DOCTOR OF PHILOSOPHY

Fine chemicals from plant extracts

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Fine Chemicals from Plant Extracts

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by

Steven William Strawson

Prifysgol Bangor • Bangor University

'Bad times have a scientific value.

These are occasions a good learner would not miss.'

Ralph Waldo Emerson

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Abstract

Saponins are a structurally diverse class of compounds occurring in many plant species possessing a number of desirable biological and pharmacological activities. In this work methodology was developed to extract, isolate and purify desirable triterpenoid saponins based on the unusual aglycone backbone hederagenin from the fruits and leaves of common ivy (*Hedera helix*). The principal saponins obtained from the fruit were monodesmosidic and composed of predominantly α-hederin; the fruit by contrast had a saponin profile consisting of a mixture of monodesmosides and bidesmosides with Hederacoside the principle saponin present. The hydrolysis of these was investigated and procedures designed for the subsequent selective hydrolysis of the glycoside portions of these saponins by acid and base hydrolysis to obtain the aglycone and hemiglycone (monodesmosidic) derivatives. By chemical modification of the glycosides and aglycone, further derivatives have been prepared so as to develop a suite of novel compounds which were used to conduct trials of their biological activities to discover any potentially useful products and identify the best means of optimising these activities. A number of new long and short chain ester derivatives of hederagenin were prepared at the two hydroxyl positions, as well as investigation into the modification of the acid and olefin functionalities of the starting material which showed both of these positions to be significantly sterically hindered and as such exhibit a very low reactivity.

The saponin extracts and hydrolysis products from both the fruit and the folium of *H. helix* together with the their hydrolysis products were shown to exhibit significant biological activity against a number of microorganisms, particularly fungi, giving rise to the potential to develop triterpene saponin based therapeutic treatments for such conditions. In addition to this, *H. helix* fruit saponin and hydrolysis products were found to exhibit activity against slugs, potato cyst nematode and potato blight which could signify a potential use of these compounds in crop protection.

Abbreviations

AHP	acid hydrolysis product
Ara	α-L-arabinose
BHP	base hydrolysis product
b.p	boiling point
n-BuOH	n-butanol
DCM	dichloromethane
DCC	N,N'-dicyclohexylcarbodiimide
DCHU	dicyclohexylurea
DMAP	4-dimethylaminopyridine
2,2-DMP	2,2-dimethoxypropane
EtOAc	ethyl acetate
EtOH	ethanol
eq.	equivalent(s)
f	furanose
FBHP	fruit base hydrolysis product
g	gram(s)
GC	gas chromatography
Glc	β-D-glucose
GlcA	β-D-glucuronic acid
LCMS	liquid chromatography - mass spectrometry
LD_{50}	median lethal dose
HPLC	high performance liquid chromatography
Hz	hertz
ir	infrared
m.c	moisture content
MeOH	methanol
MCPBA	meta-chloroperbenzoic acid
ml	millilitre(s)
µl	microlitre(s)
mmol	millimole(s)

mol	mole(s)
m.p	melting point
MS	mass spectrometry
MALDI-TOF	matrix assisted laser desorption/ionisation with time-of-flight mass spectrometer
NHS	N-hydroxysuccinimide
NMR	nuclear magnetic resonance spectroscopy
ODW	oven dried weight
PCC	pyridinium chlorochromate
PDC	pyridinium dichromate
ppm	part per million
2-PrOH	2-propanol
PTSA	para-toluene sulfonic acid
Rha	rhamnose
r.t	room temperature
sp. , spp.	species (singular and plural)
ssp. , sspp.	sub-species (singular and plural)
TAG	triacylglycerol
TBDMS	tert-butyldimethylsilane
TFA	triflouroacetic acid
THF	tetrahydrofuran
TLC	thin layer chromatography
TTS	triterpene saponin
UV-Vis	ultraviolet-visible spectrophotometry

CHAPTER 1
INTRODUCTION

1.1 Saponins

Saponins are a structurally diverse class of compounds occurring in many plant species and from a few sources in marine organisms and which possess high biological activity at low concentrations. They have been found to be present in a wide variety of plant species, a number of which are used as food by humans; examples of some of the more commonly used ones are spinach, oats, tomatoes, potatoes, asparagus, liquorice and tea in addition to a large number of legumes (such as soybeans, chick peas, broad beans and peanuts) and other plant sources used in lower quantities such as herbs and spices (e.g. thyme, garlic, nutmeg, ginseng etc.).[1] It is often the saponin components of these which impart the bitter flavour found in some foods and that render some parts of them unpalatable.

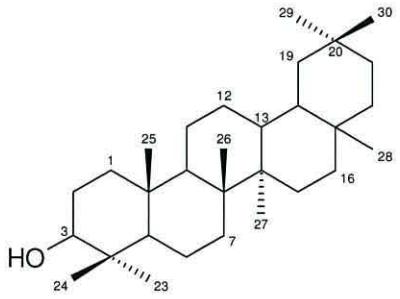
The classical definition of saponins was based on their surface activity, since many saponins possess detergent properties, form stable foams in water and show some haemolytic activity. These properties were historically used to identify and characterise this group of compounds.[2] However, due to the large number of exceptions, more recently these natural products have been defined on the basis of their molecular structure and biosynthetic origins.[3] The name 'saponin' is itself derived from this property; the Latin word *sapo* meaning soap. This reflects the historical use of saponin containing plants as soap substitutes for hundreds of years, a fact which is also apparent from some of their common names: soapwort (*Saponaria officinalis*), soaproot (*Chlorogalum pomeridianum*), soapbark (*Quillaja saponaria*), soapberry (*Sapindus saponaria*), soapnut (*Sapindus mukurossi*).[4] Although their use as soaps has now declined, saponins are still used in some situations (e.g. museums, archives) for cleaning delicate antique textiles and manuscripts where alternative surfactants would be to harsh.[5]

A related phenomenon, and one of the most common examples of the general biological activity of saponins, is the ability of saponins to cause the haemolysis of red blood cells (even at a very low concentration). This action was originally thought to be so universal amongst saponins that it was historically used as a semi-quantitative test for the determination of their identity and activity. The mode of action is uncertain but appears to be dependant on the ability of saponins to interact with lipids and cholesterol in the erythrocyte cell wall. This leads to a reduction in surface tension between the lipids and aqueous components to create an emulsion that detaches from the membrane creating holes which allow Na^+ and water to enter the cell and K^+ to leave. This flux will eventually cause the membrane to rupture and shed haemoglobin into the plasma.[6] It has been suggested that in a similar manner to this, some of the physiological importance of saponins in plants may well be to lower the surface tension of the sap.[7] Haemolytic activity varies greatly according to the structure of the saponin, i.e. the type of aglycone present, the number of saccharide chains attached and the number and type of sugars present in these chains. It has also been found that unlike originally thought, not all saponins actually display this activity: this, together with advances in analytical techniques, has led to such means of characterising and classifying saponins being abandoned.

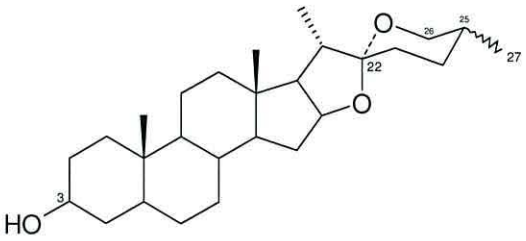
In addition to their surface activity, saponins have been constituents of many traditional plant drugs and folk medicines both from the orient and in western civilizations. It is this use which has generated a great deal of interest in these compounds and has driven the efforts at their characterization and in investigating their pharmacological and biological properties. This has allowed rapid progress to be made through the advances in isolation and structure elucidation of these compounds.

1.2 Definition and classification of saponins

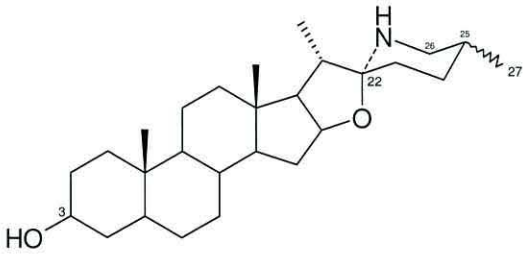
Saponins are composed of an aglycone portion attached to a sugar moiety. This aglycone portion is known as the *genin* or *sapogenin*. Saponins are commonly divided into three major classes according to the type of sapogenin present; triterpene glycosides, steroid glycosides and steroid alkaloid glycosides (Figure 1.2.1).



Triterpene class



Steroid class



Steroid alkaloid class

Figure 1.2.1 The three main classes of saponins

These aglycones are often hydroxylated at the C-3 position and certain methyl groups are frequently oxidized to hydroxymethyl, aldehyde or acid groups. All saponins will have one or more sugar moieties attached to the aglycone, generally in the form of a monosaccharide or as a polysaccharide chain. This gives rise to another type of saponin classification, monodesmosides and bidesmosides (Figure 1.2.2).

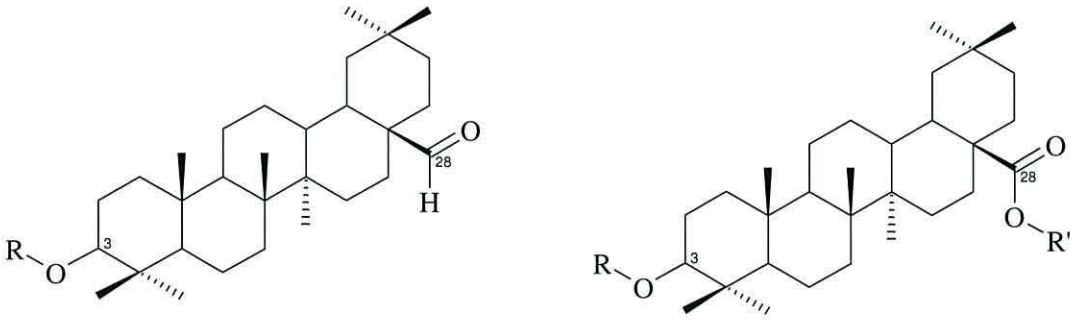


Figure 1.2.2 Monodesmosidic saponin (left) and bidesmosidic saponin (right) where R and R' represent a sugar moiety

Monodesmosidic saponins have a single sugar attached at the C-3 position through an acetal linkage. Bidesmosidic saponins contain two sugar chains, one attached at the C-3 position by an acetal linkage and another through an ester linkage at the C-28 position in triterpene saponins, as illustrated, or through an ether link at C-26 in steroid and steroid alkaloid saponins. Tridesmosidic saponins have also been found, containing three sugar moieties, although their occurrence is far less common than the other types.[8,9]

The variety of saponins is further increased by the different sugar moieties possible. The saccharide chains may be linear or branched and of varying length. Most saponins isolated so far have short, unbranched chains made up of 2-5 monosaccharide residues; however saponins have been found containing up to 11 monosaccharide units (Clematoside C from *Clematis manshurica*).[10] The most common monosaccharide units found are: D-glucose, D-galactose, D-glucuronic acid, D-galacturonic acid, L-rhamnose, L-arabinose, D-xylose and D-fucose.[11]

1.2.1 Steroid saponins

There are more than 100 known steroid sapogenins; most of these are derived from either the furostan or spirostan skeletons (Figure 1.2.1.1).

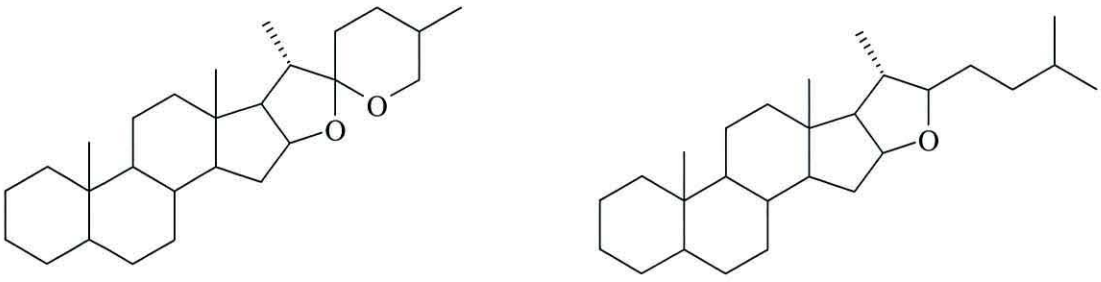


Figure 1.2.1.1 The spirostan aglycone (left) and furostan aglycone (right)

The main natural source of steroid saponins are the monocotyledons, being commonly found in plants of the families Lilacieae (especially the genera *Trillium*, *Chologalum*, *Smilax*, *Nolina*, *Agapanthus*), Dioscoreaceae (predominantly the genus *Dioscorea*) and Agavaceae (the genera *Agave*, *Yucca* and *Manfreda*). Of the dicotyledons, steroid saponins have been found in species of the Scrophulariaceae (especially in the genus *Digitalis*), Leguminosae (especially the *Trigonella*) as well as in the families Solonaceae, Simaroubaceae, Cornaceae, Zygophyllaceae, Ranunculaceae, Asteraceae and

Cruciferae.[12,13] The distribution of the two types of steroid saponins not only varies from species to species but also within the individual plants, with the spirostanol glycosides found mainly in the seeds, roots and bulbs of the plants while the furostanol glycosides are usually found in the assimilatory parts.

1.2.2 Steroid alkaloid saponins

Steroid alkaloids are characterised by an intact or modified steroid skeleton with a nitrogen atom integrated either into the ring or as a substituent or part of a side chain. Steroid alkaloids are also divided into two classes based on the sapogenin. These are the solanidans and spirosolans (Figure 1.2.2.1).

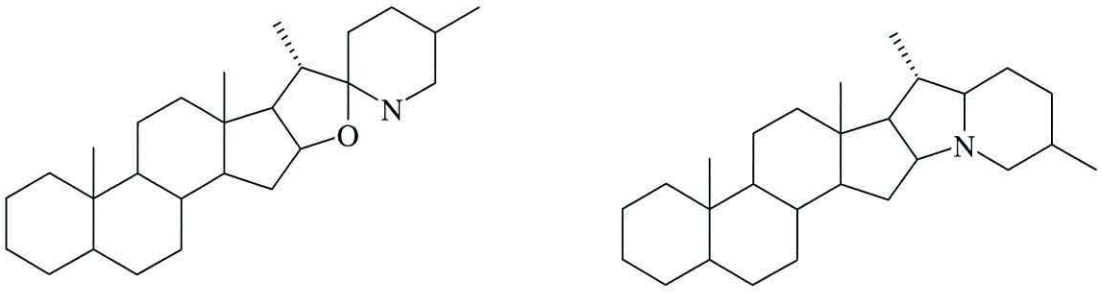


Figure 1.2.2.1 The spirosolan aglycone (left) and solanidan aglycone (right)

The naturally occurring steroid alkaloids are far lower in both number and in variety than the steroid saponins. Their distribution is limited to the Solanaceae family, which includes many important agricultural crop plants such as potato, tomato, eggplant and capsicum. The most well known, solasonine, has been found in around 200 *Solanum* species.[13] Steroid alkaloid glycosides are found in all plant organs, with highest concentrations found in the most metabolically active parts i.e. flowers, unripe berries, young leaves or shoots. They are generally toxic, but in fruits they gradually break down to form nitrogen-free non-toxic constituents (including some corresponding steroid saponins) during ripening.

1.2.3 Triterpene saponins

This subdivision is by far the largest of the three classes. The pentacyclic triterpene saponins are divided into three major classes depending on the skeleton present; these are α-amyrin, lupeol and β-amyrin (Figure 1.2.3.1). Of these, the β-amyrin class is by far the

most common (consisting of over half of the triterpene saponins). These compounds will be discussed further in the next section of this report.

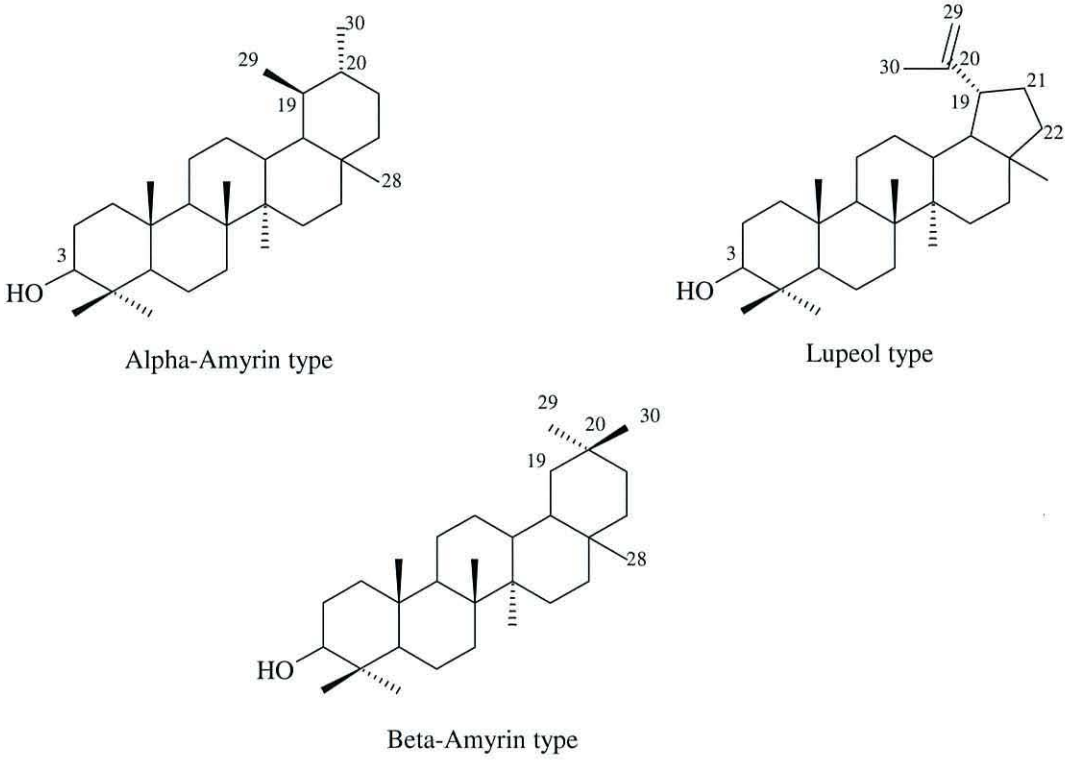


Figure 1.2.3.1 The three major classes of triterpene sapogenins

By modification of these skeletons it is also possible to obtain some other minor classes of sapogenin (Figure 1.2.3.2), although their occurrence is less common. It is also notable that all of these minor classes of triterpene sapogenins are also pentacyclic structures.

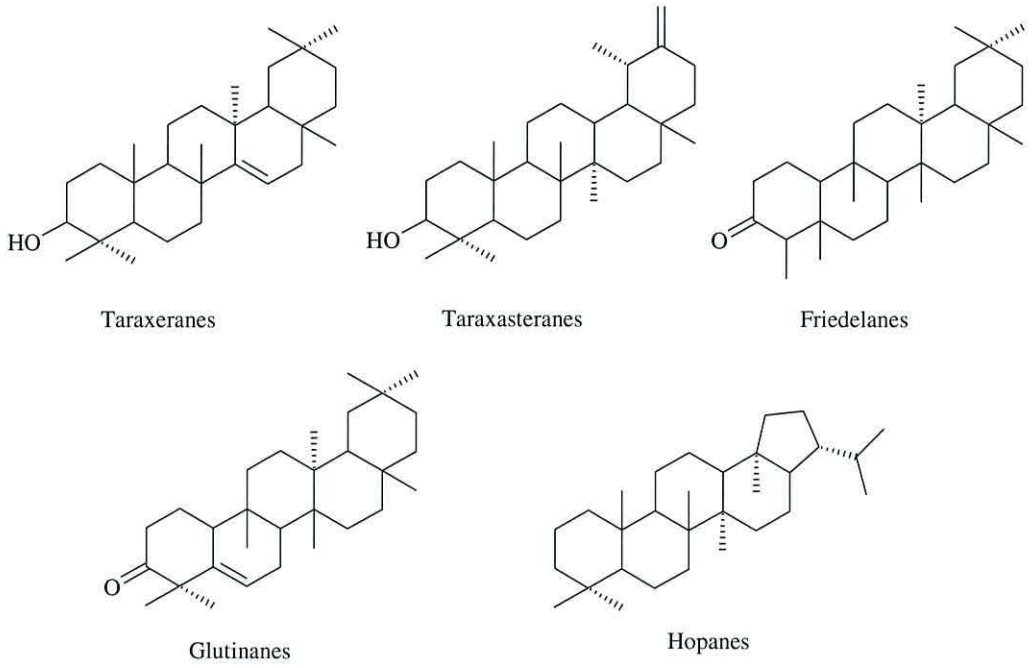


Figure 1.2.3.2 The minor classes of triterpene sapogenin, all of which are pentacyclic

The remainder of the known triterpene saponins are the dammarane class of tetracyclic triterpenes, and the lanostanes and holostanes (Figure 1.2.3.3), which are tetracyclic sapogenins recently found in some marine organisms.[14,15]

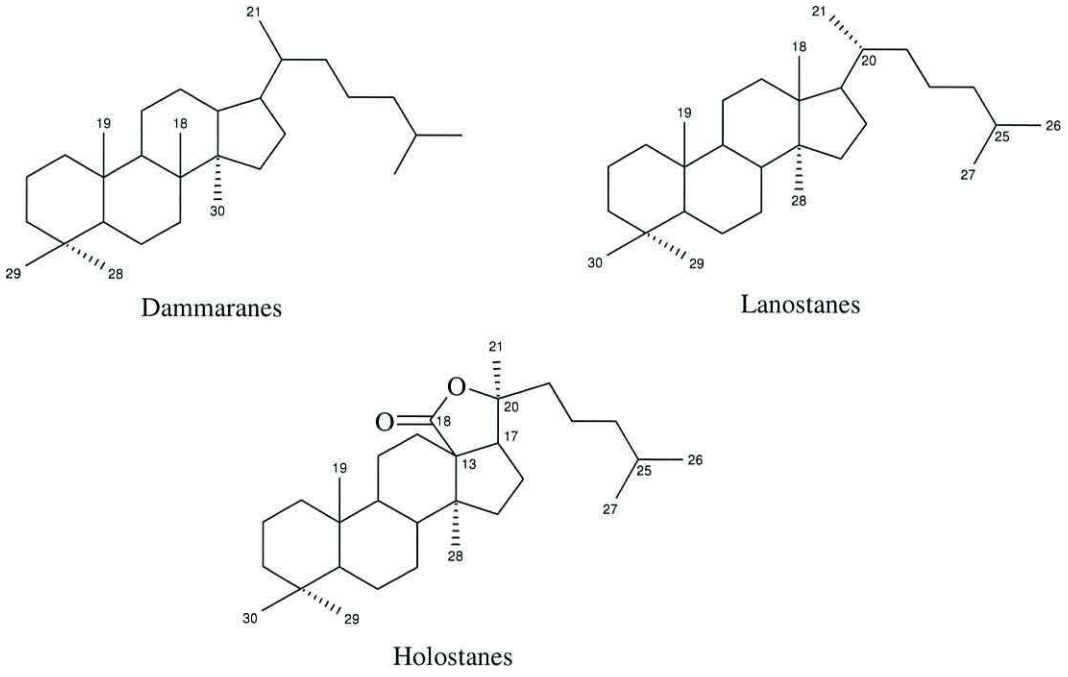


Figure 1.2.3.3 The tetracyclic triterpene sapogenins

1.3 Triterpenoid saponins

Triterpene saponins form the most extensive collection of saponins of all of the classes, with over 750 known triterpene glycosides with 360 sapogenins. These saponins vary widely, with the several types of pentacyclic skeletons illustrated earlier being possible, as well as the tetracyclic dammarane aglycones being observed. The saponins formed from these aglycones can then be mono-, bi- and even tridesmosidic. Even further diversity is created by the variety of monosaccharide units that is possible in the sugar moieties.

1.3.1 Occurrence and Distribution

Triterpene saponins are found in a wide variety of plant species. Several species of the phylum pteridophytes (ferns) contain triterpene saponins, while the gymnosperms such as conifers appear to contain almost no triterpene containing species. However, it is in the flowering plants (angiosperms) where these saponins are most predominant. These are found principally in the dicotyledons, in families such as the Leguminosae, Araliaceae, Caryophyllaceae, Asteraceae, Primulaceae, Sapindaceae and Chenopodiaceae.[16]

Although not as well represented as the dicotyledons, the monocotyledons possess saponin containing species; however these predominantly contain the steroid glycosides rather than the triterpenoid class (e.g. Liliaceae, Dioscoreaceae).

The number of known triterpenes is very large, and several review articles have provided comprehensive coverage of the isolated saponins, their structures and their plant sources. Among the most prominent of these in recent years are that of Mahato *et al.* and its subsequent updates.[17-20] These types of list demonstrate the huge diversity of this class of product (both in structure and in distribution).

It is apparent that triterpene saponins containing the oleanolic acid (**1**) and hederagenin (**2**) aglycones are the most common (Figure 1.3.1.1).

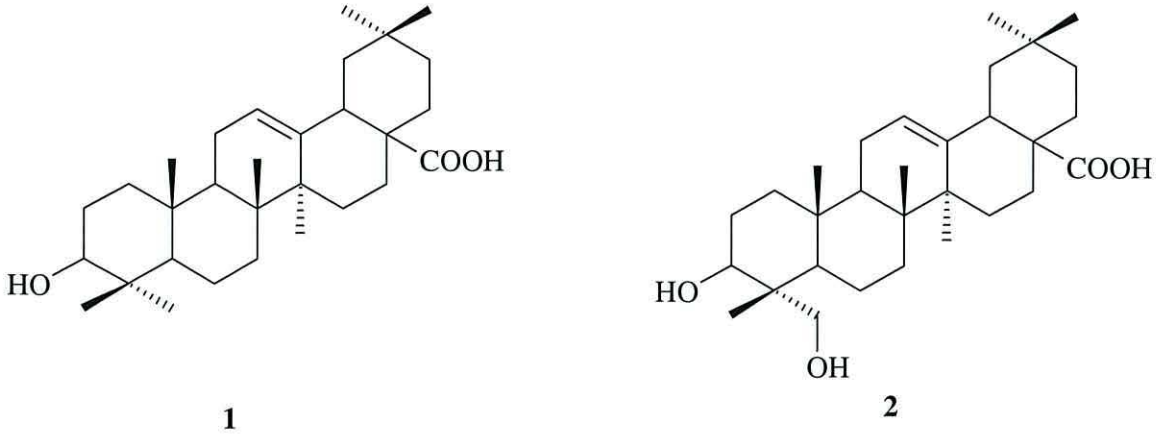


Figure 1.3.1.1 Oleanolic acid and hederagenin

The core skeleton of these oleanane aglycone and the common convention of numbering are shown in Figure 1.3.1.2. In the case of most monodesmosidic glycosides, the sugar chain is attached at the C-3 position by an acetal link; in the case of bidesmosides the second sugar chain is usually esterified to the carboxylic acid group at C-28. There are however a few examples of monodesmosidic acylglycosides (where a sugar chain is attached to the carboxylic acid position but no sugar is attached at C-3)[21-23]. These acylglycosides do not possess the usual properties of a monodesmoside, instead behaving in a similar way to that of a typical bidesmosides. This appears to suggest that it is the positioning of the sugar moiety at the C-3 position that is responsible for the properties of monodesmosides rather than merely the number of sugar moieties present.

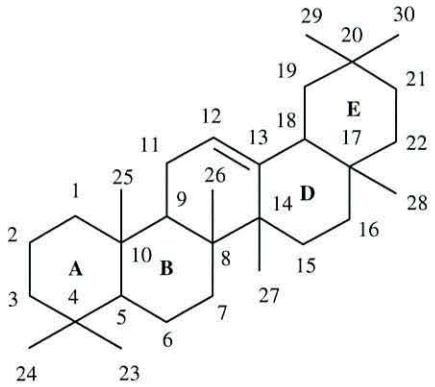


Figure 1.1.3.2 The basic skeleton of a β-amyrin type aglycone showing the system of numbering used to indicate the carbon positions and the letters corresponding to each ring system.

Bidesmosides form the majority of the known triterpene glycosides since they are not only widespread but also appear to be abundant within those species in which they are

found. Despite this fact, they have actually proved to be some of the most challenging saponins to isolate and their structural elucidation has been extremely difficult. This is due to the very complex sugar chains (often highly branched) which have been present in some of these glycosides. This difficulty is compounded by the occurrence of these saponins as complex mixtures of closely related compounds, making the identification of each individual component a rather formidable task. In the case of bidesmosides, the identification of the saponin in glycosidic form is complicated by the fact that triterpenes are frequently hydrolysed during the plant extraction, and since the sugar moiety esterified to the carboxylic acid is most readily hydrolysed their isolation can be even more difficult than that of the corresponding monodesmosides would be.

1.3.2 Properties, activities and use

As mentioned earlier, some of the properties of saponins have been known for a long time, e.g., they have been used as detergents and as piscicides for centuries. In addition to this, saponin containing plants have been constituents in a great number of herbal remedies. These discoveries have likely resulted from the widespread occurrence of saponins in plants around the world. However, little more was known than this since early scientific investigation was done using only crude saponin mixtures. Today, with the advent of sophisticated separation and characterisation techniques it is more common to work with pure saponins (or pure saponin mixtures), which has led to better definition of the properties of these compounds. Although it has since been found that there have been a number of exceptions to what were once considered the general properties of these saponins, these properties still describe the majority of saponins quite well.

Triterpene saponins are strongly surface active, form stable foams, act as emulsifying agents and form micelles in a similar way to detergents. This is all as a result of the molecules' amphiphilic nature when in solution. It has also been known for some time that saponins also form complexes with cholesterol and can cause the haemolysis of blood by emulsion of the surface lipids and consequently the destruction of the red blood cell membrane.

It should be noted that there is a vast difference between the properties of mono- and bidesmosides. Bidesmosides generally lack the biological activity of the corresponding monodesmosides or, where present, these properties are extremely reduced (with the

exception of surface activity, where this property is more pronounced in the bidesmosidc saponins and increases with the chain length of its sugar moieties).[24] An exception to this are the quillaja saponins where the monodesmosidic forms are significantly less effective haemolytic agents than the bidesmosidic forms.[25] As such, it appears likely that bidesmosidic saponins act as useful forms of storage and / or transport of these compounds within the plant until such time as the biologically active monodesmosidic forms are required for plant defence.[26,27] Despite this trend in activity, bidesmosidic saponins may also possess potent biological and pharmacological activities in animals which are completely unrelated to any aspects of plant physiology.

Useful biological activities of triterpene saponins are wide ranging but include such things as antimicrobial activity, spermicidal and contraceptive activity, insecticidal and antifeedant activity as well as use in treatments for cellulitis, hypertension and in coughs. The activities of *Hedera helix* saponins will be discussed in more detail later (See 1.6).

1.4 *Hedera helix* and its saponins

1.4.1 Hedera helix L. (Common Ivy)

Hedera (Ivy) is a genus of evergreen woody root climbing plants of the family Aralaceae; the genus is composed of approximately 15 species of ivy, which are distributed throughout Europe, North Africa, Macronesia and Asia.[28,29] The *Hedera* spp. represent an important element in European and Asian woodlands where they comprise a large proportion of the forest understory. The first recorded *Hedera* species was *H. helix*, commonly known simply as Ivy, although sometimes more specifically called English ivy or Common ivy. This species of ivy is indigenous to most of Europe and south west Asia (Figure 1.4.1.1) and is also now present in other compatible regions worldwide as a common plant due to introductions of European stock. It is cultivated in some communities as an ornamental plant for its evergreen foliage, to attract wildlife and often grown on walls for aesthetic purposes or to conceal underlying problems; numerous cultivars have now been developed displaying desirable decorative traits. In other non-native regions where *H. helix* grows, such as Australia and parts of the Pacific North West and Southern USA, it is regarded as an invasive weed as it is responsible for significant ecological damage by smothering and choking-out the native trees and plants;[30-32] many states and councils in these affected areas now encourage the removal

and destruction of English Ivy and have even in some cases made the sale, transport and propagation of the plant illegal.[33,34]

Figure 1.4.1.1 Approximate natural distribution of *H. helix* in Europe, North Africa and western Asia. Taken from Metcalfe 2005.[35]

H. helix is an evergreen climbing plant which is capable of growing to considerable heights where suitable surfaces (e.g. cliffs, trees, walls etc.) are available, but can also grow as ground cover where no vertical surfaces are present. The leaves are of two types: palmate five-lobed juvenile leaves grow on creeping and climbing stems, and unlobed cordate mature leaves grow on the fertile flowering stems exposed to full sun. The adult plant has lighter coloured leaves and produces small green-yellow flowers from late summer until late autumn as well as the fruit; small black berries ripening in late winter (Figure 1.4.1.2).

Figure 1.4.1.2 The mature leaves of Hedera helix (left) and the dark berries of fruit (right)

The species has widespread distribution through nearly all of the U.K. and Ireland (Figure 1.4.1.3) and grows profusely in areas without management or intervention. Identification is somewhat complicated by the presence of sub-species, by hybridisation between these and by the variable morphology described previously.[35] The three sub-species which have been identified are *H. helix* var. *helix*, *H. helix* var. *poetarum* Nyman (syn. *Hedera chrysocarpa* Walsh) and *H. helix* var. *rhizomatifera*.[29,35] Of these, only *H. helix* var. *helix* is indigenous to the U.K. The closely related species *Hedera hibernica* (Atlantic Ivy or Irish Ivy) is often treated as subspecies of *H. helix* and is also native to the U.K.

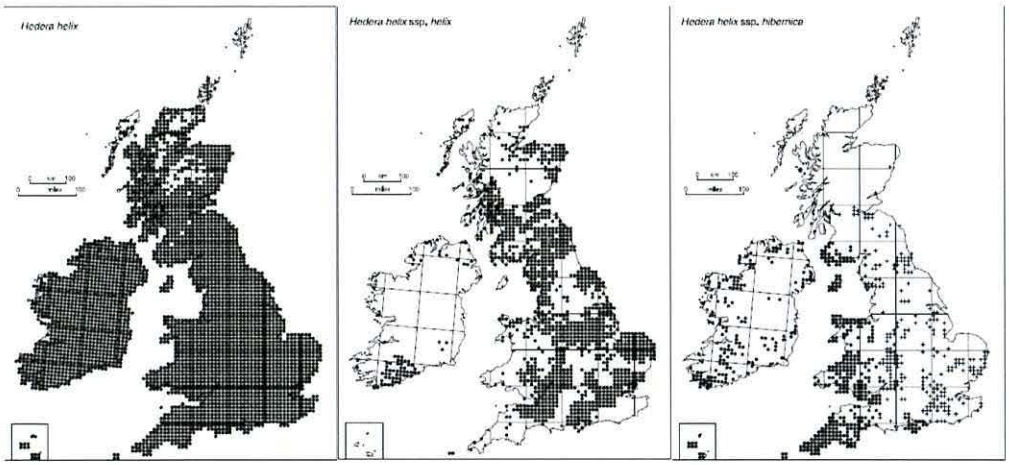


Figure 1.4.1.3 Geographical distribution of *H. helix* (left), *H helix ssp. helix* (centre) and H. helix spp. hibernica (right). Taken from Metcalfe, 2005.[35]

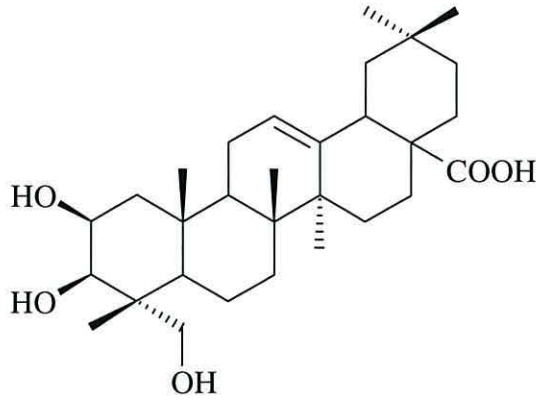
H. helix is often described in the literature as a toxic species. Some of this toxicity can be attributed to the presence of triterpene saponins which like most saponins can be considered rather toxic (the LD_{50} value for intravenous Hederacoside C is 50 mg/kg).[36] This toxicity is due to the haemolytic properties of the saponin which can result in rupture

of red blood cells, liver damage, respiratory failure, convulsions, coma and eventually death. However, as is evidenced by the large number of saponin containing food crops (the average family consume ca. 15 mg per person per day), the oral toxicity of saponins to warm-blooded animals is actually very low; the reason for this being the feeble absorption which saponins undergo in the body.[37] As a result, reported cases of poisoning by *H. helix* are extremely rare. Despite this, the overstatement of its toxicity continues; it seems this is most likely due to confusion with the unrelated North American species "poison ivy" (*Rhus toxicodendron*).[38] Skin contact with sap from this species, however, can be responsible for allergic and irritant contact dermatitis in humans. This has been found to be caused by the polyyne compounds falcarinol and didehydrofalcarinol which are present only as minor components in Ivy but had been established previously as being responsible for similar dermatitis conditions in carrot handlers.[39]

The degree of toxicity is also of note due to the well established importance of the fruits of *H. helix* as a late food source by a number of wild birds during the more severe months of the winter; it is, in fact, by means of birds eating the berries that the seeds of *H. helix* are distributed. The fruits are regularly consumed by wood pigeons (*Columba palumbus*) as well as birds of the thrush family (*Turdus* spp.), so much so that studies show that the fruits are preferentially consumed over similar berries by the thrush *(Turdus turdus)*, blackbird *(T. merula)* and songthrush *(T. philomelos)*[40] and that they comprise a significant portion of the diet of the blackbird *(T. merula)* and blackcaps *(Sylvia atriapilla)*.[41] These findings appear in contrast to other studies which propose that the intake of *H. helix* fruit is limited by the presence of the bitter tasting saponins.[42] In addition to birds there is also a wealth of literature and anecdotal evidence of the importance of *H. helix* in the diets of the larvae of some Lepidoptera; caterpillars of several species are known to consume the leaves, with the moth *Idaea seriata* (Small dusty wave) feeding exclusively on Ivy.[43]

1.4.2 Saponins present in H. helix

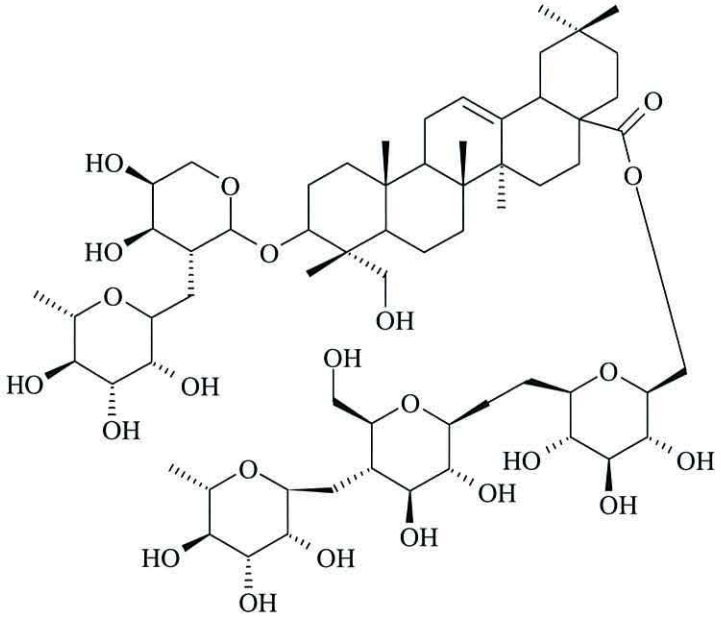
H. helix, like all Araliaceae spp. contain oleanane saponins. There has been significant reported work carried out to date in identifying and characterising the saponins present. One such study by Elias *et al.*, has shown that the saponins of the leaf contain a mixture of oleanane aglycones; this mixture is composed predominantly of hederagenin (**2**), along with oleanolic acid (**1**) and a very small quantity of bayogenin (**3**).[44]



3

Figure 1.4.2.1 The aglycone bayogenin which has a structure similar to that of hederagenin with an additional hydroxyl group at the C-2 position

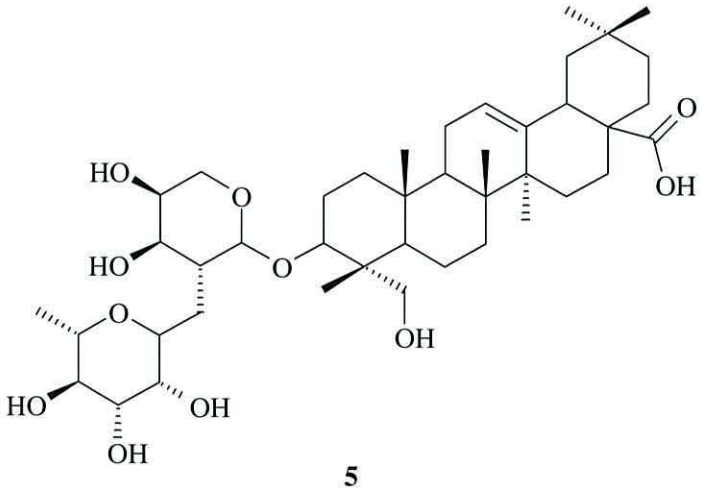
In total this study on leaves growing in Marseille characterised 8 bidesmosidic saponins. However, the composition of the saponin profile of the leaves was found to be dominated by hederacoside C (**4**). Otherwise known as hederasaponin C, hederacoside C is composed of a hederagenin aglycone together with two saccharide chains containing the uncommon sugar rhamnose as the terminal sugar of both (Figure 1.4.2.2).



4

Figure 1.4.2.2 The bidesmosidic saponin hederacoside C

The study did not, however, identify any monodesmosidic saponins as being present in this mixture; this is an unusual finding since ivy leaf had for some time been used for ethnomedicinal purposes as an expectorant (see 1.4.3) of which α-hederin (**5**) was claimed to be the active substance. This saponin was first isolated from ivy leaf by van der Haar in Bern, Switzerland who proposed the structure shown in Figure 1.4.2.3.[45] A later study did not identify this to be present in the leaves but instead found an alternative monodesmoside possessing a Glc-Ara-hederagenin structure (as opposed to the Rha-Ara-hederagenin structure of **5**), this was named hederacoside A.[46]



5

Figure 1.4.2.3 The monodesmosidic saponin α-Hederin

Van der Haar's findings were confirmed by a subsequent study in Bonn which isolated α-Hederin from dried *H. helix* leaves without identifying any hederacoside A. They proposed the structures of α-Hederin and hederacoside C to be identical to those of kalopanaxsaponins A and B, previously isolated from the bark of *Kalopanax septemlobus*.[47]

In 1991, further work on the saponins of *H. helix* leaf based in France developed a HPLC method using a light scattering detector to simultaneously determine both the bidesmosidic and monodesmosidic saponins in the mixture.[48] They once again identified 6 saponins to be present (Hederacosides C, D and B together with α-, β-, and δ-Hederin). This suggested the presence of only one bidesmoside saponin with 5 monodesmosides. However, this time the work was also able to determine the major saponins quantitatively. This showed that from their extract of *H. helix* leaves Hederacoside C was the principle saponin (7.27 %) and α-Hederin the only other major product at only 0.46 %.

In 1980, a study was conducted on the fruits of *H. helix* collected in Tüscherz, Switzerland. Hostettmann reported four principal monodesmosidic saponins to be present all of which were based on a hederagenin aglycone, α-hederin being one of them.[49] A later study on fruits collected in Northern Turkey found 6 saponins, most of which had hederagenin as the aglycone and reported a 27 % recovery of crude saponin.[50] However, only three of these were identified as the same as those reported by Hostettman and they reported the principle saponin to be based on oleanolic acid and not on hederagenin. The study did, however, agree with that of Hostettman[49] that four saponins present were monodesmosidic and two were bidesmosides. Neither study reported quantitative data on the individual saponins recovered.

Most recently, a study in Bangor reported the extraction of saponins from the dried leaf and fruit of *H. helix*.[38] A 25 % recovery of crude saponin from the fruit, with a mixture containing predominantly monodesmosidic saponins; α-hederin dominated the saponin profile. The extraction of leaf gave a 10 -15 % recovery of crude saponin, this time composed mainly of the bidesmoside Hederacoside C. In both cases the saponins were found to be based predominantly on the aglycone hederagenin, and the secondary aglycone oleanolic acid was reported only as a minor component. No other minor saponin components were reported.

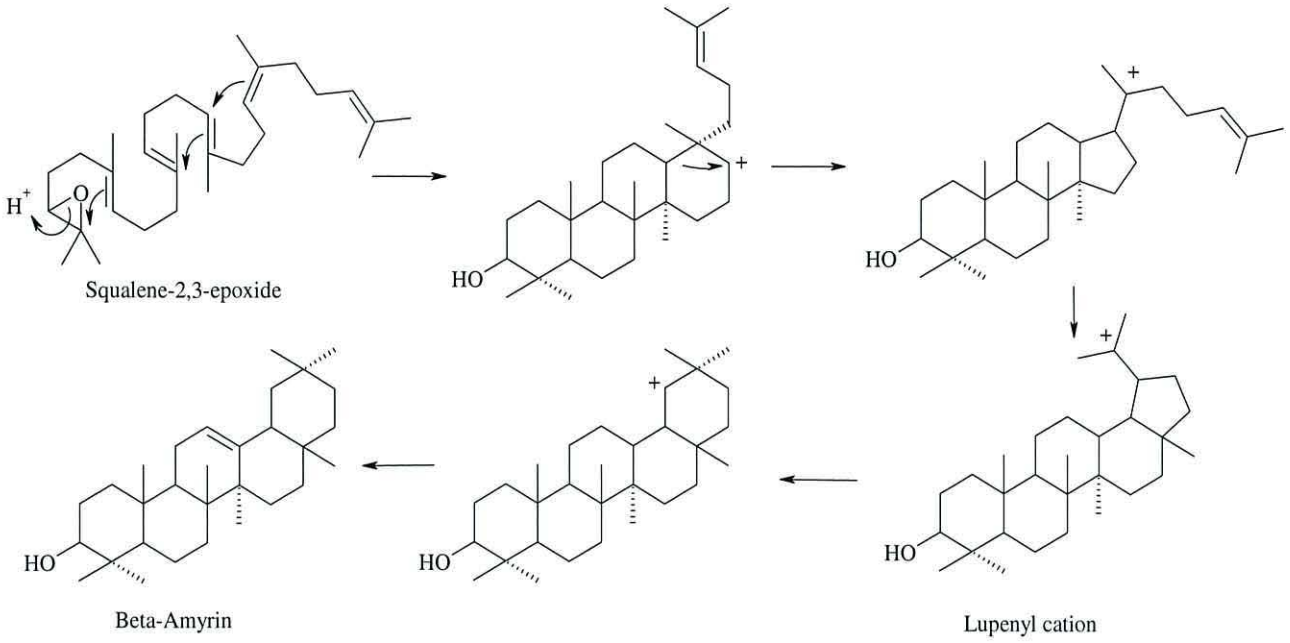
Between all of the available studies, a great deal of sometimes contradictory data has been presented, but for clarity a list of all saponins identified in *H. helix* is reported in Table 1.4.2.1.

Name	Aglycone	Saccharide at C-3	Saccharide at C-28	Reference and year
Hederaoside C (hederasaponin C / kalopanaxsaponin B / cauloside D)	Hederagenin	Ara^{2}-Rha	Glc^{6}-Glc^{4}-Rha	Tscheche *et al.* 1965[47]
α-hederin (sapinoside A / kalopanax saponin A)	Hederagenin	Ara^{2}-Rha	-	Tscheche *et al.* 1965[47] / Hostettmann 1980[49]
Hederasoponin I	Hederagenin	GlcA	Glc^{6}-Glc^{4}-Rha	Elias *et al.* 1991[44]
Cauloside G	Hederagenin	Ara^{2}-Glc	Glc^{6}-Glc^{4}-Rha	Elias *et al.* 1991[44]
Hederacoside A (hederasaponin A)	Hederagenin	$Araf^{5}$-Glc	-	Scheidegger and Cherbuliez 1965[51]
Dipsacoside B	Hederagenin	Ara^{2}-Rha	Glc^{6}-Glc	Wagner and Reger 1968[52]
δ-hederin (Hederacoside D / Hederasaponin D)	Hederagenin	Ara	-	Crespin *et al.* 1994[48]
-	Hederagenin	Glc^{2}-Glc	-	Hostettmann 1980[49]
Hederacoside B (hederasaponin B / eleutheroside M)	Oleanolic Acid	Ara^{2}-Rha	Glc^{6}-Glc^{4}-Rha	Tscheche *et al.* 1965[47]
β-hederin	Oleanolic Acid	Ara^{2}-Rha	-	Tscheche *et al.* 1965[47]
Hederasaponin F	Oleanolic Acid	OSO_3^-	Glc^{6}-Glc^{4}-Rha	Elias *et al.* 1991[44]
Hederasaponin H	Oleanolic Acid	Ara^{2}-Rha	Glc^{6}-Glc^{4}-Rha	Elias *et al.* 1991[44]
Hederasaponin D (Koelreuteria-saponin A / fatsiaside B_1)	Oleanolic Acid	Ara	-	Hostettmann 1980[49]
-	Oleanolic Acid	Ara	Glc^{6}-Glc^{4}-Rha	Elias *et al.* 1991[44]
-	Oleanolic Acid	Glc	-	Hostettmann 1980[49]
Hederasaponin E	Bayogenin	Ara	Glc^{6}-Glc^{4}-Rha	Elias *et al.* 1991[44]

Table 1.4.2.1 All reported saponins identified in *H. Helix* (N.B. where the saponin is known by more than one name the name most commonly used in the literature or in connection with this species is given with synonyms listed in brackets)

The anomalies apparent in the investigation of *H. helix* fruits may well be as a result of the geographical differences in the locations of these studies; it has been shown that phylogeographical distribution between European populations of *H. helix* exhibit a wide variability in haplotypes, which could result in such differences.[53] Similarly, natural variation between populations has been proposed to explain differences between the studies of the leaf saponins.[47] Also worth noting is the further explanation for these differences proposed by Doman and Hostettmann who suggest that the difficulty may arise from degradation caused by the different extraction processes used.[54] This concept was reinforced more recently by Oleszek and Bialy who suggested that early work in the field relied heavily on hot extraction methods which could disintegrate some labile functions and give rise to artefacts rather than genuine saponins.[55] Evidence from work on extracting saponins from the unrelated *P. dodecandra*, supports this; it showed extraction with water yielded predominantly monodesmosidic products while extraction in methanol afforded the corresponding bidesmosides; enzymatic action was found to degrade the saponins and the use of high ratios of solvent to water reduced this effect and gave the intact bidesmosides.[56]

All of the triterpene saponins present in *Hedera helix* are in the β-amyrin class (oleanane) and like all triterpene saponins are built up from six isoprene units and share a common biosynthetic origin. This class of saponins are all synthesised by the same pathway from the ring opening and cyclisation of the chair-chair-boat conformation of squalene-2,3-epoxide (Scheme 1.4.2),[57] a derivative of squalene, the biochemical precursor of all steroid and triterpene saponins. This subject was reviewed in depth by Abe *et al.*[58]



Scheme 1.4.2.1 The biosynthesis of β-amyrin from the natural product squalene

The presence of such a high proportion of hederagenin in *H. helix* is rather unusual. This aglycone, although present in a large number of Oleanane saponin containing species, is most frequently observed as only a minor constituent compared to the more prevalent oleanolic acid. This is a fact reflected in the current high market value of pure hederagenin which makes it a commercially interesting product.[59]

1.3.3 Current Applications

The leaves of *H. helix* have well documented ethnomedical use. Along with *Liquiritiae radix* (glycyrrhiza / licorice root) and *Primulae radix* (primula root), *Hederae Folium* is one of the most frequently used saponin containing plant drugs for the treatment of cough due to the expectorant properties possessed by the saponins present in the extract;[50,60] these are such that a preparation of the crude ethanol extract of dried pulverised leaves is used as a treatment of bronchial asthma and chronic obstructive pulmonary diseases. In this preparation, under the name *Hederae helicis folium* (CAS: 84082-54-2), the crude leaf saponin extract was approved by the German Health Ministry's Commission E for use as a homeopathic medicine in 1998.[61] The exact mechanism of this expectorant action was not certain, however it was believed to be a result of the general and non-specific ability of saponins to produce local irritation, especially of the mucous membranes of the nasal cavity, throat, bronchi and lungs; this effect is clearly illustrated

by the sneezing which is frequently provoked by handling saponins in powder form. It is thought that this irritation of the throat and respiratory tract will increase respiratory fluid volume by drawing more water into the bronchial secretions, which will in turn dilute the mucus and reduce its viscosity.[60] The known surface activity of the saponins could also act to make the sputum thinner, more mobile and easier to eject. More recently, it has been shown that these preparations act not only through this secretolytic action but that they also possess weak spasmolytic activity.[52,62,63] This broncho-spasmolytic action acts to help relax muscles and open up the constricted airways and further benefit the treatment of these conditions. A number of aqueous alcohol preparations are now available commercially in Europe such as Prospan®[64] and Sedotussin®.

Further evidence of the medicinal use of *H. helix* is shown by the use of its leaf and stem extracts (together with those of the related Japanese ivy species *H. rombea*) to prepare topical treatments for skin rashes in Niigata-ken, Japan.[65] Similar use of the leaf and stem extacts in Europe is observed in the cosmetics industry where it is used as a tonic, astringent and lenitive.[66] It is most commonly found in a wide range of cellulite treatments in such forms as slimming creams[67], ointments[68] and even teas[69]. The saponin constituents of these treatments have been shown to possess anti-elastase and anti-hyaluronidase activity, two enzymes identified as causative factors of cellulite development.[70] The aglycones oleanolic acid (**1**) and hederagenin (**2**) were found to show high *in vitro* inhibition of these enzymes, however hederacoside C (**4**) and α-hederin (**5**) showed only poor inhibition. There are also a number of other cosmetic products on the market, often from specialist manufacturers or retailers, containing *H. helix* extracts including shampoos and soaps which aim to utilise its beneficial properties and to market the products as more "natural" alternatives to mainstream products.

