA system.

After dialysis and concentration, the conjugate mixture was loaded onto a pharmacia S-Sepharose fast flow column equilibrated with 6 M urea and 50 mM phosphate buffer at pH 6. The elution profile consisted of two large broad peaks preceded by five poorly resolved small peaks as shown in **Figure 5.10**.

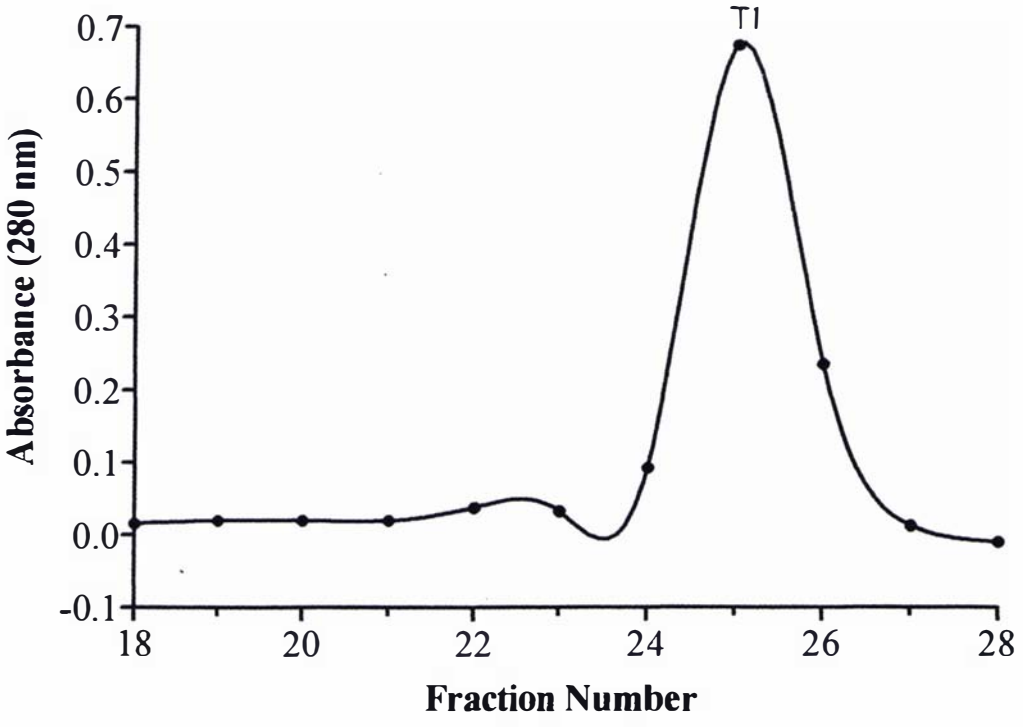
Figure 5.10 S-Sepharose fast flow profile of a HEW lysozyme-TG conjugation mixture



A comparison of this scaled up S-Sepharose fast flow profile with the Mono-S profile obtained from the total reaction mixture from a similar 1.5:1 active ester conjugation, showed that both separations had similar FPLC profiles and seven chromatographically distinct peaks were observed. The relative retention times of the earlier eluting peaks compared with the last eluting peak (i.e. the lysozyme peak) were similar between the two separations. The only major visible difference between the two profiles was that the peaks from the S-Sepharose fast flow separation were broader, which is consistent with the heavier loading used and the column's greater size.

The major T1 conjugate fractions tubes were pooled, concentrated and dialysed before loading onto a Butyl Sepharose column for further purification. The Butyl Sepharose profile (**Figure 5.11**) for T1 consisted of a single main peak.