α-Hederin, like most other monodesmosidic steroid or triterpene saponins has a very strong haemolytic activity (haemolytic index = 150000). This is to say that saponin molecules interact with membrane cholesterol of red blood cells to form pits and holes which destabilise the membrane and cause lysis of the cell.[71] In contrast to this hederacoside C and hederacoside B have an almost no haemolytic activity in a similar manner to other bidesmosides.

1.5 Chemistry of Triterpene Saponins

As has been discussed in 1.4.2, the saponins present in both *H. helix* leaf and fruit are all triterpene saponins; they exist as a mixture of both monodesmosides and bidesmosides and are predominantly based on the hederagenin aglycone with some oleanolic acid based saponins present as a minor component. Since hederagenin is usually only present as a minor component in the saponin profile of most other oleanane containing species, it has not been readily isolated in very large quantities from other plant sources. The result of this is that investigation of its chemistry has so far been fairly limited when compared to the more common oleanolic acid. Triterpene saponins, however, share some common chemical properties, and as such it is often possible to carry out similar chemistry on one type as has previously been investigated on other triterpene saponins. It is therefore of great value to observe some of the previous work in relation to the more closely related saponin products (i.e. work on oleanolic acid, ursolic acid etc.) that may be of significance.

1.5.1 Hydrolysis

Perhaps one of the most important reactions in the area of saponin chemistry is hydrolysis. The reason for this is that hydrolysis of saponins results in the cleavage of the attached sugars from the aglycone moiety, which historically has been the preferred route to structure elucidation; breaking the saponin molecule into smaller units for more ready analysis. By controlling the conditions for the hydrolysis it is possible to selectively cleave the different linkages of a bidesmoside and remove only one of the sugar moieties, which will allow its identification before cleaving the second sugar and identifying that.[72] The most common way of performing this partial / complete hydrolysis is by exploiting the properties of the two different glycosidic linkages in relation to base and acid hydrolysis conditions (Figure 1.5.1.1).

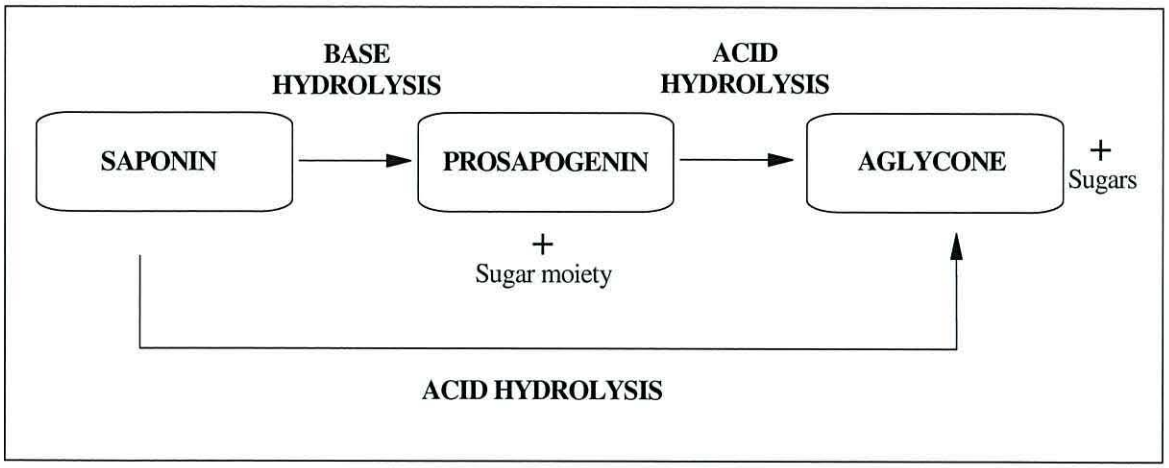
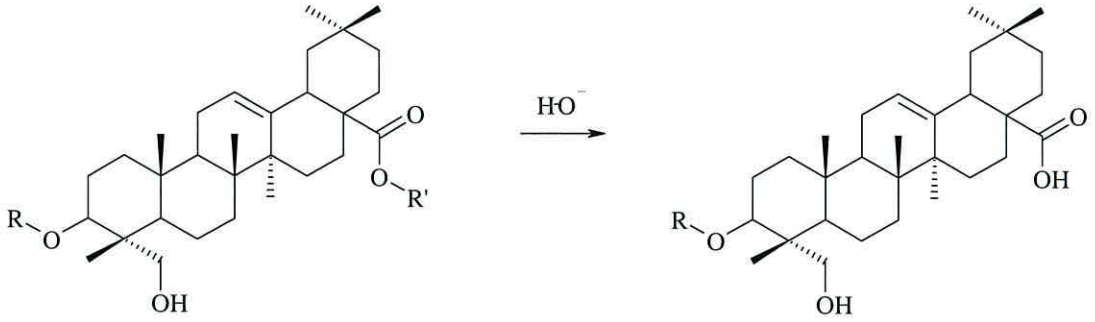


Figure 1.5.1.1 Cleavage of sugars by acid and base hydrolysis conditions

The importance of these reactions, however, no longer remains in their role in identifying saponins. Instead, they are now more significant as a preparative route to the pure aglycone or to prepare desired monodesmosidic saponins from the corresponding bidesmosides. Since the biological activity of bidesmosides has been generally observed to be significantly different to those of the monodesmosides it appears that through this type of hydrolysis it could be possible to effect changes in the biological activity of the saponins. Although work has been carried out previously to assess biological activity of the saponins of *H. helix* and to a lesser extent on the aglycone hederagenin (acid hydrolysis product) from these, there appears a dearth of literature relating to the biological activity of the base hydrolysed saponins. Since this product should possess a character more akin to the monodesmosidic saponins without the less active bidesmosides present it seems a plausible route to the facile preparation of compositions which possess higher biological activities than the crude saponin extract alone.

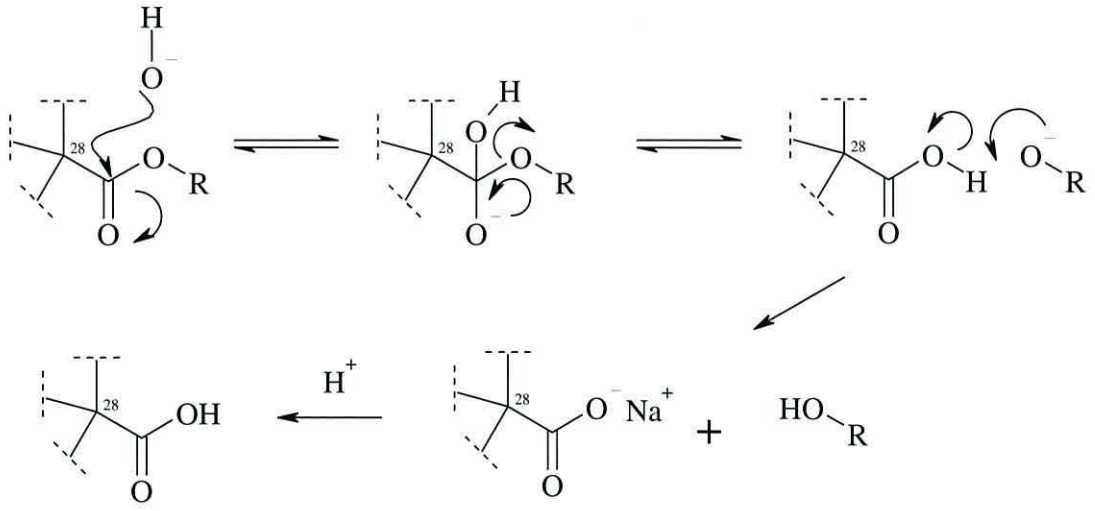
1.5.1.1 Base / Alkaline Hydrolysis

In hederagenin there are two positions where attachment of a saccharide chain is possible; the first of these is by an acetal linkage at the C-3 position, the other is by an ester linkage at the C-28 position (Figure 1.3.1.2). Of these bonds, 28-*O*-ester linkage is relatively weak and can be readily cleaved by either acidic or basic hydrolysis conditions. In the case of basic hydrolysis conditions, this will lead to cleavage of this sugar only to give the corresponding monodesmosidic hemiglycone (Figure 1.5.1.1.1), and the base stable acetal linkage at C-3 will remain unaffected by these conditions.



Scheme 1.5.1.1.1 Base hydrolysis of a generic hederagenin based bidesmosidic saponin to the corresponding monodesmosides

In the case of base hydrolysis the mechanism of this reaction is simply that of saponification (Scheme 1.5.1.1.2).[73] These reactions proceed quickly and are typically achieved by refluxing the saponin with aqueous 5 % potassium or sodium hydroxide solution for 2-5 hours.[74] Alternative methods have reported using 1-20 % methanolic solutions of base (although this is often not favoured as there is a risk of these conditions producing a methylated product in some saponins rather than the true saponin).[50]

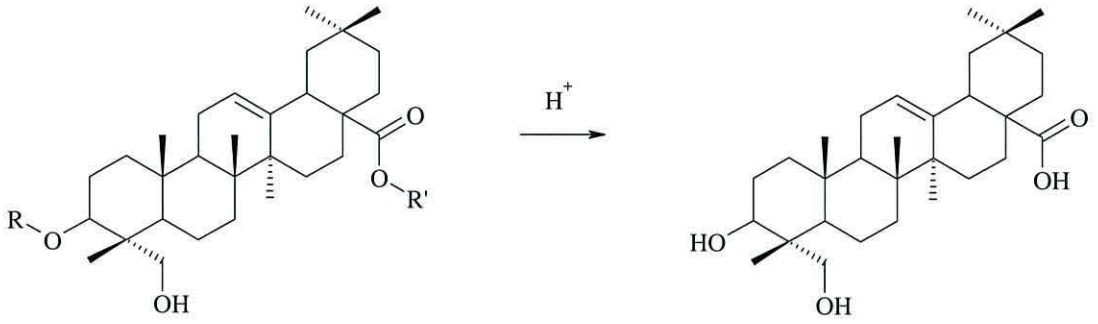


Scheme 1.5.1.1.2 The mechanism of base catalysed hydrolysis of the 28-*O*-ester linkage, the R group represents a monosaccharide or polysaccharide moiety

1.5.1.2 Acid Hydrolysis

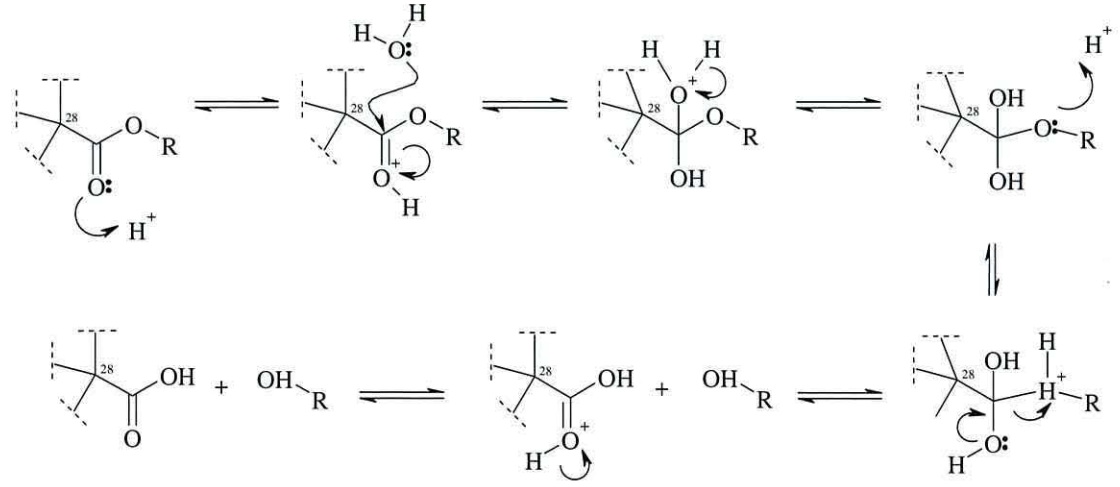
In the case of hydrolysis of a bidesmosidic saponin in acidic conditions, cleavage of both saccharide chains is possible to afford the aglycone (Figure 1.5.1.2.1). This is a slower process than that of base hydrolysis and is achieved by refluxing the saponin in acid for a fixed length of time; reports usually indicate that 2-4 M aqueous hydrochloric acid for 4 hours is sufficient to achieve complete hydrolysis however alternative methods

replacing the aqueous reaction medium with an alcohol or dioxane have also been described.[50,72,75] In addition to hydrochloric acid, sulphuric acid can also be employed for the hydrolysis of saponins; cleavage of the ether linkage is not as efficient by this method but there is a smaller chance of degradation or rearrangement of the molecule.[76]



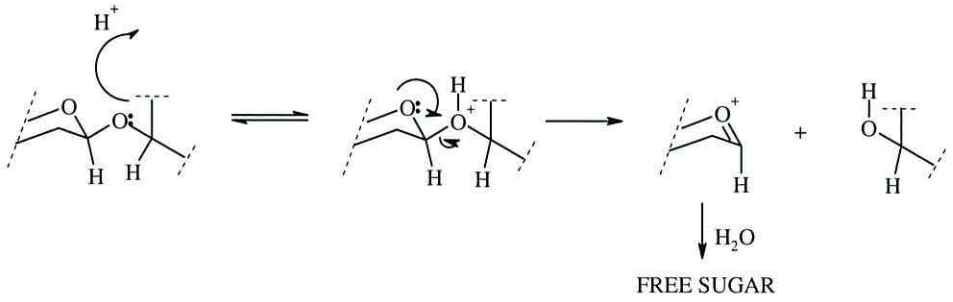
Scheme 1.5.1.2.1 Acid hydrolysis of a generic bidesmosidic saponin to its hederagenin aglycone

In the case of acid hydrolysis of bidesmosidic saponins there are two simultaneous or sequential reactions. The first reaction is the cleavage of the weaker 28-*O*-ester bond as in the case of base hydrolysis; however in acid it is the hydroxonium ion present in the aqueous acid solution which is the catalyst instead of the hydroxide ion (Scheme 1.5.1.2.2). The mechanism is that of a reverse Fisher esterification and so is an equilibrium reaction; as such this thermodynamically controlled reaction is slower than that of the base hydrolysis described above.[73] This however is of little practical consequence as the corresponding process to cleave the other sugar chain can also be slow meaning this will usually have no effect on the reaction time for complete hydrolysis.



Scheme 1.5.1.2.2 Mechanism of acid catalysed hydrolysis of 28-*O*-ester linkage, the R group represents a monosaccharide or polysaccharide moiety

The second reaction which takes place is the cleavage of the sugar moiety attached to the C-3 position of the saponin. This group is attached by an acetal which is more stable than the ester linkage since the mechanism of its cleavage first requires the oxygen bridging the sugar and the aglycone to first be protonated (Scheme 1.5.1.2.3); as such it can only be cleaved in the presence of an acid catalyst.[77] It is for this reason that monodesmosides are resistant to base hydrolysis and why that method will selectively cleave only to 28-*O*-ester bond while leaving the acetal bond intact.



Scheme 1.5.1.2.3 Mechanism of the cleavage of the 3-*O*-acetal bond

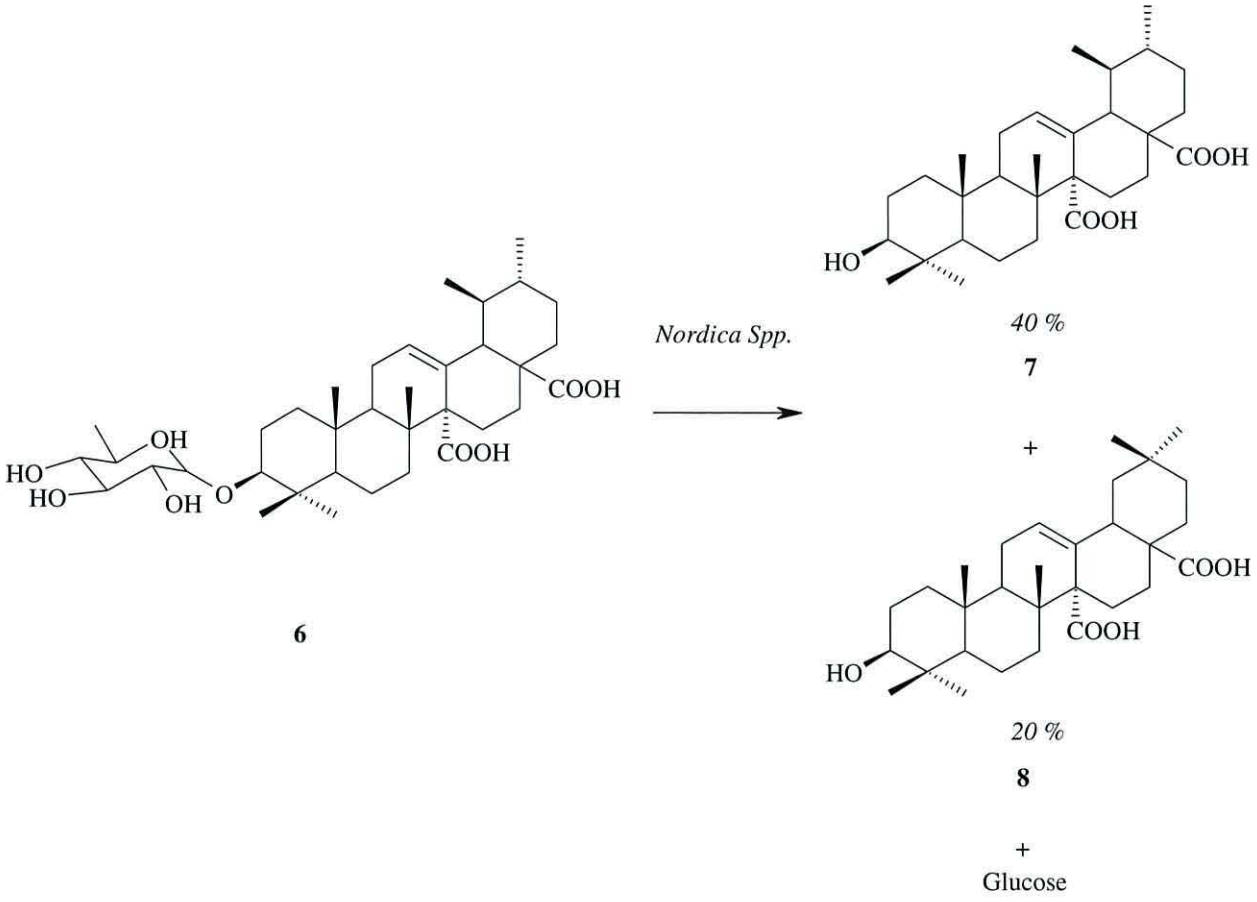
As well as bidesmosides, this method will cleave the single osidic substituent of monodesmosides or previously base hydrolysed material as is commonly employed to isolate the C-3 sugar during structure elucidation. It is also a useful method in converting sometimes very complex mixtures of mono- and bidesmosidic saponins into single starting materials which could be used for synthetic work.

1.5.1.3 Alternative means of hydrolysis

Although most hydrolysis in the literature is achieved with acid or base, there are some examples of alternative means that have been utilised. Kim *et al.* reported the hydrothermolysis of 3,28-*O*-bisglycosides to give the corresponding 3-*O*-glycosides by heating with water at 100 – 140 ° C. Depending on the saponin this can take between 10 to 140 hours.[78]

Another method for the cleavage of sugar residues from saponins is enzymatic hydrolysis. This method has the particular advantage of being very mild and so reducing the potential for degradation of the saponin.[79] This has been used successfully on saponins extracted from *H. helix* to isolate the hederagenin aglycone by using snail extract (*Helix pomatia*) as the enzyme.[80] It does however suffer the disadvantage that the relevant hydrolases for the sugars are necessary for the cleavage to occur and these are

not commercially available for all sugars. An extension of this enzymatic method is the use of microbial hydrolysis where a combination of enzymes from a microorganism are utilised directly, usually by culturing the microorganism with a medium containing a saponin as the carbon source. Once again, this is a very mild hydrolysis method but the specificity of it requires a great deal of preliminary screening in order to identify a suitable pure strain. A good example of this method is the deglycosylation of quinovic acid 3-*O*-β-6-deoxy-D-glucopyranoside (**6**), an ursane saponin from the bark of *Mitragyna intermis*, using *Nordica spp.* (NRRL 5646) to give its aglycone quinovic acid (**7**) (40 %) together with its biogenetic counterpart cincholic acid (**8**) (20 %) via a carbon skeleton rearrangement involving a methyl group migration (Scheme 1.5.1.3.1).[81]



Scheme 1.5.1.3.1 Microbial hydrolysis of 3-*O*-β-6-deoxy-D-glucopyranoside with *Nordica spp.* to its aglycone and a rearrangement product of this

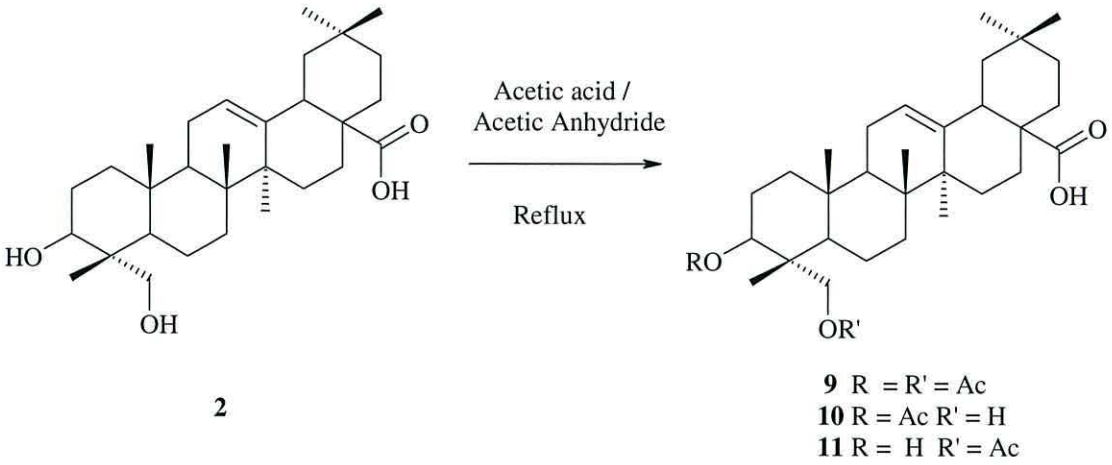
Although not common to all microbial hydrolysis, this transformation from an ursane type aglycone to an oleane type aglycone highlights both the potential advantage and disadvantage of this methodology. It can be both a valuable means of hydrolysing a saponin and modifying its aglycone simultaneously; however, unless great care is taken to

understand what is taking place the method can give unexpected artefacts that could (e.g. in the case of structure elucidation) be misleading. Also illustrated is the capability of these techniques to be used for alternative synthetic means with regards to saponins other than merely as a hydrolysis technique. Such microbial methods of hydrolysis and transformation are well reviewed by Parra *et al.*[82]

1.5.2 Esterification and modification at the hydroxyl and acid positions

Once saponins are hydrolysed to their sapogenins there are numerous reports of the derivatisation of these aglycones to afford new products, either in an attempt to change the biological activity of the material or as a means of aiding in the identification of an unknown structure. One of the most popular means of derivatisation is unsurprisingly to esterify the aglycone at the positions where the sugar moieties where previously attached.

Some of the first reports of this type of reaction are the series of papers by Jacobs in the 1920s who conducted a number of reactions on hederagenin obtained as the acid hydrolysis product of saponins extracted from shells of soapnuts (*Sapindus saponaria* L and *S. mukrossi*).[83,84] These experiments were conducted with the aim of identifying which functionalities were present in the poorly characterised aglycone in order to prove its structure. The first such esters reported were the diacetate (**9**) and mono acetate (**10** or **11**) of hederagenin prepared by refluxing the aglycone material with excess acetic acid or acetic anhydride (Scheme 1.5.2.1).[83] Given the age of the work very little characterisation of this product was performed and no yields were reported. As the structure of the molecule still remained uncertain at this time, there was no indication as to whether the primary (**10**) or secondary (**11**) isomer the monoacetate product had been prepared; they did however address the issue of their reported melting point differing to that reported for the same product prepared by Van der Haar and Tamburello[85] by suggesting that the two products were not isomeric with one another.

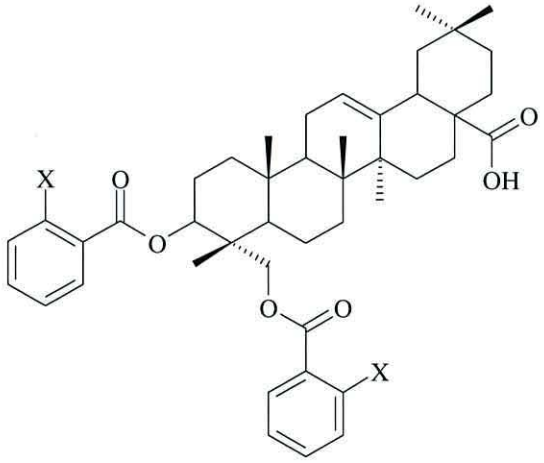


2

9 R = R' = Ac
10 R = Ac R' = H
11 R = H R' = Ac

Scheme 1.5.2.1 Jacobs' preparation of acetate derivatives of hederagenin

In the same paper, Jacobs also reports the preparation of the dibenzoate (**12**) and di-o-bromobenzoate (**13**) of hederagenin from the corresponding chloride in pyridine (Figure 1.5.2.1.). Once again, only scant data is given for these products and there was no report of any mono substituted product identified in either of these reactions, but they did show the potential to form esters at these positions.

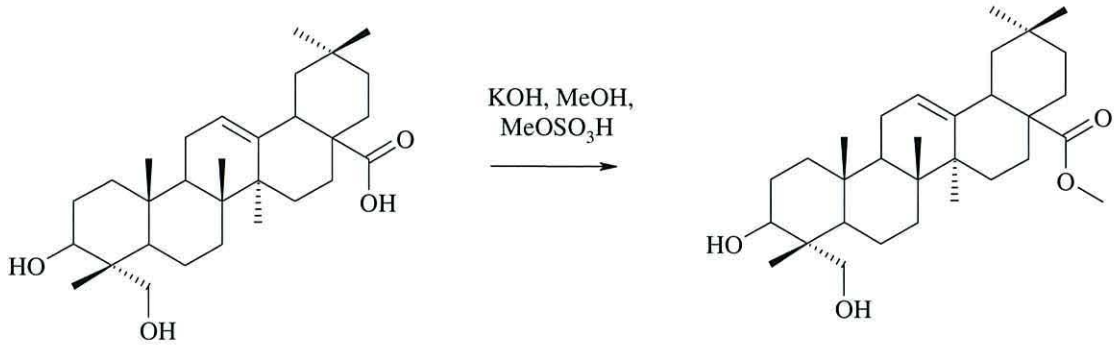


12 X = H
13 X = Br

Figure 1.5.2.1 Hederagenin dibenzoate and hederagenin di-*o*-bromobenzoate

Further to these esters, Jacobs also appears to be responsible for one of the first reports of an ester at the other position of hederagenin. He describes the preparation of the methyl ester (**14**) of the acid function of hederagenin by refluxing the aglycone with a solution of potassium hydroxide, methanol with excess methyl sulphate (Scheme 1.5.2.2).[83] In later publications he goes on to describe further derivatives based on this methyl ester by successfully repeating previous experiments on this product to form the

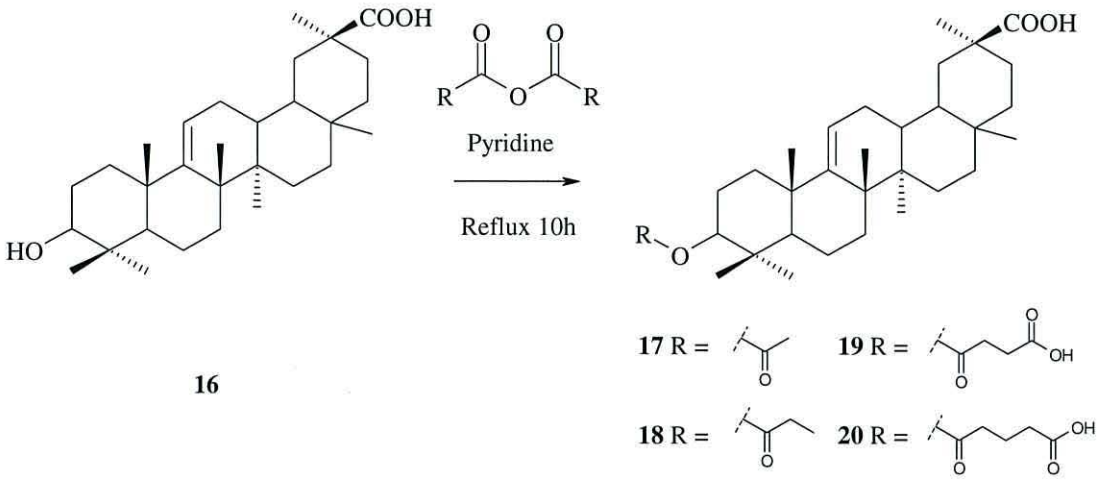
corresponding acetates, benzoates etc. as well as forming sulphites and amides of these compounds.[86]



2 **15**

Scheme 1.5.2.2 Preparation of a methyl ester of hederagenin

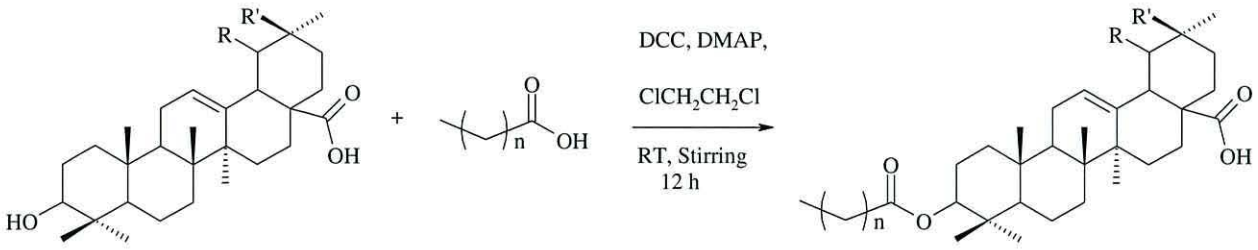
Similar reports to this are to be found preparing ester derivatives of other saponin aglycones, however little work advancing this is found until quite recently. Such work is predominantly found in patent literature,[87,88,89] where a number of esters of triterpene and steroid aglycones are reported in the claims but little is divulged of the methodology through which these are prepared. One such patent does however report amongst its claims the preparation of esters at the C-3 position of the 3-β-hydroxy-18β-olean-9-en-30-oic acid (**16**).[87] Using a methodology similar to that of Jacobs they describe refluxing the aglycone with an acid anhydride in anhydrous pyridine for 10 hours before cooling and pouring into ice cold aqueous sulphuric acid to precipitate the desired ester product (Scheme 1.5.2.3.) Through this method they report successfully preparing the acetate (**17**), propionate (**18**), succinate (**19**) and glutarate (**20**) products. All of which were also prepared as the corresponding disodium salts for pharmaceutical formulation and for biological trials as anti-ulcer treatments. A patent by the same group showed esterication methods were also successful on ursolic acid derivatives in the preparation of similar pharmaceutical products;[88] a Chinese group also report utilising the same methodology to prepare a bis-succinylated product of the steroid aglycone ruscogenin during the preparation of an immunogen.[89]



16

Scheme 1.5.2.3 Preparation of esters of 3-β-hydroxy-18β-olean-9-en-30-oic acid

Other than the above method of preparing these comparatively small or polar esters at the hydroxyl position, there have also been recent reports of preparing fatty acid ester analogues of oleanolic acid. These are of interest as they appear to be a route to preparing a lipophilic derivative of these biologically active compounds which may improve their use as agricultural treatments. One such report describes the use of a modified Hassner reaction[90] under essentially neutral conditions to synthesise the 3-*O*-fatty acid ester derivatives of oleanolic and ursolic acid in excellent yields.[91] By stirring the oleanolic acid (**1**) or ursolic acid (**21**) aglycone obtained from kendu leaves (*Diospyros melanoxylon*) with an solution of the relevant fatty acid (**22-25**), DCC and DMAP for 12 hours at room temperature it was possible to prepare the lauric acid, myristic, palmitic and stearic acid analogues of ursolic acid (**28**, **29**, **30** and **31** respectively) and the palmitic and stearic acid analogues of oleanolic acid (**26** and **27**) (Scheme 1.5.2.4). These were then tested for their activity as antifeedants for crop protection (See 1.6.1).



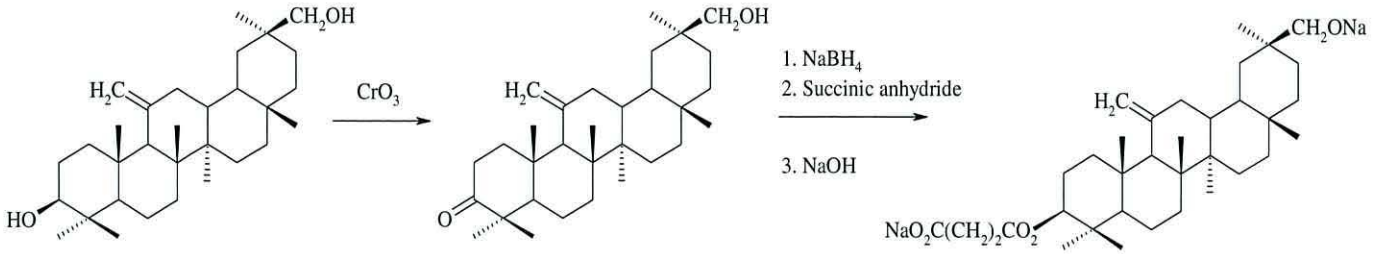
1 R = H R' = Me
20 R = Me R' = H

22 n = 10
23 n = 12
24 n = 14
25 n = 16

26 R = H R' = Me n = 14 *83 %*
27 R = H R' = Me n = 16 *84 %*
28 R = Me R' = H n = 10 *82 %*
29 R = Me R' = H n = 12 *85 %*
30 R = Me R' = H n = 14 *82 %*
31 R = Me R' = H n = 16 *80 %*

Scheme 1.5.2.4 Preparation of 3-*O*-fatty acid esters of oleanolic and ursolic acid

Other than reports of esterification type reactions, there appears to be a dearth of reliable material relating to other transformations at these positions. The exception to this is reports of the oxidation of the hydroxyl group at the C-3 position of oleane saponins to a carbonyl group. One of the earliest reports found of this was again by Jacobs's group who reported achieving the conversion of the secondary hydroxyl position of hederagenin to a hydroxyketone using potassium permanganate in acetone. As with the other work, the age of this paper results in little reported characterisation of the product. From the carbonyl product, the group go on to describe further oxidation to convert the primary hydroxyl function into a carbonyl group using chromic acid and subsequent reduction of these products.[92,93] Chromic acid has since been used to effect the transformation of the olefinic position of similar β-amyrin aglycones, where the hydroxyl group was protected as an ester to prevent reaction, to form a ketone on ring C (See 1.5.4); it therefore remains uncertain as to whether the product was actually the diketone proposed. More recent reports of this type of oxidation include one by an Italian group working on a derivative of glycyrrhetic acid (**32**) who converted the C-3 hydroxyl into its corresponding ketone (**33**) by a Jones oxidation with chromic acid (Scheme 1.5.2.5).[94] This functionality was then used in the final step in the preparation of (**34**), a glycyrrhetic acid derivative shown to have potent anti-ulcer activity.[95,96]

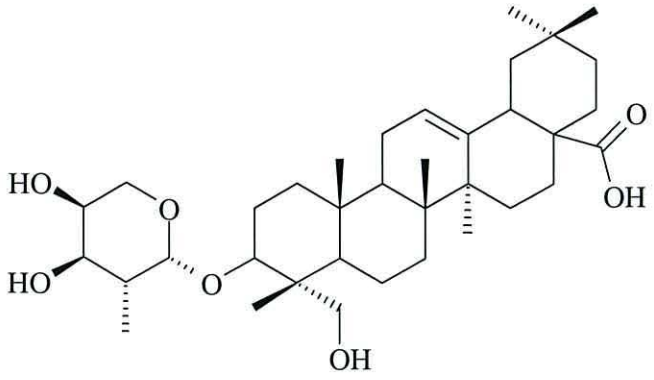


32 33 34

Scheme 1.5.2.5 A partial synthesis of 3-β-hydroxy-11-methylolean-30-oic acid which shows the successful Jones oxidation at C-3.

1.5.3 Glycosylation of triterpene aglycones

As well as attaching entirely new ester groups to the reactive positions of saponin aglycones, the other desirable chemistry in this area is to attach sugars to these positions. This type of glycosylation would allow the synthesis of new saponins from only the aglycone - either as entirely new saponins not identified in nature or potentially to prepare a desired uncommon saponin of known biological activity. This route could therefore allow previously difficult to obtain saponins to be synthesised using an aglycone obtained from the hydrolysis of a more common or easily obtained saponin source. To date, the majority of these synthetic studies have focused on steroid saponins.[97-100] The synthesis of triterpene saponins is reported in the literature, although the majority of this work has focussed only on the synthesis of saponins of oleanolic acid;[101-103] this is most likely due to the fact that saponins based on this aglycone are usually found in higher quantities in most plant species than other aglycones. Synthesis of saponins based on other triterpenes is not widespread,[104,105] and those reported for hederagenin appear to be restricted to one French group.[106,107] Through carefully protecting the acid at the C-28 position and hydroxyl at C-23 of hederagenin (**2**) and preparing suitably protected activated disaccharides they report successfully coupling the two at low temperature in the presence of a trimethylsilyl trifluoromethanesulfonate catalyst to give a protected saponin which was then converted into the synthetic saponin product. The reported saponins prepared by this means are five naturally occurring saponins including α-hederin (**5**) and δ–hederin (**35**) as well as three not present in nature, all of which are monodesmosides.[107]



35

Figure 1.5.3.1 The minor H. helix saponin δ-hederin

1.5.4 Modification of the double bond

Other than the reactive positions discussed above, the third position at which the chemistry of triterpene aglycones similar to hederagenin has been studied is the double bond present in a number of β-amyrin and α-amyrin type structures. This double bond is often observed to be somewhat unreactive due to the sometimes very significant stearic hindrance present around its position which limits access for attack (Figure 1.5.4.1); this is particularly true of α-amyrin type aglycones such as ursolic acid.

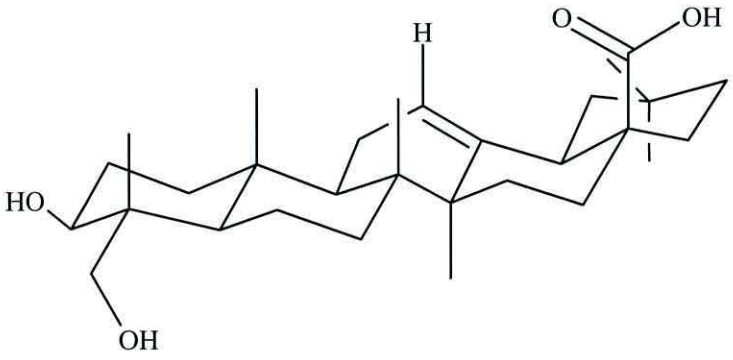
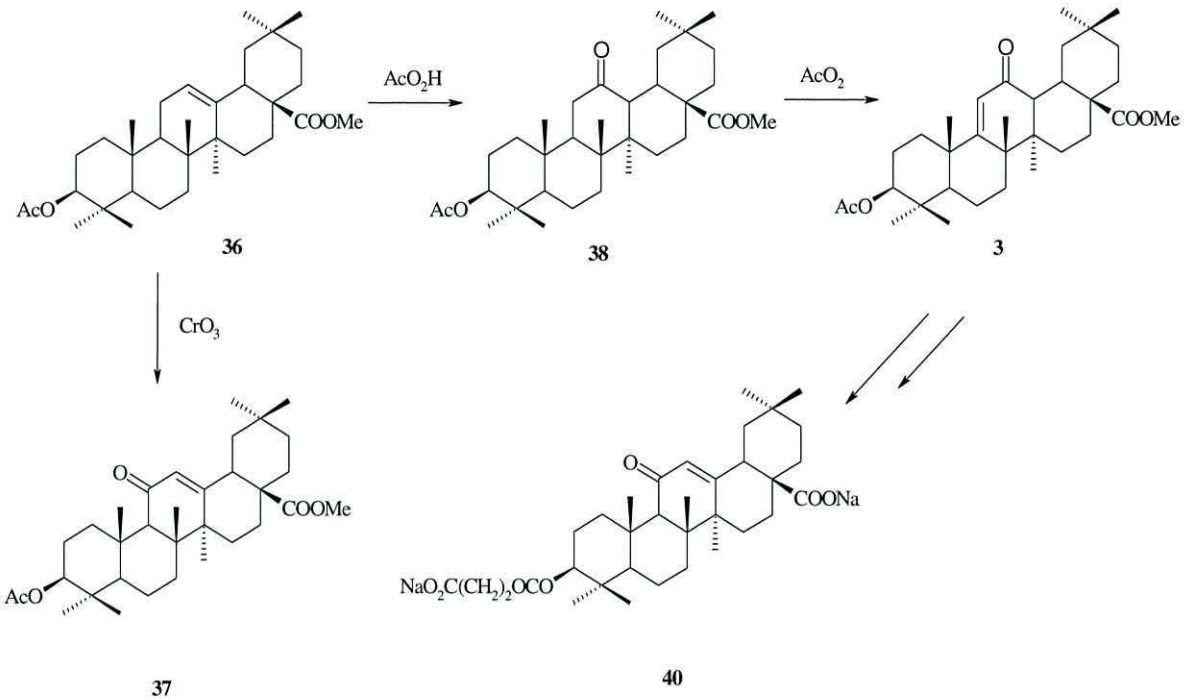


Figure 1.5.4.1 The structure of hederagenin illustrating the limited availability of attack at the double bond

This problem appears to be particularly true of hydrogenation. Of the limited reports in the literature none appear to have successfully hydrogenated the olefinic group. Jacobs commented on the inability to hydrogenate the molecule with hydrogen and a palladium catalyst when attempting to identify its structure, initially mistaking this result as evidence of it being a saturated compound.[84] Similar inertness to this type of

hydrogenation is observed in other similar saponin aglycones. In work on glycyrrhetic acid derivatives from liquorice roots (*Glycyrrhiza glabra*) this is even exploited; two studies[108,109] successfully catalytically hydrogenated a ketone group present in the molecule with platinum oxide, another work,[110] similarly used hydrogen and palladium on charcoal to hydrogenate an alcohol group in one of these compounds, there was no effect on the double bond in any of these cases.

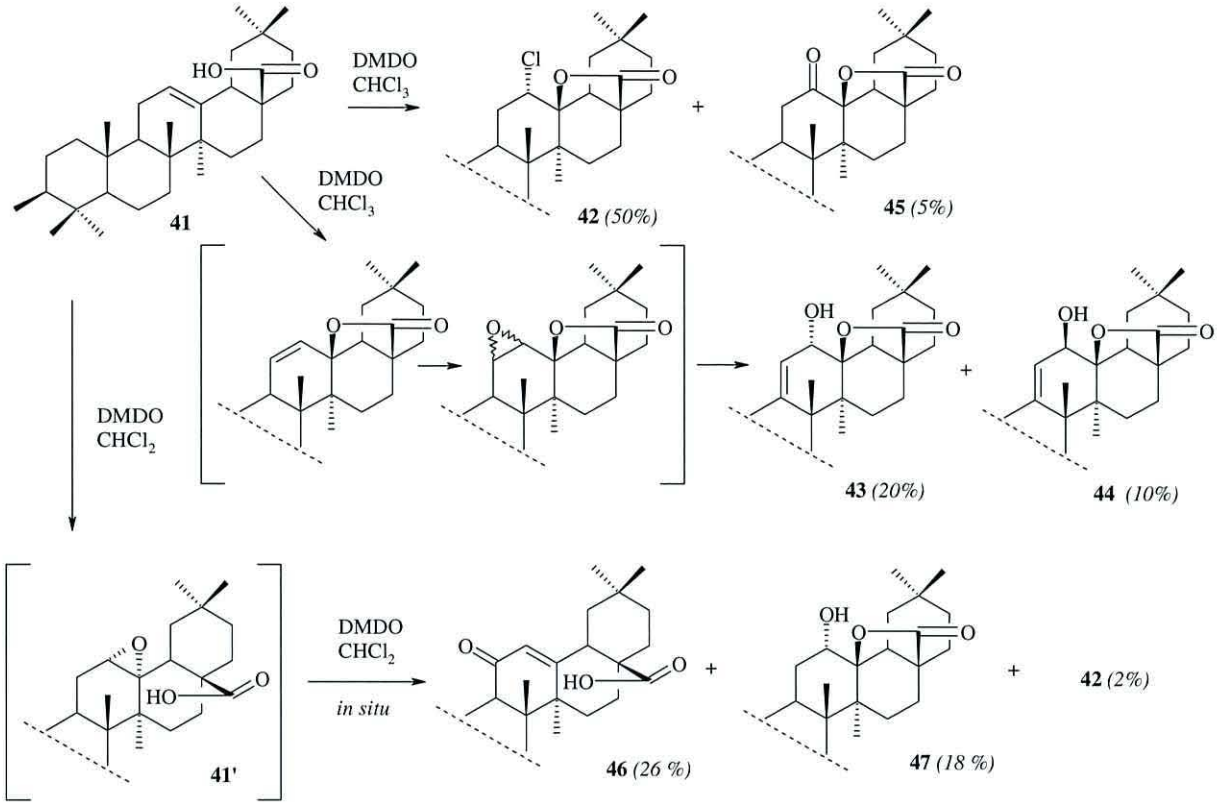
Although resistant to hydrogenation there is evidence in the literature of successful attempts to oxidise this position to give a carbonyl group. This is desirable as there are a number of naturally occurring saponins based on aglycones which possess ketones on ring C adjacent to the double bond which show high biological activity. Work by Pifferi *et al.* preparing glycyrrhetic acid derivatives utilised a peracetic acid oxidation to give the corresponding 12-oxo derivative, followed by a bromination-dehydrobromination to introduce another double bond.[94,96] The resulting end products showed such encouraging pharmacological results that the group extended this work modifying ring C to include other triterpene aglycones including ursolic acid and oleanolic acid with similar results. In the case of oleanolic acid two methods of oxidation were identified each resulting in a different regiochemistry of the product (Scheme 1.5.4.1); both were carried out on the protected methyl 3-*O*-acetyloleanolate derivative (**36**). Oxidation with chromic acid resulted in a product with an 11-oxo-Δ12(13)-enone system (**37**) while using peracetic acid gave the 12-keto derivative (**38**), bromination-hydrodebromination of which introduced a desired double bond in this product (**39**). Further modification of this allowed the preparation of disodium 3β-3-carboxypropionyloxy)olean-9(11)en-28-oate (**40**), which was found to be a very potent anti-ulcer treatment.[95,96]



Scheme 1.5.4.1 Partial preparation of disodium 3β-3-carboxypropionyloxy)olean-9(11)en-28-oate

An alternative method shown to oxyfunctionalise the double bond is by oxidation with the cyclic peroxide dimethyldioxirane (DMDO). An interesting study by Iida *et al.* showed the effect of DMDO on a number of bioactive terpenes including the triterpene saponins oleanolic acid and ursolic acid acetates.[111] This method usually results in the formation of an epoxide product but in the case of the triterpene aglycones this was not the case. When oleanolic acid acetate (**41**) was treated with DMDO for 24-36 hours at room temperature it unexpectedly afforded 3-O-acetyl-12α-chloro-oleanolic lactone (**42**) as the major product together with the 12α-hydroxy lactone (**43**), its 12β-hydroxy equivalent (**44**) and also a small quantity of 12-oxo lactone derivative (**45**) (Scheme 1.5.4.2). It was proposed that the chloride (**41**) may be the result of attack at the double bond by hypochloric acid formed from the chloroform and DMDO used in the reaction. The hydroxy lactone products (**43** and **44**) were found to form via the intermediates shown below, first forming a lactone before the double bond was converted into an unstable epoxide which degraded to the products. By contrast to the products identified in this reaction, the same starting material (**41**) was treated with DMDO generated *in situ* in dichloromethane only 2 % of the chloride (**42**) was formed, suggesting that this solvent was more resistant to the DMDO than chloroform. Aside from this chloride, both the 11-oxo product (**46**) and 12 α-hydroxy lactone (**47**) obtained by this method appear to have

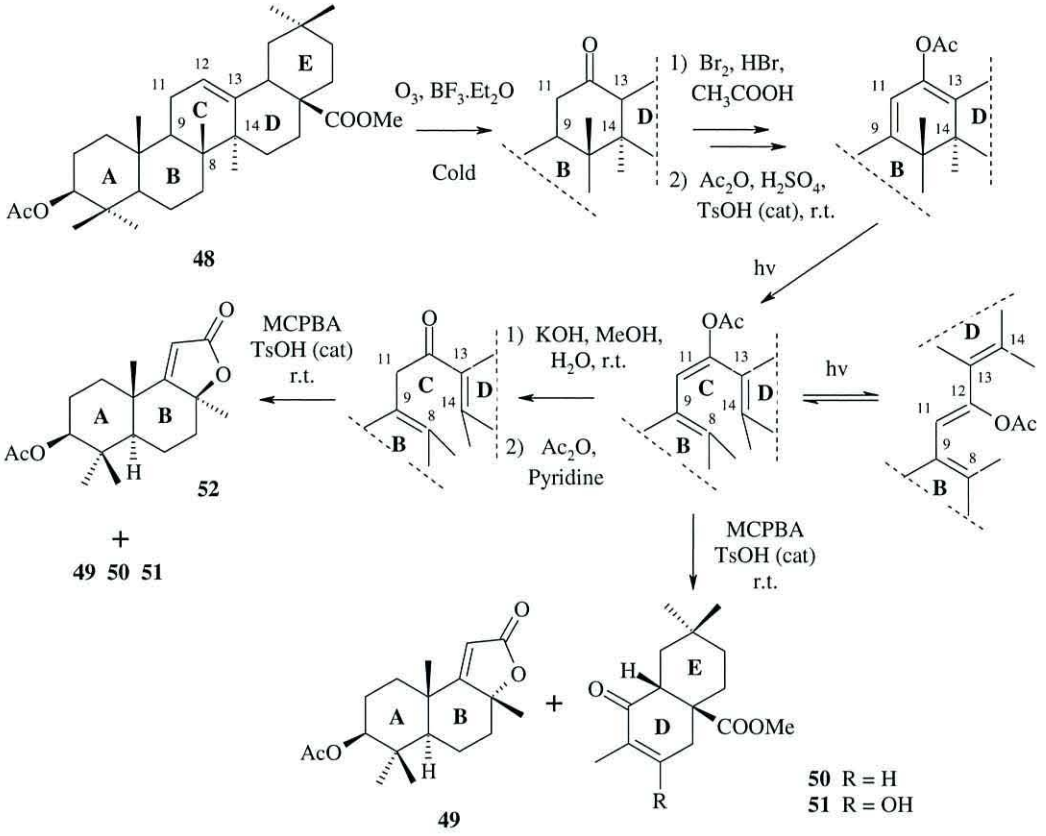
formed through allylic oxidation and ring opening respectively of the initially formed α-epoxide (**41'**).



Scheme 1.5.4.2 Oxidation of oleanolic acid acetate with DMDO

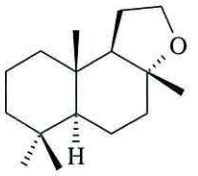
When the same reaction was performed on the α-amyrin aglycone ursolic acid acetate using the DMDO / chloroform method as for oleanolic acid acetate, the products obtained were only the 11-oxo product and 12α-hydroxy lactone (together with a small quantity of a rearrangement product of this); this suggests that the action of the hypochloric acid had been prevented by the extra stearic hindrance present in this aglycone and so allylic oxidation took place preferentially.[111]

A novel means of reaction at the double bond has also been recently proposed by a Chinese group who have established a means of cleaving ring C of the oleanolic acid aglycone as a means of generating fragments suitable for synthetic work.[112] This was carried out using the protected derivative methyl 3-acetooxyolean-12-en-28-oate (**48**) and successfully gave bicyclic fragments (**49**, **50**, **51** and **52**); although a somewhat complex process, the key stages are outlined in Scheme 1.5.4.3.



Scheme 1.5.4.3 Cleavage of an oleanolic derivative into bicyclic fragments

As shown, the method required modification of the functionalities present on ring C before ring opening could be effected by using a high pressure mercury lamp. The enol acetate product of the ring opening was then cleaved via a Baeyer-Villiger reaction with MCPBA. The presence of catalytic TsOH in this reaction was observed to inhibit the formation of epoxide or lactone products with the peracid to give only the cleavage products (**49**, **50** and **51**). Oxidation of the enol acetate first followed by a similar peracid oxidation gave the same cleavage products (**49**, **50** and **51**) together with another product **52**, the 8-epimer of **49**. Compounds **49** and **52** both possess a *trans*-decalin system which make them versatile synthons for the preparation of a number of natural products which contain this system; in this case they were used to synthesise the desirable bioactive natural products (-)-Ambrox (**53**) and (-)-9-*epi*-Ambrox (**54**) (Figure 1.5.4.4). The other fragments, **50** and **51**, both possess a *cis*-decalin framework making them potentially important intermediates for the syntheses of tricyclic triterpenoids.



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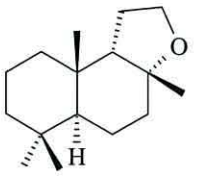

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Scheme 1.5.4.2. The novel fragments prepared by cleavage of an oleanolic acid derivative and the natural products synthesised from them

1.5 Biological activity of saponins from *H. helix* L.

Previous studies on the saponins present in *H. helix* have shown them to possess a wide range of biological activities, some of which show them to have desirable properties which could lead to their use as in plant protection treatments or even for therapeutic or pharmaceutical use. Some of these useful activities are outlined below

1.5.1 Molluscicidal activity

In what is thought may be linked to the haemolytic activity, α-hederin has been identified as having a potent molluscicidal activity.[113,114] The toxicity is believed to be as a result of the saponin binding to the gill membranes of the organism and increasing their permeability, causing a loss of important electrolytes. Hederacoside C, along with other bidesmosidic saponins, has been shown to be completely inactive as a molluscicide as the aglycone hederagenin.

This is an activity which has already been exploited in some other plant species. Molluscicidal activity of saponins was first reported in the African water-living snail *Biomphalaria glabrata.*[115] This species (along with others of the genera *Biomphalaria, Bulinus* and *Onocomelania*) is a vector of eggs from parasitic blood flukes *Schistoma* spp. which cause schistosomiasis. An opportunity was identified in Ethiopia for the local control of these snails using the saponin containing fruits of the soapberry tree (*Phytolacca dodecandra*). Local tribes-people would use the powdered berries to wash their clothes on the banks of rivers and in 1965 it was found that these people suffered less from the disease than those who did not use the berries in this way. It was established that the berries contained high levels of oleanane saponins (principally based on oleanolic acid) which when dissolved into the water courses was killing the snails and

so prevented them transmitting the disease. Investigations are now in place in Africa of numerous saponin containing species in order to find the best means of controlling schistosomiasis in areas where synthetic chemicals would be too expensive, some of which are already at the stage of field and pilot trials and show promising reductions in the infection rate.

Specifically in the case of *H. helix* saponins, evidence of their use as molluscicides is shown by two patents in the literature (both of which have since lapsed). The first, by Mason *et al.,* claimed molluscicidal and molluscal antifeedant activity using an extract of various parts of *H. helix* including the fruits.[116] This was applied to plants as a liquid extract (or formulated in an aqueous or oil carrier), a formulated water dispersible solid or as the dried powder; also described is the use of the powdered dried fruits directly. The other patent refers to the use of saponins from the fruit of *H. helix* for use as a terrestrial slug and snail repellant in Austria. This also claims the use of either powdered dry fruits directly or as the dried crude extract (with a mineral carrier, binder and fungicide for stability). Also included are claims to the use of these saponins in conjunction with extracts of other saponin containing plants (*Aesculus hippocastanum*, *Cyclamen purpurascens*, *Phytolacca americana* and *Primula eliator*).[158]

Based on this established precedent, trials were conducted at Bangor to further investigate the molluscicidal activity of *H. helix* saponins.[38] Following a successful laboratory trial and small scale field trial of crude saponin from *H. helix* fruit against slugs, three controlled assays were conducted. The first assay tested the protective action of spray treatments of aqueous fruit saponin extract at low, medium and high concentrations against attack by young slugs of three species (*Arion ater*, *A. rufus* and *Limax maximus*) on French marigold (*Tagetes patula*) plants; the assay observed only the number of slugs on or around the plant (no assessment of damage to plants or slug mortality was conducted). The results of this assay showed a repellent effect to be observed with a clear dose response between the concentrations and the control.

The trial was then repeated to quantitatively assess damage to leaf discs of spring greens (*Brassica oleracea* var. *acephala*) from the three species as previously with leaf treatments at the same concentrations as previously together with an additional ultra low treatment (0.01 %). Once again a significant repellent effect was observed (Figure

1.5.1.1). The third trial was repeated as previously to confirm the activity observed; once again, there was a higher consumption of leaf in the control discs when compared to those treated (at all concentrations)

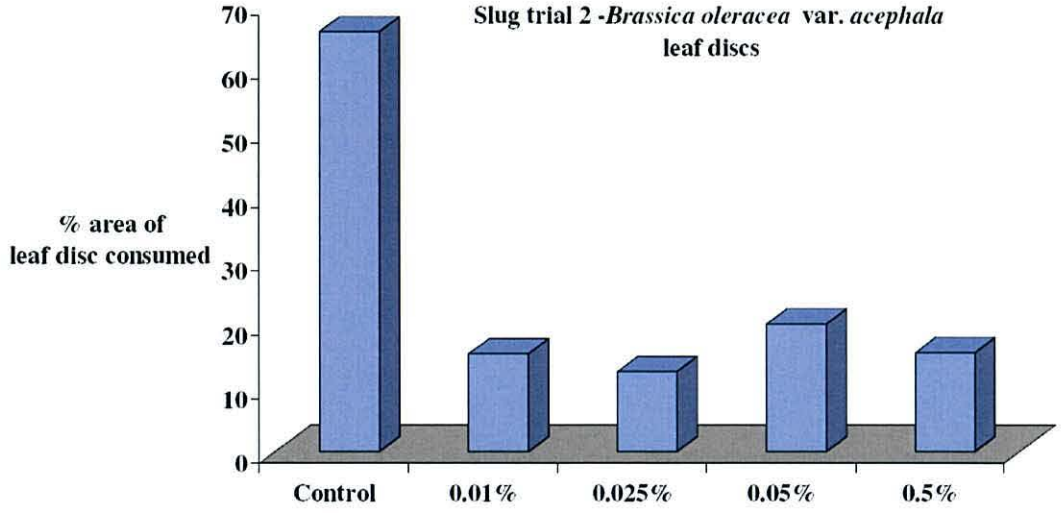

Figure 1.5.1.1 Effect of crude *H. helix* fruit saponins on slug browsing on leaf discs

Following these encouraging results, an independent controlled assay was also carried out (i2L Research Ltd.) to assess the percentage of leaf disc consumed by *Dereocereus reticulum* after treatment with crude fruit extract, together with crude leaf extract and the base hydrolysed crude fruit extract.[38] The results after 7 days are shown in Figure 1.5.1.2 and a subsequent assessment on the effect on slug health after 7 days is shown in Figure 1.5.1.3. These independent results serve to demonstrate the efficacy of the crude fruit extract while also showing the leaf extract was more effective at the lowest dose (over 80 % dead or moribund) than that of the highest dose for the crude fruit extract. However, more of the leaf disc treated with the crude leaf extract was consumed, suggesting that ingestion of the active substance is critical to efficacy. Overall the crude activity of the extracts tested on slug behaviour was pronounced and repeatable.

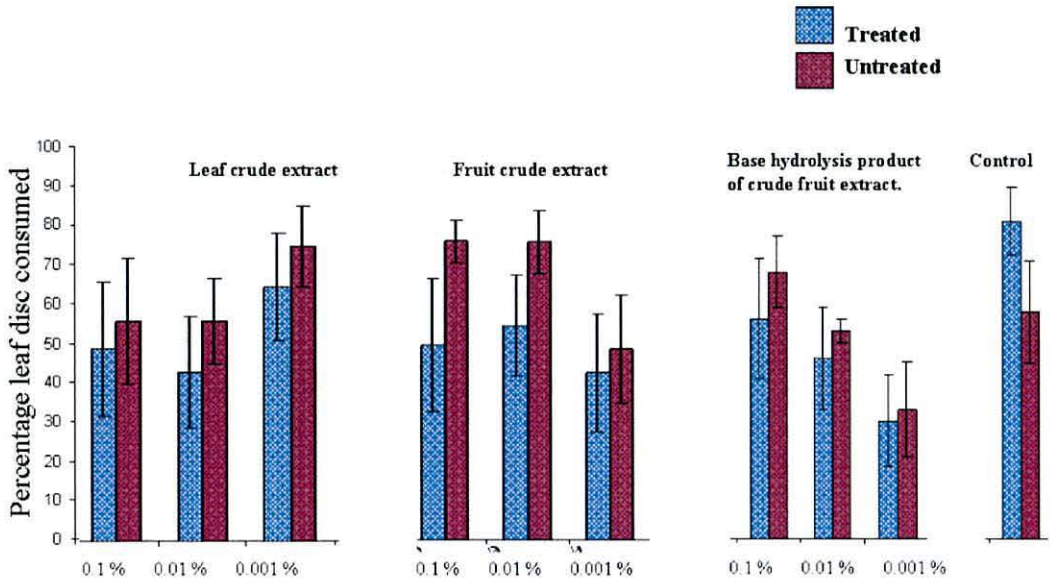

Figure 1.5.1.2 Effect of crude *H. helix* saponins on leaf browsing

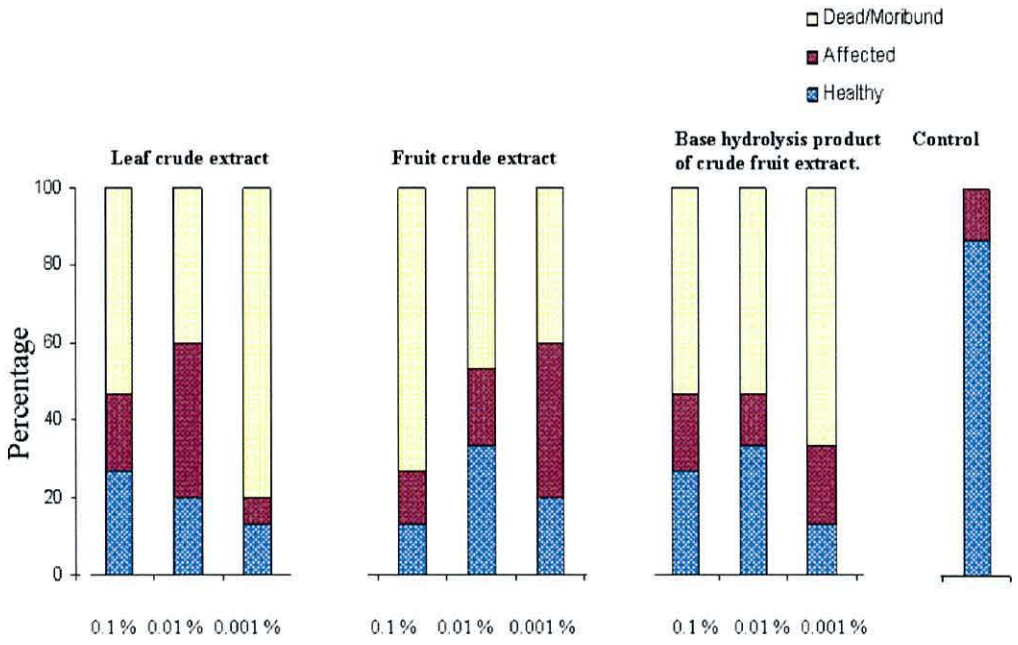

Figure 1.5.1.3 Effect of crude *H. helix* saponins on slug health

1.5.2 Antimicrobial activity

The function of saponins in plants has been somewhat uncertain. A number of theories exist, the most compelling of which is that the very high content in a number of species is as a mechanism to protect the plants against fungal attack. As there is often an increase in saponin content of the plant part under microbial attack it seems a reasonable explanation. However, it appears that it is not the bidesmosidic saponins which exist in such high abundance that are responsible for this activity but the monodesmosides, the inactive

bidesmoside form acting as a convenient water soluble storage and transport form which can be delivered from organs which are not at risk to those parts which are under attack. In the case of *H. helix*, this is shown to be the case by the main saponin Hederacoside C being readily converted through the cleavage a sugar moiety by enzymes present in the leaves into the active antifungal monodesmoside α-hederin.[117]

Reports indicating the antifungal and antibacterial activity of saponins from the leaves of *H. helix* started in 1965. The study showed that Hederacoside C showed inhibition of a number of bacterial and fungal pathogens at 10 mg / ml.[47] The bacterial pathogens *Esterichia coli*, *Pseudomonas pyocyaneus* and one strain of *Staphylococcus aureus* showed complete inhibition with a further strain of *S. aureus* showing weakened growth. The study also showed the fungal pathogens *Candida albicans* and *Aspergillus niger* to be completely inhibited with the wood rot fungus *Trichoderma mentagrophytes* slightly affected. A later report discussing the activity of α-hederin against timber decay fungi showed it to have a fungistatic but not fungicidal activity against a number of wood and other crop fungal pathogens; α-hederin showing overall high inhibitory action.[118] α-hederin has also been reported to be active against *C. albicans*, its minimum inhibitory concentration MIC reported as 25 μg / ml; suggesting that the mode of action was through degrading the cell envelope in what appears to be a similar manner to that of its haemolytic activity.[119] A recent study which isolated 5 saponin components from the fruit of *H. helix* tested each of these against *C. albicans*, *E. coli*, *Bacillus cereus* and *Staphylococcus epidermidis*.[50] Only α-hederin was found to show any activity, again against *C. albicans* with a MIC of 64 ppm at 20 μl; it also showed weak inhibition of *B. cereus* and *S. epidermidis* (both 128 ppm at 20 μl). The other saponins (all bidesmosides) and the aglycone hederagenin showed no activity in the assay.

Signficant fungistatic activity was reported against the fungal mould *Botrytis cinerea*, both from the crude leaf extract[120] and α-hederin;[121] in the latter study α-hederin also showed antifungal activity against the potato pathogen *Rhizoctonia solani*. In the case of *R. solani,* it was proposed that it was in fact the poorly soluble hederagenin which held the antifungal activity and that the saponin with its sugar chains was necessary only to transport the aglycone portion in an aqueous medium, after which fungal enzymes (β-glycosidase) would cleave the osidic chains. This theory was supported by the findings

of another study which found no antifungal activity of α-hederin when studied in the presence of aldonolactones, known inhibitors of β-glycosidase.[122]

Crude leaf extract of *H. helix* shows a reported preventative activity against *Venturia inequalis* (apple scab) and powdery mildew, fungi which are known to be problems for fruit growers. A 1 % aqueous solution of the extract gave 90 % protection.[123] *H. helix* leaf was also used successfully as part of a treatment used to control *Erwina amylovora* (fire blight),[124] another fungal condition which affects apples; a use which was protected under a German patent for a plant tonic to protect against fire blight which lists *H. helix* extract amongst its components, although this patent has since lapsed.[125] A further report of the fungicidal activity of *H. helix* leaf extracts in crop protection indicates beneficial effects against downy mildew (*Pseudoperonospora cubensis*) on cucumber plants at low concentrations and showed almost complete suppression of late blight (*Phytophthora infestans*) in tomato plants.[126]

Current active patents by the Northern Quinoa Corporation also claim antibacterial and antifungal activity of *H. helix* saponins.[127,128] Although the claims are primarily concerned with the use of triterpene saponins from *Chenopodium quinoa* as a means of plant protection against bacterial plant diseases, the sources of saponins are extended to include a number of species which includes *H. helix*. Protection by this treatment is claimed against the biological pathogens *Xanthomonas campestris* pv. *Vesicatoria*, *Corynesporia cassiicola*, and *Streptomyces scabies* together with protection against the fungal pathogens *Rhizoctonia*, *P. Infestans*, *Aphanomyces*, *Cercospora*, *Rhizopus*, *Sclerotium*, *Claviceps purpurea*, *Ascochyta*, *Fusarium*, *Anthracnose*, *Botrytis* and Dutch elm disease (*Opsiostoma ceratosystis ulmi*).

An initial detached leaf assay conducted at Bangor to assess the protective activity of the crude fruit extract of *H. helix* fruit against *P. infestans* (late blight) in potatoes (*Solanum tuberosum* var. *Bintje*) demonstrated activity at extremely low levels which were comparable to those of concentrations of commercial fungicides.[38] Following these findings, an independent field trial was conducted (Eurofins Agrisearch Ltd.) to further investigate the protective activity of *H. helix* fruit extract against *P. infestans* in potatoes. The potatoes were treated with aqueous fruit extract at concentrations of 0.1 % and 0.01

% together with control plots which were a commercial regime of fungicides and an untreated control. This trial found that the 0.1 % extract this gave total protection against tuber blight, outperforming even the commercial regime; even at 0.01 %, the extract was found to display very high beneficial activity in relation to untreated plots. The yield of tubers across the treatments (Figure 1.5.2.1) showed the crude extract having a lower yield per hectare than the commercial regime but the 0.1 % treatment was considerably higher than the untreated and the healthy yield at 0.1 % was identical to the total yield showing a high protective activity. The total yield of the 0.01 % was considerably lower than the commercial regime and comparable to the untreated; however, there was less infection in the treated than the control leading to a better healthy yield. Overall these results clearly showed a significant protective effect by the crude fruit extracts of *H. helix.*

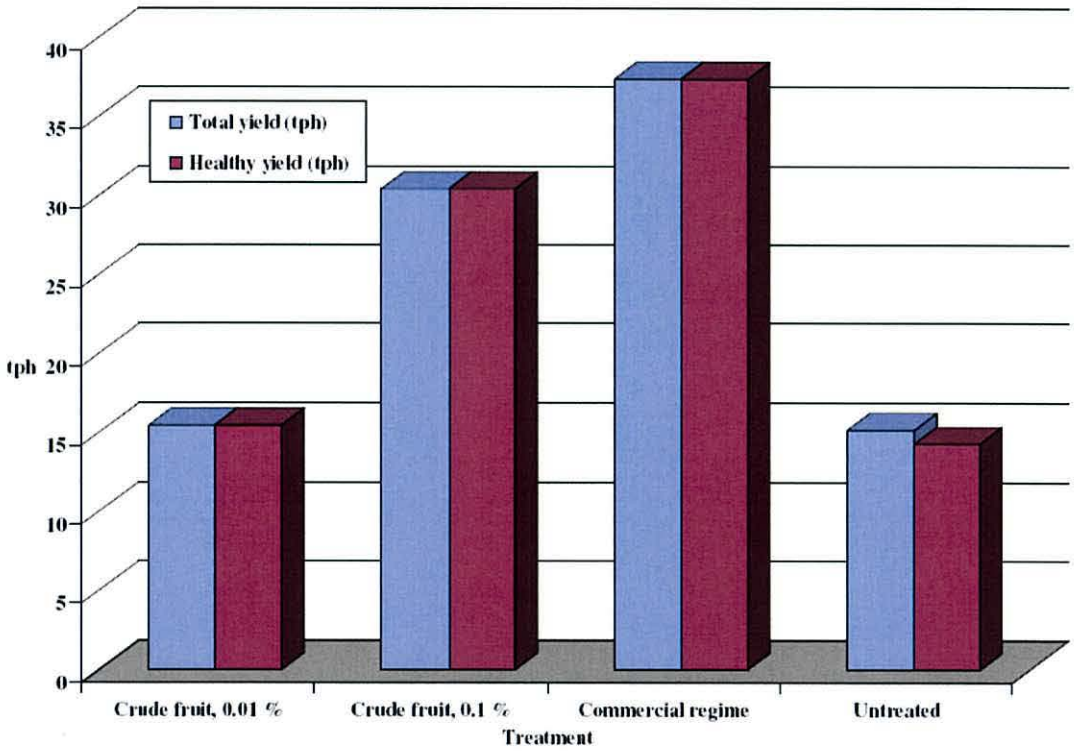

Figure 1.5.2.1 Yields in tonnes per hectare (extrapolated) of total and healthy tubers

Other fungicidal trials conducted at Bangor investigated the activity of *H. helix* fruit extract against several types of timber fungi (*Trametes versicor*, *Trichoderma viride*, *Poria placenta*, *Phanerochaete chryosoporium*, *Phlebia gigantea*, *Fibroporia vaillanti*, *Heterobasidion annosum*, *Coniophora puteana.* The first trial involved preparing malt agar plates containing suspensions of the crude fruit extract of *H. helix* at 5 different concentrations and growth of the fungus over 27 days. The results showed a significant

effect on the growth of the fungi over the period of the trial (a selection of which are shown in Figure 1.5.2.1 to indicate the trends observed). The trial was repeated using the same extract taken up first in ethanol to overcome solubility problems in the aqueous agar; the results confirmed the findings of the first assay; showing significant fungistatic action against all fungi tested over the period of the trial.

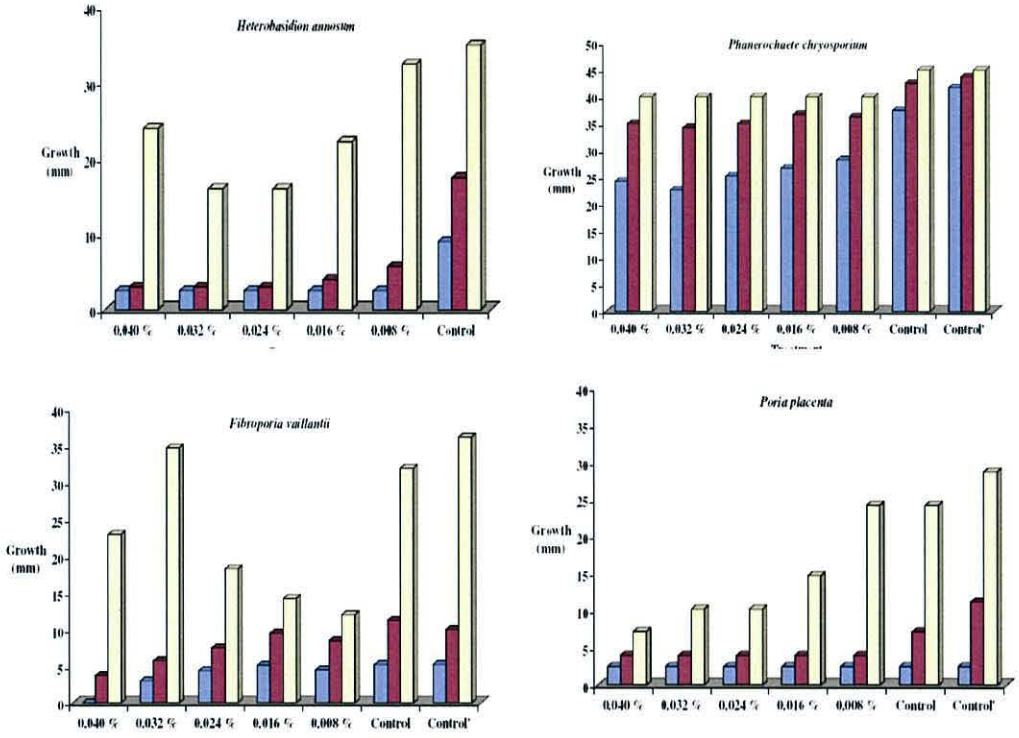

Figure 1.5.2.2 Fungistatic action against timber fungi (extrapolation over 40 mm)

An additional trial was conducted using the same methodology but instead using the potato dextrose agar as substrate in order to assess the fungistatic activity of *H. helix* fruit extract against two common fungal moulds, *Alternaria alternara* and *B. cinera*. These results, in contrast with those of the timber fungi showed far inferior activity; the fruit extract did not perform significantly better than the control against *B. cinera* and in the case of *A. alternara* performed worse than the control as it appeared to be actively feeding the organism.

1.5.3 Insecticidal activity

Although there appear to be a number of reports of insecticidal activity for saponins isolated from other plant sources, many of which are steroidal, there appear fewer cases indicating saponins of *H. helix* to posses this type of activity. One such study found α-

hederin and Hederacoside C from *H. helix* leaves showed high antifeedant activity *in vivo* when tested against the termite species *Reticulitermes flavipes*.[129] However, a conflicting report was published the same year identifying oleanolic acid as being responsible for the resisting of termite attack in Mayan wooden carvings; this report went on to test a series of saponins and found α-hederin to be inactive against termites.[130]

An extract of H. helix leaves has shown high levels of inhibition against wireworm (*Melanotus communis)*, the larvae of the click beetle / skipjack and a very damaging potato pest.[131] In these tests the larvae were observed to burrow away from treated soil and slices of treated potato indicated strong antifeedant effects. Limited activity of leaf extract was also found against another potato pest, the Colorado beetle (*Leptinotarsa decemlineata).*[132] An expired patent from the USA claimed the use of a crude *H. helix* leaf extract (the juice of the leaves and shoots) against wireworms and rootworms.[133]

High insecticidal activity (95 % mortality at 10,000 ppm) was also seen from the leaf extract of the closely related ivy species *H. nepalensis* (Himalayan ivy) against the aphid species *Aphis craccivora*.[134] The fruit extract was also tested but was found to be less effective (65 % mortality at 10,000 ppm). The leaf extract also displayed limited activity (ca. 30 % mortality) against Tobacco caterpillar (*Spodoptera litura*), Two spotted spider mite (*Tetranychus urticae*), Stem pod borer (*Helicoverpa armigera*) and Diamond black moth (*Plutella xylostella*). Another study which was studying the activity of oleanolic acid and ursolic acid saponins on *S. litura* found that the antifeedant activity observed for the two aglycones was significantly improved when they were converted into long chain fatty acid esters; even at low concentration the stearate analogue of ursolic acid (**31**) and palmitate analogue of oleanolic acid (**26**) in particular were found to be exceptionally potent antifeedants.[91]

1.5.5 Other activities of H. Helix saponins

In addition to these more established activities, a number of other potential applications for the saponins of *H. helix* have more recently been reported, most of which relate to potential pharmacological uses.

1.5.5.1 Anti-cancer activity

Most interesting of these reports are those outlining the discovery of the cytotoxic activity of hederagenin based saponins, including α-hederin against a variety of cancer lines and in vivo tumours.[71,75,135,136] This was seen previously when α-hederin isolated from *H. helix* exhibited anti-proliferative action against cancer cells *in vitro*; the mechanism was observed to be cell death as a result of membrane alterations leading to the cytoplasm filling with vacuoles in what appears to be a similar way to the mechanism of haemolysis.[137] A structure-activity relationship study of hederagenin glycosides confimed this cytotoxic activity and concluded that of those tested α-hederin was the most active and that it possessed an osidic chain with a terminal arabinose which was characteristic of the more cytotoxic saponins.[71]

In addition to these cytoxtoxic activities, saponins isolated from *H. helix* have also been found to have anti-mutagenic effects when tested against benzopyrene and concentrated smokers urine.[138] α-hederin from this source, was also tested against the chemotherapy drug and known mutagen doxorubicin, showing a significant anti-mutagenic effect.[139]

1.5.5.2 Protective role in the liver

A number of reports have observed hepatoprotective effects of *H. helix* saponins. Liu *et al.* found a number of triterpene saponins were protective against hepatotoxicity, where these compounds have acted to suppress experimental liver injury in mice; of these, α-hederin and oleanolic acid were found to be most effective.[140] It showed α-hederin to dramatically protect against the liver toxicity of carbon tetrachloride, acetaminophen, cadmium and D-galactosamine. Hederagenin was also tested but showed no protective effect against any of these, suggesting that the sugar moiety was in some way required for it to perform this function. A further study by this group looking specifically at the hepatoprotection afforded by α-hederin showed it to protect against the hepatotoxicity of acetominophen, bromobenzene, carbon tetrachloride, furosemide and thioacetamide.[141] From these experiments the group proposed the mechanism of this protection was by the suppressive effect of α-hederin on hepatic cytochrome P450 enzymes which usually biotransform these molecules and so cause liver injury. The mechanism of action for the protection against cadmium was shown to be by α-hederin inducing metallothionein production; this then binds with the Cd and reduces its availability to critical organelles and proteins.[142]

1.5.5.2 Other pharmacological activity

Numerous other activities that have been cited include an anti-inflamatory activity observed *in vivo* in rats treated with both α-hederin and hederacoside C from *H. helix*.[143] Individual saponins from *H. helix* leaves have also exhibited an anti-oxidant activity; α-hederin being the most effective of these, showing 94 % inhibition of lipid peroxidation of linoleic acid emulsion at a concentration of only 75 ρg / ml.[144] A hederagenin derivative and its pharmaceutically acceptable salt have been patented for use as a pharmaceutical composition in the treatment of nephrits.[145] The monodesmosides of H. helix leaves (α-, β- and δ Hederin) were found to be an effective control of the parasites *Leishmania infantum* and *L. tropica*, responsible for the debilitating and often fatal disease leishmania.[146] All three of these saponins were found to be as effective *in vitro* as the antileishmanial drug pentamidine against the promastigote (extracellular flagellated) forms of these strains; only the aglycone hederagenin was active against amastigote (non-flagellated intracellular) forms.

RESULTS AND DISCUSSION

CHAPTER 2

Extraction and hydrolysis of saponins from *H. helix*

2.1 Extraction of saponins from *H. helix* fruit

2.1.1 Preparation of H. helix fruit material

Ripe fruit of *H. helix* was collected from ivy plants growing wild in areas around Bangor University by the author and Dr. D. Preskett. These were supplemented by fruit from Amlwch, Anglesey and berries previously collected by Dr. Preskett from various parts of Anglesey. The fruit was then dried and milled to a fine meal as described in **6.1**. The dried fruit meal was stored in airtight containers or sealed in polythene bags at room temperature and out of direct sunlight. To minimise unnecessary moisture entering the meal, the containers were filled so as to minimise the amount of headspace as far as possible. This stock of meal was then used *ad libitum* for extraction of the fruit saponin; the current moisture content of the fruit meal identified each time an extraction was conducted, to ensure the yield of TTS could be calculated accurately on a dry weight basis. Upon storage under the conditions described there appeared to be no degradation of the material, nor did there appear to be any noticeable variation in the saponin content recovered.

2.1.2 Soxhlet extraction of saponins from H. helix fruit meal

Previous work sequentially extracting natural products from *H. helix* showed that low polarity solvents recovered very little and these extracts were composed of only fatty triacylglycerol components; increasing the polarity recovered more and the best recovery of TTS was obtained using methanol or ethanol.[38] The dried fruit meal was extracted in a Soxhlet apparatus for 5 hours using methanol as a solvent as described in **6.2(i)**. The initially purple colouration of the extraction solvent had diminished and by this time it was seen to run clear. When the solvent was removed *in vacuo* the

extract gave a purple solid of the triterpene saponins and what appeared to be a dark green oily residue of the fatty acids. It was found that adding petrol to this residue and heating at reflux for a short time dissolved the majority of the oil component of the extract while leaving the solid saponin component insoluble. As such it was possible to filter the hot suspension to recover the TTS as a purple powder; evaporation of solvent from the filtrate gave an oily tar of the TAG components. ^{1}H NMR analysis of the TTS extract was carried out and the presence of saponins identified by comparison to the spectra of commercial Hederacoside C (**4**) and α-hederin (**5**).

Having confirmed during the initial small scale extraction that this type of methodology would be suitable as a means of extracting TTS from *H. helix* fruit it was next important to adjust this to a more suitable method to obtain larger quantities of this material. As such, the same extraction was performed using a larger quantity of fruit meal in a larger Soxhlet apparatus as reported in **6.2(ii)**. The extraction was again carried out for 5 hours, this time using ethanol (IMS) at reflux as it was deemed a more suitable solvent for preparative extraction than methanol due to its lower cost and lower toxicity. The pale brown solid from this extraction was also observed to contain the same viscous green oil component as previously described and so was heated under reflux in petrol in a similar fashion to remove it. Filtration and thorough drying gave an off white solid of crude TTS products.

Using both of these methods the yield of crude fruit saponins was high, with a 27 % recovery by weight.

^{1}H NMR analysis of the ethanol extract of *H. helix* fruits confirmed the principal component to be TTS (Figure 2.1.2.1). The key analytical features being the six 3 H singlets in the region δ 0.64 - 0.12 ppm, corresponding to the CH_3 moieties present in the product, a 1 H double doublet at δ 2.75 - 2.85 ppm and a 1 H triplet in the olefinic region of δ 5.16-5.20 ppm. It is possible to identify a similar pattern of signals in the spectrum of a commercially obtained (Extrasynthese, France) standard of α-hederin (**5**) shown in Figure 2.1.2.2, which is reported to be the principal saponin in *H. helix* fruit. However, the mass of signals assigned to the mixed sugars of the osidic moiety observed in the region between δ 3 and 4 in the *H. helix* fruit extract does appear to be

more complex to that seen in the pure α-hederin, indicating that a mixture of saponins is most likely present with other sugars attached at this position. Also apparent is that the distinctive CH_3 signals of rhamnose seen at δ 2.76 and δ 2.78 in the spectrum of α-hederin do not appear dominant in that of the fruit extract, perhaps further indication of differences in the sugars present.

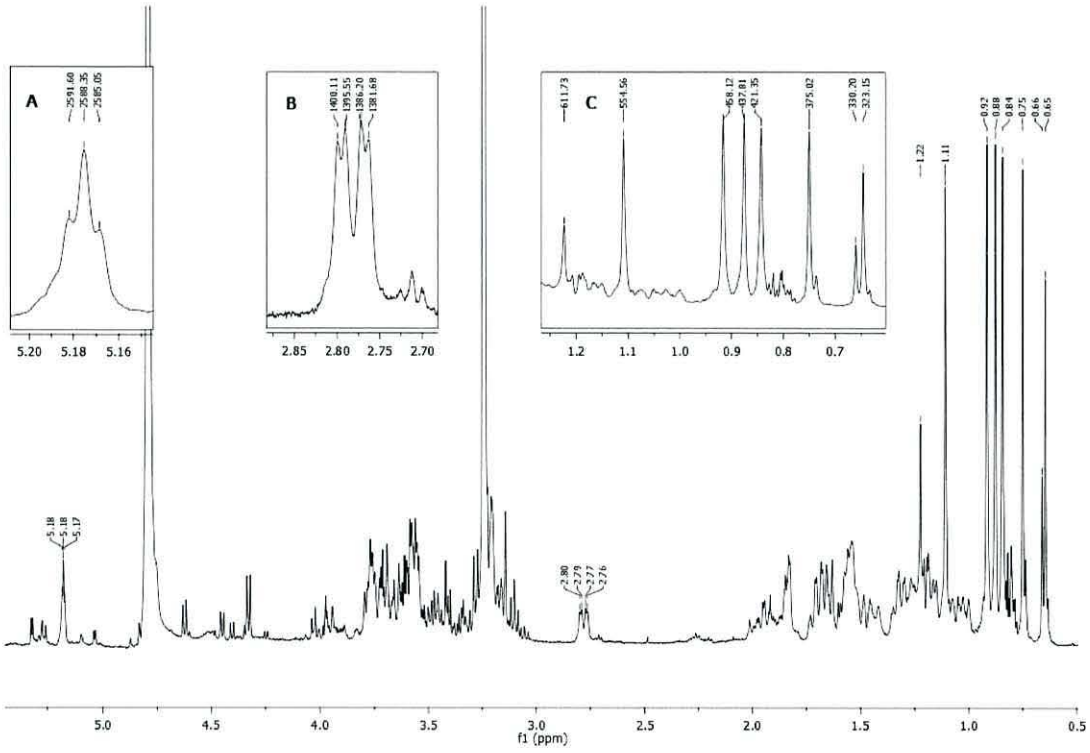

Figure 2.1.2.1 ^{1}H NMR spectrum of *H. helix* fruit extract in MeOD, the regions featuring characteristic signals are shown in expansions A, B and C

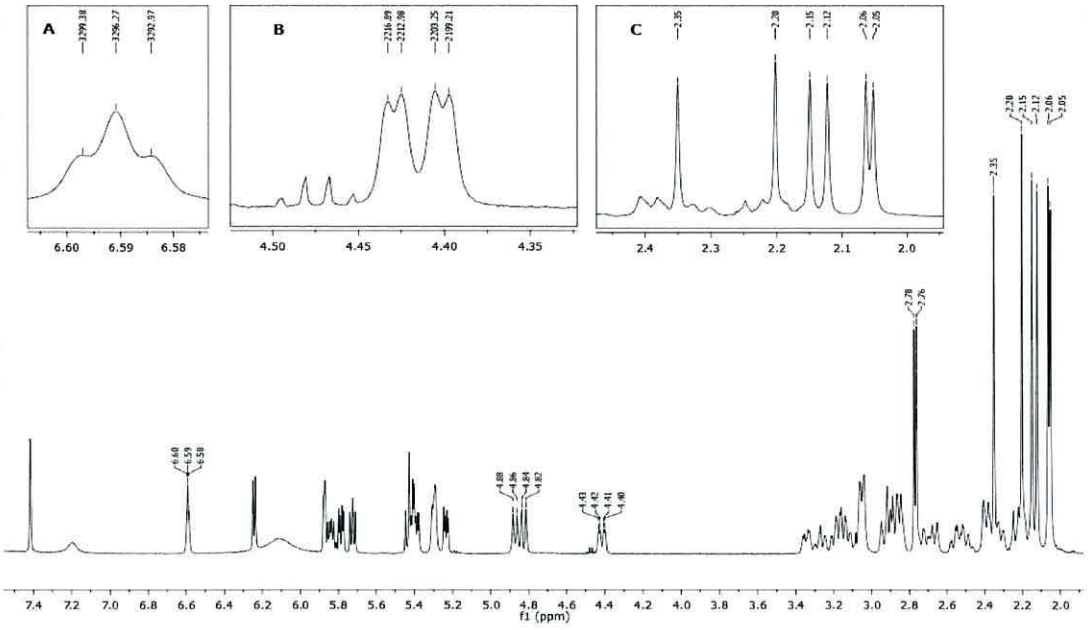

Figure 2.1.2.2 ^{1}H NMR spectrum of commercial α-hederin in D_5-Pyridine, the regions featuring characteristic signals are shown in expansions A, B and C

Close examination of ^{1}H NMR spectra also identified another difference. The *H. helix* fruit extract showed other small signals to be present, subsequently found to match the residues seen in the spectrum of the fatty acid that had been recovered from the petrol washings (Figure 2.1.2.3). Most readily observed of which were the signals of the fatty acid chains around δ 1.22 ppm as well as a small triplet at δ 2.71 corresponding to the skip conjugation of linoleic acid and a multiplet of fatty acid double bonds in the olefinic region (δ 5.55 - 5.57 ppm) just above that of the TTS.

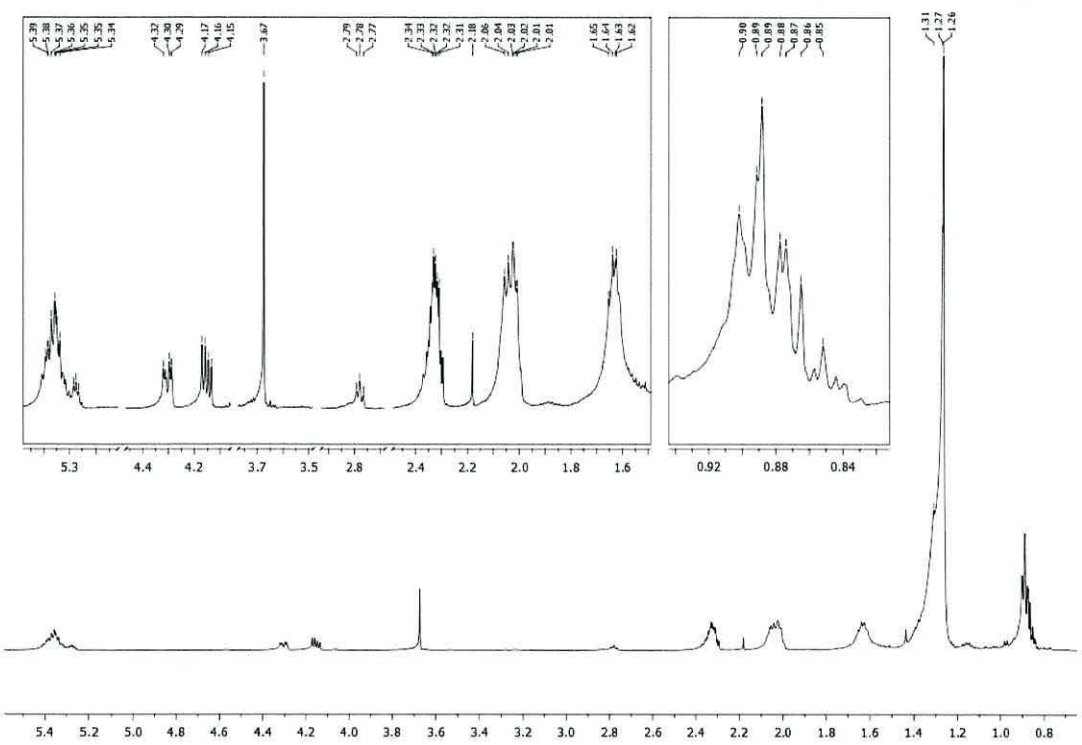

Figure 2.1.2.3 ^{1}H NMR spectrum of the fatty acid component recovered from the petrol washings (in $CDCl_3$)

This fat was essentially composed of triacylglycerol (TAG), the composition of which has been previously studied comprehensively by Dr. D. Preskett who showed it to be composed predominantly of the uncommon fatty acid petroselinic acid (**55**), together with the oleic, palmitoleic, vaccenic, palmitic, linoleic and stearic acid.[38]

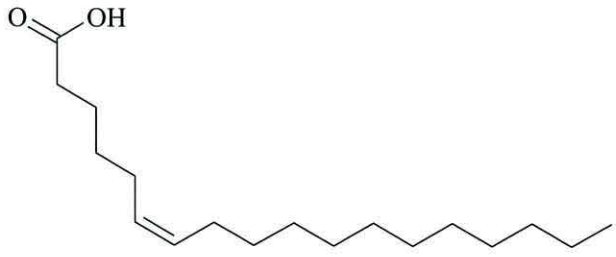

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Figure 2.1.2.3 The uncommon fatty acid petroselinic acid

This fatty impurity appeared to be present each time this method was repeated and as such it appears that this oil is well bound to the solid TTS extract. To overcome this method a strategy was developed for "defatting" the crude extract. The initial attempt to do this by recrystallising the product from 1-butanol as in **6.3(i)**, although

successful, proved rather undesirable; it offered a very poor recovery of the precipitated TTS (62 %) and that which was not precipitated was difficult to recover from this solvent by evaporation. It also proved time consuming to efficiently remove all of the solvent from the recovered solid for proper analysis. As such an alternative method was devised. Taking advantage of the petrol solubility observed previously in the crude "defatting" steps, the crude fruit TTS was ground to a very fine powder and extracted with petrol in a Soxhlet apparatus for 18 hours (**6.3(ii)**). This extensive petrol extraction was successful, the TTS was recovered in excellent yields (95 %) as a fine powder and showed no signs of fatty impurities either visually or by NMR analysis.

2.2 Extraction of saponins from *H. helix* leaf

2.2.1 *Preparation of H. helix leaf material*

Mature leaves of *H. helix* were collected from ivy plants growing wild in areas around Bangor University by the author and Dr. D. Preskett. These were collected as best possible from the same sites as those used to collect the fruit. These leaves were supplemented by others collected from Amlwch, Anglesey. The leaf was then dried and milled to a fine powder as described in **6.9**. The dried and powdered leaf was stored and used in the same manner as the fruit meal. Similarly to the fruit, the leaf material did not appear to show any degradation on storage and there was no identifiable change in the yields of TTS obtained over the period of storage. However, unlike the fruit meal, which possessed a rather coarse texture, the fine powder of the leaf material required more carful handling in bulk (in a fume cupboard or wearing a dust mask); the airborne particulates of this powder resulted in noticeable irritation, usually in the form of nasal irritation and sneezing, most likely as a result of the expectorant activity of the saponins it contained.

2.2.2 Soxhlet extraction of saponins from H. helix leaf

The dried and powdered leaf was was extracted in a Soxhlet apparatus for 5 hours using ethanol as a solvent as described in **6.10(i)**. The initially green colouration of the extraction solvent had diminished and by this time was seen to run clear. When the solvent was removed *in vacuo* the extract gave a green solid of the triterpene saponins together with a dark green tarry oil residue of the fatty acids. As with the

fruit extract, this was heated with petrol at length to dissolve the TAG residue and filtered to recover the TTS as a green powder; evaporation of solvent from the filtrate gave an oily tar of the TAG components which appeared similar by ^{1}H NMR to that obtained from the fruit. The yield of TTS obtained from this extraction was lower than that from the fruit, with a recovery of 15 % by weight. As with the fruit ^{1}H NMR analysis of the TTS extract was carried out and the presence of saponins identified by comparison to the spectra of commercial Hederacoside C (**4**) and α-hederin (**5**); this analysis once again showed traces of fatty acid to be still present in the crude TTS extract. This impurity was removed by a further "defatting" step (**6.11**) in the same manner as was used for the fruit extract.

^{1}H NMR analysis of the leaf extract showed the characteristic signals expected for TTS (Figure 2.2.2.1). A 1 H triplet was observed at δ 4.33 ppm, a 1 H double doublet around δ 2.73 - 2.80 ppm and the singlets of the CH_3 groups were also observed in the region δ 0.75 and δ 0.84 ppm. Unlike the spectrum of the fruit extract however, the leaf extract has a considerably greater intensity and complexity of signals in the region δ 3.4- 4.2 ppm. This region corresponds to the attached sugars of the osidic moieties of the saponins and as such suggests a higher proportion of sugars are present compared to the fruit TTS as well as being indicative of a mixture of different sugars being present. It has been observed that higher proportions of bidesmosidic saponins are found in the leaf than the fruit which supports this observation. It also appears likely from the pattern observed for the CH_3 singlets that more than one type of saponin is present in the extract; the expected pattern of six prominent 3 H singlets is instead more complex (Figure 2.2.2.1 expansion C). This pattern shows that two or more sets of these methyl groups are present with different shifts to one another, evidence that more than one saponin component is present. This could be the result of more than one aglycone being present at high levels (i.e. saponins based on both hederagenin (**2**) and oleanolic acid (**1**)) although this seems unlikely due to the absence of another olefinic signal and doublet doublet to correspond with this other aglycone. More likely is that both monodesmosides and bidesmosides of hederagenin are present and that the presence of the additional sugar moiety has resulted in a different shift pattern in one of these. The additional singlets downfield of these around δ 1.18 ppm are in agreement may represent the putative methyl moieties of rhamnose which is present in Hederacoside C (**4**) which is reported as the principal

saponin present in *H. helix* leaf. MALDI MS analysis of the leaf extract showed two molecular ions were present one matched the mass calculated for α-hederin, the other larger ion matched the mass calculated for Hederacoside C.

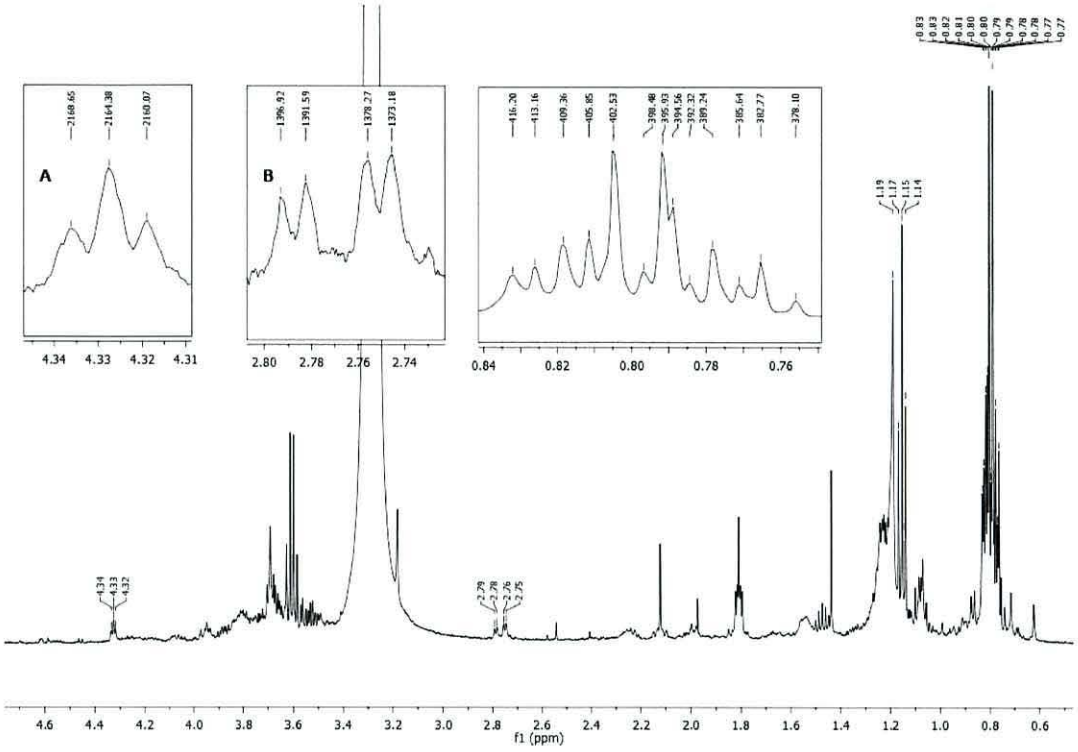


Figure 2.2.2.1 ^{1}H NMR spectrum of *H. helix* leaf extract in MeOD, the regions featuring characteristic signals are shown in expansions A, B and C

In previous extraction of H. helix leaf TTS, Dr. Preskett reports a second smaller set of signals in the ^{1}H NMR which integrated to 0.5 compared to those of the main TTS signals, evidence of multiple aglycones being present (hederagenin (**2**) and oleanolic acid(**1**)) in the extract.[38] He also identified similar signals by ^{13}C NMR. As can be seen, no such signals were identified in this extraction with the saponins present all seemingly based on hederagenin (**2**) (those based on oleanolic acid (**1**) were either not isolated by this method or were in such a concentration as to not be readily distinguished by NMR). Also reported in his extract were minor aromatic components appearing in the spectrum at δ 6.30 - 7.68 ppm; once again, these were not observed in the extract reported here.

Having successfully obtained these saponins, the extraction method was further adapted for larger scale preparation of TTS which could be used for both the fruit and

leaf. The method described in **6.10(ii)** was modified to use a larger Soxhlet apparatus which was routinely recharged with new material every 6 hours allowing the same solvent to be used for each batch of leaf material. This was possible only until the concentration of extract became enough to cause a build up of solid to form in the flask, at which point the solvent was evaporated in vacuo to afford a crude extract which was worked up in the same manner as previously. This technique proved more efficient overall. It allowed maximal return of product while limiting solvent wastage as this could be recycled several times within the Soxhlet apparatus, also reducing the time taken by combining a number of time consuming work-up and defatting steps. This method saw a modest improvement in the yield, most likely as a result of minimising losses in transfer and work-up, achieving a 17 % recovery of crude TTS.

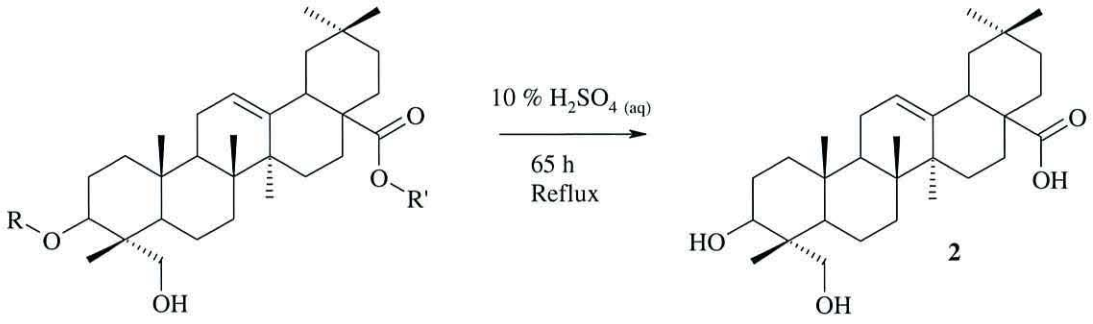
2.3 Acid hydrolysis of the TTS extract of *H. helix* fruit

The fruit saponins isolated by extraction in **2.1** appear to contain a mixture of different sugars but are all based predominantly on the aglycone hederagenin (**2**); they appear to be principally monodesmosidic. From the literature it also appears possible that some minor saponins present may be based on oleanolic acid (**1**).[50] In order to confirm which aglycones can be obtained from this source and in order to prepare a homogenous sample better suited to chemical modification than the glycosides it was necessary to identify the best means to prepare the free aglycone. In order to do so it would be necessary to carry out an acid hydrolysis of these crude saponins. The mechanism of such a hydrolysis reaction is described in the introduction (See section 1.5.1.2 and Scheme 1.5.1.2.2).

The first attempt to hydrolyse the saponin was performed using a refluxing 5 % solution of hydrochloric acid solution for 5 hours as in **6.4(i)**. However this proved to be insufficient since analysis of the recovered solid showed none of the desired product to be present. Comparison of the proton NMR spectrum to that of the TTS starting material showed them to be identical. A complex multiplet at ~ δ 3.5 ppm corresponding to the attached sugar moieties confirmed that the hydrolysis had not occurred. Repetition of this reaction confirmed that these conditions were not adequate to affect the hydrolysis of the sugar moieties from the aglycone portion. A solid remained throughout the reaction. The procedure was conducted again, this time

using a 10 % solution of sulphuric acid instead (**6.4(ii)**). The progress of the reaction was monitored by TLC and samples taken for ^{1}H NMR analysis each hour to assess if hydrolysis was taking place; all of these showed no change to the sugar signals in the spectra. Based on this it was determined the reaction time would need to be significantly extended.

For the third attempt, described in **6.4(iii),** the 10 % sulphuric acid was retained but the reaction time was extended considerably. The reaction was allowed to run continuously and followed by TLC which showed it to be complete after 65 hours. A pasty brown solid was recovered; since crude TTS extract was used, a petrol washing step was required to remove the fatty acid residue that remained and give the crude acid hydrolysis product as a pale brown solid. This was recrystallised from hot ethanol to give the aglycone hederagenin (**2**) in fair yield.



Scheme 2.3.1 Acid hydrolysis of crude triterpene saponin to the free aglycone hederagenin; where R = osidic moiety and R' = osidic moiety (bidesmoside) or H (monodesmoside).

The ^{1}H NMR spectrum of this acid hydrolysis product (Figure 2.3.1) showed characteristic signals that were almost identical to a commercially obtained (Extrasynthese, France) standard of hederagenin (Figure 2.3.2). The six methyl groups were identified as 3 H singlets in the region δ 0.91 ppm to δ 0.32 ppm (shown in expansion C). In addition, the characteristic 1 H double doublet was observed at δ 3.29 ppm (expansion B) and the proton attached to the double bond was seen as a 1 H triplet at δ 5.49 ppm (expansion C). No signals corresponding to attached sugars were observed; a doublet at δ 3.71 ppm and a multiplet at δ 4.19 ppm were the only signals seen in this region and both match corresponding signals in the commercial sample also, confirming that the aglycone isolated is hederagenin (**2**) as expected.