Figure 5.11 Butyl Sepharose elution profile for T1 HEW lysozyme-TG conjugate after initial purification by S-Sepharose fast flow chromatography

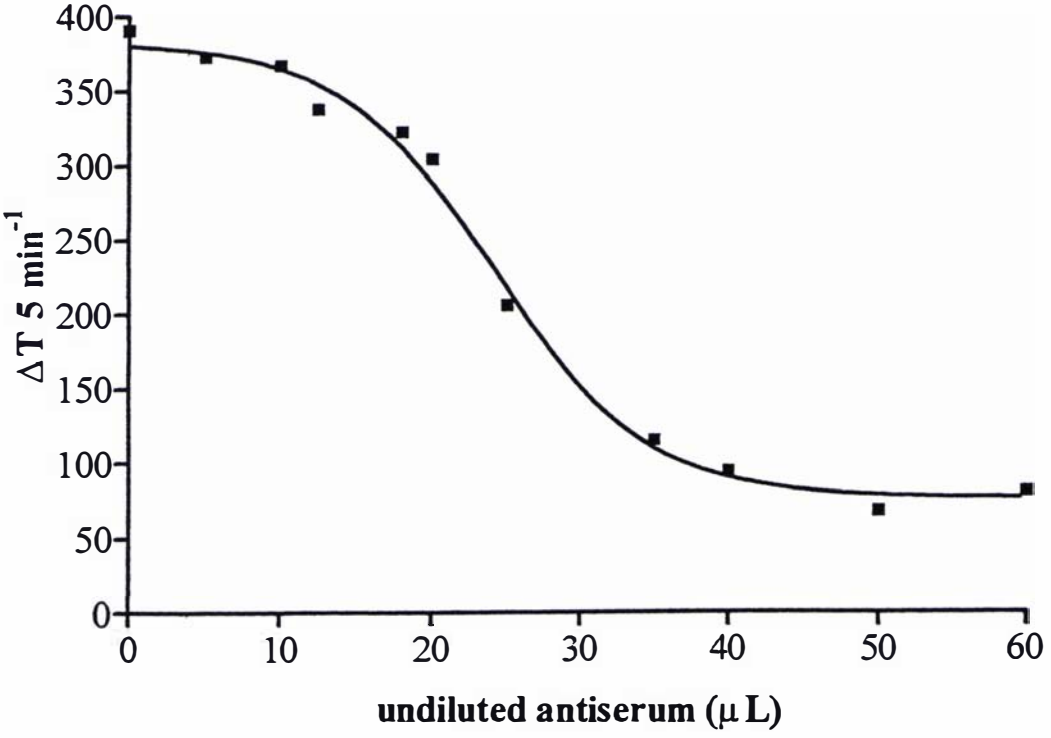


The required T1 conjugate fractions from the Butyl Sepharose separations were pooled as indicated on the absorbance profiles, dialysed against water (3 changes x 20 L) and concentrated by ultra filtration using an Amicon YM-10 membrane. The concentrated pooled fractions from the T1 Butyl Sepharose purifications showed a single peak on Mono-S chromatography under standard 7 M urea conditions suggesting that the fraction was pure.

5.3.8 Inhibition Studies (Antiserum Titration Curve For Testosterone Glucuronide)

The work described for the inhibition study was performed using the lysine residue substituted TG-lysozyme conjugate, which is referred to as T1 conjugate. T1 conjugate results obtained earlier showed that a concentration of 12 µL per assay gave a ΔT value of 347 in 5 minutes. Antiserum 880-1, raised as described in **Section 5.2.6.4** was titrated against a constant concentration of the T1 conjugate to give the titration curve shown in **Figure 5.12**.

Figure 5.12 Antiserum titration curve for HEW lysozyme-TG conjugate against anti-TG antiserum 880



An ideal curve for TG-antiserum binding would consist of two linear regions; a steep line where free TG-lysozyme exists and a shallower line when all the TG-lysozyme is bound by the antiserum. The point where the slope changes represents the point where all the TG-lysozyme is bound (the end point of the titration). From **Figure 5.12** it can be seen that at very low antiserum concentrations the curve is not linear, but this may be caused by an artefact in the Ovarian Monitor system. The titration curve shows a maximum ΔT of 383 in the absence of antiserum and gives a maximal inhibition (ΔT/5 minutes=76) at an antiserum concentration of 24 μL of undiluted antiserum per assay. Thus TG-lysozyme was 80% inhibited when 24 μL of undiluted antiserum per assay was added. This degree of inhibition is a bit lower than the desired inhibition rate of >90%, but can still be used for a generating a standard curve and for measurement of urinary TG.