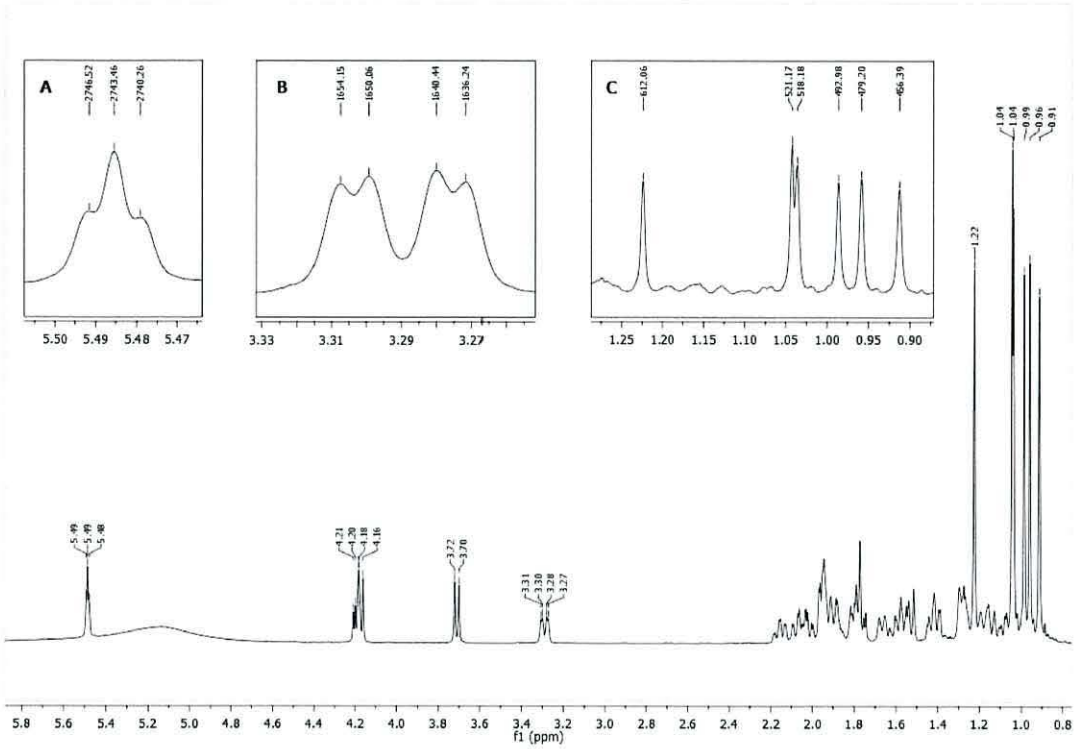


Figure 2.3.1 ^{1}H NMR spectrum of the acid hydrolysis product of *H. helix* fruit saponin extract in D_5-Pyridine, the regions featuring characteristic signals are shown in expansions A, B and C

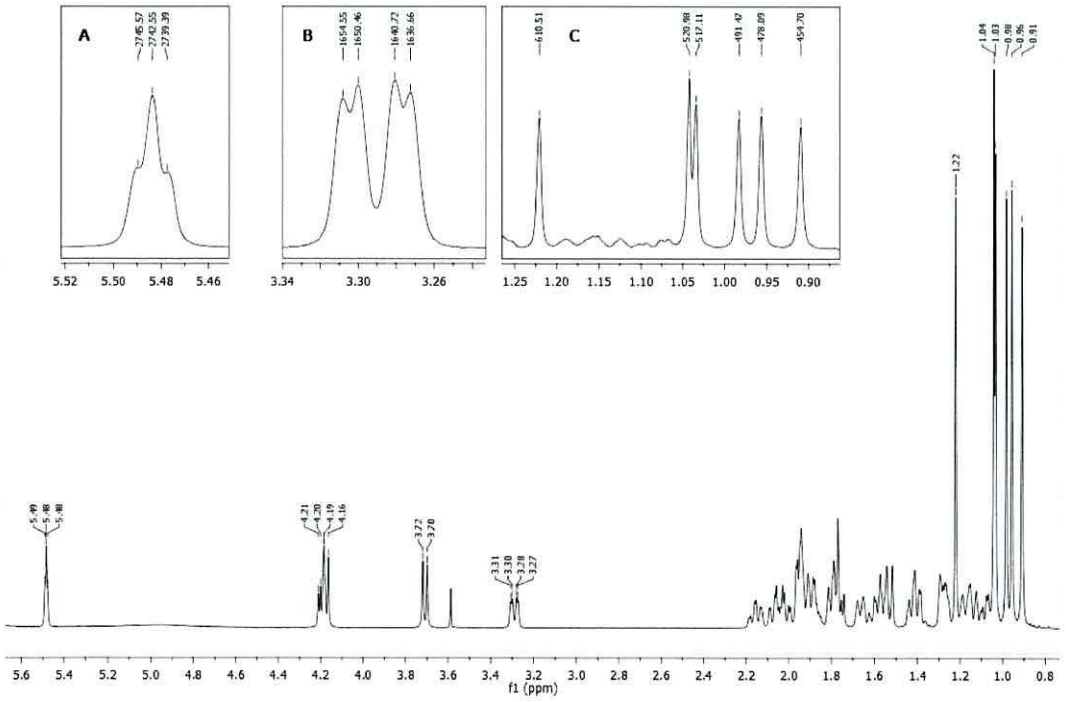


Figure 2.3.2 ^{1}H NMR spectrum of commercial hederagenin in D_5-Pyridine, the regions featuring characteristic signals are shown in expansions A, B and C

Notably, there appeared to be no signals corresponding to any other aglycones present in the spectrum, in particular oleanolic acid (**1**). Oleanolic acid has a readily distinguishable spectrum from that of hederagenin, shown in Figure 2.3.3, since oleanolic acid has seven CH_3 moieties rather than the six observed in hederagenin and thus a different pattern of singlets (see expansion C). Despite reports indicating the presence of oleanolic acid as an aglycone in the fruit saponins of *H. helix*, there appears to be no trace of this in this spectrum. MALDI MS analysis of this product showed only one molecular ion to be present at the same mass calculated for hederagenin (**2**), further supporting the suggestion that only this aglycone is present. It therefore appears from this that hederagenin is most probably the only aglycone present in the fruit saponins of *H. helix*.

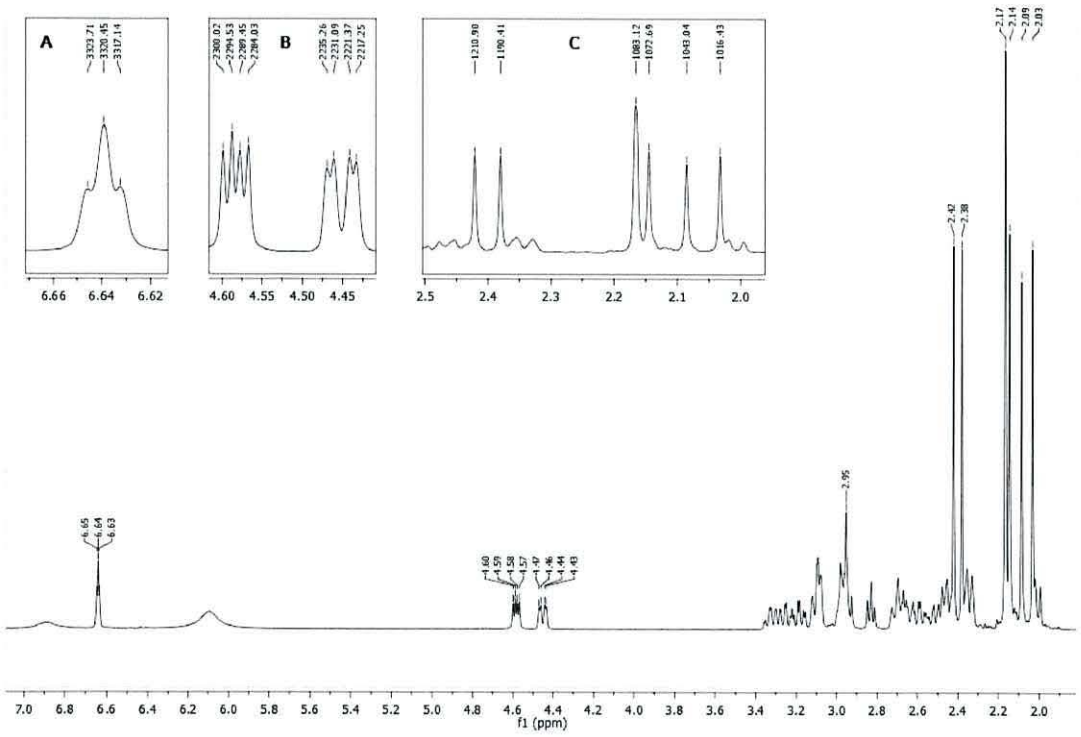


Figure 2.3.3 ^{1}H NMR spectrum of commercial oleanolic acid in D_5-Pyridine, the regions featuring characteristic signals are shown in expansions A, B and C

Having established that hederagenin could be readily prepared from the crude extract, it was scaled up using the slightly modified method described in **6.5** in order to prepare enough material to attempt synthetic modifications of the aglycone. The acid hydrolysis product was recovered in broadly similar yields to previously (ca. 18 %).

An attempt was made during the extraction procedures to achieve an *in situ* hydrolysis of the TTS extract by acidifying the extraction solution with dilute acid (**6.6**) as a facile means to obtain aglycone directly from *H. helix* fruit. This was unsuccessful and gave only unhydrolysed saponins. This is likely to be due to the long times that were found to be necessary for the acid hydrolysis to take place compared to the relativeley short time required to complete the extraction. As a result of the increased extraction time that would be required it seems that further pursuit of such a process would not be of any value.

2.4 Acid hydrolysis of the TTS extract of *H. helix* leaf

The acid hydrolysis product of the leaf extract was prepared in a similar manner to that of the fruit using 10 % sulphuric acid at reflux for 70 hours (**6.12**). An extensive "defatting" step with petrol in a Soxhlet apparatus was used to remove any remaining fatty acid residue and give the leaf acid hydrolysis product as a fine green / brown powder in fair yield (26 %).

^{1}H NMR spectrum of the leaf acid hydrolysis product (Figure 2.4.1) appeared very similar to that of the fruit acid hydrolysis product. It possesses identical characteristics signals for hederagenin (**2**) to those observed in the fruit product spectrum and that of the commercial hederagenin. No signals corresponding to the attached sugars of the starting material were observed in the region δ 3.40 - 3.80 ppm and instead the only signals observed here were those of hederagenin. Close examination of the spectrum showed it to be slightly different by comparison to that of the fruit acid hydrolysis product. Although the predominant signals were characteristic of hederagenin (**2**), there were also a number of small signals corresponding to minor impurities. Most notable of these were the singlet signals which appeared in the region δ 0.75 - 1.30 ppm; in addition to the six 3 H singlets of the hederagenin there appeared to be another smaller set of singlets perhaps indicative of a second minor aglycone present in the sample. However, no other signals were identified elsewhere in the spectrum which could correspond to such an aglycone. MALDI MS analysis of the leaf base hydrolysis product showed only one molecular ion to be present at the same mass as that calculated for hederagenin; this is further

evidence that the leaf saponins, like those of the fruit, are based on the aglycone hederagenin (**2**).

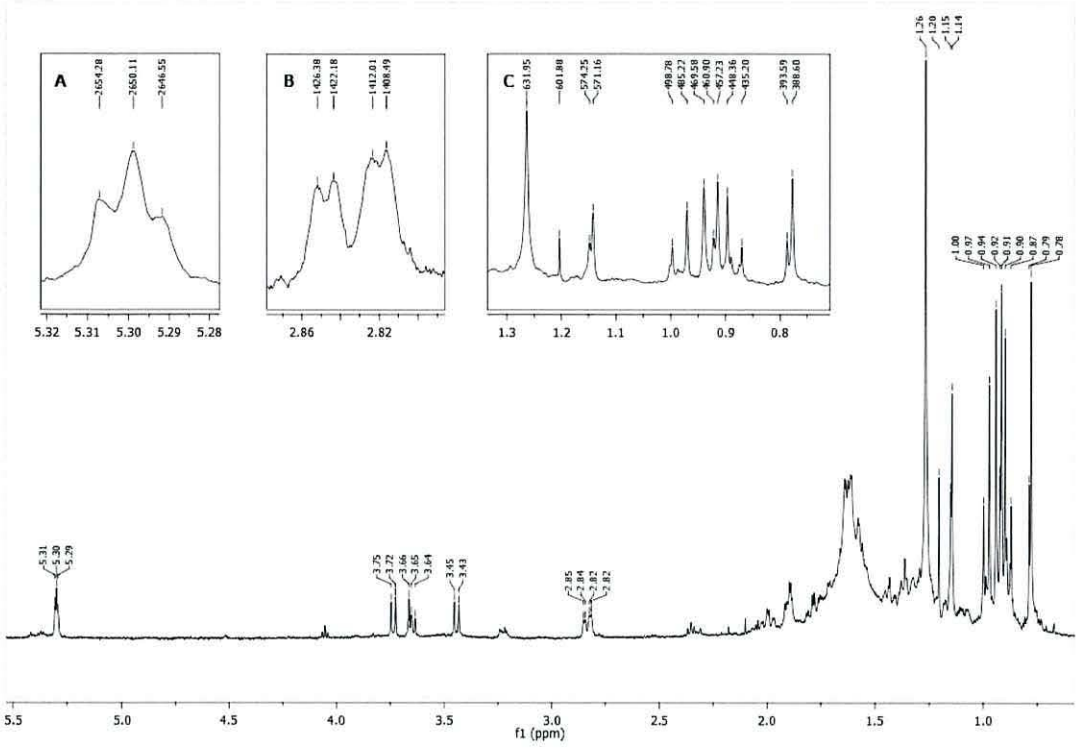
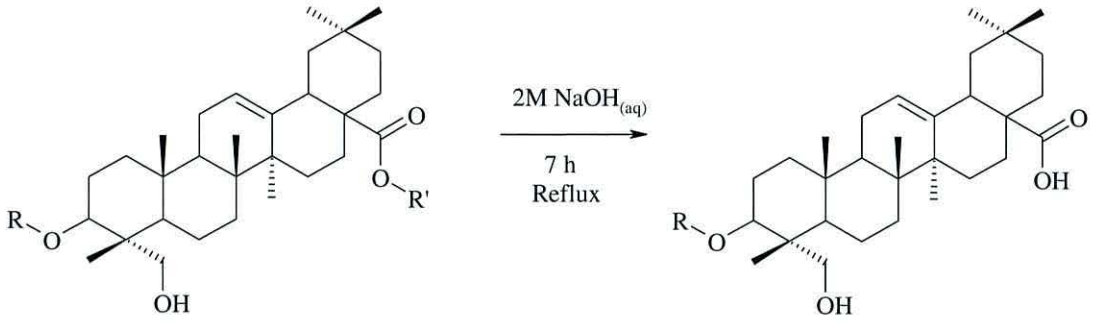


Figure 2.4.1 ^{1}H NMR spectrum of the acid hydrolysis product of *H. helix* leaf saponin extract in $CDCl_3$, the regions featuring characteristic signals are shown in expansions A, B and C

2.5 Base hydrolysis of the TTS extract of *H. helix* fruit

While acid hydrolysis was shown to remove both acetal and ester bound sugars from the crude TTS, it was expected that base hydrolysis would cleave only those bound at the C-28 ester position to give a monodesmosidic product with sugars bound only at the C-3 position. Since the fruit extract was predominantly composed of monodesmosides, this reaction should not have had any effect on these sugars. However, should any bidesmosidic saponins have been present as a minor component of the saponin profile they would have been converted into the corresponding monodesmosides. In the case of preparing compounds for biological testing this was an important transformation as it would act to homogenise the sample to give a known composition of only monodesmosidic saponins, which are generally regarded as being the more active. The mechanism of this base hydrolysis reaction is described fully in the introduction (See section 1.5.1.1 and Scheme 1.5.1.1.2).

In comparison to the rather slow acid hydrolysis the base hydrolysis appeared to take place far more readily. Base hydrolysis of the fruit extract of *H. helix* was first achieved as decribed in **6.7(i).** The crude fruit TTS extract was heated at reflux with sodium hydroxide solution (2 M) for 7 hours. The solid product was collected from the neutralised solution by filtering it under reduced pressure through a large Buchner funnel. This solid was "defatted" with refluxing dichloromethane to remove the remaining fatty acid component and decolourised with activated charcoal to afford the pure hydrolysis product as an off-white solid in good yield (72 %). The product was then extensiveley "defatted" with petrol as described in **6.8** prior to characterisation.



Scheme 2.5.1 Base hydrolysis of crude triterpene saponin to the monodesmosidic saponin only; where R = osidic moiety and R' = osidic moiety (bidesmoside) or H (monodesmoside).

The ^{1}H NMR spectrum of the base hydrolysis product (Figure 2.5.1) showed signals similar to those seen in the initial fruit extract, possessing the six 1 H singlets of the methyl groups in the region δ 0.70 - 1.20 ppm, the same 1 H double doublet at δ 2.86 ppm and the 1 H triplet at δ 5.26 ppm corresponding to the olefinic proton. The signals from the sugar moieties were observed between δ 3.40 and δ 4.00 and appeared similar to those of the fruit extract, if somewhat less complex. These signals once again suggest that a mixture of sugars is likely to be present in the saponins of this product and remain far more complicated than those of the pure α-hederin (**5**). The molecular ion identified by MALDI MS was shown to be the same mass as that calculated for α-hederin.

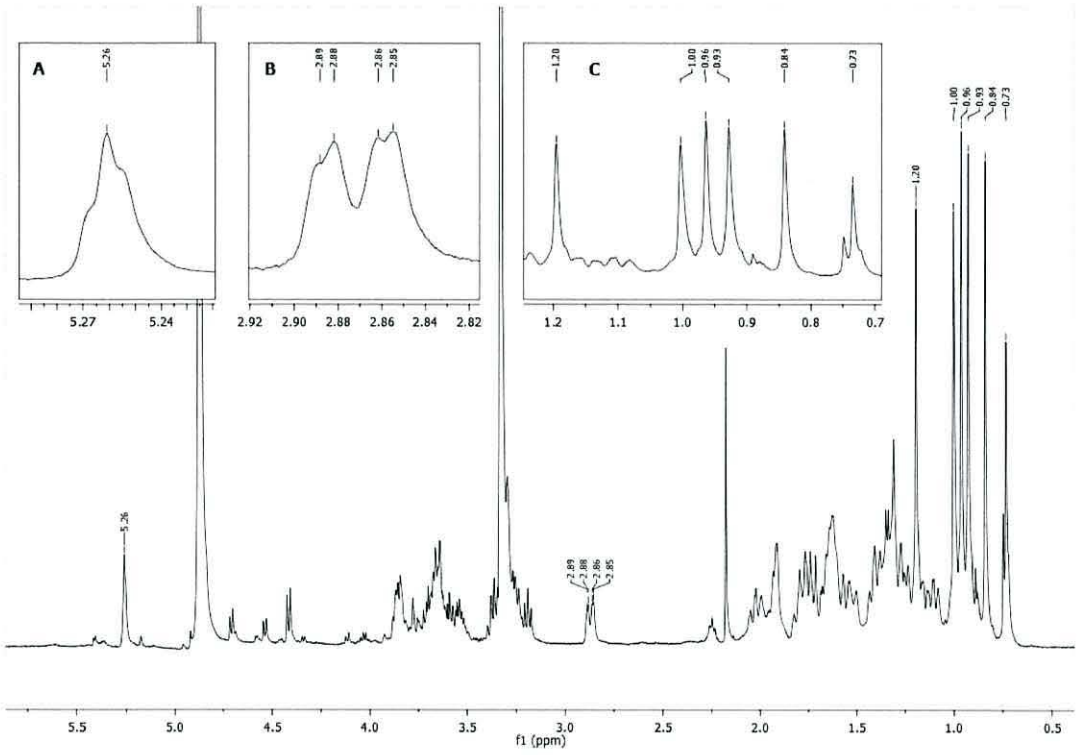


Figure 2.5.1 ^{1}H NMR spectrum of the base hydrolysis product of *H. helix* fruit saponin extract in $CDCl_3$, the regions featuring characteristic signals are shown in expansions A, B and C

The filtration stage of this reaction was very slow and it seemed that the fine powder was settling on the surface of the filter paper and in doing so prevented the remaining suspension filtering efficiently. This proved to be very time consuming and required the collecting solid to be regularly agitated by spatula. As a result of this rather inelegant process, the same hydrolysis procedure was repeated but instead of passing through a filter the mixture was transferred into centrifuge tubes and centrifuged for 30 minutes (**6.7(ii)**). This effectively separated the contents into a large solid pellet of the crude product and a supernatant solution of the neutralised base and the free sugars cleaved from the saponins. The solution could then simply be decanted and the solid residues combined, dried and purified as in the previous method to give a product of comparable purity and in similar good yields.

2.6 Base hydrolysis of the TTS extract of *H. helix* leaf

Unlike the fruit extract, which is predominantly composed of monodesmosides, the majority of saponins present in the leaf extract are bidesmosidic. Base hydrolysis of this extract should therefore have a more pronounced effect, cleaving the ester bound

sugars of these bidesmosides to give a monodesmosidic product more akin to the saponins of the fruit extract. Since the bidesmosides are widely regarded as biologically less active than the monodesmosides it could prove a useful means of producing more desirable compounds from the otherwise inferior leaf saponins.

The reaction was carried out on the crude leaf extract as described in **6.13**, using the same method as was developed for the fruit extract base hydrolysis, this time with the separate defatting step incorporated into the procedure. A fine green powder of the leaf base hydrolysis product was obtained in good yield (77 %).

Analysis of the product by ^{1}H NMR gave a spectrum (Figure 2.6.1) very similar to that observed for the fruit. The same 1 H double doublet was observed at δ 2.64 ppm (expansion B), and six 3 H singlets were observed in the region δ 0.49 - 1.13 ppm corresponding to the six methyl groups present in the hederagenin backbone. Additional singlets were identified downfield of these but appear disimilar to the pattern expected for additional groups of another aglycone, instead they appear most likeley to correspond to the CH_3 groups of rhamnose sugars present in the osidic moiety. The pattern of singlets is not as complicated as that seen in the crude leaf extracts, perhaps suggesting that the additional signals observed in that were not the result of an alternative aglycone but may have resulted from a different shift pattern of these groups in the mono- and bidesmosidic saponins; cleavage of one moiety would then give only the monodesmosides and as such simplify the signals to show only six CH_3 signals in the hydrolysis product. The mass of signals assigned to the mixed sugars in the region δ 3.10 - 3.80 ppm was still present in this product, however the pattern appeared less complex and less intense, again a result of the cleavage of ester bound sugars to leave only the acetal bound sugar moiety. The only other significant difference observed in the spectrum of the leaf base hydrolysis product is shown in expansion A; together with the expected 1 H triplet at δ 5.03 ppm of the proton attached to the double bond. A second olefinic signal slightly upfield at δ 4.95 ppm is present which most likely corresponds to a saturated fatty acid being present as a minor impurity, since the conditions required for base hydrolysis of the TTS would also be likely to hydrolyse any residual TAG into such a free fatty acid. When the MALDI MS of the leaf base hydrolysis product was conducted it showed a molecular

ion to be present of the same mass as both the base hydrolysis product of the fruit and that calculated for α-hederin.

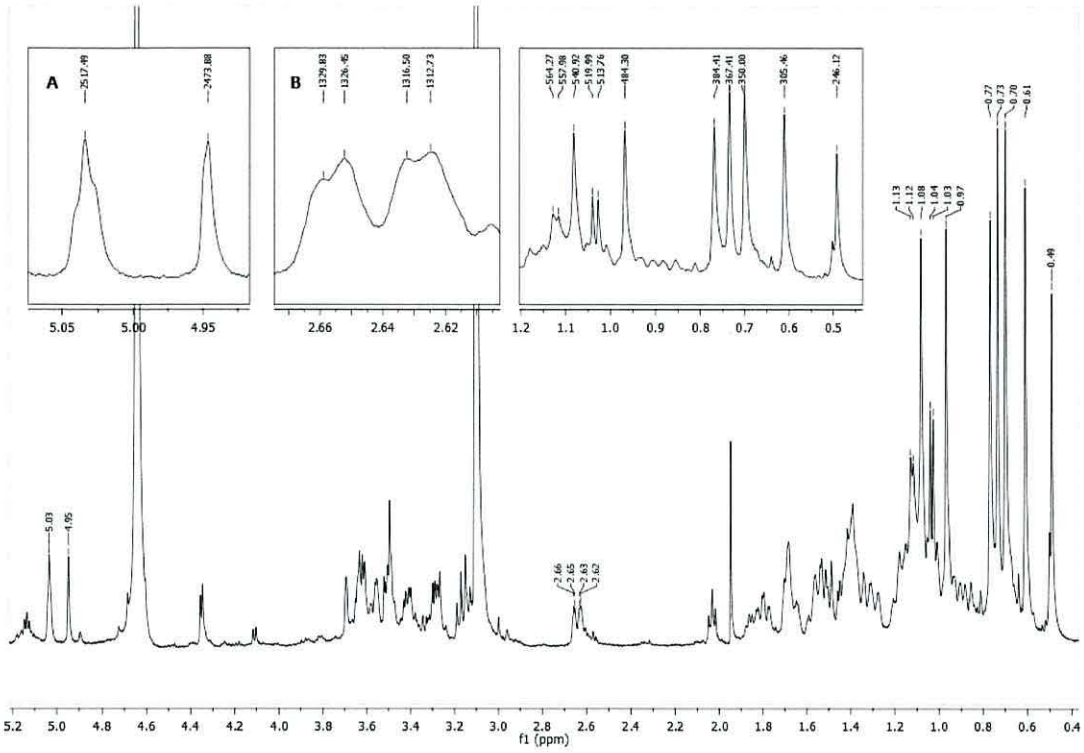
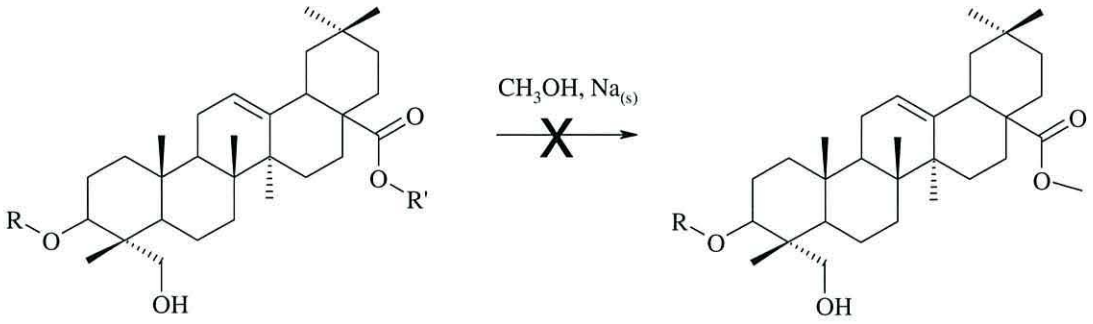


Figure 2.6.1 ^{1}H NMR spectrum of the base hydrolysis product of *H. helix* leaf saponin extract in $CDCl_3$, the regions featuring characteristic signals are shown in expansions A, B and C

2.7 Attempts at the transesterification of TTS

A common method to prepare esters in lipids is to transesterify them with an alcohol using sodium methoxide. It was therefore possible that a similar technique could be used to transesterify the C-28 position of the crude fruit TTS and replace the sugar moiety with an alternative ester. This appeared to be a means of preparing a methyl ester at the C-28 position of the aglycone while simultaneously cleaving the sugars from this position (Scheme 2.7.1). It also provided the potential to produce a range of longer chained esters by using the same methodology with a number of different alcohols. The reaction was attempted twice on the crude fruit saponin, using methanol in the first instance to try and prepare the methyl ester, then ethanol to attempt to prepare the ethyl ester. Neither of these reactions yielded the desired products. Some changes had taken place to the starting material as was observed by the proton NMR spectra, but these changes were not those expected to be observed

for the formation of the respective methyl or ethyl esters. Instead the changes in the spectra appear to be a result of the sugar moieties beginning to hydrolyse in the basic reaction conditions.



Scheme 2.7 Attempted trans-esterification of crude triterpene saponin to the methyl ester of the monodesmosidic saponin; where R and R' are osidic moieties

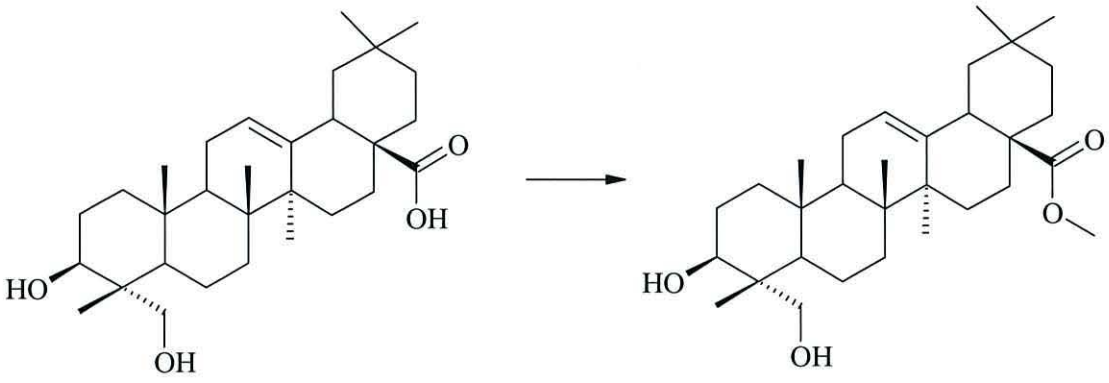
CHAPTER 3

Modification of the Hydrolysis products of saponins from *H. helix*

3.1 Preparation of the methyl ester of hederagenin and base hydrolysis product

3.1.1 Preparation of the methyl ester of hederagenin

Having prepared hederagenin (**2**) by acid hydrolysis of the extracted fruit saponins, the first attempt to functionalise it was to protect the carboxylic acid function as its corresponding methyl ester (**15**) (Scheme 3.1.1.1).



2 **15**

Scheme 3.1.1.1 Preparation of hederagenin 28-*O*-methy ester

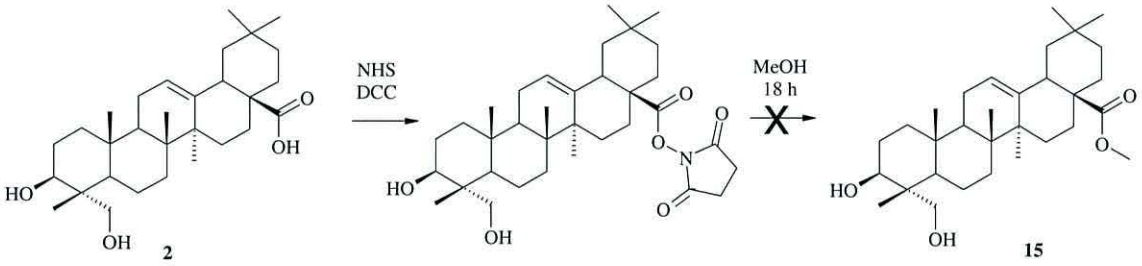
This was first performed on a small scale by treating the hederagenin with an ethereal solution of diazomethane. This gave the desired product (**15**), as identified by the single spot observed by TLC at a retention time different to that of the starting material, in near quantitative yield (97 %). The proton NMR spectrum confirmed the identity of the product. Characteristic signals similar to those of the starting material were observed; once again the methyl groups attached to the triterpene were visible as six 1H singlets at δ 0.73 ppm, 0.90 ppm, 0.93 ppm, 0.96 ppm and 1.13 ppm. Signals were also observed at δ 2.88 ppm (1 H double doublet) for the proton at C-3 and a 1 H signal at δ 5.31 ppm for the olefinic proton. However, unlike the starting material, a

new 3 H singlet was observed at δ 3.63, this corresponds to the methyl ester that has been formed from the carboxylic acid attached at the C-28 position. Formation of a methyl ester by this means is common, and usually proceeds very rapidly; in this case, however, the reaction appeared to be rather sluggish and had to be stirred with the diazomethane solution for a considerable time to allow complete conversion into the methyl ester.

Having established that a methyl ester could be prepared it was necessary to find a preparative method to produce a larger quantity of the product. The first attempt used methanol and sulphuric acid in a manner similar to that used to prepare fatty acid methyl esters. Although this appears to be an efficient method in those cases, it did not appear to work for this compound. No change was apparent in the retention time of the recovered triterpene by TLC when compared to the unreacted starting material. Both the ^{1}H and ^{13}C NMR spectra also showed only signals consistent with those of the starting material. The 3 H singlet at approximately δ 3.6 ppm that was observed in the methyl ester formed by the previous method was not apparent in the proton NMR spectrum either, confirming that none of the desired product was obtained.

An alternative method was attempted, this time by reacting the aglycone with an excess of methyl iodide in the presence of a catalytic ammount of potassium carbonate. When allowed to react for 16 hours this method proved to be successful and the purified product was obtained in modest yield (41 %). The proton NMR spectrum appeared free of starting material and gave characteristic signals identical to those observed in the spectra of the methyl ester prepared with diazomethane. Furthermore MALDI MS identified the molecular ion corresponding to that expected for the desired product. This method of preparing a methyl ester would be expected to occur more rapidly than was observed and to give a higher yield of product, but once again the reaction was sluggish much as was the case with the diazomethane method. Even with a somewhat lengthy reaction time the yield remained only modest. However, despite these problems it still appears that this method is preferable for preparing these derivatives since the reaction conditions do not appear to cause any other change to the hederagenin. As such, the unreacted starting material could be easily recovered from the product by column chromatography and used for further reactions to acquire more of the desired product.

Finally a fourth approach was attempted, this time by attempting to first activate the acid function as an NHS ester in a manner similar to that used in preparing esters or amides in protein chemistry. To do this, hederaganin was reacting with N-hydroxysuccinimide in the presence of the coupling agent DCC in order to prepare the highly unstable activated acid (Scheme 3.1.1.2). This intermediate could then have been treated with a large excess of methanol in order to give the methyl ester. However, when the intermediate was prepared and treated with methanol for 18 hours none of the desired product was obtained. The recovered solution was filtered to remove the excess DCHU by-product present (analysis of the filter cake showed no triterpene product to be present in the filtered solid), the filtrate was evaporated to a white solid which was observed to also contain significant DCHU impurities. As such, this residue was purified by column chromatography to obtain only the triterpene component of this mixture. The purified material showed NMR spectra consistent with the hederagenin starting material and it appeared the same by TLC. No signal was identified by ^{1}H NMR consistent with the 3 H singlet expected for the new methyl ester group, confirming that the reaction was unsuccessful.



Scheme 3.1.1.2 Attempted preparation of the methyl ester of hederagenin via an active ester intermediate

The above method was attempted again, but this time using ethanol in place of methanol in order to identify if it was this that had prevented the success of the coupling. However, the reaction was once again unsuccessful and none of the corresponding ethyl ester was obtained either.

All of these difficulties appear to indicate the presence of some form of hindrance which would reduce the reactivity at this position. In order to identify this a 3-D

model of hederagenin (**2**) was constructed and the geometry optimised by molecular mechanics (Using the Tinker molecular modelling software with the MM3 force field and using the 3-21G basis set). The model (Figure 3.1.1.1) indicates a rather complex structure which actually leaves some functions sterically hindered and blocks attack of these reaction centres from certain directions. In the specific case of the carboxylic acid, which is shown in greater detail in Figure 3.1.1.2, it was calculated that the C-23 atom has access blocked from one side by the triterpene skeleton; the proximity of the C-26 and C-29 methyl groups is also notable in this model, which may also impart steric hindrance on the acid by limiting access to the position. This limit on the viable angle of attack would help to explain the sluggish nature of the reactions observed at this position. Furthermore, while there appears adequate space near the acid to accommodate the methyl ester, the shape of the molecule may prevent the access of more bulky groups and as such might account for the failure of the NHS ester reaction. If this is the case, it would prove a significant barrier to the preparation of other novel esters at this position.

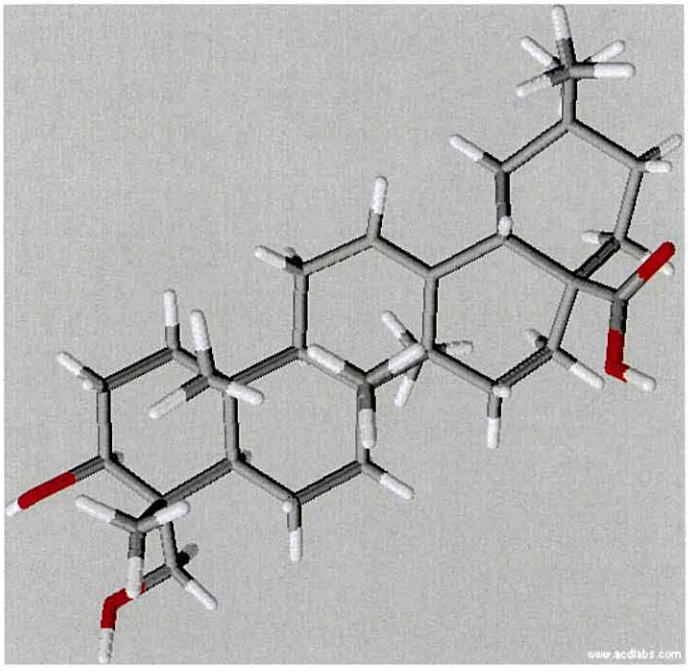


Figure 3.1.1.1 The 3 dimensional structure of hederagenin with geometry optimised by molecular mechanics; atoms coloured grey correspond to carbon, white to hydrogen and red to oxygen.

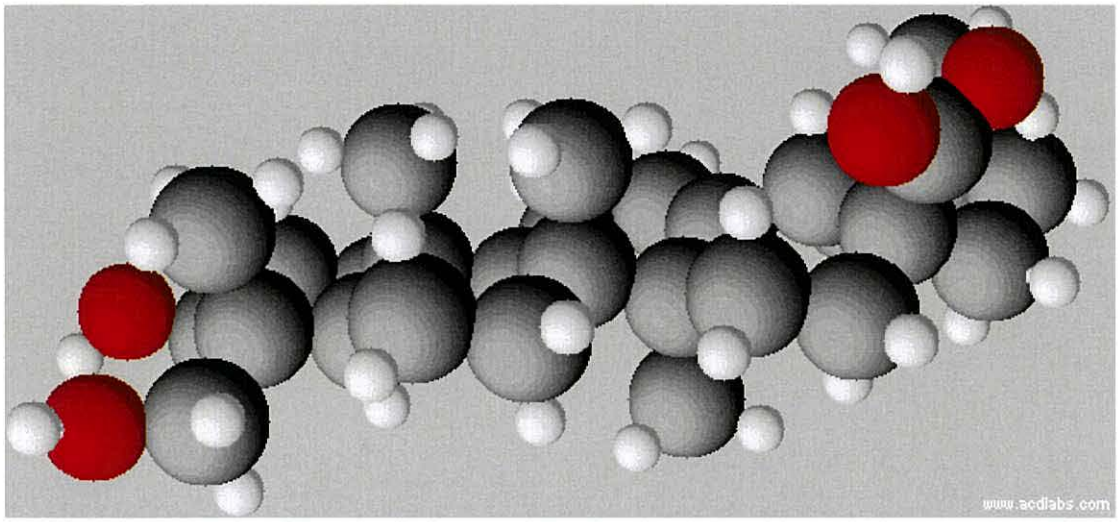
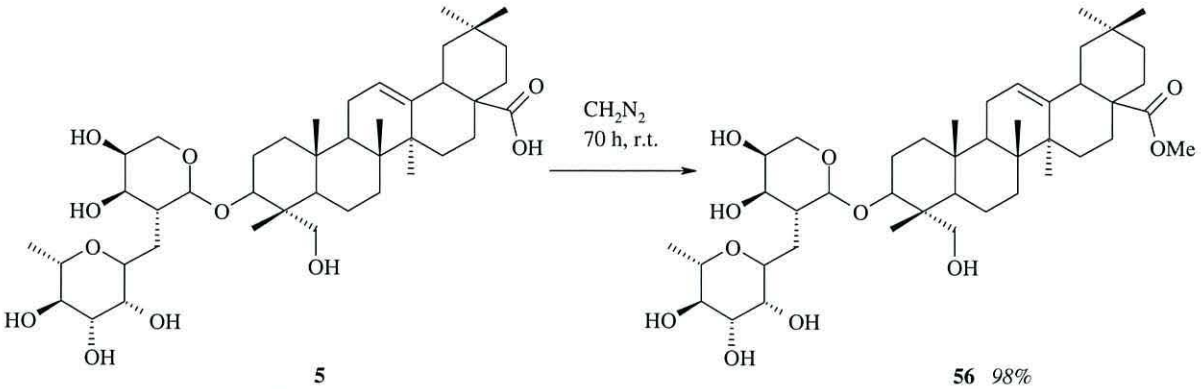


Figure 3.1.1.2 View of the 3 dimensional model of the structure of hederagenin, showing the steric hindrance surrounding the C-23 acid function

3.1.2 Preparation of the methyl ester of base hydrolysate

In order to prepare a methyl ester of the base hydrolysis product it was treated with diazomethane in the same manner as used previously with hederagenin. This afforded a yellow / off-white solid of the methyl ester product (**56**) in near quantitative yield (calculated based on the molecular weight of the principle saponin, α-hederin **5**). This appeared pure by TLC analysis and gave a shorter retention time than that of the starting material. Its identity was confirmed by comparison of its proton NMR spectrum to that of the unreacted base hydrolysis product; as expected it showed many similarities. It possessed the usual six 3 H singlets of the methyl groups, as well as the double doublet at δ 2.78 and olefinic proton at δ 5.11. It also had a multiplet from the attached sugar moiety at δ 3.38-3.55. However, a new signal was observed compared to that of the starting material; a 3 H singlet at 3.61 ppm corresponds to the new ester methyl group present. Also of note was a significantly lower and sharper melting point when compared to the starting material.



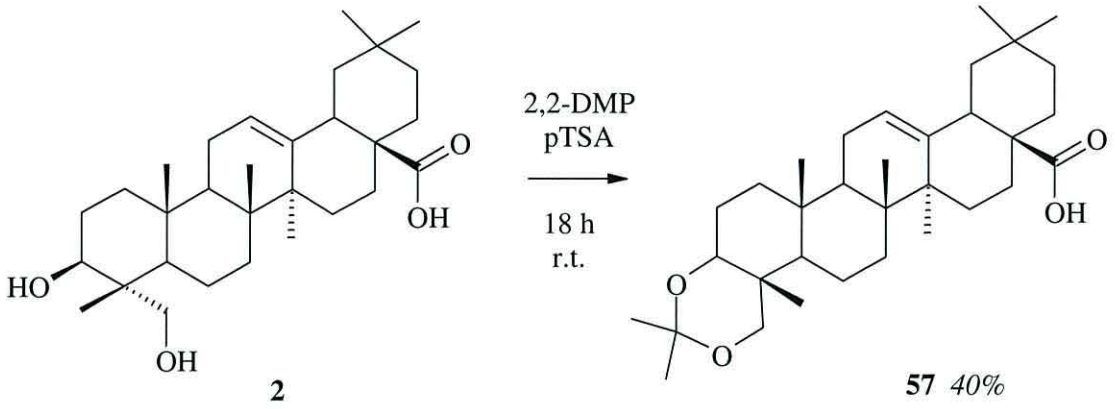
5 **56** *98%*

Scheme 3.1.2.1 Preparation of methyl ester of base hydrolysis product. N.B. The scheme illustrates only the principal saponin (α-hederin) of this composistion

Having successfully formed the methyl ester of the base hydrolysis product by this means an attempt was made to scale this up in the same manner as was done for the acid hydrolysis product (**2**). The same two methods were attempted as were used in that case: refluxing methanol and sodium hydroxide catalyst (this was used rather than the acid catalyst attempted in the case of hederagenin in order to eliminate the possibility of any unwanted cleavage of the sugar moiety) or with methyl iodide and potassium carbonate. In both cases, the reaction was found to be unsuccesful with only the unreacted starting material recovered.

3.2 Protection of hederagenin hydroxyl groups as an acetonide

Having established a means of readily protecting the acid function of the aglycone it was then deemed desirable to identify a means of protecting the hydroxyl groups by formation of an acetal. The first means of doing this was by forming an acetonide group across the two hydroxyl groups[147] (Scheme 3.2.1).



2 **57** *40%*

Scheme 3.2.1 Preparation of 3,23-*O*-acetonide protected hederagenin

This was achieved by stirring a solution of hederagenin (**2**) with an excess of 2,2-dimethoxypropane and a catalytic ammount of p-toluenesulfonic acid. The reaction was somewhat sluggish (18 hours) but proceeded successfully without heating to give the desired product (**57**), which upon purification by column chromatography, was obtained in modest yield (40 %). The proton NMR spectrum gave a number of signals similar to those of the aglycone, as expected. The distinctive pattern of 3 H singlets was seen at δ 0.53, 0.72, 0.75, 0.83, 0.94, 0.96 ppm identifying the methyl groups of the triterpene as well as the characteristic double doublet at δ 2.61 and the olefinic signal at δ 5.07. In addition to the usual signals of the aglycone a pair of 3 H singlets at δ 1.20 and 1.24 were observed; these signify the two non-equivalent methyl groups present within the acetonide group which has been formed. MALDI MS analysis of this product confirmed the identity of the molecular ion as that corresponding to the acetonide product. It is also noted that by IR spectroscopy that the very broad signal observed at 3300 cm^{-1} in the starting material corresponding to these hydroxyl groups was no longer apparent. Similarly to the aglycone, the acetonide protected product (**57**) was modelled, using molecular mechanics to calculate the geometry of the structure (Tinker software, using MM3 force field and 3-21G basis set). The 3-D structure of the acetonide chair is shown in Figure 3.2.1.

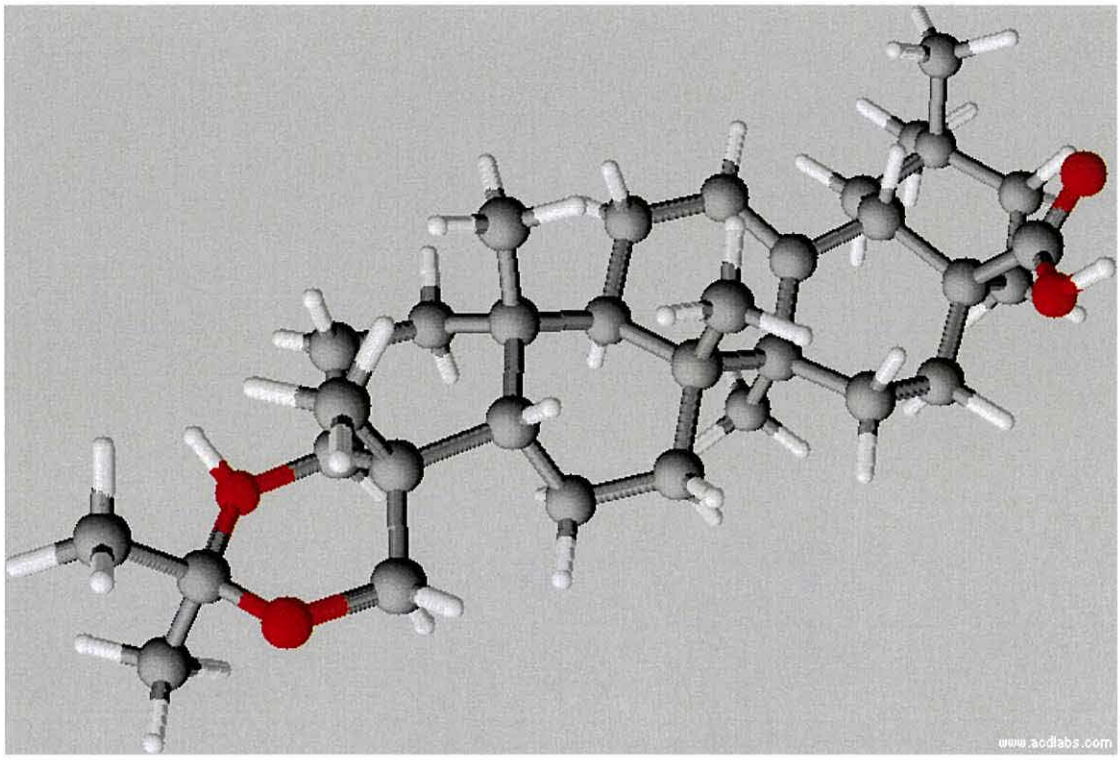
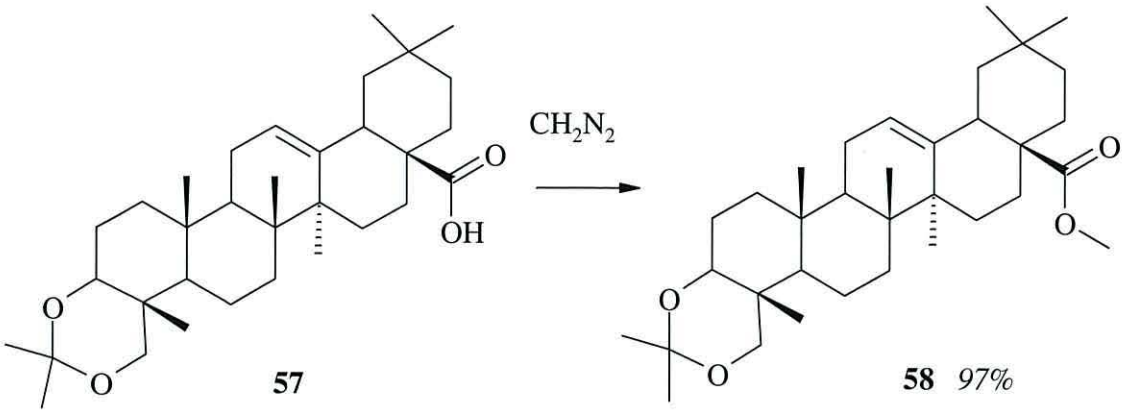


Figure 3.2.1 Molecular model showing the optimal geometry of the 3,23-*O*-acetonide of hederagenin with the two oxygen atoms shown in red (atoms number 40 and 42)

3.2.1 Preparation of methyl ester of acetonide

The acetonide protected hederagenin (**57**) when treated with diazomethane gave its corresponding methyl ester (**58**) (Scheme 3.2.1.1) in a similar manner to that discussed for the unprotected compound (**3.1**). Once again, after 16 hours, a pure product was obtained in near quantitative yield (97 %). By TLC analysis a single spot at a retention time different to that of the starting material was observed. The proton NMR spectrum appeared consistent with that expected for the compound showing the characteristic signals for the methyl groups of the triterpene at δ 0.64, 0.83, 0.86, 0.89, 0.98 and 1.07 ppm, as well as those of the acetonide methyl groups at δ 1.34 and 1.38 (all 3 H singlets). The double doublet at δ 2.79 and the triplet at δ 5.31 were also retained. This time however a rather prominent 3 H singlet also appeared in the spectrum at δ 3.55 ppm, indicating the presence of the new methyl ester attached to the C-28 position. The molecular ion identified by MALDI MS also confirmed the identity of the desired methyl ester product.

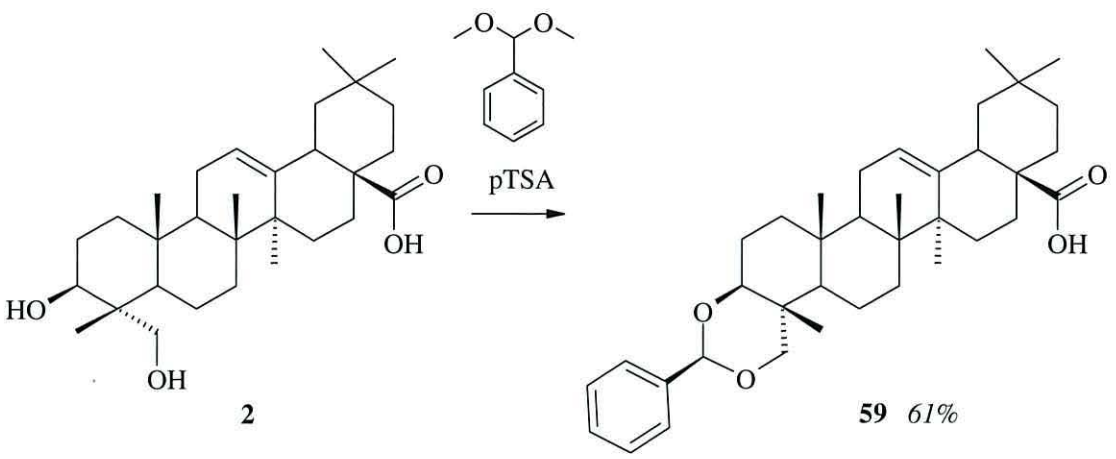


Scheme 3.2.1.1 Preparation of 28-*O*-methyl ester of acetonide protected aglycone

The same compound (**58**) was prepared on a larger scale with methyl iodide and potassium carbonate in the same manner as the methyl ester of hederagenin was previously (3.1). This method afforded the purified product in very good yield (80 %) compared to the same reaction performed on the unprotected compound; this is more than likely as a result of the improved solubility observed of the acetonide compound in the reaction solvent when compared with the unprotected hederagenin. The ^{1}H and ^{13}C NMR data was identical to the product prepared using diazomethane and again showed a new 3 H singlet at δ 3.64 ppm in the proton spectrum to indicate complete esterification. Furthermore, the MALDI MS data corresponded both with the calculated mass for the desired product and with that of the product prepared by the previous method.

3.3 Protection of hederagenin hydroxyl groups as a benzylidene acetal

The second means investigated to form an acetal across the two hydroxyl positions was to form a benzylidene acetal derivative[148] of hederagenin (**2**) (Scheme 3.3.1).