5.3.9 Standard Curve For Testosterone Glucuronide

A standard curve suitable for the determination of urinary testosterone glucuronide levels was established as described in **Section 5.2.7.5**. The assay was

carried out with variable amounts of TG standards and the results (of ΔT 5 min^{-1}) were summarised in the table below.

Table 5.2 Summary of the data obtained for the TG standard curve

µmol TG 24 hr^{-1}	ΔT 5 min	ΔT average
0.001	120	120
0.004	105, 106	106
0.01	90, 120	105
0.04	90, 120	111
0.1	119, 124	122
0.4	147, 127	137
1	166, 156	151
4	242, 241	242
10	295, 259	277
40	335	335
100	366, 351	359
400	-	-
1000	-	-
4000	357, 341	348

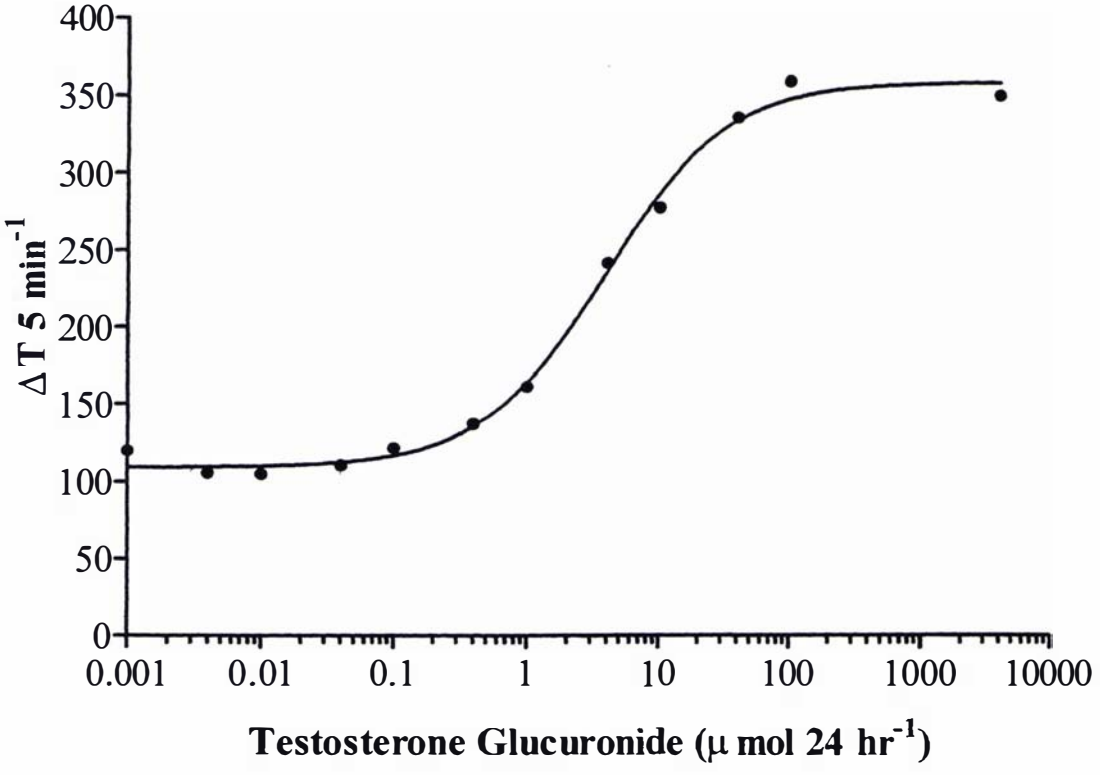
The data in the above summary table relating lytic rates and the concentration of TG were used for the testosterone glucuronide standard curve in the graphics Program

PRISM. The standard curve was fitted by the sigmoidal dose response curve with a variable slope using the equation shown below.