2 **59** *61%*

Scheme 3.3.1 Preparation of 3,23-*O*-benzylidene acetal protected hederagenin

The benzylidene acetal derivative (**59**) was prepared by a similar means to the acetonide, this time by stirring a solution of hederagenin (**2**) with an excess of benzaldehyde dimethyl acetal in the presence of a catalytic amount of p-TSA for 24 hours. After this time no further reaction was observed by TLC and the crude product was purified by column chromatography to afford the benzylidene acetal product in fair yield (61 %). Proton NMR analysis of this product showed the expected signals characteristic of the triterpene backbone, including the ubiquitous pattern of 3 H singlets at δ 0.80, 0.94, 0.96, 1.00, 1.19 and 1.20 ppm identifying the six methyl groups, as well as the characteristic double doublet at δ 2.88 ppm and the olefinic signal at δ 5.32 ppm. In addition to these signals several new ones were observed which corresponded to the new benzylidene acetal that had been formed. Most notable are the two 2 H multiplets and a 1 H multiplet observed in the aromatic region of the spectrum which identify the 5 protons attached top the aromatic ring of the benzylidene; these are accompanied by a new 1 H singlet observed at δ 5.55 ppm corresponding to the proton attached to the carbon bridging the protecting group and the two oxygen. From this spectrum it appeared that only one stereochemistry of the product was present. In order to identify which stereoisomer would be formed, with the phenyl group up or down, a molecular model of the product was made and the geometry calculated by molecular mechanics (Figure 3.3.1). This was done using the Tinker software under the same conditions used previously for the aglycone model (MM3 force field, 3-21G basis set). This model showed that the more favourable geometry of the product would be that with the phenyl group of the acetal in the equatorial position (*cis*- to the 4-related methyl) and the proton down. The structure

of the region surrounding the acetal clearly explains why only this isomer was formed.

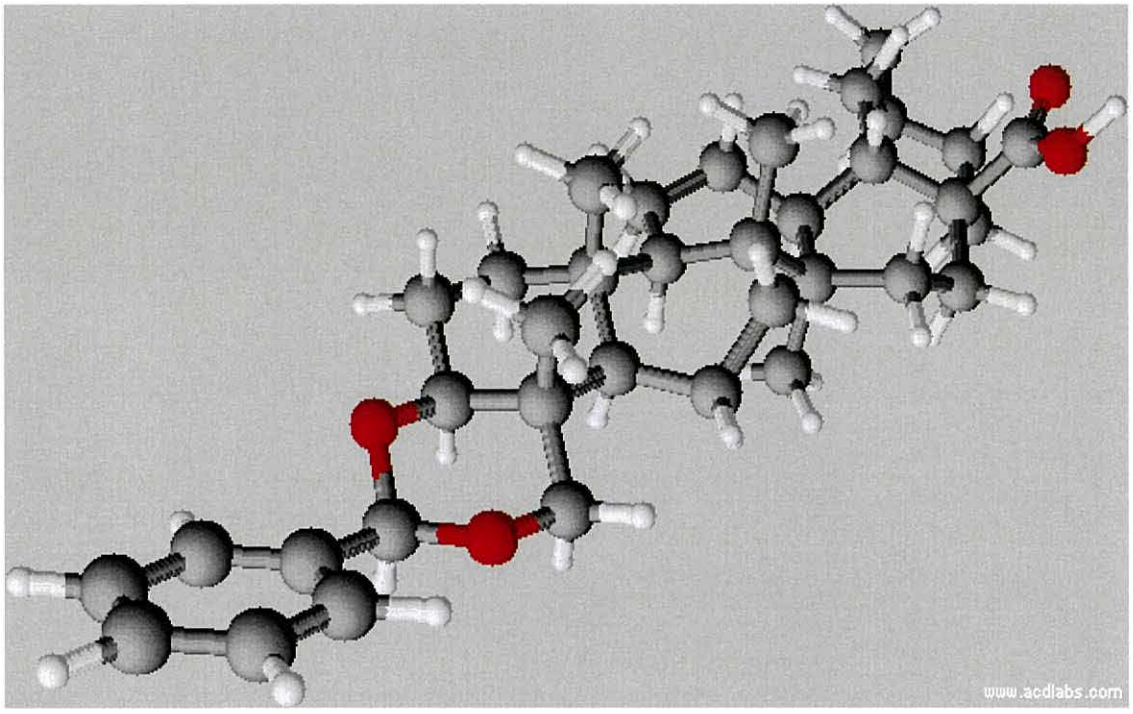


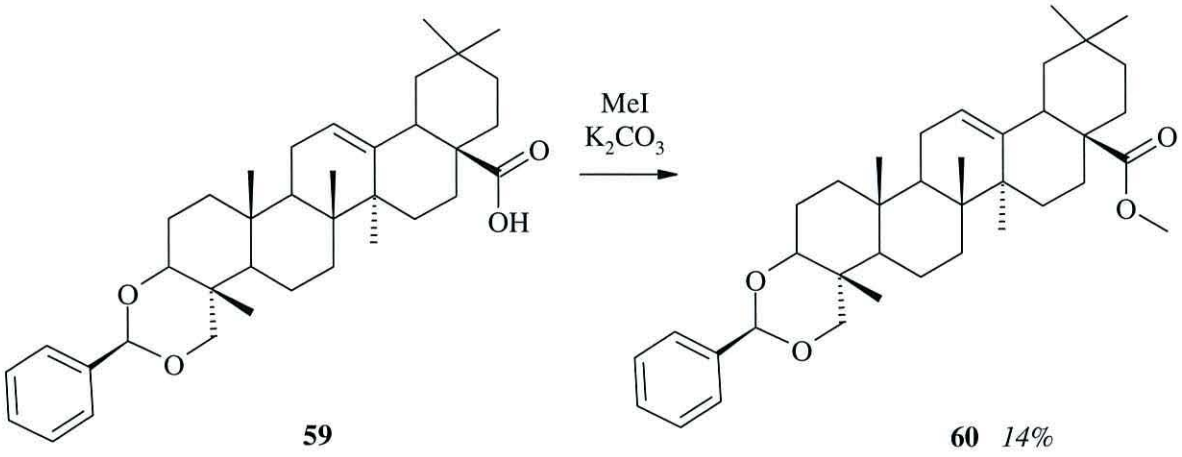
Figure 3.3.1 Molecular model showing the optimal geometry of the 3,23-*O*-benzylidene acetal of hederagenin; showing the most favourable stereochemistry has the phenyl group up relative to the triterpene chairs

Further to the NMR data, MALDI MS analysis of this product confirmed the identity of the molecular ion as that corresponding to the benzylidene acetal product. It is also noted that the IR spectrum of the product, although largely similar to the starting material does show some additional C-H stretches in the region between 3160 – 3000 cm^{-1} which represent bonds in the new aromatic group.

3.3.1 Preparation of methyl ester of benzylidene acetal

The methyl ester of the benzylidene acetal derivative (**60**) was prepared with methyl iodide and potassium carbonate in the same manner as the methyl ester of hederagenin (**3.1**) and its acetonide were previously (**3.2.1**). This method afforded the purified product in only poor yield (14 %). By TLC analysis a single spot at a retention time different to that of the starting material was observed. The proton NMR spectrum appeared consistent with that expected for the compound showing the

characteristic signals for the methyl groups of the triterpene at δ 0.80, 0.94, 0.96, 1.00, 1.19 and 1.20 ppm, the double doublet at δ 2.88 and the olefinic triplet at δ 5.32, as well as the signals of the benzylidene acetal group (several signals for the aromatic protons between δ 8.15 ppm and 7.36 ppm and a 1 H singlet at δ 5.55 ppm). This time however, a new 3 H singlet also appeared in the spectrum at δ 1.44, indicating the presence of the new methyl ester attached to the C-28 position. The molecular ion identified by MALDI MS also confirmed the identity of the desired methyl ester product.



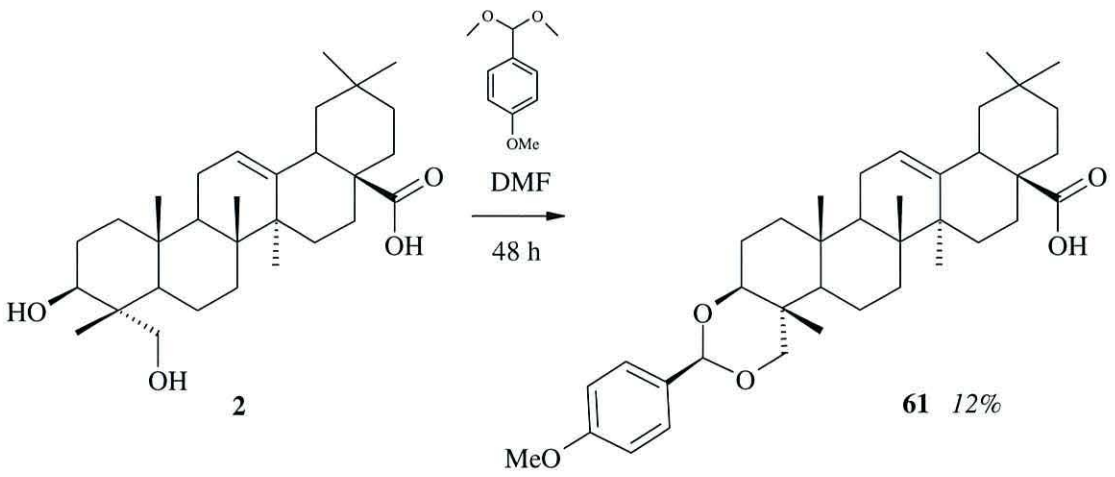
59 **60** *14%*

Scheme 3.1.1.1 Preparation of 28-*O*-methyl ester of benzylidene acetal protected hederagenin

3.4 Protection of hederagenin hydroxyl groups as a p-methoxybenzylidene acetal derivative

In a similar manner to the benzylidene acetal, the final acetal derivative prepared was the p-methoxybenzylidene acetal (**61**) (Scheme 3.4.1). This time a solution of hederagenin (**2**) in dry DMF was stirred at room temperature for 48 hours with methoxybenzaldehyde dimethyl acetal in the presence of catalytic p-TSA. At several stages during the work up and purification a white solid of the unreacted starting material was recovered, which accounts for the poor yield (12 %) of the purified methoxybenzylidene acetal product obtained. However, taking into consideration the amount of pure starting material that could be recovered, it appears that the effect of this low yield can be to some extent mitigated by returning this material into repeat reactions in order to prepare further product. The product obtained showed signals in its proton NMR spectrum very similar to those of the benzylidene acetal prepared

previously. However the notable difference between the two spectra is only observed in the aromatic region, this time only two aromatic signals are observed, two 2 H broad doublets appear at δ 7.44 and 6.89 coupled to one another. As this time only four protons are present in the aromatic ring of the protecting group, it is these that are identified by these doublet signals, the symmetry present in this ring leading to this coupling pattern, strictly an AA'BB' system. These are accompanied by a 1 H singlet at δ 5.50 corresponding to the proton attached to the acetal carbon bridging the protecting group. As with the benzylidene acetal (**60**), only one isomer was observed in the NMR, which appears to be that with the methoxy phenyl group equatorial and *cis*- to the 4-methyl was identified as the optimal geometry by molecular modelling of this structure. Analysis by MALDI MS confirmed the identity of the molecular ion as that corresponding to the methoxy-benzylidene acetal product. As with the benzylidene acetal, the IR spectrum showed the same additional C-H stretches in the region between 3160 – 3000 cm^{-1} which represent bonds in the new aromatic group.



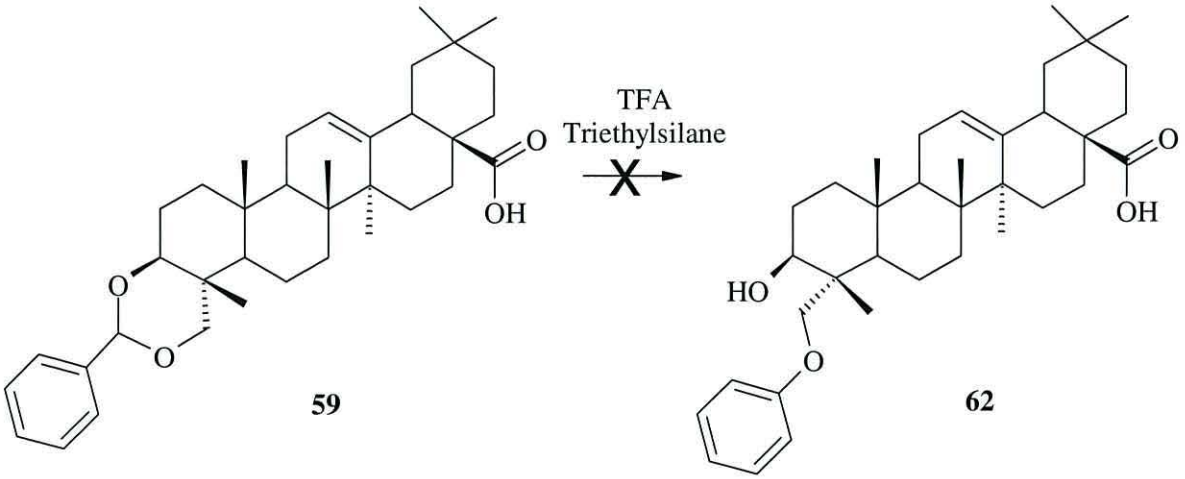
Scheme 3.4.1 Preparation of 3,23-*O*-p-methoxybenzylidene protected hederagenin

3.5 Attempted partial deprotection of hederagenin acetal derivatives

Literature shows that it is possible in some cases, where a diol has been protected as a benzylidene acetal or a methoxybenzylidene acetal, to perform a deprotection step in a way that deprotects only one hydroxyl group while leaving the protecting group attached to the other.[149-151] Such a method would prove a useful means of obtaining a selectively protected aglycone molecule that could then be used to prepare esters at specific positions or to direct the attachments of sugars if they were to be coupled to the molecule.

3.5.1 Attempted partial deprotection of benzylidene acetal

Treatment of a solution of a benzylidene acetal and excess triethylsilane with an excess of triflouroacetic acid in a drop-wise manner at 0 ° C is reported to partially remove the protecting group and leave the primary benzyl and a secondary hydroxyl group.[151] Attempts were made to carry out this deprotection on the benzylidene acetal of hederagenin (**59**) in order to prepare such a mono-substituted product (**62**) (Scheme 3.5.1.1) but the reaction was found to be unsuccessful. Even when the conditions were very carefully controlled and the reaction followed by TLC it was only possible to isolate two triterpene components from the reaction mixture. The first was consistent by ^{1}H and ^{13}C analysis with the unreacted benzylidene acetal (**59**), the second gave signals corresponding to unprotected hederagenin (**2**). It therefore appears that the cleavage of the protecting group, where it did occur, took place to completion and that the desired singly protected product may not have been stable enough to obtain without it too being deprotected by the reaction conditions.

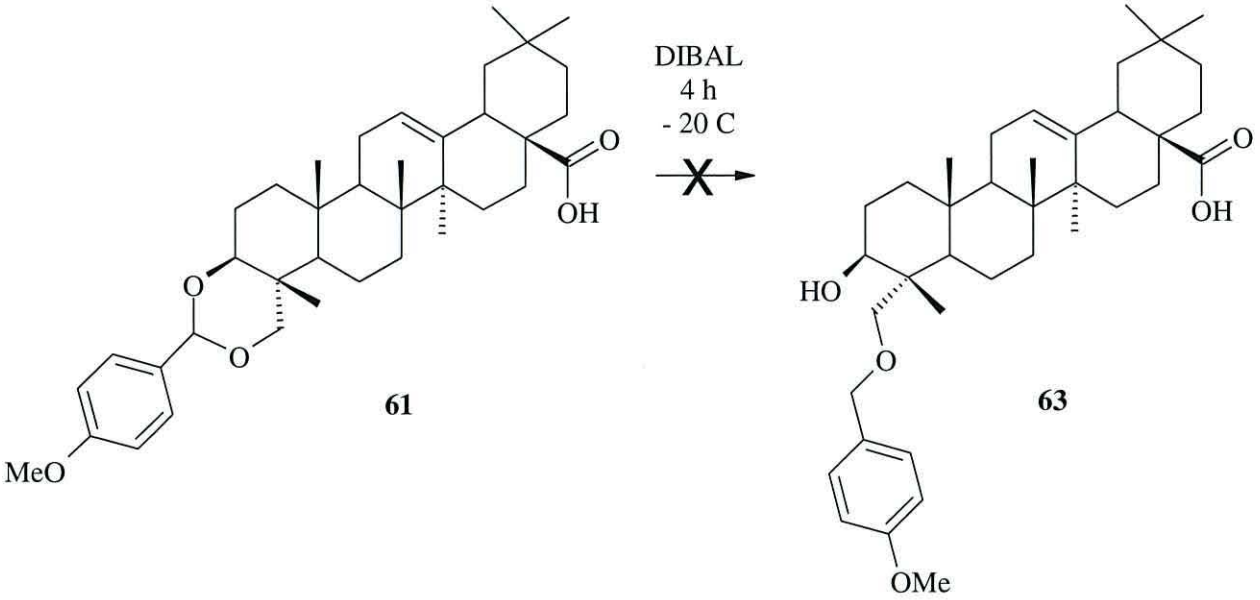


59 62

Scheme 3.5.1.1 Attempted partial deprotection of 3,23-*O*-benzylidene hederagenin

3.5.2 Attempted partial deprotection of p-methoxybenzylidene acetal

Having failed to isolate the partially deprotected the benzylidene acetal an alternative method was found for a similar deprotection of p-methoxybenzylidene acetal to its corresponding mono-substituted product.[152] An attempt was made to ring open the methoxybenzylidene acetal (**61**) by treating a cold (- 20 ° C) solution of the acetal with an excess of DIBAL for 4 hours in order to remove the protecting group so as to leave a product (**63**) with a primary p-methoxybenzyl group and a secondary hydroxyl group (Scheme 3.5.1.1). Once again though, the reaction was unsuccessful and the entire protecting group was instead cleaved completely. ^{1}H NMR analysis of the crude product showed only signals corresponding to unprotected hederagenin and to the free aromatic fragment cleaved by the reaction. No signals were identified for the desired mono-substituted product, nor were there any consistent with the methoxybenzylidene acetal starting material either, indicating that it had all been deprotected to hederagenin.



Scheme 3.5.2.1 Attempted partial deprotection of 3,23-*O*-p-methoxybenzylidene hederagenin

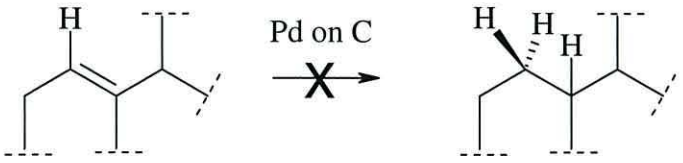
3.6 Reactions at the olefinic position of hederagenin and its protected derivatives

Of the main sites at which it was deemed possible to modify the hederagenin, the initial attempts at protecting the acid and hydroxyl functional groups had indicated that (although difficult in some cases) it was possible to successfully modify these positions. With this in mind it next seemed important to identify whether the carbon-carbon double bond present in the molecule was a viable position at which to modify this molecule. The reason for trying to react at this position were two-fold; firstly as a route to prepare further novel derivatives of hederagenin and secondly, since modifying this olefinic group would in turn alter the shape of the hederagenin molecule, whether this change in structure was a significant factor in terms of the activity of hederagenin.

3.6.1 Attempted hydrogenation of double bond in aglycone and acetonide

The first logical means to modify the olefinic portion of the molecule was by what appeared to be the simplest transformation of this type of bond, a hydrogenation (Scheme 3.6.1.1). The first attempt at this was to hydrogenate the double bond with hydrogen gas in the presence of a palladium catalyst. This was performed on both the hederagenin (**2**) and its acetonide protected derivative (**57**). Despite being allowed a considerable amount of time to react, the reaction was unsuccessful for both compounds. Proton NMR analysis of the products appeared to indicate that only

unreacted starting material was recovered; the 1 H triplet at around δ 5.3 ppm was conclusive evidence that the proton attached to the double bond remained unchanged.



2 64

Scheme 3.6.1.1 Attempted hydrogenation of hederagenin double bond

Since other reactions of hederagenin were observed to be sluggish it seemed that this problem may also be due to low reactivity. It appeared that it might be possible to overcome this by carrying out the reaction in a high pressure vessel. Given the large capacity of the reactor available it was deemed necessary to carry out the reaction at a larger scale than done previously in order to ensure the efficiency of the reaction. It was also required that for safety reasons the reaction be conducted using water as solvent in place of the ethyl acetate used previously. Due to these factors it seemed sensible to carry out this experiment initially on the TTS extract rather than on hederagenin as it was more readily available in higher quantity and would be soluble in the water without having to use the organic solvents needed to prepare a similar solution of the aglycone. Having established this, an aqueous solution of the TTS extract was prepared and reacted with hydrogen under high pressure (10 bar) and with heating (40 °C) in the presence of a palladium on charcoal catalyst for 4 hours, after this time a portion of the reaction mixture was analysed by proton NMR analysis and showed that the characteristic triplet of the olefinic proton was still present. The hydrogenation was therefore continued under the same conditions for a further 5 hours before working up the resulting reaction mixture. The white solid recovered was analysed again using proton NMR spectroscopy; once again the olefinic proton was still clearly visible in the spectrum, furthermore there was no indication of any other TTS signals corresponding to the hydrogenated product. Given this observation it appears that even under these conditions, the double bond appears resistant to hydrogenation by this type of technique, likely due to the steric hindrance of the rest

of the molecule preventing the double bond readily accessing the surface of the heterogeneous catalyst.

A final attempt was made at hydrogenating these compounds, this time using a method without a palladium catalyst. Instead, solutions were prepared of both hederagenin (**2**) and of its more soluble acetonide derivative (**57**) and these cooled solutions treated with diimide (prepared from dipotassium azodicarboxylate and acetic acid) for 26 hours. However when the reaction was worked up none of the desired product (**64**) was obtained. In the case of both the hederagenin and the acetonide the 1 H triplet of the olefinic proton was still observed in the proton NMR spectrum, indicating that the recovered triterpene was only unreacted starting material and that the hydrogenation did not take place and instead the diimide which had visibly been consumed must have instead degraded in preference to reacting with the double bond in the desired manner. This is not an unexpected result, as this method of hydrogenation is less powerful than that previously attempted using hydrogen gas and palladium; however it seemed a worthwhile route to investigate as the diimide may have been more capable of accessing the hindered double bond (although it is itself still a fairly large molecule and it seems that it too may have been affected by the same steric hindrance). The 3-D model of hederagenin calculated earlier shows this to be the case (Figure 3.6.1.1 and 3.6.1.2). The structure of the triterpene chairs and methyl groups effectively blocks access to the bond from one face; the other face, while accessible also appears to be subject to some hindrance from nearby methyl groups which reduces the viable angle of attack. These structural details could account for the inability of these large reagents and catalysts to successfully access and hydrogenate the alkene bond.

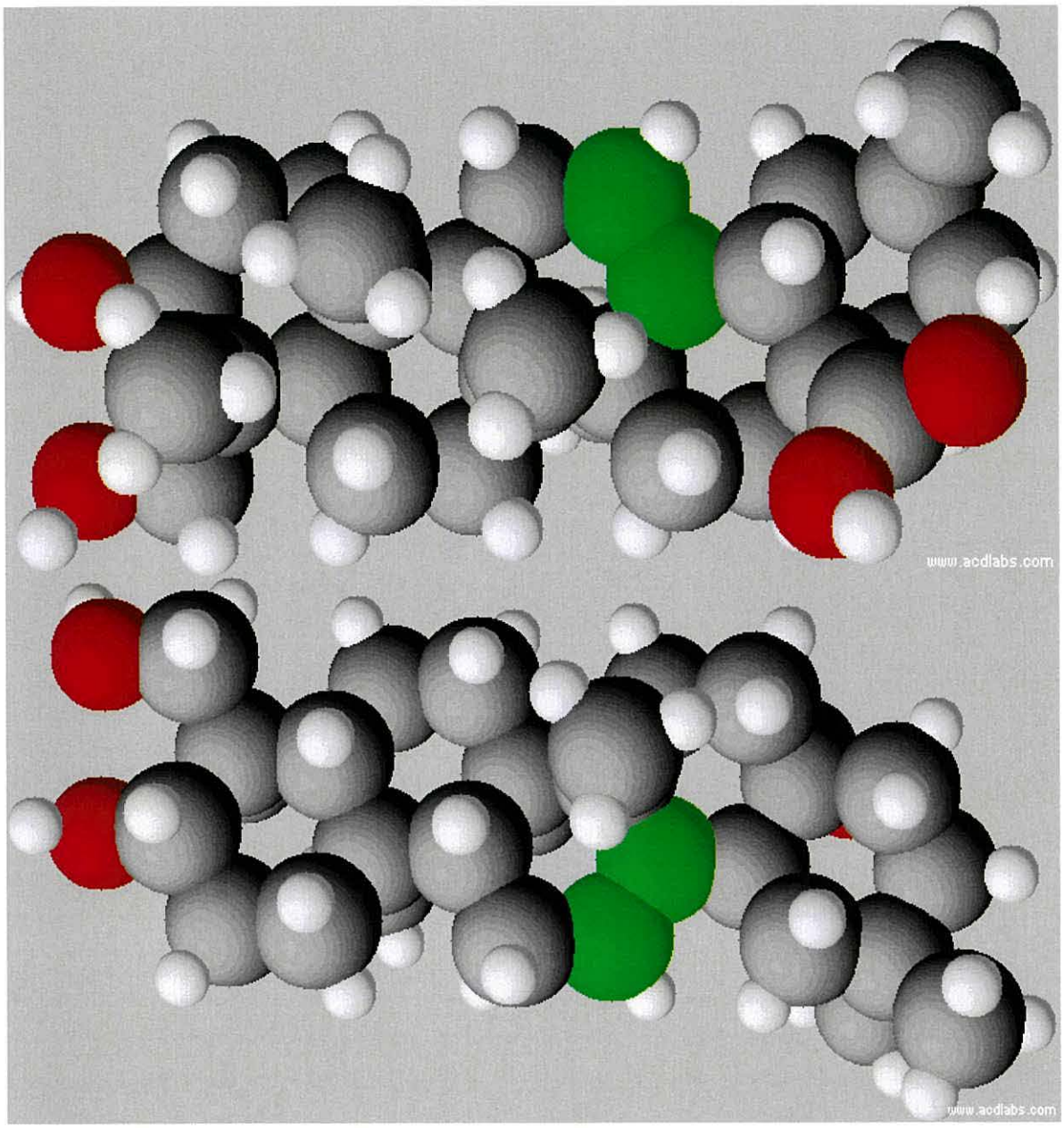


Figure 3.6.1.1 Molecular model of hederagenin showing the geometry of the structure surrounding the double bond from above and below the plane of the triterpene rings

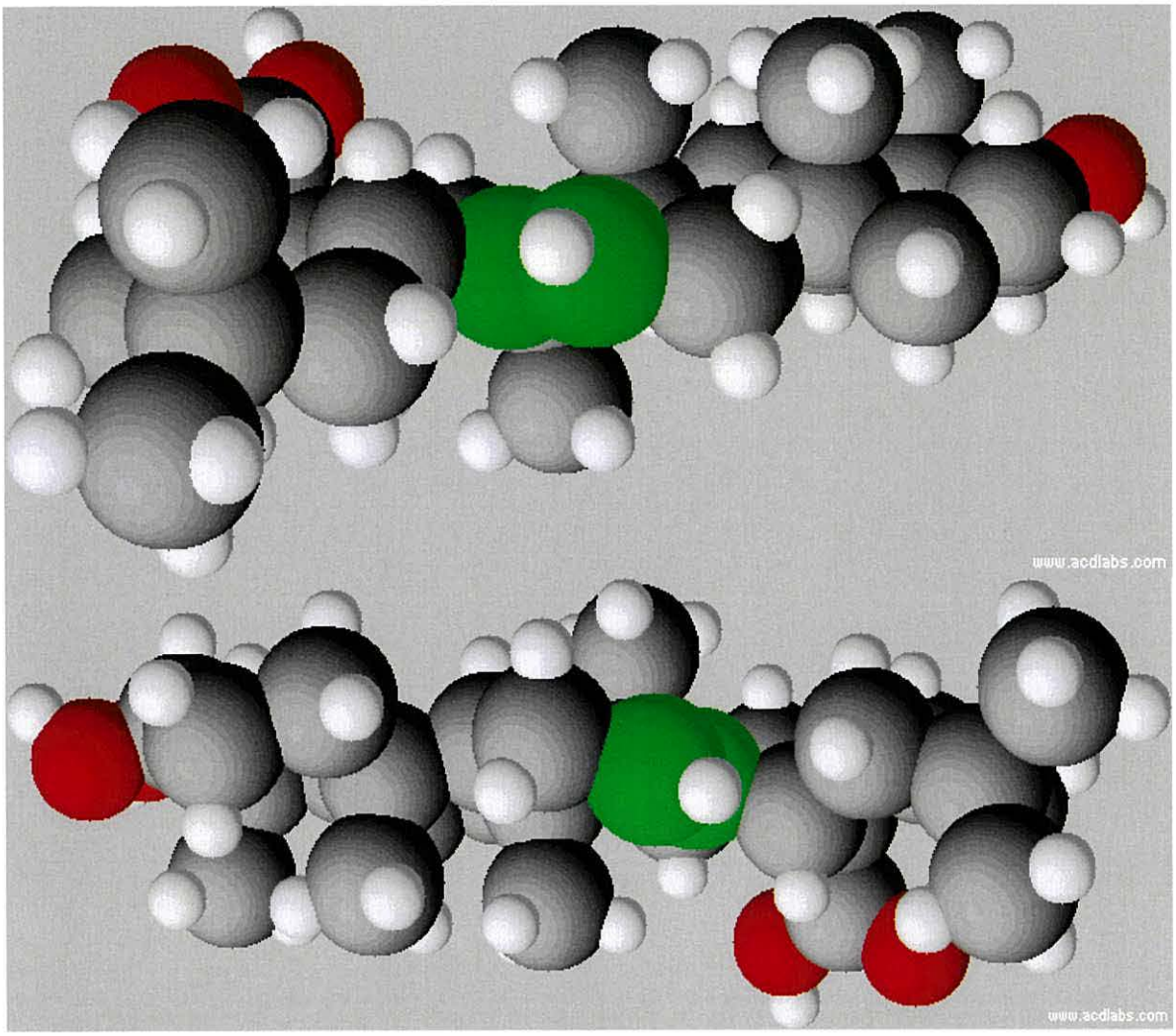
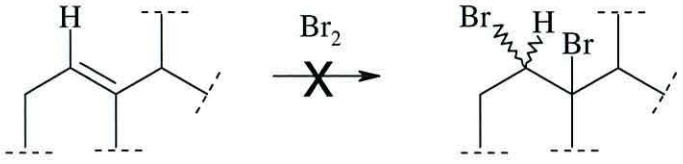


Figure 3.6.1.2 Molecular model of hederagenin showing the geometry of the structure surrounding the double bond from perpendicular the triterpene rings

3.6.2 Attempted bromination of double bond in aglycone and acetonide

Having failed to identify a means of hydrogenating the carbon-carbon double bond present in these compounds, an attempt was made instead to carry out another standard reaction of olefins and to try to add bromine to it (Scheme 3.6.2.1). This was done by treating a dichloromethane solution of hederagenin (**2**) with a single equivalent of bromine and stirring for 48 hours, after which time it was expected the orange solution would have been decolourised by the reaction; instead the colour remained. Upon working up the reaction and purifying the residue, only one triterpene fraction was identified by TLC with the same retention time as hederagenin

(**2**). Analysis of this by proton NMR showed signals consistent with the unreacted starting material, including that characteristic of the unchanged olefinic proton; no signals were identified for the brominated product (**65**). When the same procedure was repeated for the acetonide (**57**) protected derivative of hederagenin, the results also indicated that no reaction had taken place.

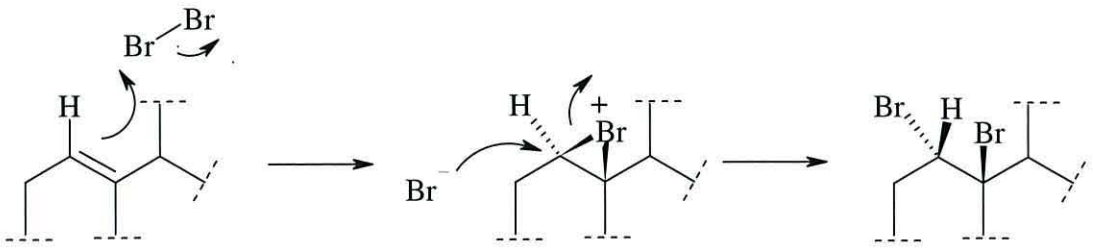


2 **65**

Scheme 3.6.2.1 Attempted bromination of the olefinic position of hederagenin

Since there is evidence to indicate that halogenation reactions can sometimes be optimised by carrying out the reaction at cold temperatures the bromination was attempted once more, this time with constant cooling to ensure the reaction remained below - 35 °C. After two hours there appeared to be no visible change in the colour of the bromine solution and no change was identified by TLC analysis. The cooling was ceased and the reaction mixture stirred at room temperature for a further 48 hours, but again no reaction was observed and only starting material was recovered.

These results appear to further indicate the low reactivity of this position, likely as a result of similar steric hindrance as discussed for the hydrogenation in 6.3.1. Further to this is the complication which this hindrance results in when considered in terms of the mechanism of the bromination (Scheme 3.6.2.2). Bromination of the alkene proceeds by the formation first of the bromonium ion by electrophilic addition before this is opened in an S_N2 reaction that results in inversion to give the *trans*-stereoisomer as shown. However, due to the geometry of the molecule, this product would be very unfavourable as the downward position is subject to significant steric hindrance from the C-27 methyl group which would prevent the bromine atom moving into this position.



Scheme 3.6.2.2 Mechanism of the bromination of the olefinic position of hederagenin showing the electrophilic addition of bromine to give an intermediate bromonium ion followed by inversion to the product

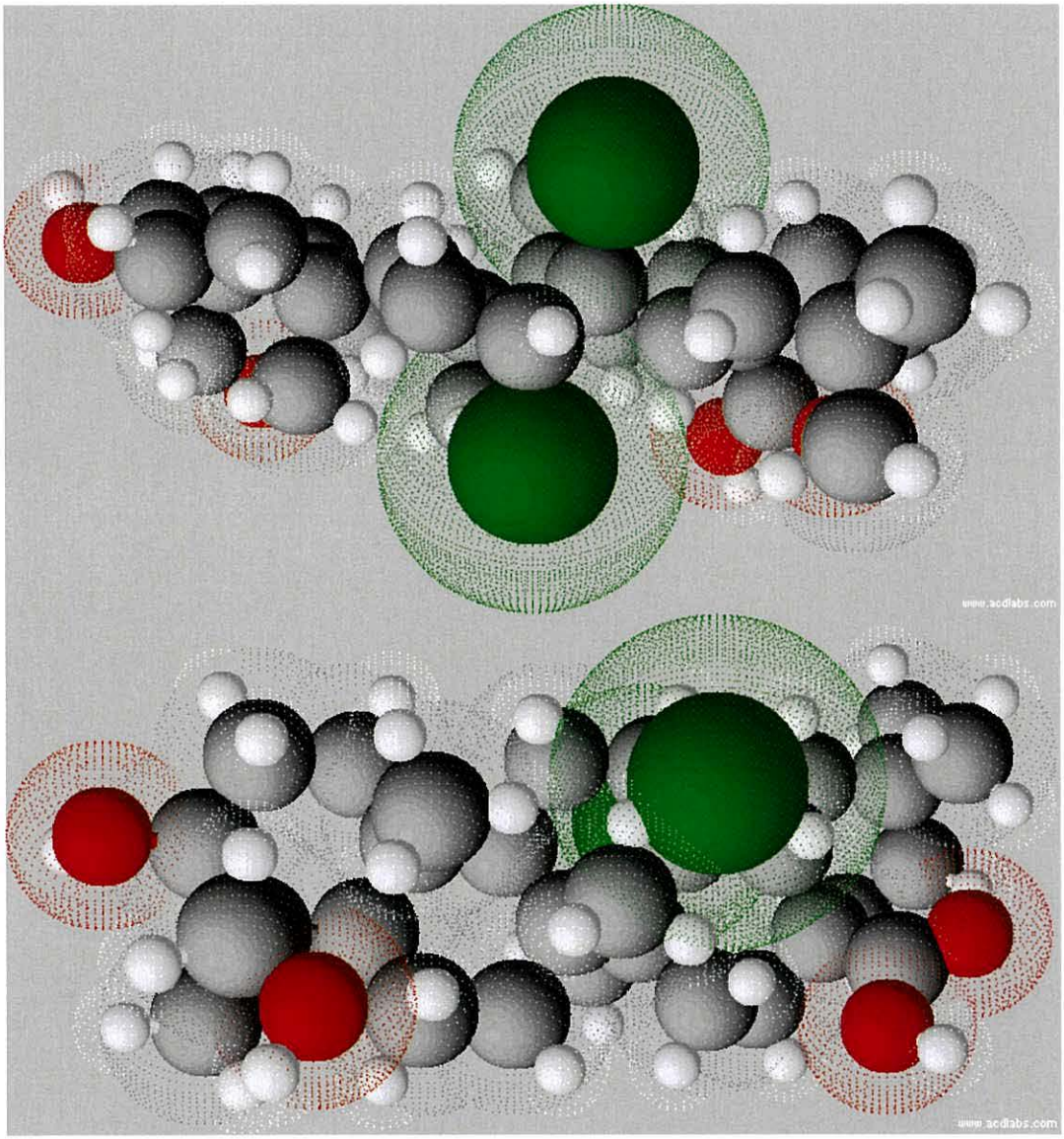


Figure 3.6.2.1 Molecular model of the trans structure of the dibromide product demonstrating the unfavourable proximity of a bromine atom to the C-27 methyl group; the bromine atoms shown in green

When the lowest energy geometry of the product was calculated by molecular mechanics (using Tinker, MM3, 3-21G basis set), it was found to be that of the cis-stereoisomer in which the bromine atoms were both on the less hindered face of the chair (Figure 3.6.2.2), therby maximising the angle between the bromine atom and the methyl group. Since the mechanism of bromination does not allow for the formation of this product it further explains the failure to successfully form the dibromo-product.

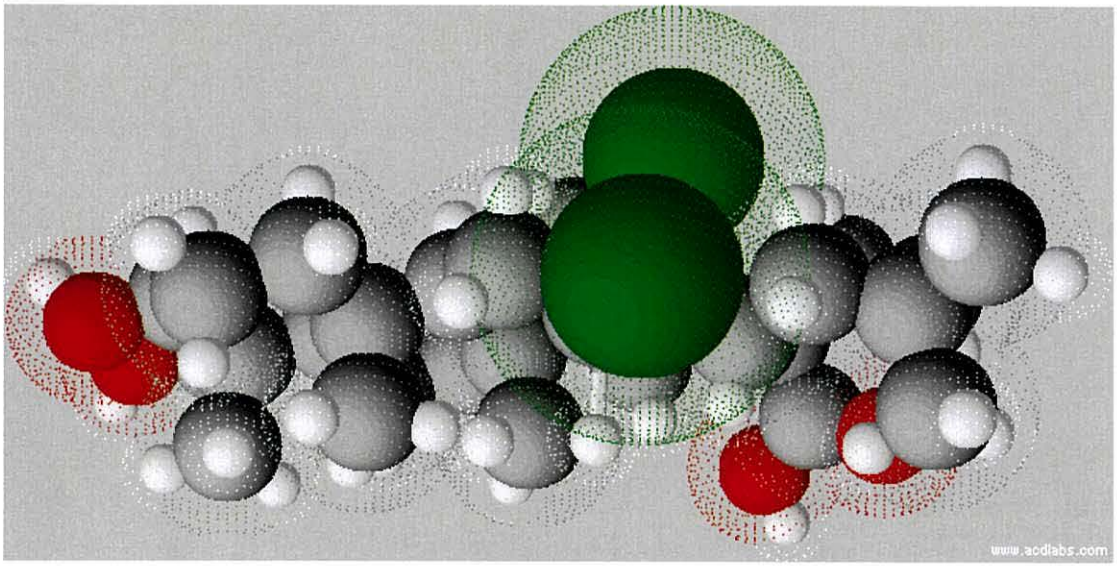
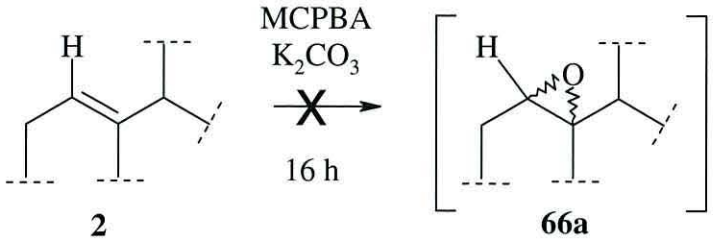


Figure 3.6.2.2 Molecular model of hederagenin showing the lowest energy structure of the cis-dibromide product; the bromine atoms shown in green

3.6.3 Epoxidation of double bond in aglycone and acetonide

Another attempt was made to attack the carbon-carbon double bond, this time by forming an epoxide with excess MCPBA and sodium hydrogen carbonate. Once again, both hederagenin (**2**) and its acetonide (**57**) were used as starting materials. The reactions were initially carried out in a routine manner using only a slight excess of MCPBA in order to minimise the difficulty of removing the unreacted reagent from the recovered products. However, after 16 hours there appeared to be no signs of reaction as indicated by TLC analysis of the crude reaction mixtures. The mixtures were worked up and the recovered material analysed by proton NMR spectroscopy which gave spectra corresponding only to the unreacted starting materials with no signals present for the desired epoxide products (**66a**) or any corresponding decomposition products.



2 **66a**

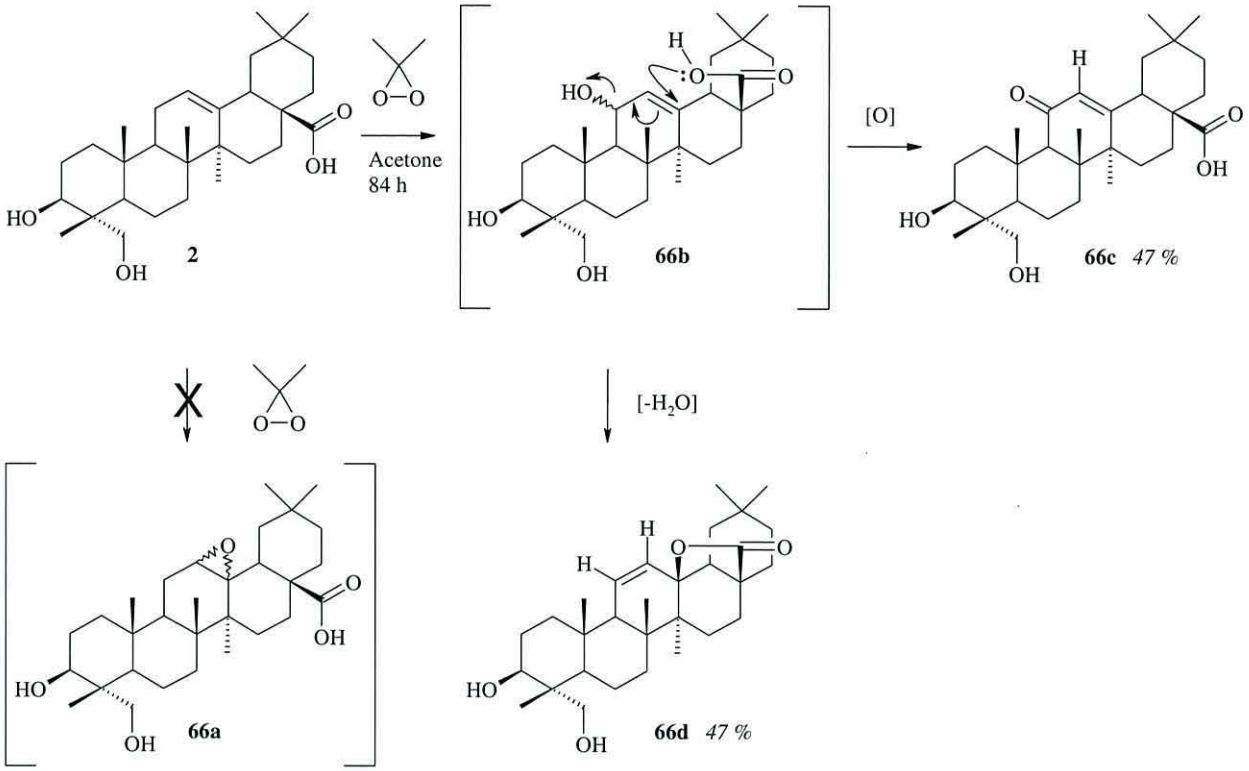
Scheme 3.6.3.1 Attempted epoxidation of the olefinic position of hederagenin with MCPBA

Given that this approach was unsuccessful the same method was again attempted, this time using a large excess of MCPBA (10 mol equivalents). The reaction was again followed by TLC analysis, but after 16 hours there was still no evidence of any reaction taking place. Upon work-up and purification to separate the triterpene from the excess of MCPBA it again appeared that only the hederagenin starting material was recovered with no epoxide present.

Since the previous method had failed to successfully epoxidise hederagenin or its acetonide derivative it seemed that an alternative route would be required. Furthermore, it had appeared from all of the previous attempts to attack this hindered position that no modification had been successful at this bond using the largely bulky reagents available. For this reason, the final attempt to form an epoxide at this position used was carried out using the small dioxirane compound DMDO as reagent (Scheme 3.6.3.2). A fresh portion of DMDO was prepared by the reaction of acetone with oxone. The resultant DMDO solution in acetone was added directly to a portion of hederagenin (**2**) and allowed to stir at room temperature for 84 hours to ensure the reaction was complete. As the only by-product of this reaction is acetone the reaction mixture was removed *in vacuo* with no need for any work up. The resulting solid of crude product was first dried and then crystallised from ethyl acetate with ice cold petrol to recover a triterpene product giving a single spot by TLC. MALDI MS analysis of this product identified a single molecular ion with a higher mass than the starting material but different to that expected for the epoxide (**66a**). ^{1}H and ^{13}C NMR data also showed no signals expected for the epoxide product; instead it appeared that two alternative products had formed in approximately 1:1 ratio. This was evident as there appeared to be a large number of different carbon environments present in the ^{13}C NMR spectrum and two distinct sets of signals in the ^{1}H NMR

spectrum integrating to 1 and 0.5 respectively. The first of these products appeared to be an enone (**66c**). This was indicated by some changes to the NMR spectra of the product when compared to hederagenin. The proton NMR spectrum of the product showed that the signal corresponding to the proton attached to the double bond was no longer visible as a 1 H triplet but instead as a singlet as it was no longer adjacent to CH_2, shifted downfield to δ 5.70 ppm as it is now part of the conjugated α,β-unsaturated system of the enone (**66c**). The equivalent compound of oleanolic acid (**46**) prepared by Iida et al. reported a similar signal for this proton (1 H singlet at δ 5.54 ppm).[111] The second product identified was a lactone (**66d**), which showed a 2 H multiplet at δ 4.8 ppm. Overlapping signals for each compound were also seen as a 2 H multiplet at ca. δ 3.92 and two doublets at 3.70 and 3.69 ppm corresponding to the protons at C-23 as well as broad double doublets at δ 3.10 and 3.00 ppm representing the proton at C-18 of the two products. Further evidence of the formation of these new compounds was seen in the ^{13}C NMR spectrum which showed signals corresponding to carbonyl carbons of the lactone (**66d**) at δ 184.0 ppm and of the enone (**66c**) at δ 179.8 ppm, far further downfield than any signals in the starting material. The molecular weight of both products was identical to that of the molecular ion identified by MALDI MS.

It seems likely that both of these new products were formed from oxidation of hederagenin to first form an alcohol intermediate (**66b**) rather than the expected epoxide (**66a**) (Scheme 3.6.3.2). This would then be further oxidised in the reaction mixture to afford the enone product (**66a**) or undergo lactonisation by the mechanism shown followed by dehydration to the corresponding product (**66b**)



Scheme 3.6.3.2 Epoxidation of hederagenin with DMDO and subsequent conversion to the enone and lactone product

The products are consistent with those identified in the literature where oleanolic acid and ursolic acid were treated with DMDO under similar conditions.[111] In these experiments, the enone and lactone products such as these were also observed to form as a result of allylic oxidation by the DMDO and not epoxidation. In the case of ursolic acid (an alpha-amyrin type saponin, Figure 1.2.3.1, having one methyl on C-19 and one on C-20 rather than two methyl groups on C-20), the corresponding products were the only oxyfunctionalised products obtained; the proposed explanation was that steric hindrance posed by the C-29 methyl group (which is attached at the C-19 position of this aglycone rather than the C-20 as is the case of hederagenin) had prevented epoxidation and so resulted in the alternative oxidation taking place instead. In the case of oleanolic acid, which does not possess such great hindrance from the methyl group on C-19, an epoxide intermediate was claimed to form but the resulting ring opening products appear inconsistent with anything observed in the hederagenin reaction.

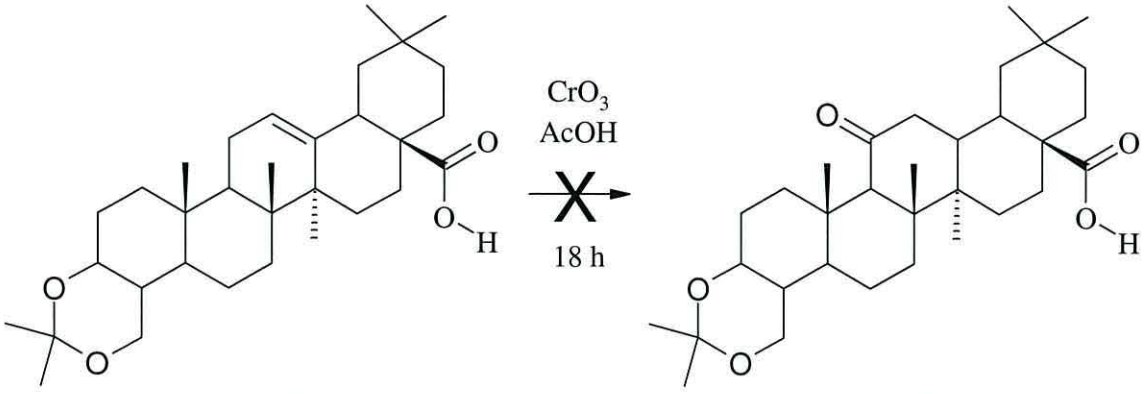
The same procedure was carried out on the acetonide protected aglycone (**57**) using DMDO solution from the same batch; however upon analysing the residue from this

reaction it appeared to contain only unreacted starting material. The reason for this, given the success of the unprotected aglycone remains unclear.

Given the facile means of preparing oxidation products by this route, as well as the high combined yield obtained (owing largely to the absence of any need for work-up to obtain a mixture of the compounds with no by-products) this appears to be a very desirable means of modifying the olefinic function of hederagenin. However, it seems clear from the fact that the corresponding reaction on the protected material failed to prepare a similar product that there may be other factors to be explored. Since none of the epoxide (**66a**) or allyl alcohol (**66b**) intermediates were isolated, further investigation would be beneficial in order to confirm the mechanism of the reaction,

3.6.4 Attempted oxidation of acetonide derivative of aglycone

Literature reports show that in the case of some triterpene saponin aglycones possessing a double bond at the same position as hederagenin are susceptible to oxidation at this position.[94,96,153] It therefore seemed plausible that by attempting a similar method on this compound may result in a similar oxidation to give a new carbonyl function. However, due to the presence of the two hydroxyl positions in hederagenin it seemed preferable to first protect it as the acetonide derivative (**57**) and attempt the oxidation on this compound so as to avoid the conditions instead attacking these positions instead. This was first attempted using chromium (VI) oxide in acetic acid (Scheme 3.6.4.1) according to a method similar to that reported to be used to prepare the corresponding oxidation product of oleanolic acid.[96] However, after 18 hours the reaction showed no indication of product by TLC and, following work up, only a colourless solid which was identified by ^{1}H NMR analysis as unreacted starting material. No signals corresponding to the carbonyl product (**67**) were observed.



57 67

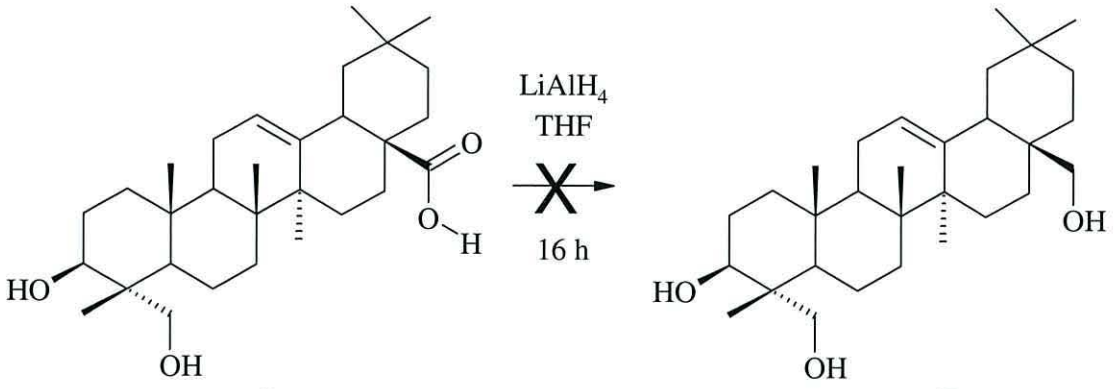
Scheme 3.6.4.1 Attempted oxidation of the acetonide protected aglycone to impart a new carbonyl function

Having been unsuccessful by this method an alternative approach was attempted; this time the chromium (VI) oxide in acetic acid was supplemented with the oxidising agents PCC and PDC.[153] Once again, this proved unsuccessful and only starting material could be recovered. No signals corresponding to the ketone product (**67**) were observed in the proton NMR spectrum. Given these difficulties, it did not appear worthwhile pursuing this route to modification any further.

3.7 Attempted reduction of hederagenin and its derivatives at the C-28 position

Attempts were made to reduce the carboxylic acid functional group in these compounds as a means of preparing the corresponding alcohol. This would not only prove a worthwhile modification in its own right but also offer an alternative route to prepare further novel derrivatives by esterifying the new alcohol formed.

This was attempted using excess lithium aluminium hydride (Scheme 3.7.1) and was done for both hederagenin (**2**) and its acetonide derivative (**57**), but after 16 hours no reaction was visible by TLC and only starting material was observed to be present in the proton and carbon NMR spectra with no new signal for the protons expected to be seen atached at the C-28 position of the desired products (**68**) were identified.