$$Y = Bottom + \frac{Top - Bottom}{1 + 10^{(LogEC50 - Log\ X)\ .\ Hill\ Slope}}$$

In this equation, X represents the concentration of TG in terms of urinary excretion rates, Y the rate of the assay, and Bottom and Top the rate at the bottom and top plateau of the standard curve. EC50 is the X value halfway between the bottom and top plateaus and thus is equal to the TG excretion rate that gives a response halfway between the minimum and maximum rates. The Hill slope variable controls the gradient of the curve. If the Hill slope is equal to 1, then the equation generates the standard dose-response curve and the response shifts from 10-90% of the maximal rate over two log units. A Hill slope less than 1 results in a shallower standard curve and a Hill slope greater than 1 results in a steeper standard curve.

Figure 5.13 Ovarian Monitor testosterone glucuronide (TG) standard curve



The key parameters for this standard curve were: Bottom of standard curve=109 ΔT 5 min^{-1}; Top of standard curve= 358 ΔT 5 min^{-1}; EC_{50} i.e. mid-point= 0.585 µmol 24 hr^{-1}; Hill slope = 0.942 (1= 10-90% of the change over 2 log units; >1= steeper gradient; <1= shallower gradient). The working range of the curve was 0.001 µmol 24 hr^{-1} to 4000 µmol 24 hr^{-1}.

5.3.10 Conclusion

The standard curve for TG is obviously suitable for the measurement of urinary TG at physiological concentrations using a 5 minute assay. If lower concentrations of TG need to be determined the assay time, and hence the sensitivity, can be increased. Clearly work with clinical samples related to physiological conditions now needs to be carried out to determine the usefulness of the assay. It is a then simple enough matter to convert this assay into a dried assay tube version and hence concentrations of TG in urine can be determined using the same assay systems and protocols as used for the home testing of fertility using urinary E1G and PdG levels. The way is now clear for the development of HEIA's for a variety of analyte glucuronides, including (±) methoxy equol glucuronide and entry into on-site phytoestrogen testing. All that is required to justify further work is a clinically relevant analyte and the ability to synthesise the glucuronide by either chemical or enzymatic methodology (**Section 2.1.10**, i) as discussed in this thesis. All the other critical steps listed (**Section 2.1.10**, ii-v) have been validated in this thesis and a multi-purpose home monitor is thus a reality with exciting commercial and practical applications and opportunities.

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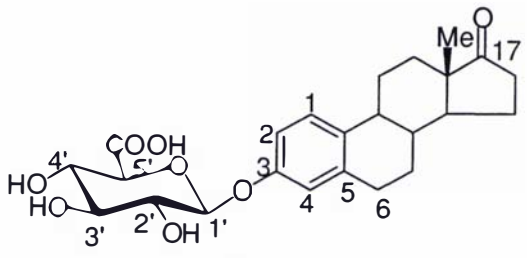
Figure 5.14 COSY Spectrum Of **5.1**