2 **68**

Scheme 3.7.1 Attempted reduction of the acid at the C-28 position of hederagenin to its corresponding alcohol

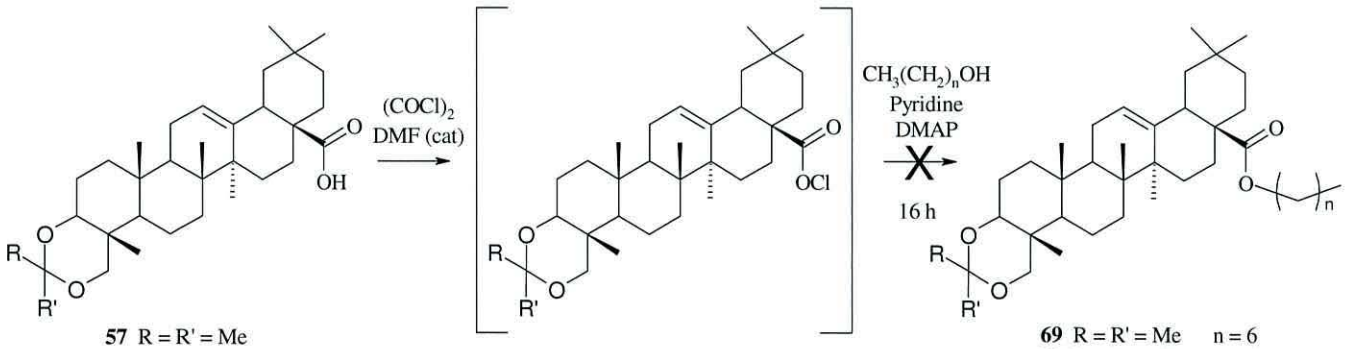
The reaction was even attempted on a portion of the acetonide methyl ester (**58**) which should have been more readily reduced to an alcohol than its corresponding carboxylic acid (**57**) would, but again this reaction yielded no product and only starting material could be recovered.

3.8 Esterification of the acid chloride derivatives of protected hederagenin with alcohols

Although it has had been possible to modify the acid functionality at the C-28 position of hederagenin to form a methyl ester, it was also deemed to be desirable to identify a means of forming a longer chain ester at this position which might enable a more lipophilic derivative to be formed. One powerful method to prepare an ester from a carboxylic acid is to first convert it to an acid chloride, before coupling it to an alcohol by nucleophilic addition-elimination. This is possible because the acid chloride derivatives are extremely reactive and are open to attack by nucleophiles such as the oxygen present in alcohols.

This method was attempted on both of the protected forms of the aglycone - the acetonide (**57**) and the benzylidene acetal (**59**). These were selected over the unprotected hederagenin in order to prevent the possibility of the hydroxyl groups of the hederagenin reacting with the acyl chloride derivative rather than that of the long chain alcohol; an unlikely outcome due to what would appear to be significant steric hindrance to the formation of a dimer, however a source of uncertainty which was

readily eliminated by using the protected compounds. The acid chloride derivatives of the protected compounds were prepared by reaction of with oxalyl chloride and a catalytic amount of DMF. Since the acyl chloride intermediate is susceptible to attack by water, the reaction was carried out in anhydrous conditions in dry toluene. The prepared chlorides were then reacted directly with an excess of heptanol in the presence of catalytic pyridine and DMAP (Scheme 3.8.1). In the case of both the acetonide (**57**) and the benzylidene acetal (**59**) protected hederagenin the preparation of the acid chloride intermediatate appeared to take place successfully, however after 16 hours of stirring with the alcohol there appeared no indication of the ester products (**69** and **70** respectively) by TLC comparison to the starting material. When the reaction mixture was worked up the only triterpene containing signals observed to be present in the ^{1}H and ^{13}C NMR spectra were those of the unreacted starting materials. In the case of the acetonide, a portion of the acid chloride derivative was reacted with an alternative longer chain acid behenic alcohol using the same method but once again only the unreacted starting material (**57**) was recovered and none of the esterified product (**71**).



57 R = R' = Me
59 R = Bz R' = H

69 R = R' = Me n = 6
70 R = Bz R' = H n = 6
71 R = R' = Me n = 22

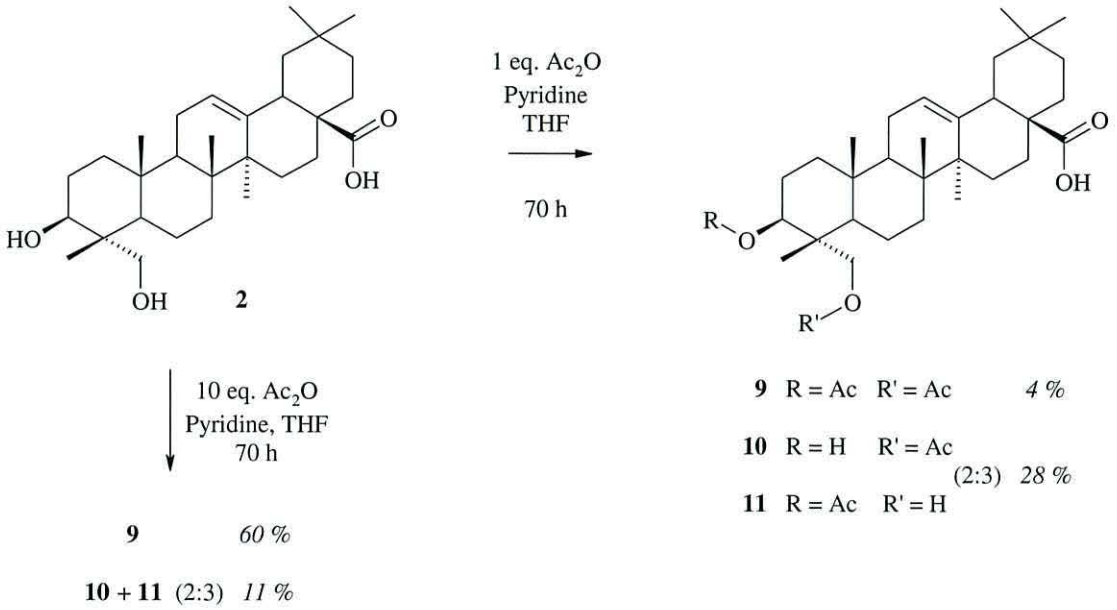
Scheme 3.8.1 Attempted preparation of ester at the C-28 position of hederagenin via an acid chloride intermediate

3.9 Esterification of hederagenin with acid anhydrides

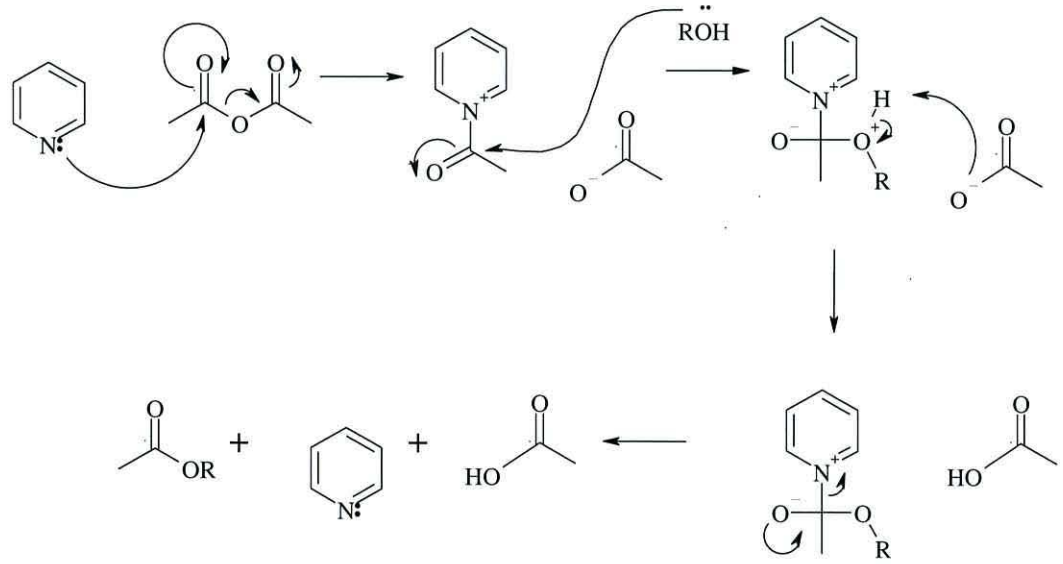
Having found the C-28 acid function of hederagenin to be rather difficult to esterify successfully to anything longer than a methyl ester, possibly due to the steric hindrance of the position, it was apparent that an alternative route may be required to prepare derivatives that possessed larger groups or longer chains. It was observed that

the hydroxyl groups at the C-3 and C-23 positions were significantly less hindered and as such should more readily react to give ester products. As discussed in 1.5.2, a number of groups have reported successes in preparing esters at the C-3 position of other triterpene aglycones by using acid anhydrides.[87-89,91] It is however noteworthy that these reports all refer to triterpene aglycones such as oleanolic acid (**1**) or ursolic acid (**20**) which only posses a single hydroxyl group at the C-3 position rather than hederagenin (**2**) which has an additional alcohol function at the C-23; the chemistry of the C-23 hydroxyl group on reaction with acid anhydrides has therefore remained largely unexplored.

The first attempt at this reaction was to treat the hederagenin aglycone with the simplest available acid anhydride - acetic anhydride. This was done by stirring a solution of hederagenin (**2**) in anhydrous THF with acetic anhydride and a catalytic amount of pyridine for 70 hours at room temperature (Scheme 3.9.1). The presence of the pyridine was required in order to convert the anhydride into a labile ion pair and allow the hederagenin alcohol functions to readily attack by the mechanism illustrated in Scheme 3.9.2. As can be seen in Scheme 3.9.1, the reaction was performed twice. In each case the conditions were the same, with only the number of molar equivalents of the anhydride changed; in the first case using only a single equivalent and in the second a large excess (10 equivalents). In both cases the same three products were formed, a bis-substituted product (**9**) with an acetate group attached at both the C-3 and C-23 positions and two mono-substituted products one with an acetate group at the C-3 position (**10**) and the other with an acetate attached at the C-23 position (**11**). It was, however, found that the ratio of these products was controlled by the number of equivalents of anhydride that were used. When treated with only a single equivalent of acetic anhydride the mono substituted products (**10** and **11**) were favoured (28 % yield) with only a small amount of the bis product (**9**) obtained (4 %). Conversely, when a large excess was used, the bis-substituted product was recovered in fair yield (60 %) with a far smaller proportion of the mono substituted products (11 %).



Scheme 3.9.1 Esterification of hederagenin with acetic anhydride



Scheme 3.9.2 Mechanism of pyridine catalysed esterification of an alcohol with acetic anhydride

The products from this reaction were separated by column chromatography in order to isolate the individual components. The bis-acetate (**9**) was successfully separated from the mono-acetate products (**10** and **11**) by this means due to the difference in polarity between these components; however, despite repeated efforts, it was not possible to successfully separate the two mono-acetate products from one another. Despite this it was possible to identify the ratio of these products by the integration of each in the ^{1}H NMR spectrum; in each case, for the single equivalent reaction and that

with an excess, the ratio of products was seen to be 2:3 C-3 substituted (**10**) to C-23 substituted (**11**). This result is logical, since it appears that the hydroxyl group attached to the C-23, a primary alcohol, would be a more favourable position for attack than the secondary alcohol at the C-3 position as it is further removed from the triterpene skeleton and so more accessible to attack. The molecular model of optimal geometry calculated for the aglycone showed this to be the case, with the C-23 position subject to less steric hindrance (Figure 3.9.1). This preference in regiochemistry displayed in the acetate products is in contrast to that observed in the 3-*O*-sugar attachment of the natural saponins via their biosynthesis.

Modelling of the bis-substituted acetate product (**9**) in a similar manner (Figure 3.9.3) confirms this, displaying the geometry of the two ester groups to differ in the steric hindrance that they were subject to and consequently their relative ease of substitution. The effect of this is likely to be more prevalent as the size of the substituent is increased.

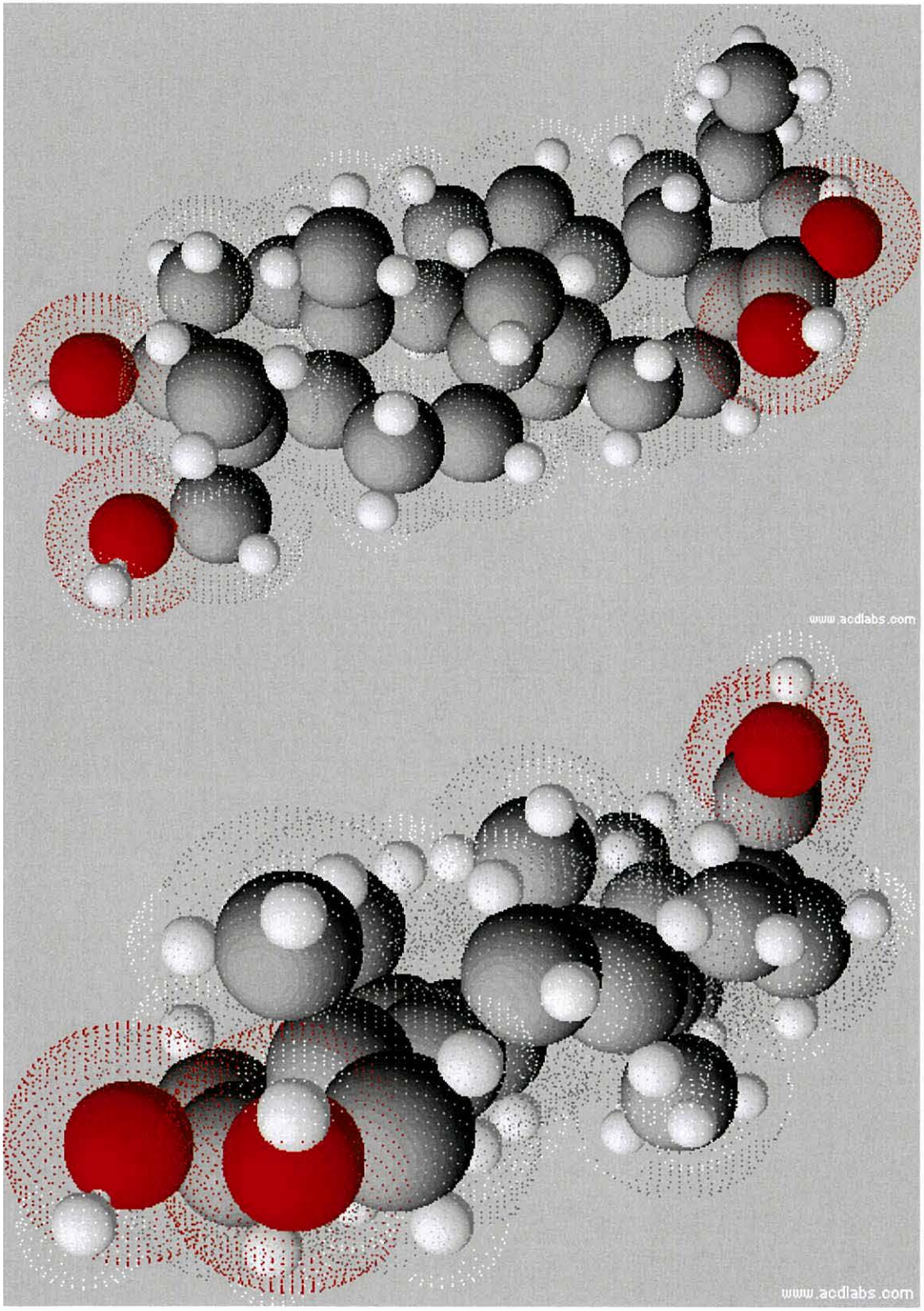


Figure 3.9.1 Molecular model of hederagenin showing the geometry of the hydroxyl positions at C-3 and C-23; the oxygen atoms are shown in red

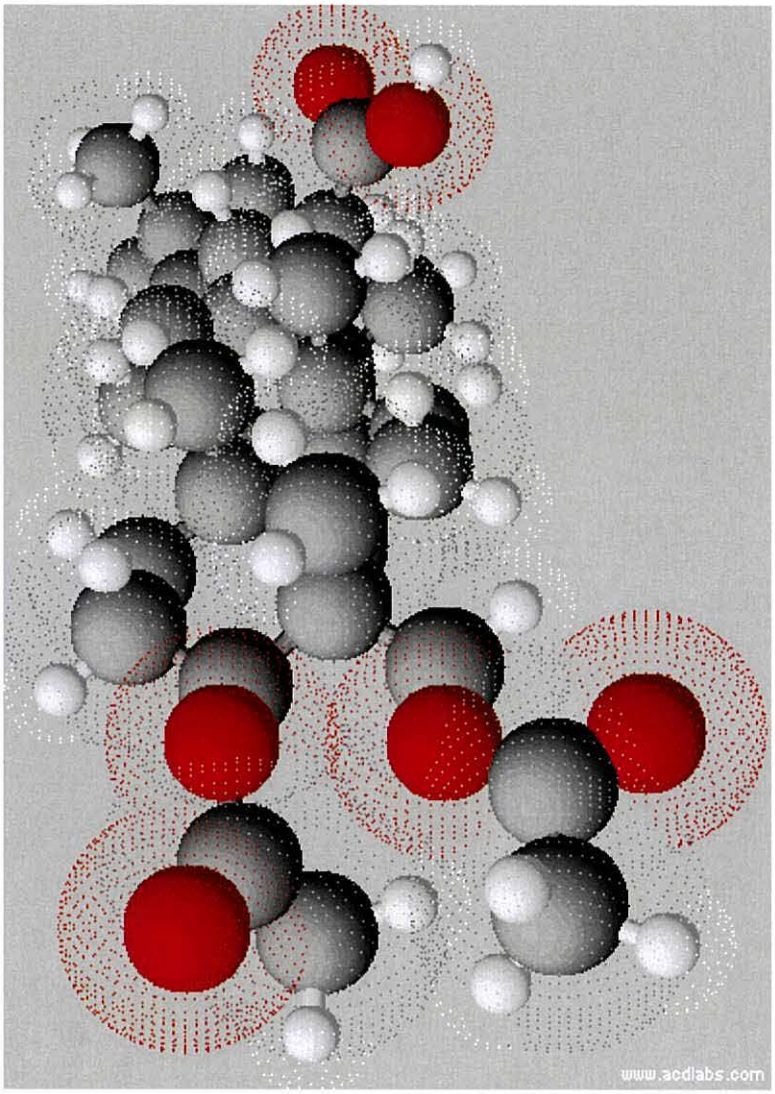


Figure 3.9.2 Molecular model of the 3,23-*O*-bis acetate of hederagenin showing the geometry of the two acetate groups relative to the triterpene skeleton

Products from these reactions were identified by ^{1}H NMR analysis and gave distinctive patterns which were later seen in all of the subsequent esters prepared by this method; given this, the spectra of these products are included to further identify these characteristic signals. Furthermore, MALDI MS analysis of the products showed the masses of the molecular ions to be identical to the calculated masses.

The ^{1}H NMR spectrum of the bis substituted product (**9**) (Figure 3.9.1) showed a number of signals similar to those of the hederagenin starting material (**2**), the spectrum of which is discussed in 2.3. As can be seen, the distinctive pattern of 3 H singlets corresponding to the methyl groups is still present, as are the 1 H, double doublet at 2.83 and 1 H triplet at 5.29. These signals are, however, supplemented with an extra set of peaks. A double doublet was observed at δ 4.80 ppm (See

expansion A) which corresponded to the proton attached to the C-3 which is geminal to the new secondary acetate group, its multiplicity the result of being split by both the axial and equatorial protons of the neighbouring C-2 and its shift noticeably downfield of those signals seen in the starting material as a result of the proximity of the highly electronegative acetal. The presence of the other ester group was confirmed by two new doublets at δ 3.88 and δ 3.70 ppm (see expansion B) which were coupled to one another (*J* 11.4 Hz). These doublet signals corresponded to the two protons attached to the C-23 carbon; these protons are not equivalent and as such they are split by one another resulting in their multiplicity as doublets (the adjacent C-4 being a quaternary carbon) which is confirmed by the high value of the coupling constant which is consistent with this kind of geminal coupling. The shift of these signals is again further downfield than the corresponding signals seen in the starting material as a result of the nearby acetal linkage. The final new signals visible in the spectrum were two 3 H singlets around δ 2.05 ppm which represent the two CH_3 groups of the new acetyl groups, the C=O bond on the adjacent carbon causing a shift of these downfield from the signals of the pre-existing hederagenin methyl groups.

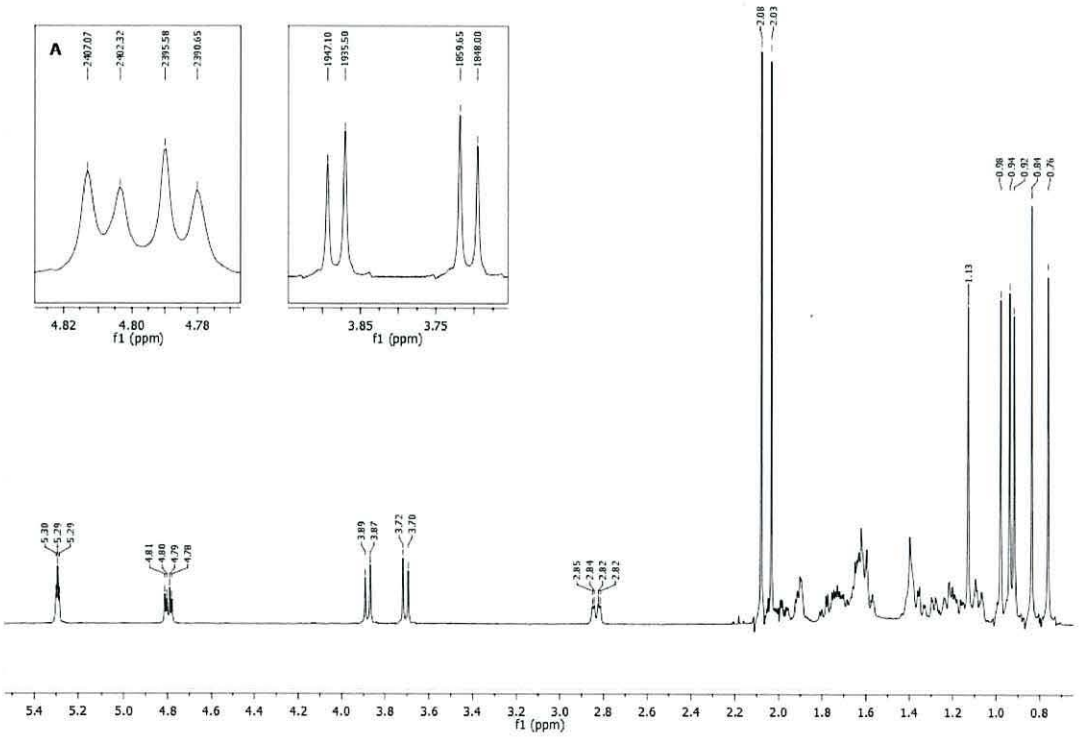


Figure 3.9.2 ^{1}H NMR spectrum of a mixture of the 3,23-*O*-acetate product of hederagenin recorded in $CDCl_3$. The regions of interest are shown in expansions A and B.

The mixture of mono-substituted products (**10** and **11**) again showed new signals in this region (Figure 3.9.3), both isomers also showed a number of common signals which are similar to those seen in both the bis-acetate (**9**) and hederagenin (**2**). The new signals however did not integrate equally to these common signals, instead integrating as a proportion of their composition in the mixture i.e. those of the primary substituted product (**11**) integrated to 0.6 H relative to the 1 H double doublet at δ 2.82 ppm, while those of the secondary substituted product (**10**) integrated to 0.4 H. The secondary substituted product (**10**) was identified by three characteristic signals in its ^{1}H NMR spectrum, a double doublet at δ 4.89 representing the proton on the C-3 position (see expansion A); this was accompanied by two 0.4 H doublets at δ 3.38 and 2.91 which represent the protons on the C-23 position (see expansion C). While the proton signal close to the substituted group appears very similar to that described for the bis-substituted product (**9**), these doublets near the unsubstituted hydroxyl group possess the same geminal coupling pattern but their chemical shift is upfield of the equivalent signals seen in the bis-acetate. The primary substituted product (**11**) also showed three new signals, two 0.6 H doublets at δ 4.19 and 3.82 coupled to one another (see expansion B) representing the two geminal protons on C-23 together with what appears to be a 0.6 H triplet (although this is more likely a double doublet as well which has not been completely resolved) at δ 3.43 which corresponds to the proton geminal to the unsubstituted hydroxyl group on C-3(see expansion C). This triplet is upfield of the equivalent signal for the bis-acetate (**9**) or the secondary mono-acetate (**10**). Further to these signals, two new singlets were also observed at δ 2.11 and δ 2.09 ppm resulting from the CH_3 groups present in the secondary mono-acetate (**11**) and primary mono-acetate (**10**) respectively.

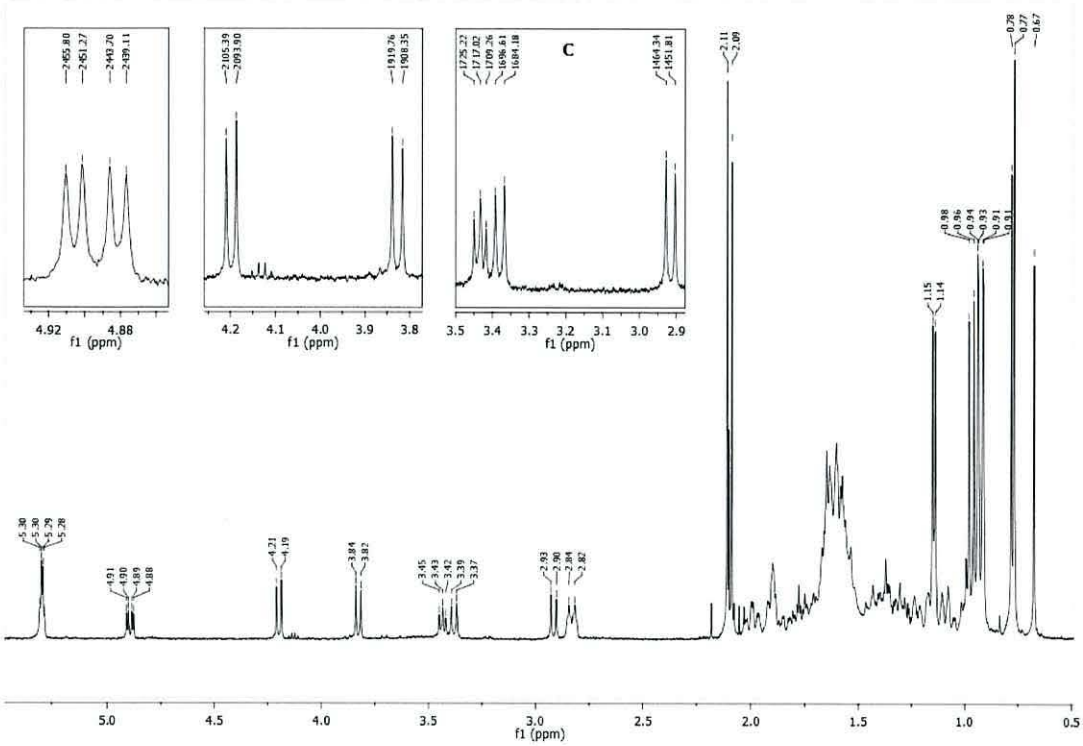
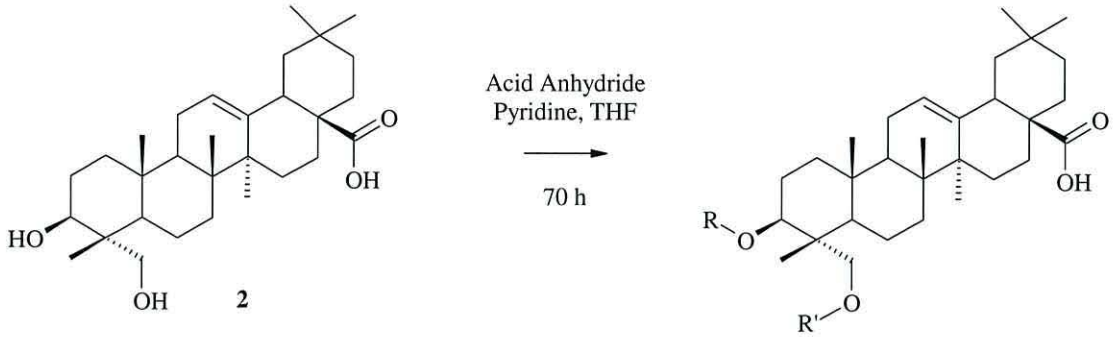


Figure 3.9.3 ^{1}H NMR spectrum of a mixture of the 3-*O*- and 23-*O*- mono acetate products of hederagenin recorded in $CDCl_3$. The regions of interest are shown in expansions A-C.

Having established a suitable esterification method with these acetic anhydride experiments, the technique was repeated using other anhydrides in order to prepare a range of novel compounds. In addition to this, by continuing to use both a small quantity of the anhydride and a large excess it was also possible to further investigate the amount of control which can be exerted on the regiochemistry. The results of these experiments are presented in scheme 3.9.1.



Anhydride	Equivalents Used	Bis substituted product			Primary mono substituted product				Secondary mono substituted product			
		No.	R and R'	Yield	No.	R	R'	Yield	No.	R	R'	Yield
Butyric	1	**72**	$-COCH_2CH_2CH_3$	0 %	**73**	H	$-COCH_2CH_2CH_3$	73 %	**74**	$-COCH_2CH_2CH_3$	H	15 %
	2.2			10 %				0 %				0 %
	10			32 %				0 %				0 %
Benzoic	1	**12**	Bz	7 %	**75**	H	Bz	12 %	**76**	Bz	H	0 %
	2.2			26 %				0 %				0 %

Scheme 3.9.1 Esterification of hederagenin with acid anhydrides

The ester products formed with butyric anhydride exhibited similar trends to those seen with the acetate products. The ratio of bis-substituted product (**72**) to mono-substituted products (**73** and **74**) again changes according to the excess of anhydride used in the reaction. With a single equivalent of butyric anhydride none of the bis-substituted product was obtained, with a very good recovery of monosubstituted products. Once again, the mono-butyrate products had very close retention times such that they could not be readily separated from one another by column chromatography. As with the acetate products, it was the primary substituted product (**73**) that was favoured in this reaction (76 % yield) with the secondary substituted product apparently a more minor product (15 %). As the excess of anhydride was increased there was a change in the composition of the product, with 2.2 equivalents of butyric anhydride no mono-substituted product was obtained, instead only a small amount of the bis-butyrate product (**72**) could be recovered (10 %). A further experiment with a large excess of butyric anhydride (10 equivalents) yielded a modest return of this product (32 %) again without recovery of any mono-butyrate products (**73** or **74**); however it proved to be increasingly difficult to purify the desired product from this reaction to remove the large excess of butyric acid (a problem not encountered in the case of acetic anhydride where the equivalent by-product was acetic acid which was

readily removed during the work-up procedure). As such it appears it may be better practice to use a smaller excess of these larger anhydrides to prevent such difficulties. Once again the identities of these products were confirmed by MALDI MS and by ^{1}H NMR analysis which gave the same pattern of signals for the C-3 and C-23 protons as described for the bis-acetate and mono-acetate products. The bis-butyrate (**72**) showed a 1 H double doublet at δ 4.80 and two geminally coupled 1 H doublets at δ 3.88 and 3.70. The mixed mono-butyrate products showed a new 0.4 H double doublet at δ 4.87 and two 0.4 H doublets at δ 3.36 and 2.89 for the secondary substituted product (**74**) together with two 0.6 H doublets at δ 4.16 and 3.82 and a 0.6 H triplet at δ 3.43 from the primary substituted product (**73**).

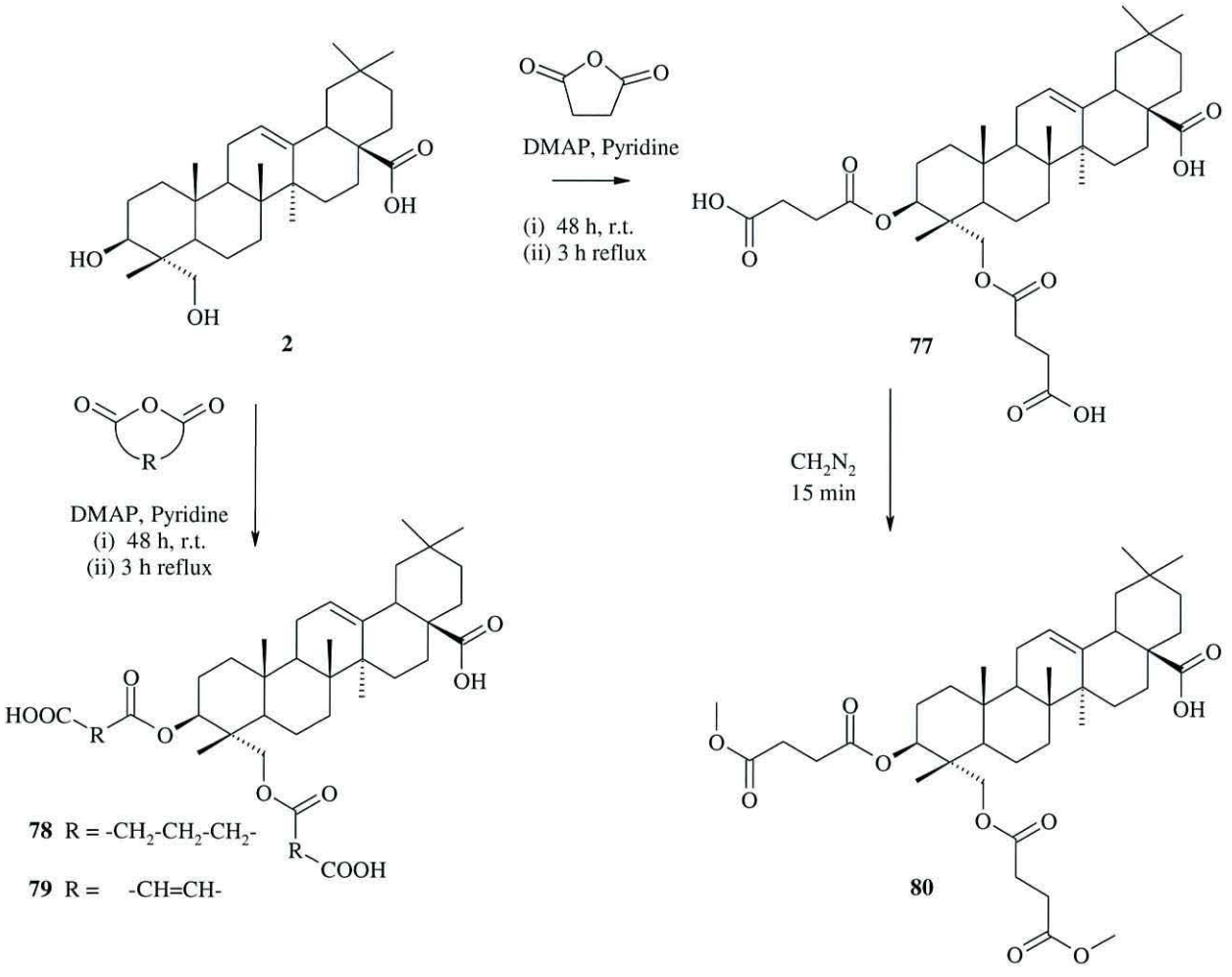
Benzoic anhydride again performed in a similar manner to the other anhydrides. When the aglycone (**2**) was reacted with only a single equivalent of the anhydride it gave a mixture of the bis- and mono-benzoate products, with the mono- being favoured. Purification by column chromatography isolated the bis-benzoate product (**12**) as the minor product (7 %) and the primary substituted mono-benzoate (**75**) as the major product, albeit still at a relatively low yield (12 %); no secondary substituted mono-benzoate (**76**) was identified in the mixture. Since, in the cases of the other anhydrides, the primary alcohol was observed to esterify preferentially compared to the more hindered secondary alcohol it appears likely that this hindrance was even more pronounced using the bulkier benzoic anhydride therefore preventing the formation of an ester at this position without using a larger excess of anhydride to encourage substitution of both hydroxyl positions. When the reaction was repeated with an excess of benzoic anhydride this was found to be the case, giving an improved yield (26 %) of the bis-benzoate (**12**) and none of the mono-substituted products. The identities of these products were once again confirmed by MALDI MS and ^{1}H NMR, the spectra of which again showed the same characteristic pattern of peaks for the protons attached to the C-3 and C-23 positions. As well as these signals and the common signals of the aglycone backbone another set of new peaks were observed in the aromatic region between δ 8.11 and 7.23 which corresponded to the protons on the benzene rings attached to these products.

Some subsequent efforts to prepare further esterified products using the same method did, however, encounter problems. Attempts were made to prepare products

with more polar ester groups by using succinic or maleic anhydride or with the similarly structured cyclic imide compound succinimide; however all of these reactions proved to be unsuccessful with none of the ester products recovered. It therefore appeared that while the above technique was adequate to readily prepare esters with less polar moieties, it needed to be adapted in order to successfully prepare those containing polar functionalities such as acids or alcohols. Such a derivative could be highly desirable as biologically active saponin molecules possess polar functionalities at these positions in the form of sugar chains; preparing an alternative polar function here might help to mimic this action and help to increase the water solubility of the otherwise poorly soluble aglycone. These types of esters e.g. succinates have also been synthesised for other triterpene saponin aglycones which could then enable the preparation of pharmacologically acceptable salts of these compounds.[87]

An alternative method was developed to carry out this type of esterification of hederagenin to prepare succinate, maleate or glutarate products, this time by treating the aglycone with the acid chloride in excess pyridine together with an additional nucleophilic catalyst DMAP (the mechanism of this catalysis is the same as that of pyridine in Scheme 3.9.2). The mixture was stirred for 48 hours at room temperature before being heated at reflux for 3 hours in order to ensure the reaction was complete; the reaction mixture poured into sulphuric acid and cooled in order to precipitate the crude ester product. This, as with the previous method, was carried out using differing quantities of the anhydride in order to identify what effect this had on the regiochemistry of the reaction. When conducted using a very large excess of succinic anhydride (10 equivalents) the bis-substituted product (**77**) was recovered in modest yield (44 %); however, it appeared likely that the actual yield was probably higher than this but difficulties experienced in purifying the product from the excess succinic anhydride may have led to some losses. With this in mind the reaction was repeated using a smaller excess (2.2 equivalents) of succinic anhydride to once again prepare the bis-substituted product (**77**), this time in a higher yield of recovered product (61 %). No mono-substituted products were recovered in either case. The purified bis-succinate (**77**) of hederagenin prepared by this means was identified by MALDI MS and by ^{1}H NMR analysis. Its spectrum contains signals matching the pattern identified previously for the bis-substituted products (a 1 H double doublet at δ 4.82

and a pair of 1 H doublets at δ 3.94 and 3.69). Treatment of the crude product with diazomethane gave the corresponding methyl ester; this was purified by column chromatography to recover the methyl ester of the bis-succinate (**80**). This was identified by ^{1}H NMR which gave a spectrum identical to that of the bis-succinate (**77**) except for two new 3 H singlets at δ 3.70 and δ 3.68 ppm corresponding to the methyl groups of the new esters of the succinate methyl ester and another 3 H singlet at δ 1.14 ppm representing the methyl ester of the acid at the C-28 position. In an effort to try and prepare and isolate a mono-succinate product the reaction was repeated again using only a single equivalent of the anhydride, although in this case no ester product was recovered.



Scheme 3.9.2 Esterification of hederagenin with succinic and glutaric anhydride and DMAP catalyst

The absence of any mono-substituted product in any of these attempts, even as a minor component was unexpected; as such it may have been a result of the

precipitation and purification methods used which were selectively only recovering the bis-substituted product. Due to the high polarity of these products it was not possible to purify them by the same means of column chromatography used for the previous esters; instead, the best means identified to purify them was to precipitate the product from ether by the addition of petrol. It was thought that it may therefore be the case that the relative solubilities of the bis- and mono-succinate products differed enough to precipitate only the bis-substituted product while other products remained in solution. Study of the mother liquor of these precipitations in the reactions with excess anhydride showed no indication of any other esterified products. In the case of the single equivalent method, there did appear to be signals corresponding to an ester product mixed with unreacted starting materials. Treatment of this crude residue with diazomethane formed the methyl ester products which could be purified by column chromatography to isolate a 1:1 mixture of the methyl esters of the mono-substituted products (**81** and **82** in Figure 3.9.4).

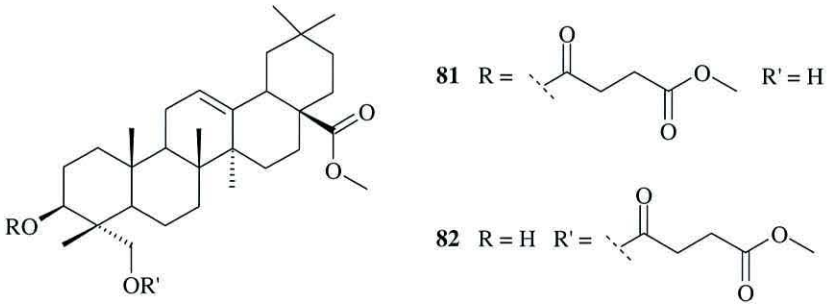
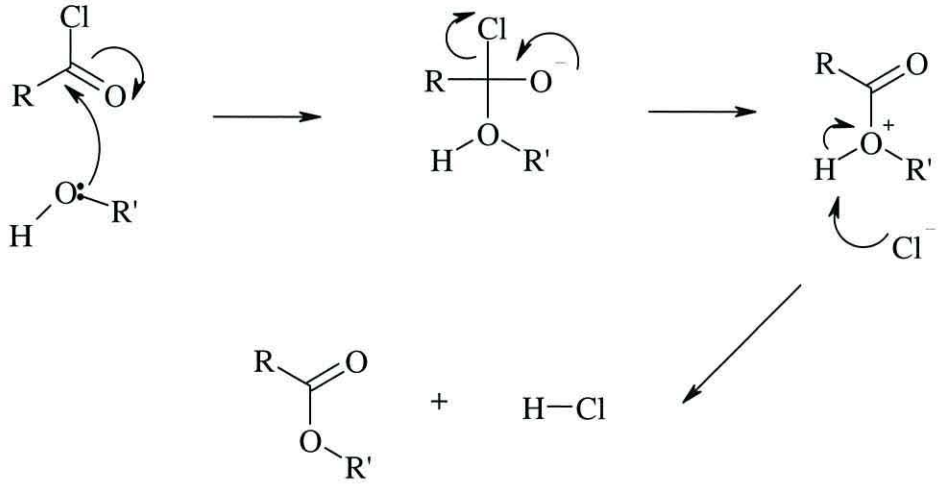


Figure 3.9.4 Methyl esters of the two mono succinate derivatives of hederagenin

Using the same method as with the succinate, similar esters were prepared with glutaric anhydride and maleic anhydride (Scheme 3.9.2). Given the difficulties experienced in recovering the mono-substituted products of the succinate, these reactions were only attempted using an excess of the anhydride. With 10 equivalents of anhydride, the bis-glutarate (**78**) was prepared in comparable yield to the succinate (27 %) while the bis-maleate (**79**) was recovered in a comparably higher yield (40 %). The identities of both of these products were confirmed by MALDI MS and ^{1}H NMR.

3.10 Esterification of hederagenin with acid chlorides

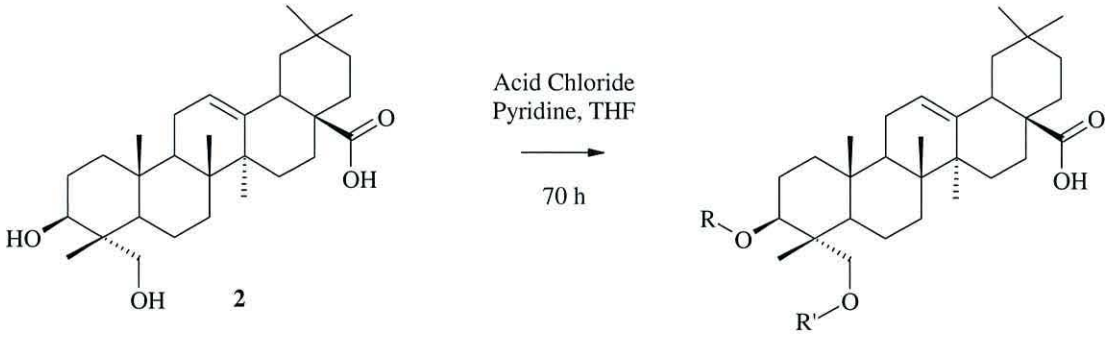
Although attempts to prepare an ester. The highly electronegative oxygen and chlorine atoms leave a partial positive at the C-28 position by preparing its acid chloride and treating it with an alcohol were not successful, it seemed more likely that the same methodology could be used in a different way in order to esterify these compounds at the C-3 and C-23 positions instead. This time, the acid chloride was selected such that it possessed the desired chain or substituent and the two hydroxyl groups of the aglycone acting as the nucleophile charge on the carbon atom they are attached to which can readily undergo nucleophilic addition by an alcohol; this subsequently undergoes an elimination to expel hydrogen chloride and leave a new ester product by the mechanism shown in Scheme 3.10.1. This method was carried out under the same conditions as were used previously to perform the esterification with acid anhydrides. Since the range of commercially available acid anhydrides are often somewhat limited in terms of the chain lengths available, the use of acid chlorides instead offers an opportunity to prepare longer chain esters at the C-3 and C-23 than had been prepared previously; such an ester would be an interesting molecule to test for biological activity because the presence of a lipophilic group or groups could be beneficial in applications such as plant protection treatments.



Scheme 3.10.1 Mechanism of the nucleophilic addition - elimination reaction of an alcohol and acid chloride as occurs in the esterification of hederagenin with acid chlorides

The reaction was attempted on using a range of commercially available acid chlorides. Hederagenin (**2**) was treated with the acid chloride and pyridine in THF

and stirred at room temperature for 70 hours. In all cases the reactions were performed twice in the same manner as was used for the esterification with acid anhydrides; once using a single equivalent of the acid chloride, the second using an excess of the reagent. Once again, this differential in concentration was expected to control the regiochemistry of the reaction to give mono- and bis-substituted products respectively. The results of these experiments are presented in 3.10.2.



Acid Chloride	Eq.	Bis substituted product			Primary mono substituted product				Secondary mono substituted product			
		No.	R and R'	Yield	No.	R	R'	Yield	No.	R	R'	Yield
Palmitoyl	1 2.12 10	**83**	$CH_3(CH_2)_{14}CO$	1 % 52 % -	**84**	H	$CH_3(CH_2)_{14}CO$	13 % 4 % -	**85**	$CH_3(CH_2)_{14}CO$	H	9 % 4 % -
Oleoyl	1 2.12	**86**	$CH_3(CH_2)_7$- $CH=CH(CH_2)_7CO$	9 % 26 %	**87**	H	$CH_3(CH_2)_7$- $CH=CH(CH_2)_7CO$	19 % 5 %	**88**	$CH_3(CH_2)_7$ - $CH=CH(CH_2)_7CO$	H	19 % 5 %
Trimethyl Acetyl	1 2.2	**89**	$(CH_3)_3CCO$	22 % 12 %	**90**	H	$(CH_3)_3CCO$	7 % 47 %	**91**	$(CH_3)_3CCO$	H	0 % 0 %
Isobutyryl	1 2.2	**92**	$(CH_3)_2CHCO$	6 % 10 %	**93**	H	$(CH_3)_2CHCO$	14 % 11 %	**94**	$(CH_3)_2CHCO$	H	0 % 3 %

Scheme 3.10.2 Esterification of hederagenin with acid chlorides

Across these reactions it can be seen that similar trends are observed to those identified in the acid anhydride esterifications. Overall, the reactions with an excess of acid chloride favoured the preparation of more bis-substituted product while the single equivalent experiments tended to favour the preparation of more mono-substituted products. The ratios of the primary and secondary mono-substituted products were somewhat more variable. All of the products were identified by NMR and MALDI MS; the ^1H NMR spectrum displaying an identical pattern of common

signals and those of the new esterified positions to those observed of the esters prepared in 3.9

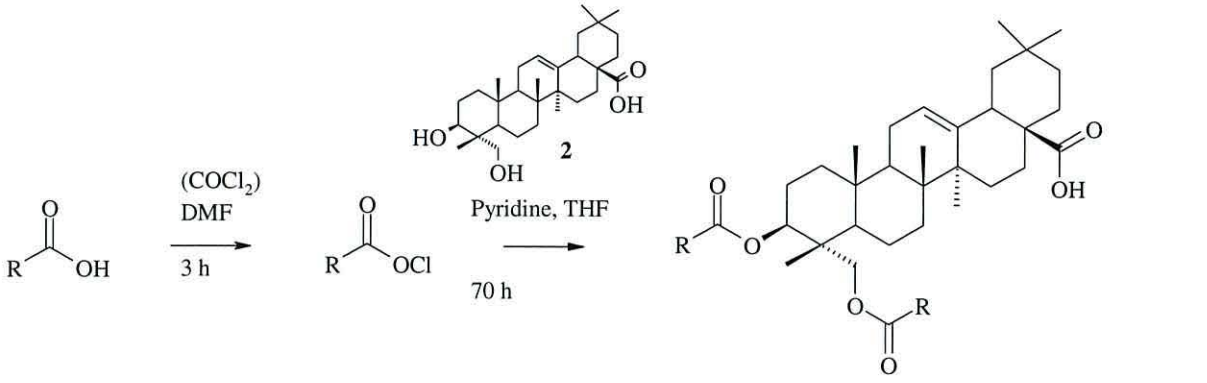
In the case of the first of these, the reaction of hederagenin (**2**) with palmitoyl chloride, the reaction was attempted initially with a very large excess (10 equivalents). This reaction was however unsuccessful as all attempts to purify the product by column chromatography failed to successfully separate the products from the excess of palmitic acid. In order to reduce this difficulty the palmitoyl chloride used was reduced to around 2 equivalents. Under these conditions the mixture of products formed consisted predominantly of the bis-substituted (**83**) product obtained in fair yield (52 %) and a 1:1 mixture of the mono-palmitate products as a minor product (8 %). The reaction using only a single equivalent of the acid chloride reagent gave the mono-palmitate products as major products (22 %), in this case the primary substituted product was favoured over the secondary, though only in a ratio of 3:2. The only significant difference observed in the proton NMR spectra to those previously observed for esters being the large signals around δ 1.25 corresponding to the conjugated protons of the hydrocarbon chains.

The reaction with oleoyl chloride showed very similar results to that of the reaction with the structurally similar palmitoyl chloride. With an excess of the reagent the mixture of products was predominantly the bis substituted product (**86**) which was obtained in a modest yield (26 %) together with a 1:1 mixture of both the primary (**87**) and secondary (**88**) mono-substituted products. The same products were all obtained when only 1 equivalent of oleoyl chloride was used although in this case it was a 1:1 mixture of the two mono-substituted products (**87** and **88**) in modest yield (37 %) with the bis-substituted oleoyl ester (**86**) at a lower yield (9 %). The ^{1}H NMR spectra of the bis- and mono- substituted products appeared nearly identical to those of the palmitate products with the major difference observed being an extra olefinic signals at around δ 5.30 ppm corresponding to the double bonds of the oleoate esters.

Both the trimethylacetyl chloride and isobutyryl chloride reactions gave very similar products to one another. They both gave a mixture of bis-substituted products and mono-substituted products at all concentrations of the reagent. In the case of the trimethylacetyl chloride (1 equivalent) reaction the bis-substituted product (**89**) was

the major product (22 %) with the primary mono-substituted product (**90**) was the only other product isolated. With an excess of this reagent only a 12 % yield of the bis-ester (**89**) was obtained but this time the primary mono-substituted product (**90**) was obtained in far higher yield (47 %). No secondary mono-substituted product (**91**) was identified in either case. In the case of the isobutyryl chloride there was no significant difference between the composition of the mixture of product obtained at either concentration of the reagent. In each case it was the mono-substituted product which was the major product (14 % in each case) with the bis-substituted product recovered in a lower yield (6 % with the single equivalent and 10 % with the excess). Once again, within the mono-substituted products it was the primary ester which was favoured, being the only mono-product in the 1 equivalent reaction and the largest component of the mixture (4:1) when treated with the excess.

In order to investigate the possibility of preparing other longer chain ester products, two long chain fatty acids, linoleic acid and behenic acid were first converted to acid chloride by treatment with oxalyl chloride and DMF for three hours before treating an excess of these freshly prepared chlorides with a solution of hederagenin (**2**) in pyridine and THF (Scheme 3.1.3). After being stirred at room temperature for 70 hours these reactions were worked up and purified by column chromatography. In the case of the reaction with linoleic acid, a product identified by MALDI MS and NMR as the novel bis-substituted product (**95**) was obtained in a modest yield 27 %. Similarly, only the bis substituted product (**96**) was obtained with behenic acid chloride, this time in a lower yield (16 %). No mono-substituted products were identified.

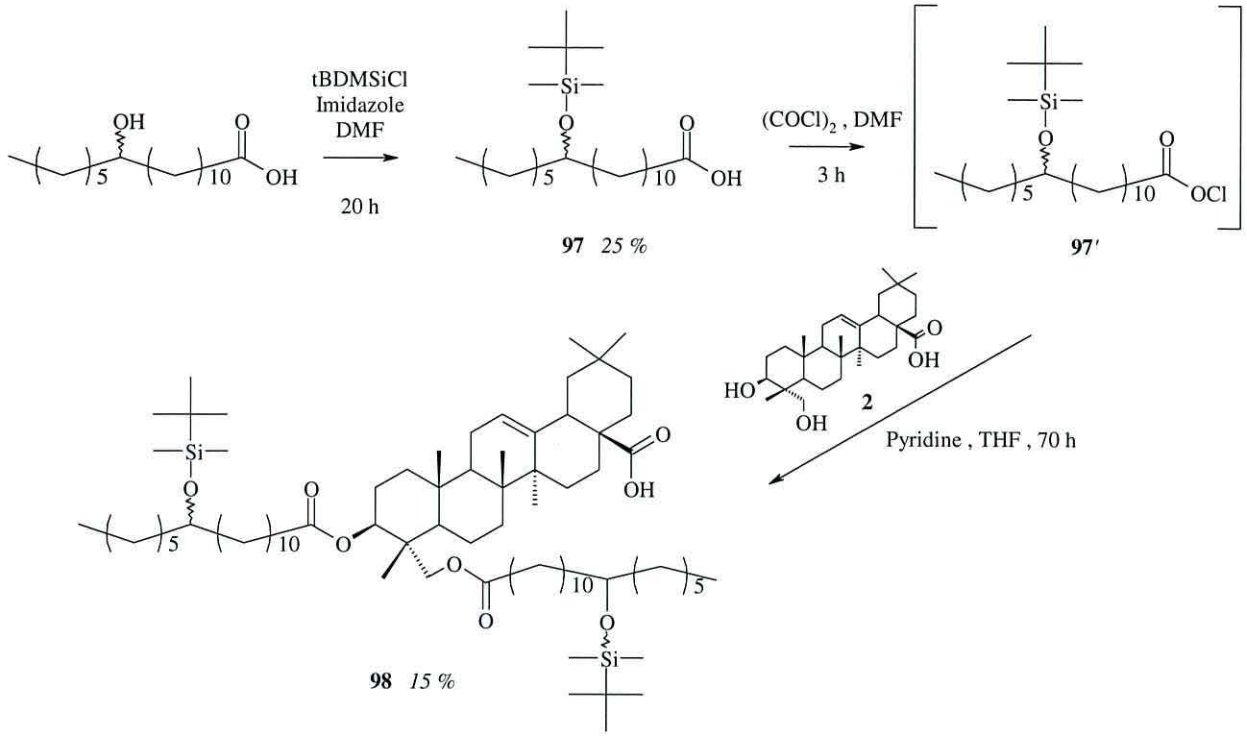


95 R = $CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COO$- *27 %*

96 R = $CH_3(CH_2)_{20}COO$- *16 %*

Scheme 3.10.3 Preparation of acid chlorides of two long chain acids and the subsequent esterification with hederagenin

One final attempt was made to prepare another new ester by this method, this time by esterifying the C-3 and C-23 positions of hederagenin (**2**) with a β-hydroxy acid. This would provide a new functional group which could provide an opportunity to carry out further modification of the product. The acid selected for this purpose was 12-hydroxystearic acid. In order to successfully prepare this ester it was deemed necessary to first protect the hydroxyl group present. This compound was protected by preparing the t-butyldimethylsilyl ester (**97**) by treatment with the corresponding chloride, Imidazole and DMF for 20 hours (Scheme 3.10.4). The protected compound was then converted to an acid chloride (**97'**) with oxalyl chloride in the same manner described previously. This active acid chloride was finally treated with hederagenin (**2**) in pyridine and THF with stirring for 70 hours. The resulting bis-substituted product (**98**) was obtained in poor yield (15 %) and as such was characterised with the protecting groups still attached. There is a great deal of literature describing means of removing this type of silyl ether protecting group;[154] as such it appears plausible that this compound could be subsequently deprotected to free the OH functions and to enable the compound to be further modified.



Scheme 3.10.4 Protection of 12-hydroxystearic acid, preparation of acid chloride and subsequent esterification with hederagenin

CHAPTER 4

Biological assays of saponin products from *H. helix*

4.1 Slug and nematode control activity of *H. helix* saponins and hydrolysis products

4.1.1 Independent evaluation of molluscicidal treatments in field studies on potatoes

An independent field trail was conducted to examine the prophylactic use of the base hydrolysis product of *H. helix* saponins (prepared as in **6.8**) as molluscicidal treatments. The trial was directed by Dr. C. Whaley, i2L Research Ltd., Cardiff, an organisation which holds ORETO status (Officially Recognised Efficacy Testing Organisation) accredited by the Pesticides Safety Directorate under the Plant Protection Product Regulations;[155] it was kindly sponsored by Branston Ltd. The trial was conducted on a field site in Heddon-on-the-wall, Northumberland containing the susceptible variety of potato *King Edward* and species of slug known to attack potatoes, in particular *Arion distinctus*, *Deroceras reticulum* and *Tandonia budapestensis*. Treatments of the fruit base hydrolysis product (FBHP) were applied as a foliar spray application at a 600 g / ha in 300 L / ha water; applications were delivered every 2 weeks in one regime and at 4 timed intervals over the duration of the trial (05.06.08 – 20.10.08). Data is also included for two competitor treatments that were also studied during the field trial; the first of these (Alt 1) was a current chemical treatment (Draza Forte 3.75 kg / ha at 50 - 70 % crop cover and again after 3 weeks) and the other (Alt 2) a biological control (Nematode at 1N, monthly applications).

The results of the field trial showing the mean number of damaged tubers expressed as a percentage of the total is summarised in Figure 4.1.1.1. It shows that highest percentage of damaged tubers was recorded within the untreated control with 13.4 % of tubers exhibiting signs of slug damage. By comparison, the crop treated with fruit base hydrolysis product (FBHP) at 2 week intervals showed a significant reduction in the percentage of damaged tubers with only 5.4 % of tubers exhibiting slug damage, the same treatment applied at four timed intervals over the period failed to provide crop protection when compared to the control (10.2 % of tubers exhibited slug damage). Both FBHP

treatments were more efficient than the biological control tested (Alt 2), significantly so at 2 week intervals, but neither was as active as the chemical pellet treatment (Alt 1).

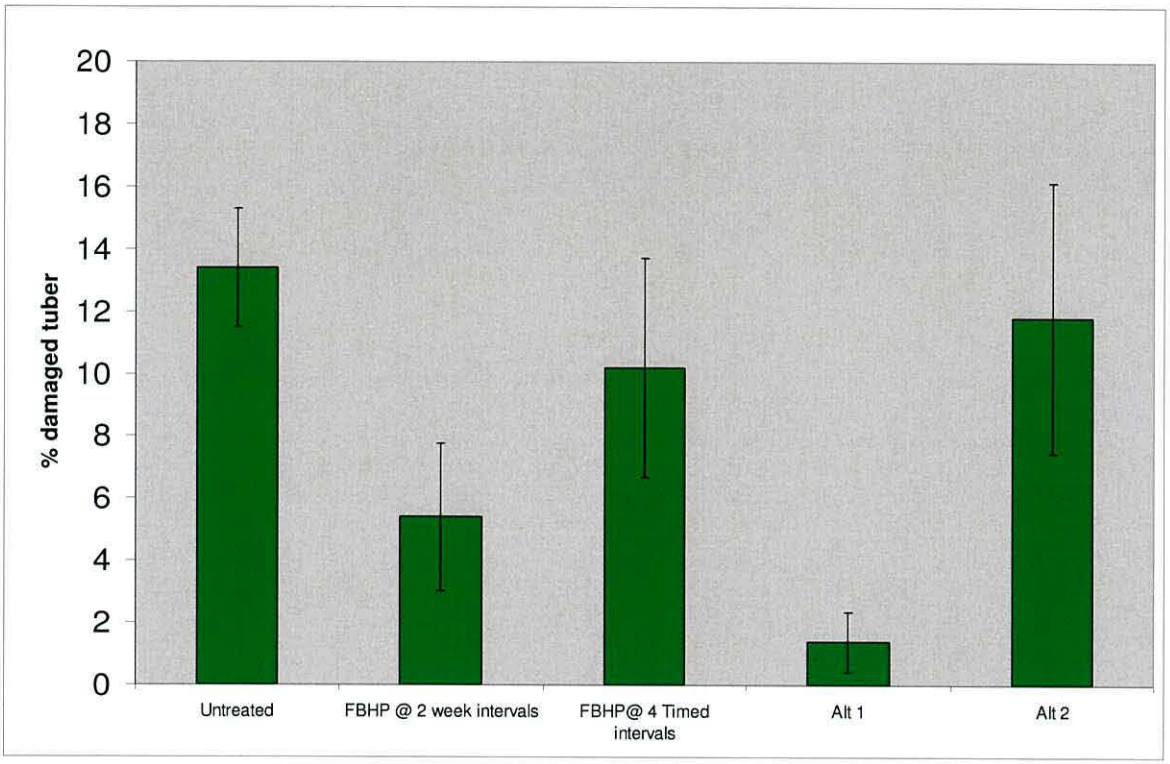


Figure 4.1.1.1 Percentage of damaged tubers from test plots following application of different molluscicidal treatment regimes

In order to identify more subtle differences in these treatments, analysis was conducted to assess the level of damage sustained by individual tubers. This is important to study since potatoes classed as damaged in a control plot may have suffered severe hollowing whereas tubers found in a treated plot may have only sustained a slight or insignificant degree of grazing damage. The damage scores assigned to 100 sampled tubers from each plot were totalled to produce a total damage score per plot. The mean total damage score for each treatment is illustrated in Figure 4.1.1.2 and shows a similar trend to the percentage of tubers damaged. Once again, the highest total damage score belonged to the untreated control (mean sore of 21.6). The FBHP applied every 2 weeks was again found afford significant levels of control (mean score 9.4) while this treatment delivered at four timed intervals failed to show a statistically significant effect (mean score 15.8). In comparison to the commercial competitors tested the saponin treatments were inferior to the chemical treatment (Alt 1) while outperforming the biological control (Alt 2). This data is broken down to show the percentage of tubers falling into each damage class in

Figure 4.1.1.3. These results indicate that all treatments contained a higher number of undamaged tubers when compared to the control; however, statistical analysis showed that FBHP applied at four timed intervals and Alt 2 both contained statistically similar numbers of undamaged tubers compared to the control. The FBHP treatment at 2 weeks also showed considerably fewer tubers falling into damage score categories 3, 2 and 1 than the control while even the less frequent treatment regime showed some effect in this way by reducing the number of tubers exhibiting heavy damage (damage score 3). By comparison to these, the chemical competitor treatment (Alt 1) showed greater control with more undamaged tubers and fewer tubers in damage score 1 or 2; no tubers treated with this regime exhibited damage scores of 3.

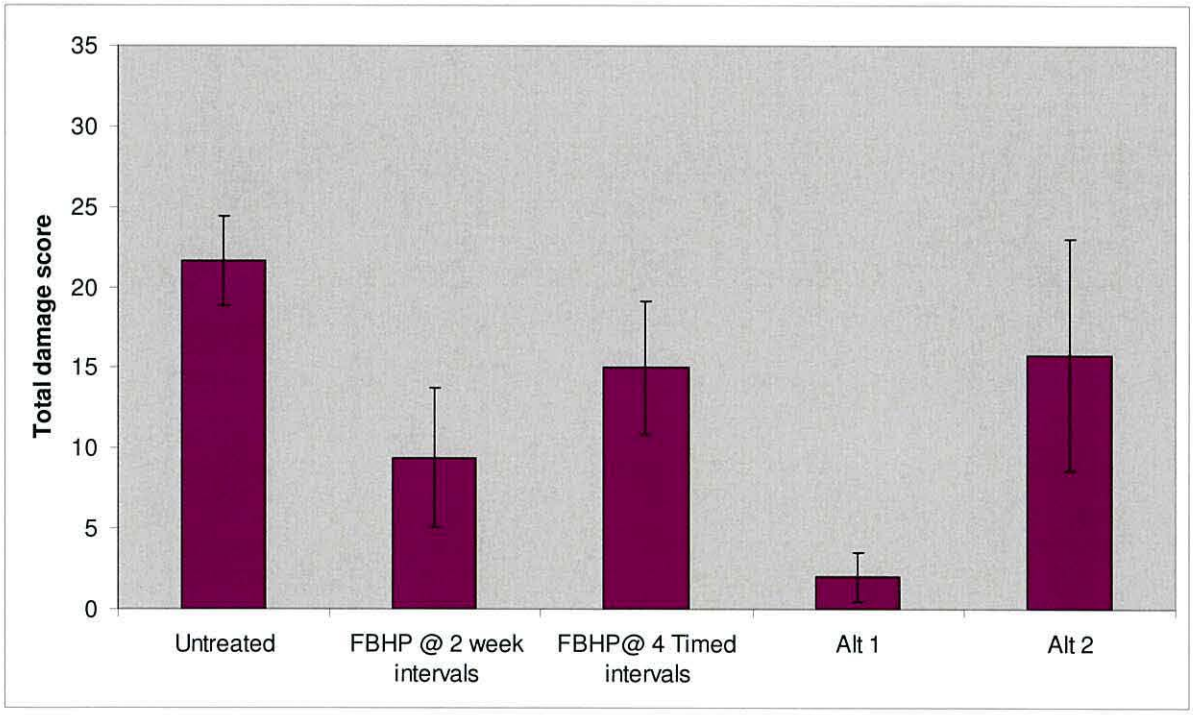


Figure 4.1.1.2 Total Damage Score of tubers following application of different molluscicidal treatment regimes

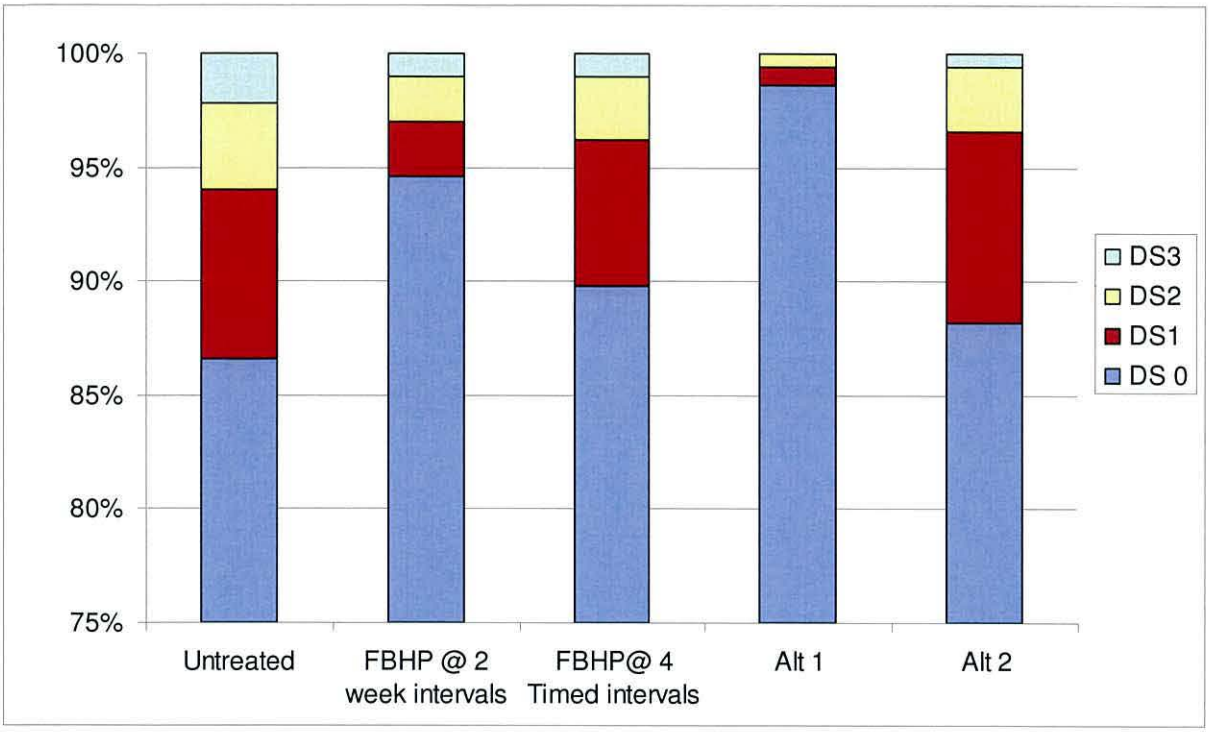


Figure 4.1.1.3 Percentage of tubers falling into each damage score category following application of various molluscicidal treatment regimes

Overall these results are encouraging with regards to the use of *H. helix* saponins as a means of controlling slug damage to crops. Control of slug damage to potato tubers is often expensive and labour intensive and most commercial regimes of pellets require frequent repeat applications throughout the season. Effective crop protection by this means is difficult as feeding damage occurs below ground and control depends upon slugs encountering pellets on the soil surface. The fruit saponin base hydrolysis product, in contrast, was applied as a foliar application in the form of a spray treatment. Although shown to be less effective than a current commercial chemical treatment, the activity identified for the saponin derived treatment applied in 2 week intervals was still significant and for an un-optimised compound indicate that it may have considerable potential for this type of treatment. There may also be interest in this type of slug control treatment as it is prepared from a natural plant extract rather than from synthetic chemicals in the manner of most treatments available at present

4.1.2 Independent evaluation of cidal activity against potato cyst nematode (PCN)

As well as slugs, potatoes can be susceptible to damage from other pest species; one such pest is the potato root nematode or potato cyst nematode (PCN). PCN are small roundworms belonging to the genus *Globodera* which comprises around twelve spp. and

are currently regarded as one of the most important potato pests in the UK.[156] Native to South America, it is now widespread in the UK with populations present in the majority of potato fields as well as in gardens and allotments; this leads to a shortage of clean land suitable for potato production. The eggs of PCN may survive in the soil in the form of a cyst for up to 10 or more years and, if potatoes are planted in infested soil, a large proportion of larvae emerge, stimulated by a range of substances (including glycoalkaloids) diffusing from the potato root. Hatched juveniles will invade the root and feed on the cells, causing damage to the roots which limits the uptake of water and minerals. Symptoms include stunted, wilting and yellowing plants in patches where populations are high. Through both the growth retardation in all infected plants, as well as serious root damage in severe cases, PCN can result in significant reductions in yield (up to 60 % in high population densities). As such suitable control measures, especially those not based on synthetic pesticides, are highly desirable.

An independent assay was conducted by Branston Ltd to assess the effect on mortality of PCN following treatment with varying concentrations of four compositions derived from *H. helix* saponins. The samples tested were: the crude fruit saponin extract from **6.3(ii)**; the base hydrolysis product of the fruit saponins from **6.8**; the acid hydrolysis product of the fruit saponins from **6.5**; the leaf base hydrolysis product prepared in **6.13**. Hatched juveniles of PCN were exposed to compositions comprising varying concentrations in water and the percentage mortality calculated from four replicates of each concentration shown each time. The results (shown in Figures 4.1.2.1 - 4.1.2.3) indicate that the mortality rate of the larvae treated with all four of the *H. helix* saponin compounds was higher than observed in the untreated control. The fruit base hydrolysis product performed best at all concentrations; showing a particularly high activity at a concentration of 0.1 % (Figure 4.1.2.1) where it resulted in 53.8 % mortality after 5 hours. The activities of the crude fruit saponins and fruit acid hydrolysis product were generally similar to one another at the two higher concentrations (Figure 4.1.2.1 and Figure 4.1.2.1), although the acid hydrolysis product appeared to perform better at the lowest concentration (Figure 4.1.2.3). The leaf saponin extract performed poorest overall of the four compositions tested, however at the highest concentration it showed a mortality rate of 25 % after 5 hours which is still significantly higher than the equivalent mortality rate of the untreated control.

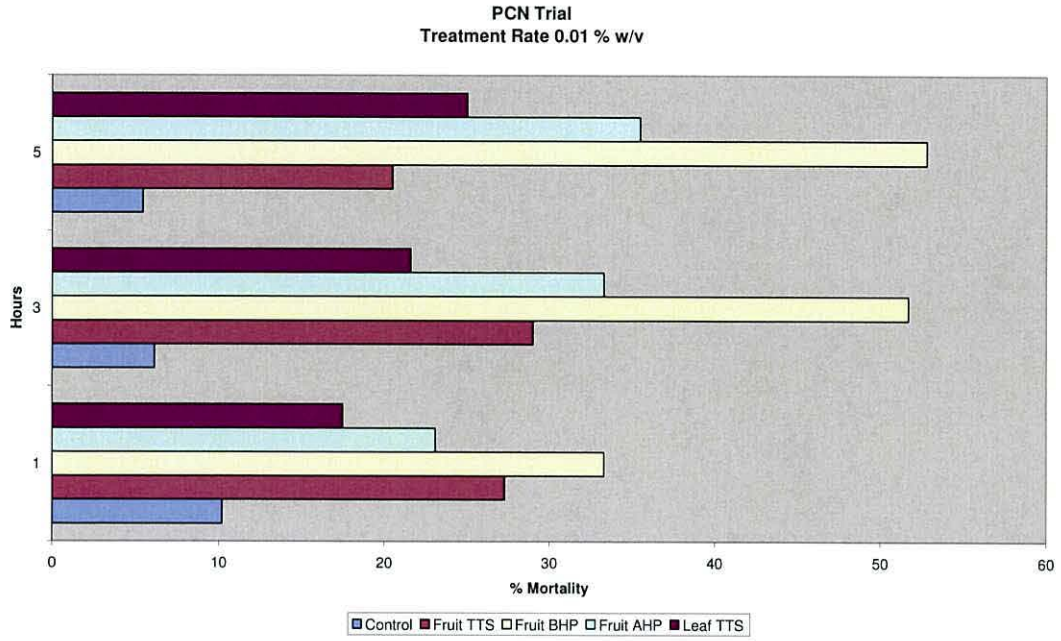


Figure 4.1.2.1 The effect on mortality of PCN following treatment with 0.01 % w/v solutions of four compositions derived from *H. helix* saponins

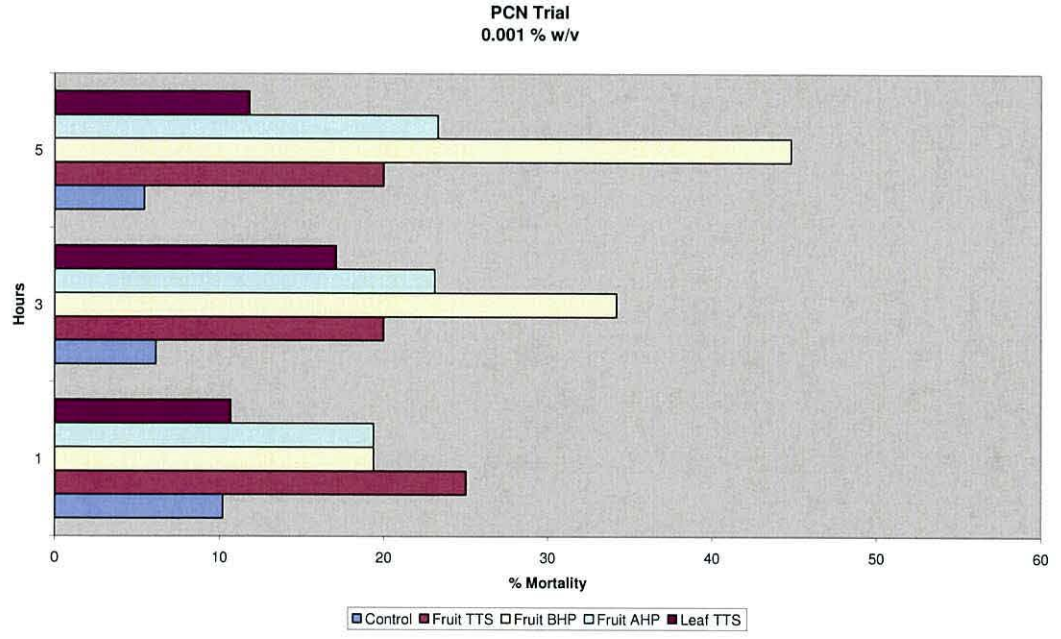


Figure 4.1.2.1 The effect on mortality of PCN following treatment with 0.001 % w/v solutions of four compositions derived from *H. helix* saponins

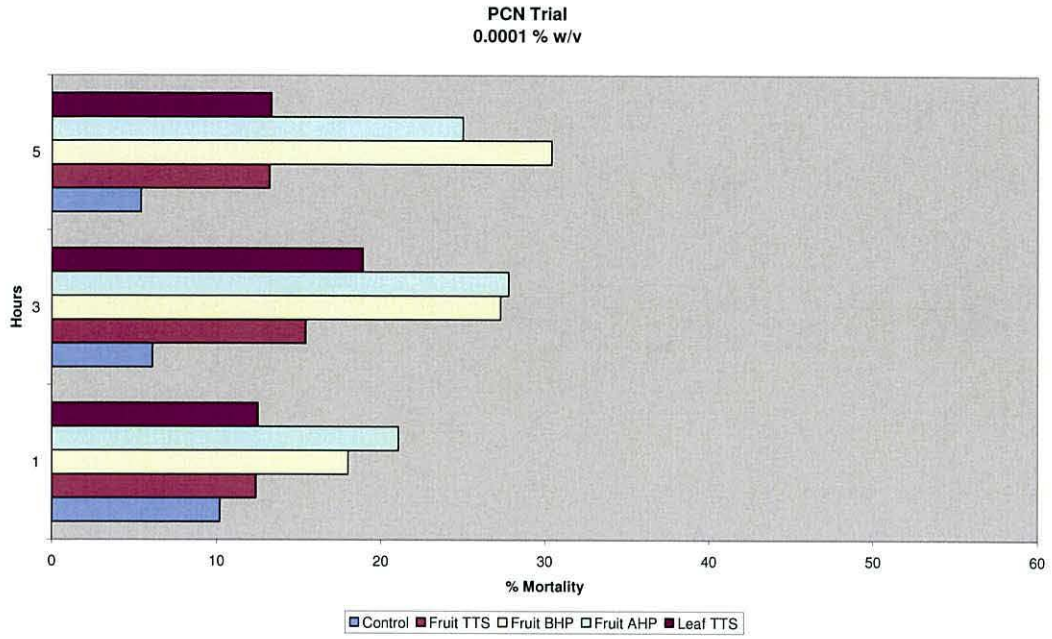


Figure 4.1.2.1 The effect on mortality of PCN following treatment with 0.01 % w/v solutions of four compositions derived from *H. helix* saponins

4.2 Antibacterial and antifungal activity of *H. helix* saponins and the corresponding hydrolysis products

Independent preliminary assays were conducted (Dr. P. Warn, Euprotec, Manchester) to assess the antibacterial and antifungal activity of four samples derived from *H. helix* saponins. These samples tested were: the crude fruit saponin extract (S1) from **6.3(ii)**; the base hydrolysis product of the fruit saponins (S2) from **6.8**; the acid hydrolysis product of the fruit saponins (S3) from **6.5**; the leaf base hydrolysis product (S4) prepared in **6.13**. In all of these assays, only one replicate was performed (n = 1) which is not sufficiently robust to draw any absolute conclusions of the medicinal efficacy of these compounds but is sufficient to identify which compunds are of interest to take forward for more rigorous biological testing.

4.2.1 Independent assay of the in vitro inhibitory and cidal activity against 3 bacterial strains and 2 fungal isolates

The *in vitro* inhibitory and cidal activity of the four *H. helix* saponin products against the bacteria *Pseudomonas aeruginosa*, *Eschericha coli*, and *Staphylococcus aureus* as well as the fungal strains *Aspergillus fumigatus* and *Candida albicans* was assessed. The cultures were grown on Sabouraud agar (*C. albicans* and *A. fumigatus*) or Nutrient agar (*P.*

aeruginosa, *E. coli* and *S. aureus*) and inoculated with 0.5-2.5 $x10^5$ per ml of suspension (bacteria) or 0.5-2.5 $x10^4$ per ml of suspension (*Candia* and *Aspergillus*). These were treated with a series of dilutions of the samples to be tested (512mg/L to 1mg/L for S1, S2 and S4 and 128mg/L to 0.25mg/L for S3). The plates were read visually with the endpoint taken as the lowest concentration of the sample that inhibited growth by 50 % compared to the drug free control; the minimum inhibitory concentrations (MIC_{50}) were then determined.

The endpoint MIC_{50} values for the fruit saponin extract (S1) against all strains is shown in Figure 4.2.1.1; this was identical to the results observed with the fruit base hydrolysis product (S2). This is not unexpected, since the fruit saponins are composed predominantly of monodesmosides and the saponin profile after base hydrolysis would not be expected to be dramatically different. The endpoint MIC_{50} values for the fruit acid hydrolysis product (S3) against all strains is shown in Figure 4.2.1.2 and those for the leaf base hydrolysis product (S4) are shown in Figure 4.2.1.3.

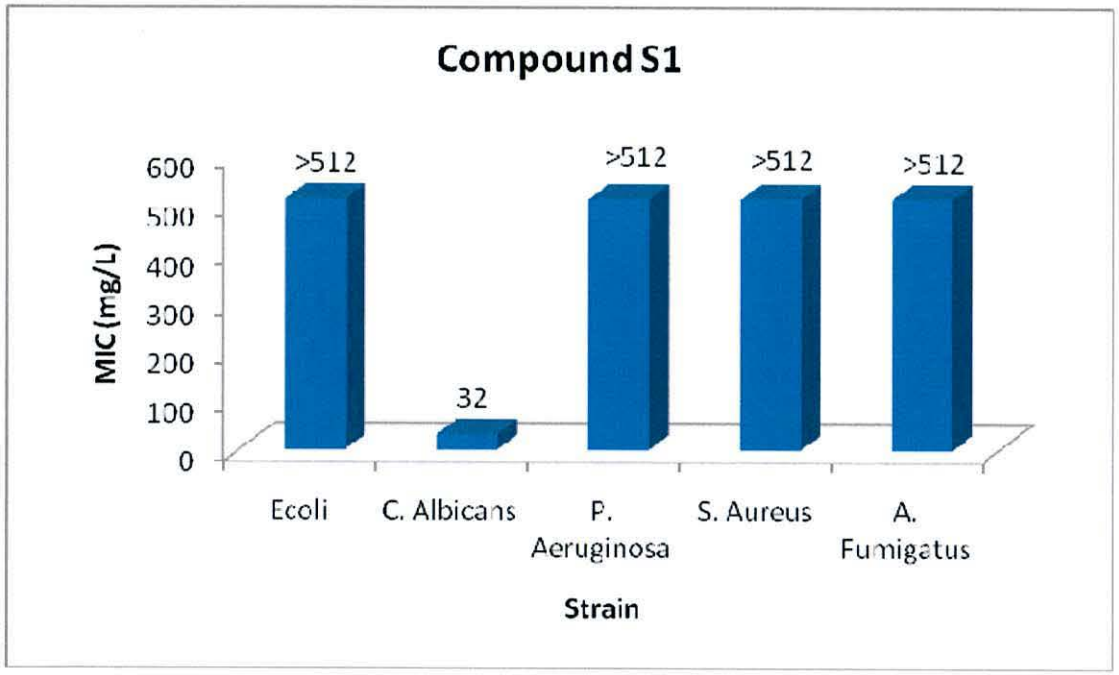


Figure 4.2.1.1 MIC_{50} values for fruit saponin extract (S1) and fruit base hydrolysis product (S2) against all strains

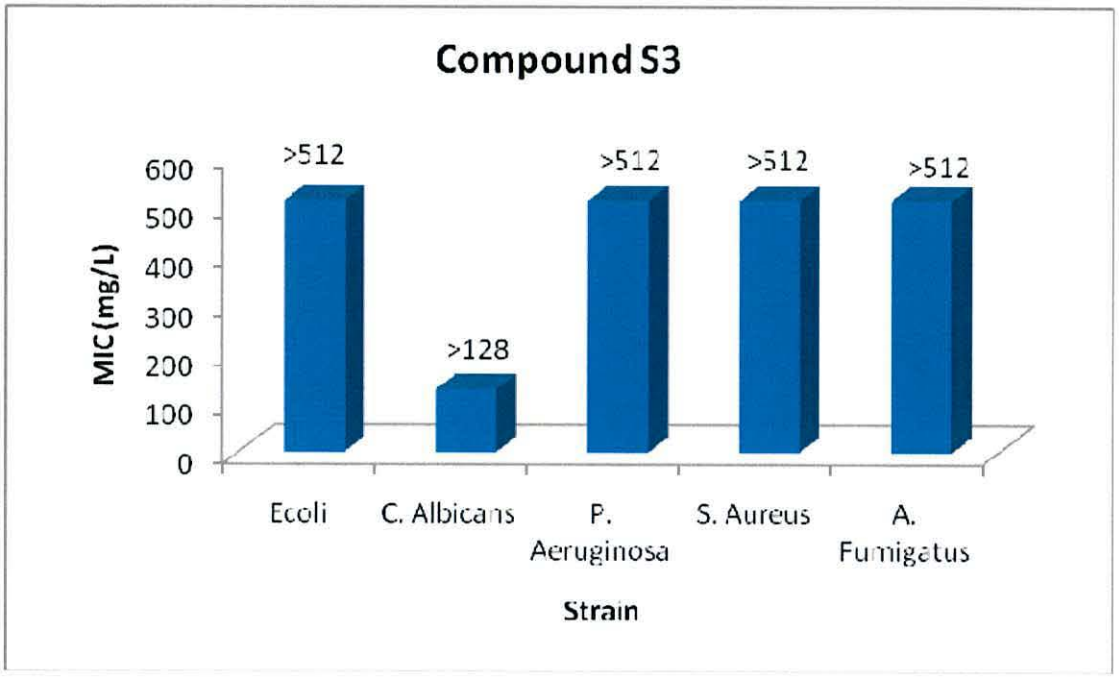


Figure 4.2.1.2 MIC_{50} value for the fruit acid hydrolysis product (S3) against all strains

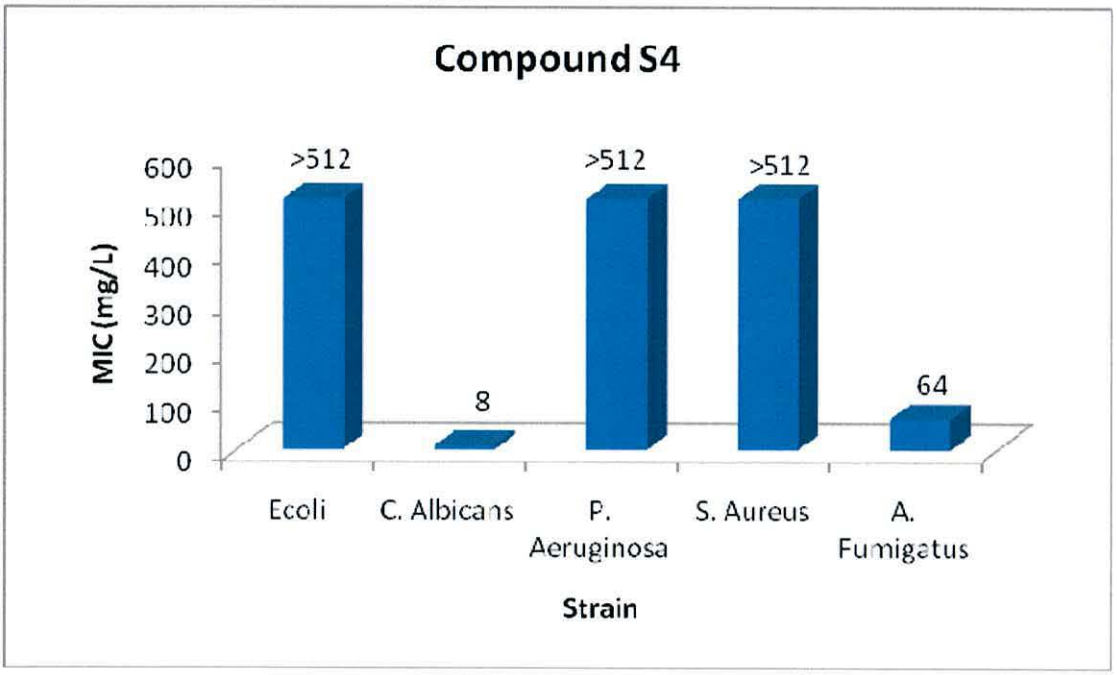


Figure 4.2.1.3 MIC_{50} value for the leaf base hydrolysis product (S4) against all strains

All of the samples 1-4 had no effect against either *E. coli* or *P. aeruginosa*. The *H. helix* fruit saponin extract (S1) and the fruit and leaf base hydrolysis products (S2 and S4) were effective against the yeast fungus *C. albicans* (Figure 4.2.1.4); the leaf base hydrolysis was the most potent, inhibiting 100 % of growth at 8 mg/L while the fruit extract and base hydrolysis product inhibited 100 % of growth at 32 mg/L. The acid hydrolysis product showed had no effect against this fungus, indicating that it is likely that the osidic chains

(particularly those at the C-3 position) were significant in the activity in some way. *C. albicans* is responsible for a number of oral and genital infections in humans (e.g. thrush) and can even lead to morbidity and mortality in immuno-compromised patients. Very few compounds exhibit a narrow spectrum activity against it and current topical therapies can easily be over 100 mg/L so the activity shown by these compounds is impressive for compounds which have not been optimized.

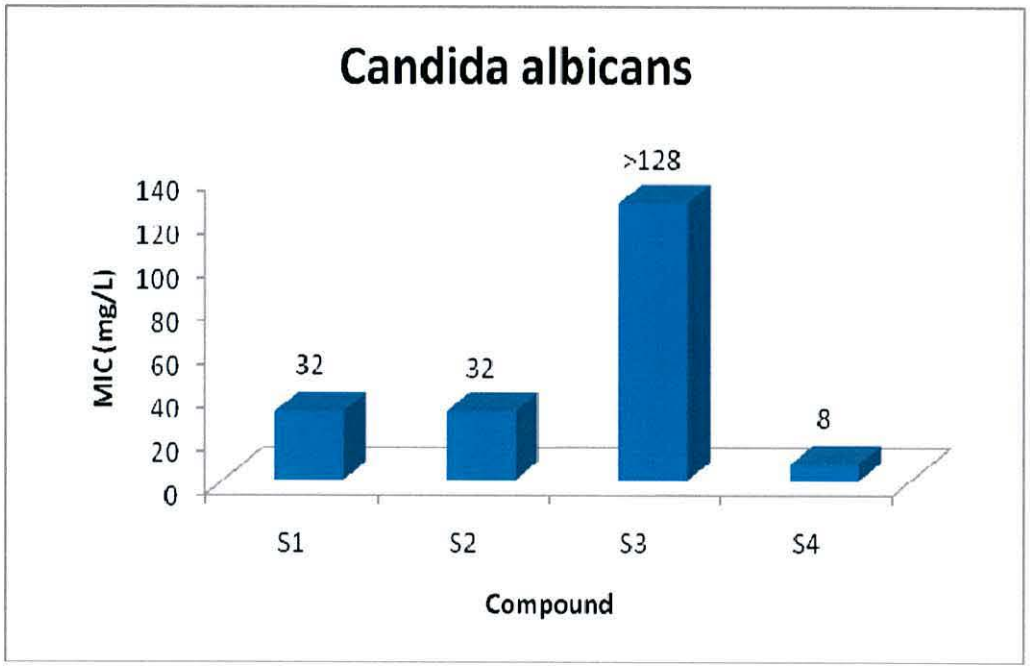


Figure 4.2.1.4 MIC_{50} values for S1, S2, S3 and S4 against *Candida albicans*

Only the leaf base hydrolysis product (S4) was effective against *A. fumigatus* with an inhibitory concentration of 64 mg/L, the other samples showing no activity (Figure 4.2.1.5). This is a fungus widespread in nature which is usually eliminated by the immune system, however in immuno-supressed patients it can become pathogenic; Aspergillosis of the lungs and deeper tissues can even lead to potentially fatal complications.

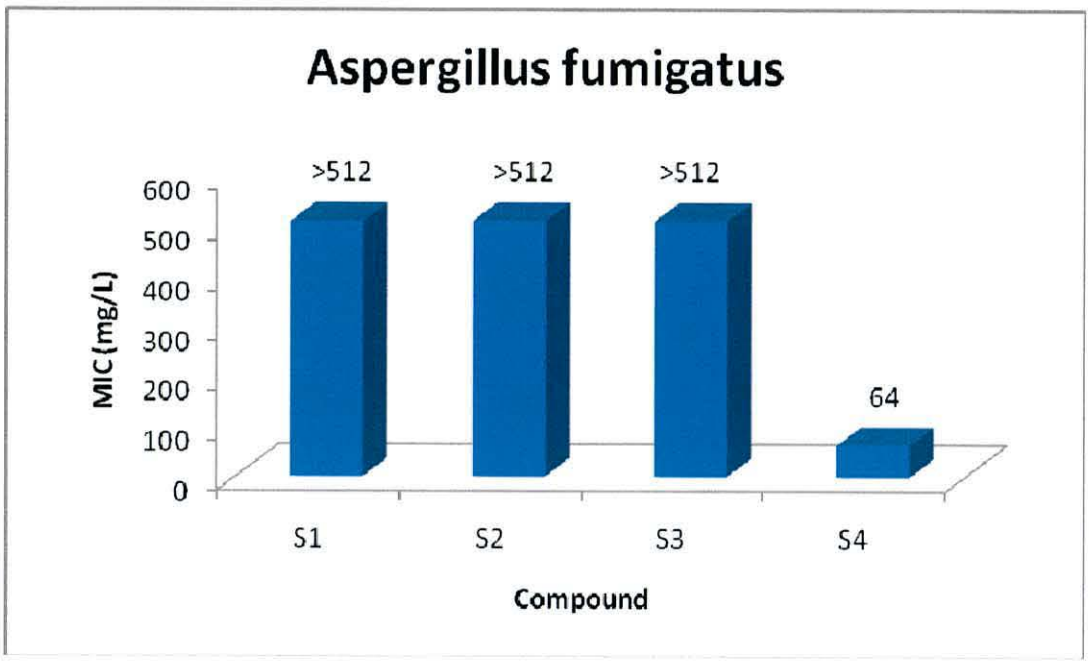


Figure 4.2.1.5 MIC_{50} values for S1, S2, S3 and S4 against *Aspergillus fumigatus*

None of the samples tested showed any effect on the MIC_{50} values for *S. aureus*, however some activity was observed. As well as determining the endpoint MIC values the cultures were incubated in a microplate reader and the wells read every 10 minutes for 24 hours to generate growth curves, determine the time of lag phase growth, and the maximal growth rate. The fruit acid hydrolysis product (S3) significantly reduced the maximal growth rate of *S. aureus* and increased the lag phase in a dose dependent manner over the dose range tested (8-128 mg/L). This can be seen in the growth curves below (Figure 4.2.1.6) when you compare the control maximal growth rate (brown) to that observed in the presence of increasing amounts of S3 with the highest concentration having the largest effect (dark blue). A minor effect on maximal growth rate of *S. aureus* was shown in the other three samples, but it was not observed to the same extent.

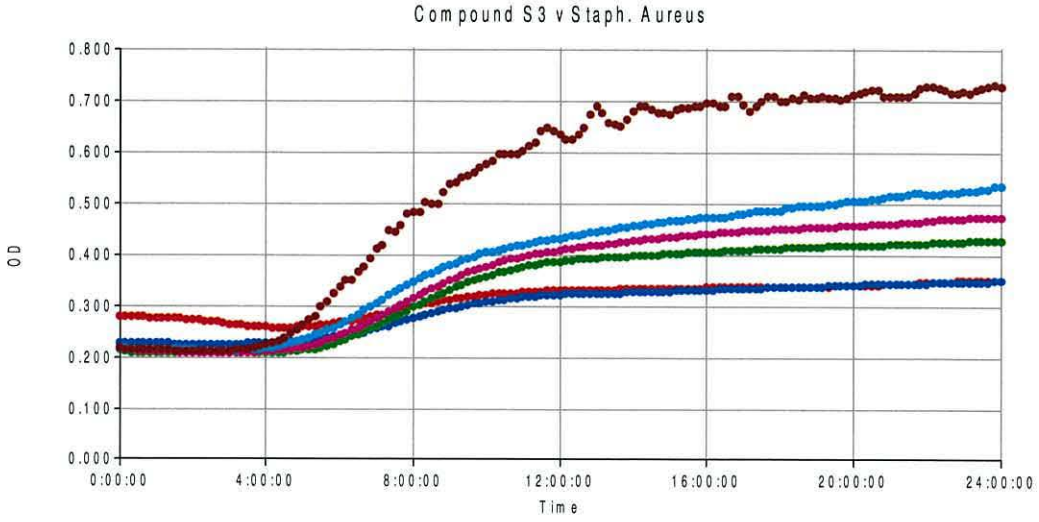


Figure 4.2.1.6: *Staphylococcus aureus* growth curve in presence of S3

No effect was observed on either lag phase growth or maximal growth rate with any of the samples 1-4 against *Pseudomonas aeruginosa*, a representative growth curve for S2 against *P. aeruginosa* is shown below (Figure 4.2.1.7). Similar results were observed for the growth curves of samples 1-3 against *E. coli*; however the curve of S4 (Figure 4.2.1.7) did show some activity, a result which is inconsistent with the observed MIC_{50} result reported earlier (Figure 4.2.1.3) suggesting an error was made in one of these experiments.

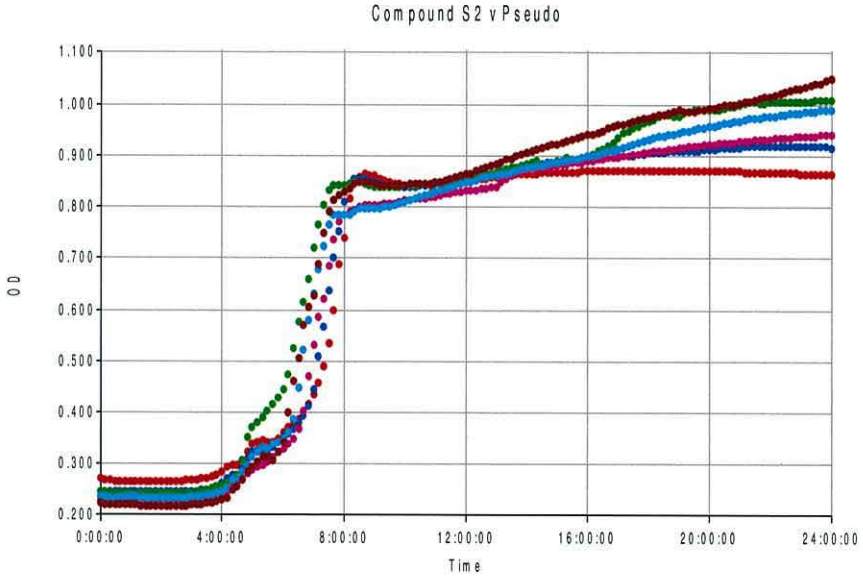


Figure 4.2.1.7: *Pseudomonas aeruginosa* growth curve in presence of S2

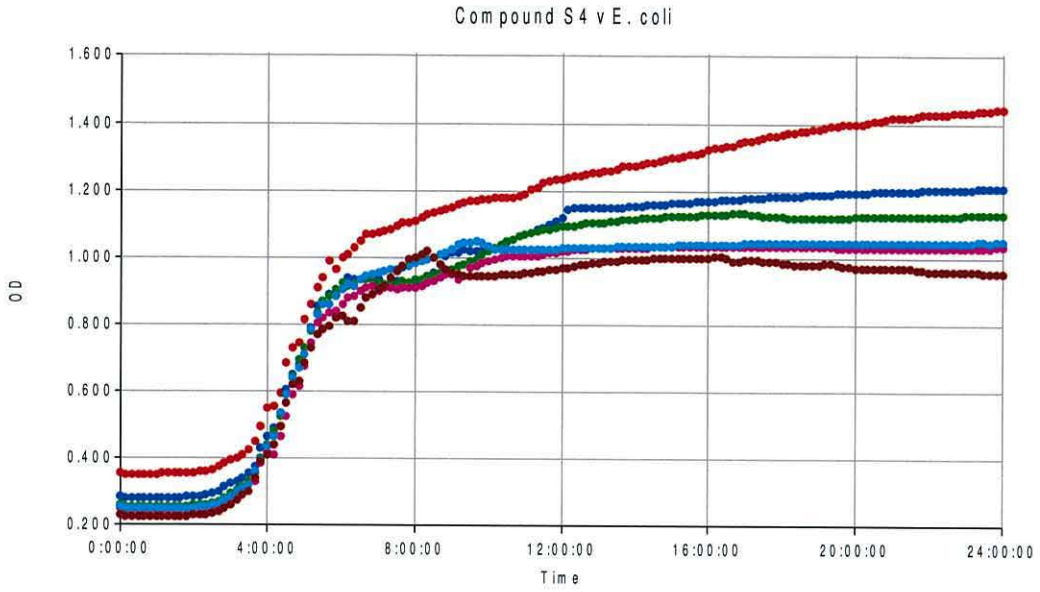


Figure 4.2.1.8: *E. coli* growth curve in presence of S4

4.2.2 Independent assay of the in vitro inhibitory and cidal activity against two strains of the fungus Malassezia furfur

The *in vitro* inhibitory and cidal activity of the four *H. helix* saponin products against two *Malassezia* isolates (*Malassezia pachydermatis* CBS 6536 and *Malassezia globosa* CBS 7966) was assessed. The cultures were prepared in either a modified Leeming notman media or a modified Christensen's urea media. These were treated with a series of dilutions of the samples to be tested. The plates were read visually with the endpoint taken as the lowest concentration of the sample that inhibited growth by 50 % (Leeming Notman media) compared to the drug free control (MIC_{50}). For Christensen's urea media the endpoint was taken as the first well that inhibited hydrolysis of urea (MIC).

MICs demonstrated that these compounds had some inhibitory effects against *M. pachydermatis* and *M. globosa*, and the results were similar in both growth media used. The assay conducted in Leeming Notman media showed no growth of *M. globosa* in plates treated with any of the four samples; similarly, all samples inhibited growth of *M. pachydermatis* at 32 mg/L (Figure 4.2.2.1).

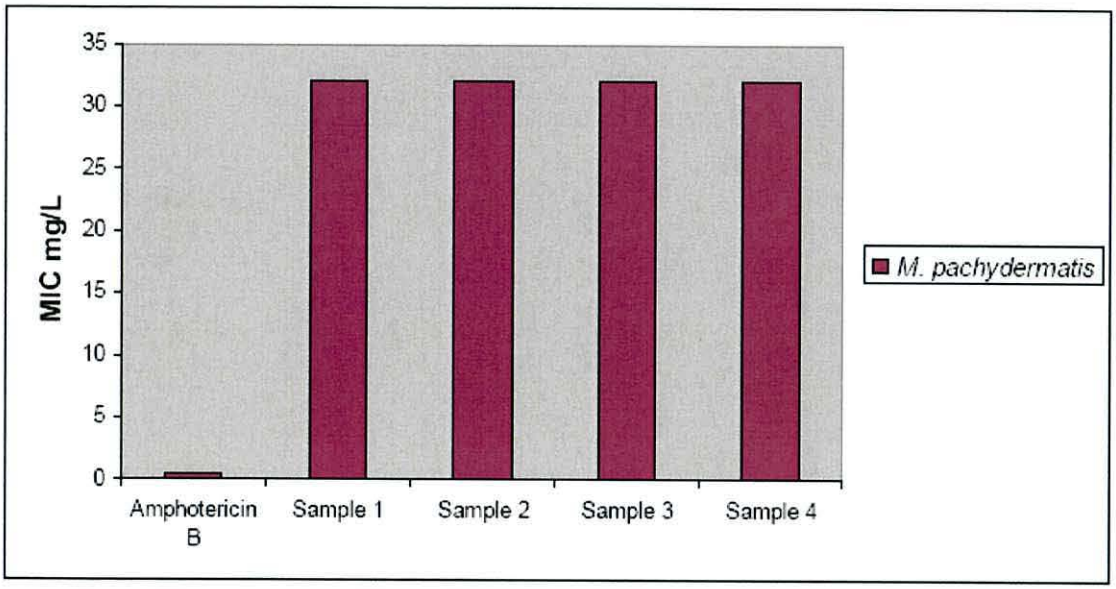


Figure 4.2.2.1 MIC_{50} values for S1, S2, S3 and S4 against *Malassezia pachydermatis* in Leeming Notman media

The assay conducted in Christensen's urea media showed all of the hydrolysis products (S2, S3 and S4) to inhibit the growth of *M. globosa* at 32 mg/L while the crude fruit extract (S1) was more active, inhibiting growth at 16 mg/L; in this medium, it was the fruit acid hydrolysis product (S3) which appeared most active against *M. pachydermatis* at 32 mg/L while the others inhibited growth at 64 mg/L (Figure 4.2.2.2).

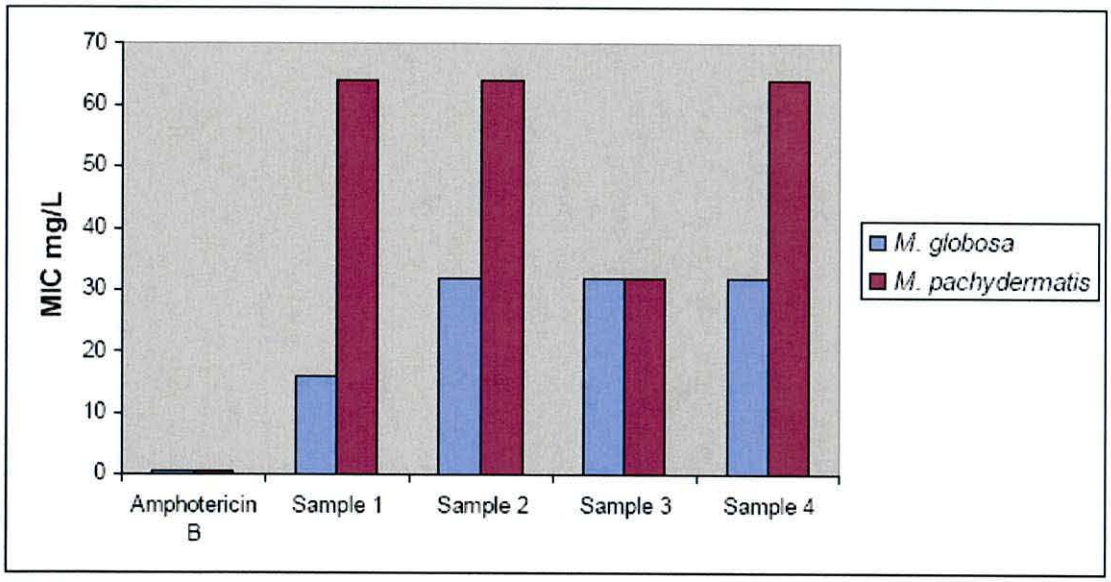


Figure 4.2.2.2 MIC values for S1, S2, S3 and S4 against *Malassezia* in Christensen's urea media

Overall this assay showed that all four compounds were effective against both strains of the yeast fungus *Malassezia* with little to choose between the samples tested in terms of

efficacy. MICs for all samples tested were in the range 16-64 mg/L (a control of Amphotericin B gave MICs of 0.5 mg/L. *Malassezia* fungi are naturally found on the human skin surface of humans and can result in opportunistic infection; it is the cause of most human skin disease. M. globosa in particular is the main cause of dandruff and soborrhoeic dermatitis. The MIC values shown for these compounds are within the range suitable for topical therapy for this type of fungal condition.