5.1

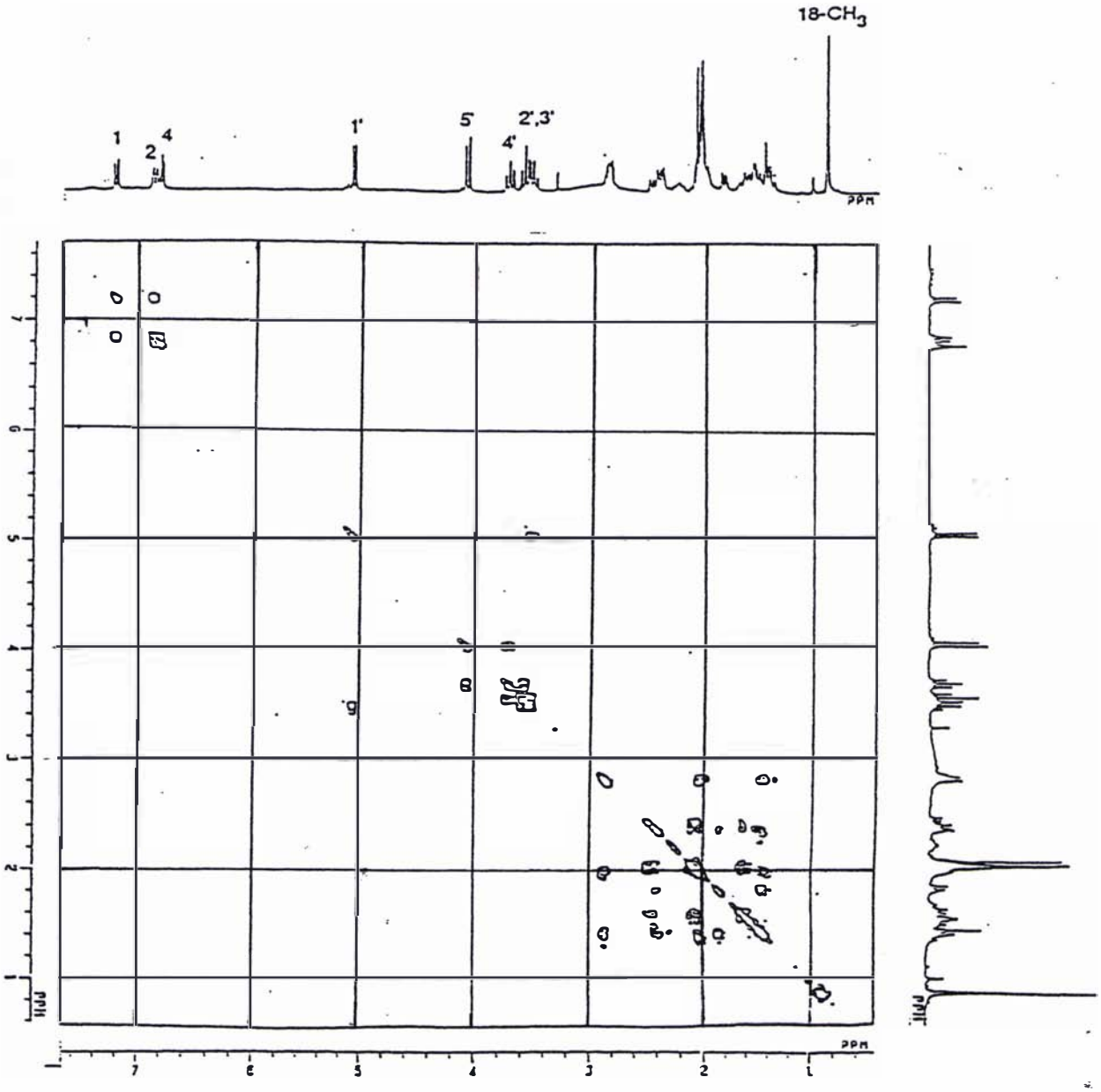
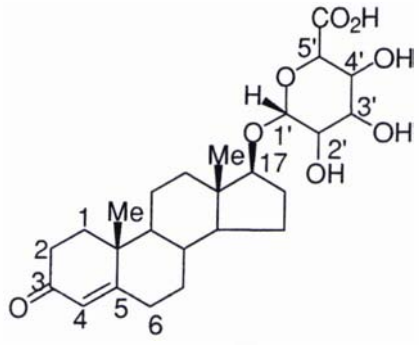


Figure 5.15 COSY Spectrum Of **5.2**



5.2

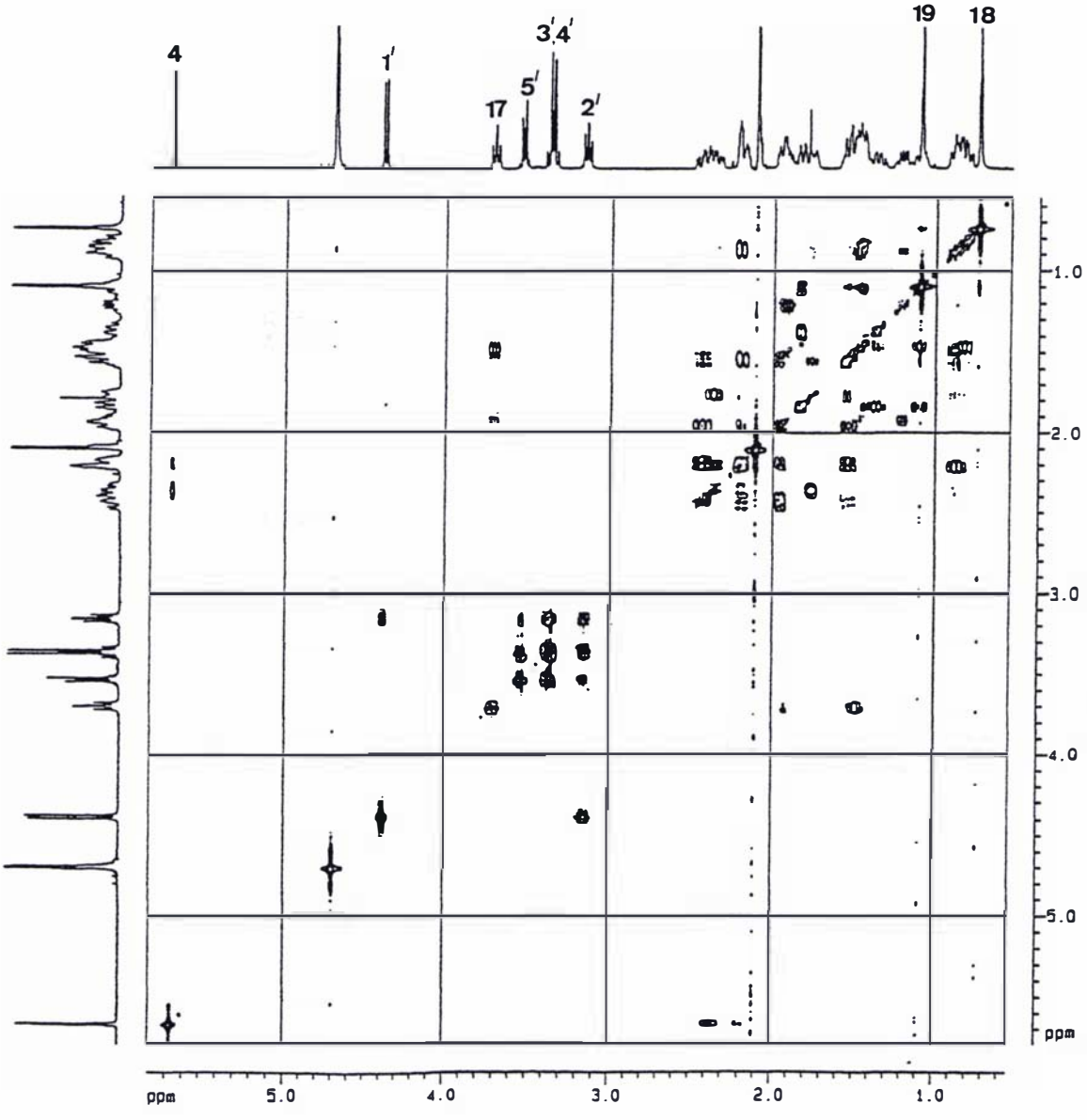
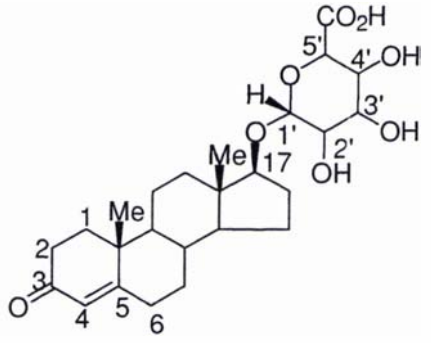


Figure 5.15 HETCOR Spectrum Of **5.2**



5.2