4.2.3 Independent assay of the in vitro inhibitory and cidal activity against the fungus Fusarium oxysporum

The *in vitro* inhibitory activity of the four *H. helix* saponin products against the crop pathogenic fungus *Fusarium oxysporum* was assessed. The cultures were grown on a RPMI-1640 growth medium and inoculated with 2 x 10^5 per ml of a suspension of *F. oxysporum*. These were treated with a series of dilutions of the samples to be tested (512 mg/L to 1 mg/L for S1, S2 and S4 and 128 mg/L to 0.25 mg/L for S3). The plates were read visually with the endpoint taken as the lowest concentration of the sample that inhibited growth by 50 % compared to the drug free control; the minimum inhibitory concentrations (MIC_{50}) were then determined. The wells were also read with a microplate reader every 10 minutes for 40 hours to generate growth curves, determine the time of lag phase growth and the maximal growth rate.

The endpoint assay showed fruit saponin extract (S1), fruit base hydrolysis product (S2) and leaf base hydrolysis product (S4) were effective against *F. oxysporum* with the fruit extract being the most potent, inhibiting 100% of growth at 64 mg/L, while the others inhibited growth at 128 mg/L. There was an effect observed on the maximal growth rate of *F. oxysporum* in the presence of these samples which resulted in increased lag-phase and lower final burden in the test wells. An example of this can be seen in the growth curve below (Figure 4.2.3.1) when comparing the control maximal growth rate (dark blue) to that observed in the presence of increasing amounts of fruit saponin extract (S1), with the highest concentration having the most significant effect (dark green), i.e. a dose-dependent response. The acid hydrolysis product (S3) showed no inhibition of *F. oxysporum* in the concentration range studied and no effect on maximal growth rate was observed either (Figure 4.2.3.2).

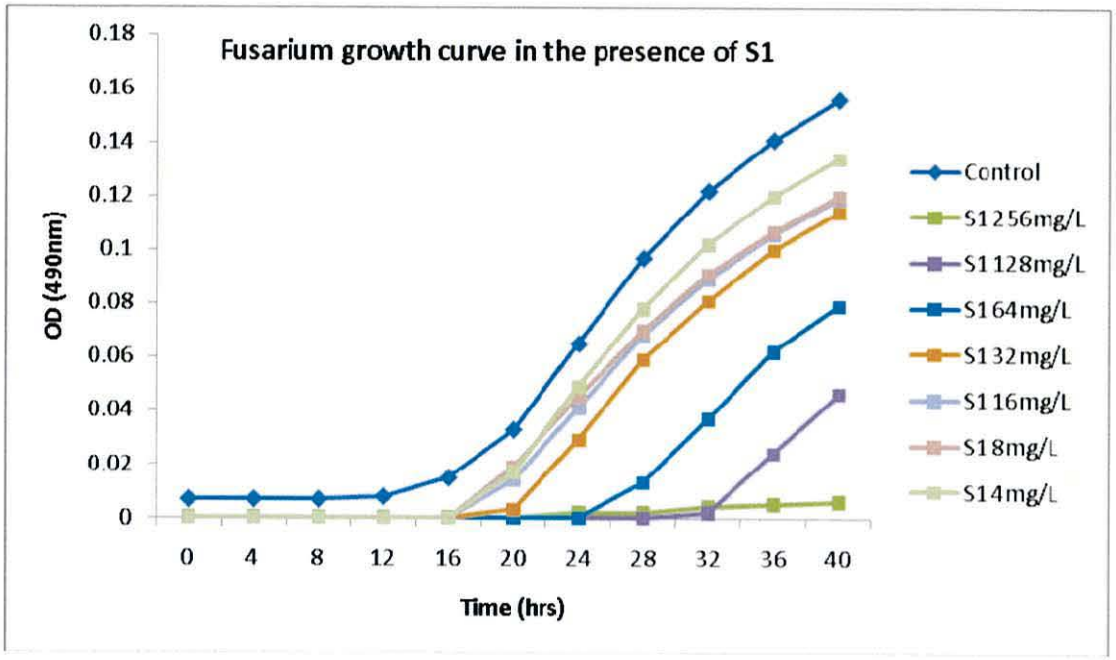


Figure 4.2.3.1: ***Fusarium*** **growth curve in the presence of fruit saponin extract (S1)**

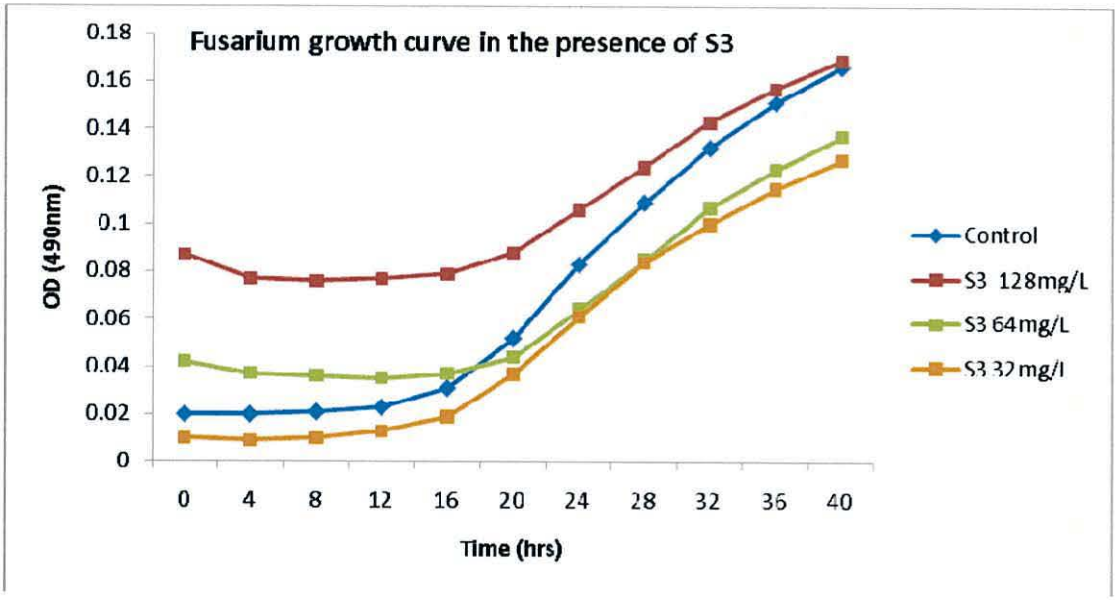


Figure 4.2.3.2: ***Fusarium*** **growth curve in the presence of acid hydrolysis product (S3).**

4.3 Activity of *H. helix* fruit saponins as crop treatments against late blight in field potatoes

Phytophthora infestans is an oomycete or water mould responsible for the serious potato disease known as late blight or potato blight. Spores of this mold develop on the leaves

of potatoes and other *Solanaceae* species such as tomatoes, spreading through the crop when temperatures are above 10 °C and humidity is over 75% for 2 days or more. Rain can wash spores into the soil where they infect young tubers. They can also travel long distances on the wind. These spores survive over winter on infected tubers, particularly those that are left in the ground after the previous year's harvest and are spread rapidly in warm and wet conditions, often resulting in devastating effects by destroying entire crops. Symptoms of blight include the appearance of dark blotches on leaf tips and plant stems with the appearance of white mould under the leaves in humid conditions, after which the whole plant may quickly collapse. Infected tubers develop grey or dark patches that are reddish brown beneath the skin; secondary bacterial rots will usually set in causing them to rapidly decay to a foul-smelling mush. As a result of the severe damage it causes to the infected tubers and the rapid spread of the disease through the foliage which in turn stops tuber growth, late blight is a major problem for commercial potato growers and can significantly reduce yields of healthy tubers for sale. Control of *P. infestans* is difficult, even using modern methods, often requiring a number of fungicide treatments to be sprayed on to the crops, sometimes weekly, in order to protect them. Alternatively switching to other varieties of more blight resistant potatoes is a means of minimising losses; however consumer demand for traditional varieties such as King Edward and Maris Piper makes this solution less feasible for most growers.

An independent field trial was conducted to determine the efficiency of four compositions from *H. helix* saponins for the control of foliar blight in potato. The samples tested were: the crude fruit saponin extract from **6.3(ii)**; the base hydrolysis product of the fruit saponins from **6.8**; the leaf saponin extract from **6.10(ii)**; the leaf base hydrolysis product prepared in **6.13**. The trial was directed by Dr. O. Scrimshaw, Eurofins Agroscience Services, an organisation which holds ORETO status accredited by the Pesticides Safety Directorate under the Plant Protection Product Regulations;[155] it was sponsored by Branston Ltd. The trial was conducted on a field site at Barrow on Trent, Derbyshire containing the blight susceptible variety of potato *Maris Piper* which had previously been used for growing Winter wheat. Treatments of the crude fruit saponin extract together with a synthetic latex bonding adjuvant were applied as a foliar spray application at a rate of 6 kg / ha as a 0.14 % solution v/v in water; applications were delivered at a spray volume of 1000 l / ha at 100 % plant emergence (rosette stage) with

subsequent applications at 7 day intervals. Disease inoculation took place 10 days after the initial treatment using a bulk inoculum of an A2 (B13) strain and an A1 strain of *P. infestans* in water at a concentration of approx. 5 x 10^5 / ml. Data is also included for the untreated control as well as for one of the commercial competitor treatments that were also tested in the same field trial.

Unfortunately, weather conditions during May and June 2008 were not conducive to foliar blight development. Rainfall during July and August were supplemented with additional irrigation, giving 270 % of the average monthly rainfall for the two months, in order to manipulate in-crop humidity and leaf moisture levels to encourage blight infection. This, together with increasing night time temperatures during late July improved conditions for blight development and the disease spread rapidly in untreated plots in early August.

Throughout the trial problems were encountered with mixing and application of the leaf saponin extract and the leaf base hydrolysis product. During the formulation of these compounds with bonding agent, they were found to be difficult to get into suspension both during mixing and then during application; nozzles and filters tended to block which reduced the spray patterns during the application. These problems were not encountered when using the other formulations. In all cases, no phytotoxic symptoms were observed in the plants at any stage of assessment; this is indicative that the crop safety of these formulations is such that they could be used in an agricultural treatment on this type of crop.

Foliar blight remained low throughout July and all treatments were observed to achieve full control of the disease. Once infection in the untreated plots began to spread rapidly from early August a more pronounced effect was observed (Figure 4.3.1); infection reached 45 % in the untreated plots by 12th August while in comparison all of the treated plots exhibited significant foliar blight control up until 18th August when the infection became epidemic; after this point all of the treatments failed to exhibit control of the disease with exception of a standard commercial mixed fungicide regime (shown in red) which exhibited 87 % control at the final assessment timing. Of the saponin treatments it was the fruit saponin extract that achieved the best foliar blight control, giving 40 % control on the 18th August, whilst the fruit base hydrolysis product achieved 28 % and

both leaf samples approximately 20 % control; the reduced activity observed with the leaf products may have been compounded by the formulation problems mentioned earlier. A number of alternative treatments were assessed in the same trial, an example of which (Alt) is also shown. This treatment was shown to exhibit a similar control to the leaf saponin extract and leaf base hydrolysis product and was found to be less effective than either the fruit saponin or fruit base hydrolysis product.

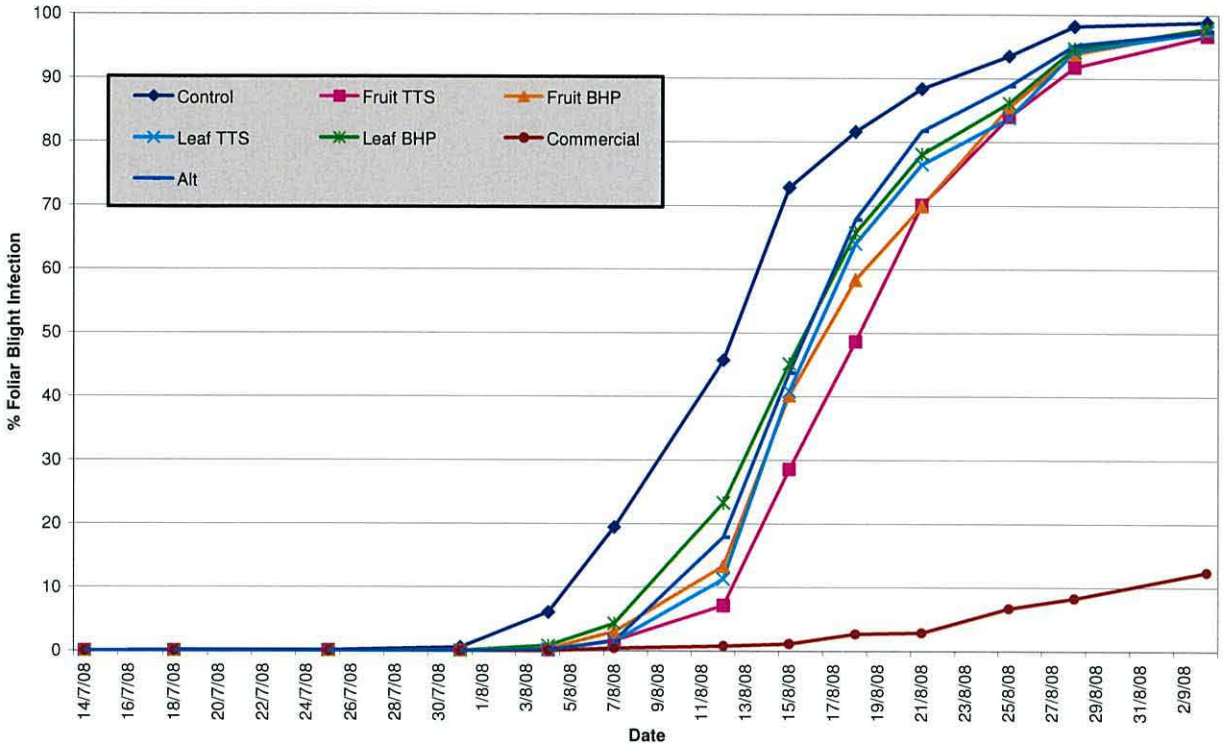


Figure 4.3.1: Foliar blight infection of potatoes over the duration of the field trial following applications of different treatment regimes

The trial also attempted to assess the incidence of stem blight. This was done in the middle of August but little stem blight was apparent on the untreated plots and there was no difference apparent in the treated blocks. Only the standard fungicide regime was observed to reduce the amount of stem blight. However, due to the very low incidence of stem blight observed overall it is difficult to assess how significant this control was compared to the other treatments.

No tuber blight was observed on any of the treatments or the untreated control at harvest. This may have been due to any infected tubers having degraded in the soil prior to harvest, partially down to heavy rainfall in September and flooding in the vicinity of

the trial, which delayed harvest until ground conditions were more suitable. As such, the total yield and healthy yield were identical to one another for all treatments (Figure 4.3.2). All of the treatments except the fruit base hydrolysis product and leaf saponin extract achieved an increase in marketable yield compared to the untreated.

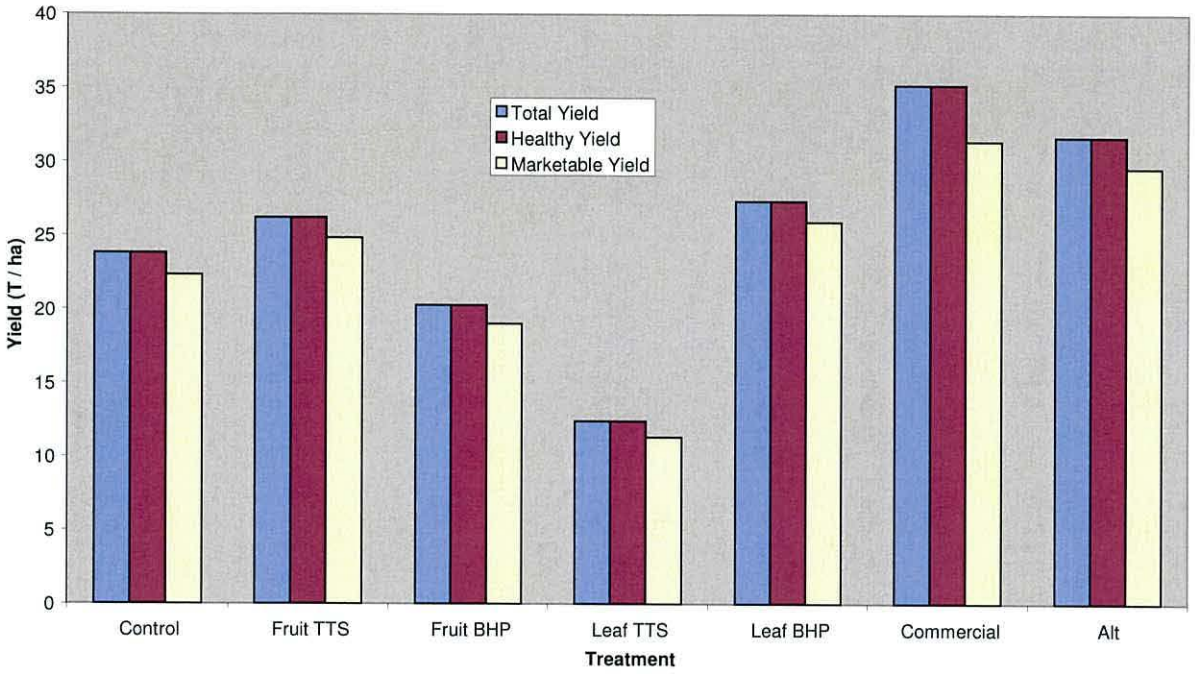


Figure 4.3.2: Total, healthy and marketable yield of potatoes (tonnes per hectare) following applications of different treatment regimes

As has been highlighted previously, weather conditions throughout the field trial appear to have had serious effects on the growth of blight and on the assessment of infection in some cases. Despite this some evidence was shown to indicate the activity of these compounds as a control of late blight in potatoes. The failure of these towards the later part of the trial was shown to coincide with a similar failure of all of the other new treatments tested (e.g. Alt) and as such may not be a true reflection of the effectiveness of the *H. helix* saponin treatments under more normal climatic conditions.

4.4 The activity of *H. helix* saponins and a number of derivatives as timber preservatives

Timber decay as a result of fungal attack is a serious problem; dry rot (caused by *Serpula lacrymans*) and wet rot (caused by *Coniophora puteana*) are the most common form of decay in buildings in the UK. These types of fungus break down break down

timber elements in buildings, particularly in areas were ventilation is poor and humidity can become high. This reduces the strength of the timber until it finally becomes structurally compromised. Since this can be financially costly, not to mention the safety implications associated with buildings which have suffered such decay, there is an increasing demand to identify suitable treatments which can be applied to the wood to prevent such a fungal attack.

A series of independent assays were conducted (Dr. Morwena Spear, Biocomposites Centre, Bangor) to assess the potential of a number of compositions as potential timber preservatives.

4.4.1 Mini-block test of timber treated with H. helix saponin extracts

An accelerated decay test was conducted using a widely accepted method, presenting treated and untreated wood blocks to cultures of pure fungus on agar. Three brown rot fungi were used – *Coniophora puteana*, *Serpula lacrymans* and *Poria placenta*. These are common in the decay of timber in buildings. Scots pine miniblocks (5 x 10 x 30mm) were impregnated with a crude saponin extract from leaves of *H. helix* from **6.8**; crude fruit saponin extract from **6.3(ii)**; and the base hydrolysis product of the fruit extract from **6.5**. The miniblocks were arranged in the petri dishes so that three treated and three untreated blocks alternated around the dish. All three extracts showed a significant decrease in weight loss after exposure to the three brown rot fungi. The weight loss was suppressed to negligible levels in the blocks exposed to *Poria placenta*, and the difference in colonisation by the mycelium was clearly visible, as shown in Figure 4.4.1.1.

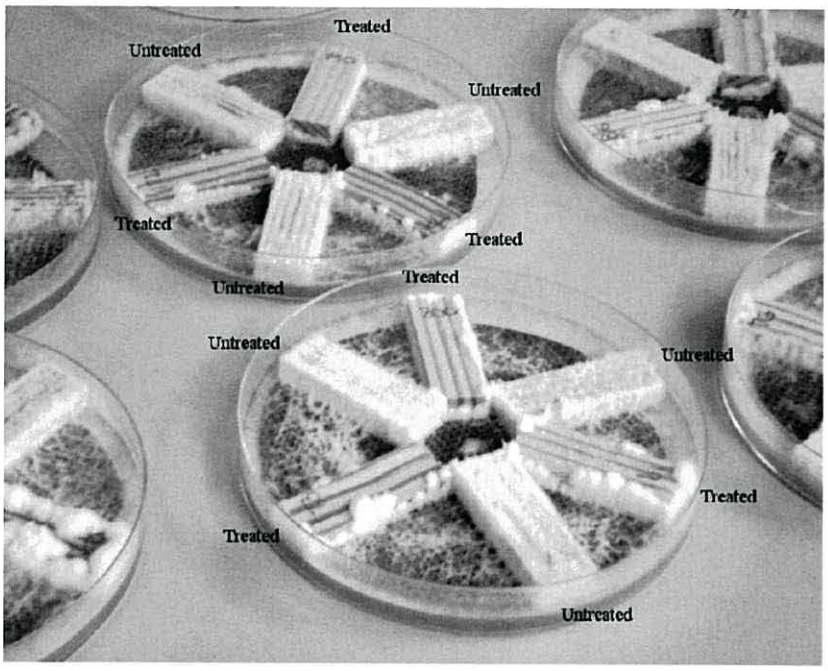


Figure 4.4.1.1 The difference in colonisation by the mycelium between treated and untreated miniblocks following exposure to *Poria placenta* (shown in black and white for better clarity)

4.4.2 Testing of timber treated with H. helix saponin extracts

Following the positive findings of this initial miniblock trial a further study was carried out to assess in more detail the preservative action of these compounds against timber fungi using a method conforming to that set out by the British Standards Institution (BS EN 113). The samples tested were: the crude fruit saponin extract from **6.3(ii)**; the base hydrolysis product (FBHP) of the fruit saponins from **6.8**; the acid hydrolysis product (FAHP) of the fruit saponins from **6.5**; the leaf base hydrolysis product (LBHP) prepared in **6.13**. Extracts were made up as solutions or fine suspensions in ethanol, at a concentration of 0.625 % (w/w), to allow impregnation into timber test samples. The impregnated blocks were then dried at 105 °C for 2 days to drive off the solvent and the weight of retained preservative was determined. They were then conditioned at 20 °C and 65 % humidity for 2 weeks before being sterilised by irradiation. Sterilised samples were placed into jars in which a fungal culture was already established upon an agar medium. Four fungi were tested, the three brown rot fungi tested in the miniblock trial (*Coniophora puteana*, *Poria placenta* and *Serpula lacrymans*) and one white rot (*Trametes versicolor*). The jars were maintained in constant conditions (25 °C, 70 % relative humidity) for a 16 week period; the jars containing *S. lacrymans* were observed to be faring poorly under these conditions so in order to encourage better growth the jars were moved to a chamber maintained at 22 °C at week 8 of the test and the test period for

this strain extended by 1 week in an attempt to revive the fungus. At the end of the test, the blocks were removed from the jars, cleaned of surface mycelium, dried and reweighed to determine the weight loss experienced due to timber decay. This was assessed to determine whether it met the levels required by BS EN 113.

The data gathered for *T. versicolor* was not used for the BS EN 113 test due to insufficient levels of decay being observed in the control samples (20 % weight loss is required by the standard for this fungus on beech). However, despite not being robust enough for this assessment, the results obtained did show a small suppression of weight loss in the treated blocks compared to the untreated (Figure 4.4.2.1). Although these decreases in weight loss by decay are small, statistical analysis of them by a paired t-test showed that the crude fruit extract and FBHP showed significant differences at the 95 % confidence level. The effects of the crude leaf and FAHP, despite appearing to show a similar magnitude of difference, were found to be non-significant.

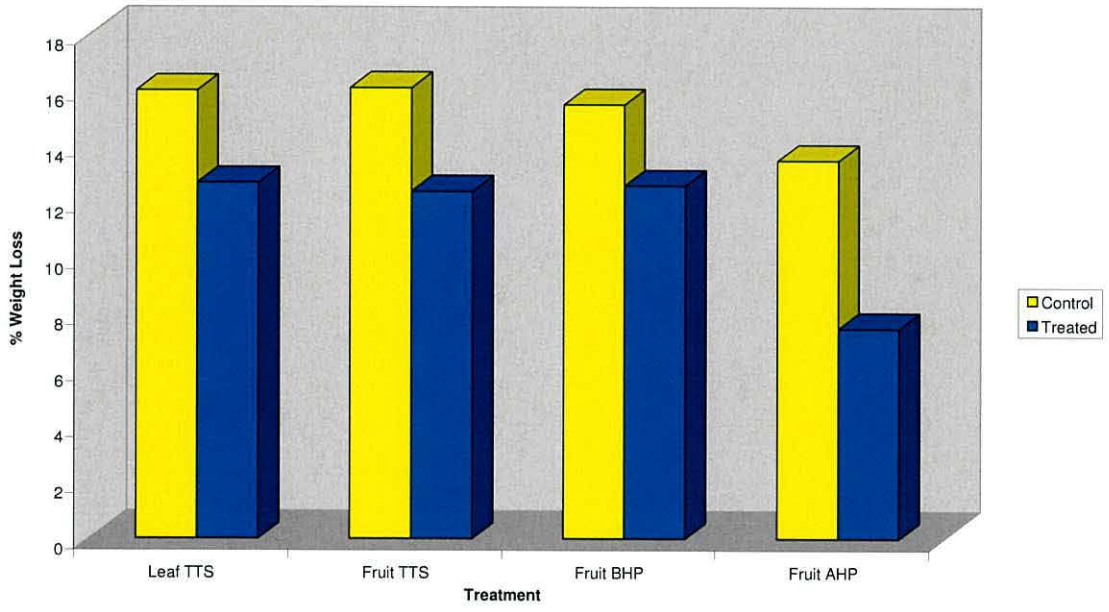


Figure 4.4.2.1 Mean percentage weight loss of treated timber blocks and untreated controls on exposure to *Trametes versicolor*

The tests did not show a suitable level of decay resistance for any of the four compositions tested against *C. puteana* and *P. placenta* to meet BS EN 113. Statistical analysis of the data did however show a significant difference in weight loss in exposed

test specimens. Statistical analysis of weight loss in exposed test specimens showed a significant difference for the crude leaf extract, but no significant difference for the other three extracts with *C. puteana*. A visible change was also observed for timber treated with all four compositions in tests with *P. placenta* (Figure 4.4.2.2) with the mean percentage weight loss seen to be reduced in the treated blocks when compared to the untreated blocks; of these changes, those for crude leaf extract, crude fruit extract and FBHP were observed to be statistically significant.

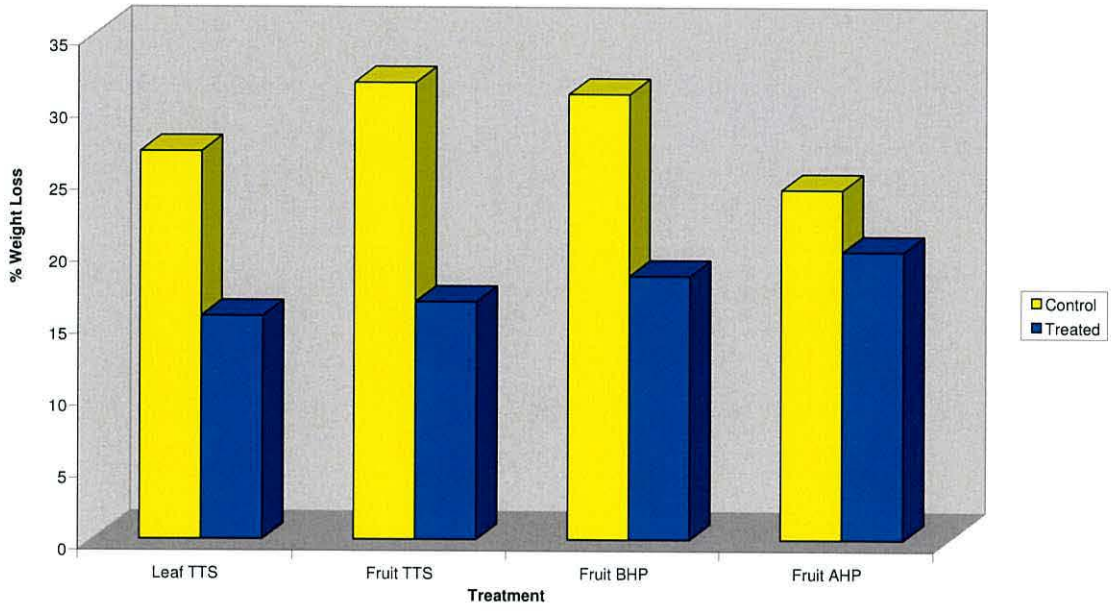


Figure 4.4.2.2 Mean percentage weight loss of treated timber blocks and untreated controls on exposure to *Poria placenta*

The technical difficulties described earlier prevented suitable data being obtained in the case of *S. lacrymans*. The very poor colonisation of the jars by this fungus resulted in only a very small weight loss of the control blocks and as such the difference between this small weight loss and the negligible weight loss of the test specimens was not analysed further.

For both of the fungi that the treatments were most effective against (*T. versicolor* and *P. placenta*), it appeared that although there was a diminished level of degradation during the course of the test, the fungus was still able to colonise the timber and inflict decay at the levels of treatment tested (0.625 % w/w solution). Since this concentration was close

to (or above) the solubility limit of these samples it does not appear feasible to further increase this dose while using the same methodology.

The use of the acid hydrolysis product as a treatment showed no significant difference in tests with any of the selected fungi; this sample did however, give a lower level of weight gain when the timber was impregnated than was observed for the other treatments which might have contributed to this problem.

4.4.3 Assessment of the activity of hederagenin against timber fungi

Agar plates of the acid hydrolysis product of *H. helix* fruit saponins (FAHP) were prepared at a range of concentrations between 0.008 – 0.128 % together with untreated control plates and reference plates of the fruit base hydrolysis product (FBHP) and crude fruit saponins at a concentration of 0.064 % to allow cross correlation with results from the previous study. Twelve different fungi were grown on the plates which represent many white and brown rot basidiomycete fungi, and one mould fungus.

All three groups of fungi showed a decrease in mycelium growth rate as a result of the acid hydrolysis product present in the agar, although in some cases there appeared to be a threshold where a minimum quantity of the compound was required before the effect was observed.

Growth of the mould fungus *Trichoderma viride* on the control plate and on the plate treated at 0.008 % was rapid. As the concentration increased there was a decrease in the growth rate of the fungus up to the highest concentration of 0.128. %. Comparison of the 0.064 % concentration with those of the other saponin compositions at this concentration showed the growth rate of *T. viride* was slightly faster in the FAHP than the crude fruit extract and slightly slower than the FBHP.

The white rot fungi *Trametes versicolor* and *Pycnoporous sanguineus* both showed growth influenced by the concentration of acid hydrolysis product on the agar plates. The growth rate of both fungi showed a clear concentration response from 0 - 0.064 %. Comparison with the other saponin compositions showed the FAHP had a greater inhibitory effect than either the crude fruit saponin extract or FBHP against both of these fungi. The growth rates of two other white rot fungi (*Pleurotus ostreatus* and *Phlebia*

gigantea) were found to be inhibited by FAHP, although in these cases a marked difference was observed between the low concentration plates and the high. It appears the fungi were able to tolerate low levels of the FAHP but growth became stunted above a threshold at 0.64 %. The FAHP performed well against these fungi compared to the FBHP and crude extract references. The final white rot fungus tested was *Heterobasidon annosum*; this showed a reduction in growth rate at all concentrations above 0.016 % FAHP. The growth rates of *H. annosum* in the presence of FAHP and FBHP at 0.064 % were similar, where both were faster than the crude fruit reference.

The growth rate of *Coniophora puteana*, a brown rot fungus, showed a concentration response to FAHP, with mycelial growth being dramatically reduced at 0.064 % and 0.128 %; this composition caused slower growth of *C. puteana* than the FBHP and crude saponin extracts. A very strong trend to reduce growth rate was seen for *Serpula lacrymans* and *Poria placenta* on FAHP plates, with little difference visible in growth between the FAHP, FBHP and crude saponin extract plates of either of these fungi. Other brown rots, *Gloeophyllum trabeum* and *Lentinus lepideus* showed a retardation in growth at the higher concentrations tested; growth rates were shown to be reduced similarly for all composition types at 0.064 % and FAHP at 0.128 %, the lower concentrations behaved similarly to the untreated control plates.

4.4.4 Screening study of six modified saponin products for activity against timber fungi

Using a similar method to the previous study, agar plates were prepared containing six derivatives of hederagenin from *H. helix* saponins at a range of concentrations between 0.008 – 0.032 %. The modified compounds tested were the 3,23-*O*-acetonide protected hederagenin (**57**) prepared in **6.19**; the 3,23-*O*-benzylidene protected hederagenin (**59**) prepared in **6.25**; 3,23-*O*-bis succinate (**77**) from **6.42(iv)**; 3,23-*O*-bis acetate (**9**) from **6.39(ii)**; a 1:1 mixture of 3-mono acetate (**10**) and 23-mono acetate (**11**) prepared in **6.39(i)**; 3,23-*O*-bis palmitate (**83**) from **6.51(iii)**. This time six different fungi were used the mould *Trichoderma viride*, three brown rot fungi common in buildings (*Coniophora puteana*, *Serpula lacrymans*, *Poria placenta*) and two white rot fungi common in timber (*Trametes versicolor* and *Pleurotus ostreatus*).

The results of the screening study showed little fungistatic effect for any of the six compounds. It was evident that the *T. viride*, *T. versicolor* and *P. ostreatus* mycelium developed much more rapidly than the other fungi and that there was no trend in the

growth rate of these fungi in relation to the treatments. In the two brown rot fungi the benzylidene acetal compound (**59**) showed the greatest potential of the six. At the strongest concentration tested (0.032 %) a strong inhibitory effect was observed in each case. However, the level of growth observed with *P. placenta* was only equivalent to that observed previously for the unmodified aglycone and greater than that shown for the crude leaf and fruit saponin extracts after 14 days. As such it appears that none of the extracts tested from this batch would be suitable for further investigation or development as wood protection chemicals.

The interaction of fungi with saponins is widely believed to be related to the sugar digestion processes available for the given fungus.[157] As such, fungi will often cleave sugars from a saponin to liberate the aglycone inside the fungus. Since the aglycone hederagenin was shown to possess some activity against this type of fungus it appears from these results that the replacement of these sugars with ester chains or acetals corresponds with a lower activity than observed in the unmodified compounds; that is to say that protecting these positions with a function that cannot be cleaved by the fungus might be the cause of this reduced activity. The exception to this being the benzylidene acetal which showed similar activity to the unmodified aglycone, as such this may be a compound of interest in attempting to identify the mechanism of this inhibitory activity. It also seems likely that the effect of these modifications on activity might also be simply a result of the effect of these groups on the permeability of the compounds through the lipid bilayer of the fungal cell walls (all of these modified compounds have significantly higher ClogP values than those of the more active extracts and hydrolysis products).

CHAPTER 5

Conclusions and further work

5.1 Extraction and hydrolysis

Prior to this work, it had been identified that most growing structures of common ivy (*H. helix*) provide a source of biologically active saponins based on the aglycone hederagenin.[38] Through this work improved methods of facile extraction and purification have been developed in order to obtain large quantities of saponins from both the fruit and the leaf. The yields of these saponins were found to be very high from the *H. helix* fruit (ca. 27 % on a dry weight basis) and modest from the leaf material (ca. 15 %) using a fairly simple alcoholic extraction in a Soxhlet apparatus which could be readily scaled to accommodate larger scale preparation of these materials. The saponins isolated from the fruit were found to be predominantly monodesmosidic while those of the leaf were mainly bidesmosides with some monodesmosidic components also present. There is presently a body of work in the literature discussing the leaves of *H. helix*, however little currently exists for the fruits.

In order to obtain pure samples of these saponins to use for biological testing or for synthetic work a protocol was also developed to separate the fatty acid components that were co-extracted with the triterpene components. These fatty acid components were not investigated further in this work, but previous investigation of these showed them to be composed of triacyl glycerols (ca. 35 % of the dry matter in the fruit) as well as the uncommon fatty acid petroselinic acid.[38] Should commercialisation of the saponin extractives be developed it may prove prudent to return to these other components and further investigate their use as replacements for current mineral oils or more specifically investigated for high value components possessing biological activities or nutritional benefits. This would provide a second revenue stream and maximise the value of ivy as a crop species.

Further to the extractions, methods were also developed for the efficient acid and base hydrolysis for both the fruit and leaf saponins in order to prepare quantities of the hemiglycone (monodesmoside) and aglycone products. Base hydrolysis of material from both sources afforded products in good yield (ca. 75 %). In the case of the aglycone, this was found to be composed solely of hederagenin (**2**) and was obtained in fair yield from both fruit and leaf TTS (ca. 18 %). Methods were also developed to further purify these crude products of remaining fatty acids to provide suitable materials to use as platform molecules to carry out synthetic work as well as for conducting biological trials.

In order to further develop the techniques used for hydrolysis it may prove necessary to establish a means to further scale up the reactions. Another route which might prove worthy of investigation may be that of attempting these types of hydrolysis by using an enzymatic or microbial method; such techniques, if identified, might enable these reactions to be carried out more efficiently under comparatively very mild conditions.

As a source of these natural compounds this species seems like a very good option commercially. This plant is essentially unused in the UK at present, despite the fact that it is available in great abundance and grows readily here in its native climate. As such, the species possesses great potential to be exploited should a commercial use be developed for its saponin extractives by initially utilising existing wild populations and, depending on demand, by developing programme to grow the plant as a commercial crop. This project has focussed mainly on the use of the fruit saponins as a source to obtain hederagenin, due to the higher saponin content and smaller number of minor impurities which are co-extracted. However. it seems feasible that the leaf may prove a better source of material for a larger scale extraction project as it would overcome the major disadvantage of the fruit, its seasonality. The fruit of *H. helix* is only available to harvest during the late autumn and early winter months. Conversely, being an evergreen leafy shrub, the leaves of *H. helix* would allow for year round cropping which could make it more attractive. This in itself gives rise to one other area of further work, since the composition of the saponins varies somewhat from month to month and at different geographical locations. In order to better understand the implications of this, it may be an area which could be studied further to identify what differences are present in the saponin profiles of the fruit and leaves over the course of the year.

5.2 Modification

Using hederagenin (**2**) prepared by acid hydrolysis of the saponins of *H. helix* fruit as starting material, a series of modifications were attempted in order to develop a number of novel triterpene derivatives. The majority of these were formed at the two hydroxyl positions at C-3 and C-23 of hederagenin. These positions appeared to be reasonably reactive and a method was developed to prepare esters at these positions by treating them with acid anhydrides or acid chlorides in the presence of a catalyst. Both bis-substituted and mono-substituted products were prepared; it was found that by adjusting the quantity of acid chloride or anhydride used from a single equivalent to an excess it was possible to impart a fair amount of control over the proportions of which products were formed. Although it was not possible to control in the same way, there was also a significant difference within the mono-substituted products between the primary and secondary substituted products. The primary alcohol at the C-23 position was found to react preferentially over the secondary alcohol at C-3. Furthermore, the magnitude of this difference was observed to increase as the size of the ester substituent was increased, with bulky or longer chain esters often forming only the primary substituted ester and the bis ester with no secondary substituted product recovered. The bis- and mono-ester products were found to be readily separated from one another by column chromatography, but where a mixture of mono-substituted products was formed it was found that the retention times of these esters were too close to separate them successfully. As such these would have to be used as a mixture or an alternative purification method will have to be developed in the future. In the case of the more polar esters which possessed extra acid functions (e.g. succinic anhydride) a more facile means of purification was developed by simply precipitating the pure product from a mixed solvent system. Although a number of novel esters were formed by this means the possibilities of further ester products that can be prepared using the same method is vast. Not all of these products have yet been tested for biological activity, but using the results from these types of assays should help to guide the direction in which future attempts at forming more esters might take; identifying which groups (if any) impart desirable activities or properties (solubility, lipophilicity etc.) and attempting to expand the suite of compounds to include other analogous groups. Another potential use for these methods may be in the protection of these compounds in the preparation of synthetic saponins. Since these methods preferentially substitute the primary hydroxyl group they could be used to from an ester at

the C-23 position while leaving the C-3 position available for the attachment of new sugars.

Other compounds formed at these hydroxyl positions were those that were prepared by bridging the two OH groups as an acetal. All of the acetonide (**57**), benzylidene acetal (**59**) and p-methoxy-benzylidene acetal (**61**) were formed, in each case steric hindrance from a proximal methyl group resulted in a single stereoisomer of the product being formed. All of these derivatives act as reasonably stable protecting groups for hederagenin while attempting modifications on other parts of the molecule. Since all of these acetals were formed by reaction with aldehydes (or their gem-ether) future development of this may be the investigation the reactions of other aldehydes in this manner. Using a long chain aldehyde, or those possessing an interesting group may provide an alternative means of functionalising hederagenin at these positions.

Reactions at the other positions of the hederagenin molecule proved more difficult to carry out and where they were achieved often gave only low yields and had very long reaction times. In order to account for these problems hederagenin was modelled by molecular mechanics in order to observe the geometry of the structure. This showed these positions to be subject to significant steric hindrance. As such, the acid at the C-28 position was successfully converted into the corresponding methyl ester (**15**) (as were the acetal protected derivatives); however, all attempts to synthesise longer chained esters at this position were unsuccessful, likely as a result of the 3-dimensional structure of the triterpene backbone preventing sufficient access for larger molecules, or requiring the esters to form at angles which were energetically unfavourable. Similarly other attempts at reactions at this position (e.g. reduction) were also not found to be possible.

Modelling the aglycone molecule also indicated that there was substantial steric hindrance of the alkene function of hederagenin. Attempts by several routes to hydrogenate the group showed an unexpectedly high resistance to this, even at very high pressure and temperature. This and a number of other problems encountered when attempting to modify the group are believed to be caused by the significantly hindered structure around the bond; although allowing some access, the site was most likely sufficiently blocked to prevent the bulky reagents or the surface of the palladium catalyst to get sufficiently close to enable a reaction. Similar failures were also encountered when

attempting bromination, oxidation and epoxidation using peracids. The attempt to epoxidise the hederagenin with DMDO did however result in new products. Although none of the desired epoxide product was obtained, it appeared instead that an oxidation occurred instead through an alternative mechanism to form an allyl alcohol intermediate which was converted *in situ* into a novel enone product (**66c**, by further oxidation of the alcohol) and to a lactone (**66d**). Both were obtained in good yield. It appears that this route to oxyfunctionalisation was favoured over epoxidation. The reason for this was not established but as any modification of this alkene would alter the shape of ring C of the triterpene from a flattened shape to a regular chair shape it seems probable that such a compound might be energetically less favourable, while the products of this oxidation both retain an alkene function. The same reaction on the acetonide protected compound (**57**), however, was unsuccessful. It therefore seems important that this reaction is investigated, not only to properly identify the mechanism of the reaction which is taking place, but as it was the only means identified in this project through which this position could be functionalised. Imparting a functional group such as a ketone into this ring could with more time to investigate it, provide opportunity to prepare further derivatives at this position by relatively simple means e.g. oxidation, reduction, Esterification etc..

Overall, hederagenin (**2**) has been found to be a challenging compound to work with, with steric factors resulting in the low reactivity of some of its functionalities while influencing the regiochemistry at others. These issues, although not ideal, should not detract from the potential which still remains. It is important that its chemistry is further studied and the suite of novel derivatives expanded so that more biological testing can be conducted to better define the structure activity relationship of these compounds. Furthermore, very little work was done to modify the base hydrolysis product of ivy saponins other than early preparations of the methyl ester. As the base hydrolysis product reains a great deal of biological activity due to the attached sugars, it is believed further endeavours must be made to find suitable methods to modify this compound and protecting strategies so as modifications of the triterpene could be carried out without causing damage to the existing sugars.

5.3 Biological activity

A high biological activity has been demonstrated for a number of crude saponins and their hydrolysis products which suggests their utility in a number of fields. The activities in many cases found at very low concentrations which would be within or close to the range of those suited to commercial use.

These compounds, the fruit base hydrolysis product in particular, appear to be very good candidates for further investigation as crop protection agents for potatoes. The fruit BHP performed very well as a protective treatment in a field trial against potato damage by slugs as well as being shown to have strong activity against potato cyst nematode. The trial against potato blight gave somewhat disappointing results for the extracts – showing much smaller effects than an earlier trial; however, this was true for all treatments in the trial, including those of the commercial regimes, likely resulting from climatic conditions. However since there is evidence of some considerable activity when all the trials are considered together, there could be a major commercial advantage for a protective treatment based on *H. helix* saponins which was shown to effective against all three of the major problems afflicting potato growers today. However, development of such a treatment would require a considerable amount of formulation and further testing in order to fully assess not only the efficacy of the compound but also the safety of it use, since the broad spectrum cytotoxicity exhibited by some of these compounds may mean their activities might not just be restricted to plant pathogenic species.

As well as in agricultural treatments, a number of the assays conducted indicated potential applications as biocides in fungal control and in animal and human health. The in vitro trials showed very good activity against a number of pathogenic microorganisms, predominantly fungi, but also some effect on bacteria as well. In the assays against fungi, samples of the saponins and base hydrolysis products all showed activities often at very low concentration. The acid hydrolysis product in the human pathogenic cases displayed no equivalent activity, suggesting that the presence of the sugar moieties is significant to this effect. However, it did show some effect when tested against wood rot fungi. A series of modified derivatives of the aglycone were also tested against timber fungi but none showed significant activity, the benzylidene acetal (**59**) performing best of those

tested but still only at a level comparable to untreated hederagenin; it may, however, still prove to be a useful compound in identifying the mechanism of this fungicidal activity.

Worth noting at this stage is the apparent relationship of the activities observed to the permeability coefficients (logP) of the compounds. This is a property often used in medicinal chemistry to assess the druglikeness of a molecule since it strongly affects how easily a molecule can reach its target, how effective it will be once it reaches its target and how long it will remain in its active form within the body. In the case of these saponin derived compounds, those which have shown the greatest activity are the fruit saponin extract, the fruit base hydrolysis product and leaf base hydrolysis product which have a calculated permeability coefficient (ClogP) of 5.8. In contrast to this the more polar ester and acetal products tested so far have far higher ClogP values (see Figure 5.3.1) and as such would have very different solubility properties to the monodesmosides as well as very different permeability through the lipid cell walls and membranes of any intended target organisms. As such it may be desirable in any future work to take the logP values into account when selecting compounds for assay as well as in designing new derivatives as the distribution of the molecules in a biological system are reliant on this value.

Compound	Number	C log P
Leaf TTS	**4**	1.9
Fruit TTS / Fruit BHP / Leaf BHP	**5**	5.8
Fruit AHP / Leaf AHP	**2**	7.3
3-*O*-acetate / 23-*O*-acetate products	**10 + 11**	8.2
3,23-*O*-succinate	**77**	8.3
3,23-*O*-acetate product	**9**	9.1
3,23-*O*-acetonide product	**57**	9.3
3,23-*O*-benzylidene acetal product	**59**	9.9
3,23-*O*-palmitate	**83**	21.0

Figure 5.3.1 Table of calculated permeability coefficients of the tested compounds

Further laboratory testing is also currently underway to test the activity of 12 other modified products prepared during this project against a number of bacteria and fungi, some early results of which have shown modified compounds prepared in this project (the

longer chain bis-substituted ester products in particular) to be active against stains of Leishmania.

Before much further testing is carried out on these compounds it will also be necessary to assess for cytotoxicity of the extracts and products against mammalian cell lines such as HS27, HepG2, Hela cells, RAW or THP1. Such studies would identify whether these substances would be worth pursuing for therapeutic purposes, since cytotoxicity identified at this stage would rule out their use in pharmacology.

CHAPTER 6
EXPERIMENTAL

General Experimental Procedures

Unless stated, reagents were obtained commercially and were used as received. The boiling point of petroleum used was in the range 40 – 60 °C. Silica gel (Merck 7736 silica gel) used in column chromatography was obtained from Fisher Scientific. Organic solutions were dried with anhydrous magnesium sulphate and solvents removed by rotary evaporation at 14mm Hg unless otherwise stated. All refluxing solutions were heated with an oil bath. Those reactions carried out at low temperature were cooled using a methylated spirit and liquid nitrogen bath. Air and moisture sensitive reactions requiring anhydrous conditions were performed in oven dried glassware and carried out under a slow stream of nitrogen from a balloon and septum. Anhydrous diethyl ether and THF were prepared by drying over sodium wire prior to use and storing under a slow stream of nitrogen from a balloon and septum until use. Anhydrous pyridine, toluene and DMF were used as received from a commercial source with no further drying.

Weights of ivy fruit and leaf meal are given on an oven dried weight (ODW) basis; this was established by drying a small sample in a 105 °C oven for 24 hours and the moisture content (m.c.) calculated by:

m.c. = [(wet weight – dry weight) / wet weight] x 100

This value of m.c. was subtracted from the weight of plant material used to give the ODW.

Analytical Techniques

i. Thin layer chromatography

All TLC was carried out on glass backed silica gel plates obtained from Fisher Scientific. Detection of chromatograms was carried out under ultraviolet light or by a brief immersion in 5 % phosphomolybdic acid solution in methylated spirits followed by heating at 250 °C with a hot air gun.

ii. Infra red spectroscopy

All infrared spectra were recorded on a Perkin-Elmer 1600 FTIR spectrometer and were obtained as thin films (liquids / oils), in chloroform (solids). In all cases region scanned was 4000 – 450 cm^{-1}.

iii. NMR Spectroscopy

Proton and carbon NMR spectra were run in $CDCl_3$ solution (unless otherwise stated) and recorded on a Bruker Advance 500 spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C. For all ^{13}C-NMR the acquisition was with 1024 scans and a two second delay between scans with no special considerations. All chemical shifts are quoted as parts per million downfield of TMS.

iv. Mass Spectrometry

MALDI-TOF mass spectrometry was performed on a Bruker Reflex IV instrument operating in reflectron mode and calibrated against inulin. Approximately 5 mg of sample was dissolved in chloroform, suspended in a matrix of α-cyano-4-hydroxycinnamic acid, spotted on the target plate and dried.

v. Other analytical Data

The specific optical rotations were recorded on either an Optical Activity ltd. PolAAr 2001 polarimiter or on a Bellingham + Stanley ltd. ADP440 polarimeter as solutions in chloroform. All melting point values were uncorrected and measured by a Griffin Edu Lab Melting Point Apparatus.

6.1. Preparation of Ivy fruit for extraction

Ripe fruit of the *H. helix* plant was collected and air-dried at room temperature for 18 hours. These fruit were then placed in a 50 °C drying oven for 65 hours to remove the remaining moisture content. The dried fruit matter was ground using a hammer mill fitted with a 0.25 mm sieve plate to afford a coarse meal. The meal was passed through a hand grinder to break this coarse solid into a finer meal with a smaller particle size. All of this milled fruit material was then transferred to dried plastic containers and sealed with screw cap lids and parafilm to minimise exposure to atmospheric moisture until use.

6.2. Soxhlet extraction of dried, prepared *H. helix* fruit meal

(i.) Ivy fruit meal (21.38 g) was extracted in a Soxhlet apparatus using methanol (250 ml) for 5 hours. The solvent was removed *in vacuo* to recover a purple solid and a dark green oily residue. The oil was dissolved in petrol (100 ml) by refluxing at 65 °C for 15 minutes. The solvent was filtered through a Buchner funnel under reduced pressure and the solid on the filter paper washed with petrol (3 x 30 ml). The solvent was removed *in vacuo* from the filtrate to recover the dark green oily tar of triacylglycerol fatty acids. The solid on the filter paper was transferred to a round bottomed flask with with acetone (100 ml) and the solvent removed *in vacuo*; this was repeated with the addition of further acetone (2 x 100 ml), the solvent removed each time in order to dry the solid. The solid was then ground to a fine powder with a pestle and mortar and placed under vacuum for 16 hours with constant stirring in order to remove any remaining moisture. This gave the desired crude TTS extract (5.67 g, 27 %) as a purple solid which showed δ_H (500 MHz; D_4-MeOD): 5.14 (1 H, t, J 3.5 Hz), 3.37-3.8 (10 H, m), 2.91-3.35 (8 H, m), 3.70 (1 H, d, *J* 10.4 Hz), 2.75 (1 H, dd, *J* 4.4 / 13.9 Hz), 1.31-1.98 (6 H, m), 1.10-1.40 (9 H, m), 0.88 (3 H, s), 0.84 (3 H, s), 0.80 (3 H, s), 0.77 (3 H s), 0.71 (3 H s), 0.70 (3 H, s). By comparison with a commercial sample of hederacoside C it was possible to identify the presence of the saponin components in the crude residue.

(ii.) Ivy fruit meal (120.59 g) was added to a cellulose extraction thimble and extracted in a Soxhlet apparatus with refluxing ethanol (2000 ml) for 5 hours, at which point the solvent was running clear. The heating was ceased and the resultant solution evaporated *in vacuo* to obtain a pale brown solid residue containing a dark tarry oil. This mixture was treated with petrol (1000 ml) and stirred for 6 hours, after which time the mixture was passed through a Buchner funnel under reduced pressure to collect a pale brown solid of the very crude fruit saponin (48.63 g). The filtrate was evporated to recover only a viscous green /

black oil, ^{1}H NMR analysis of which showed only signals for similar fatty components to those observed in the previous extraction of the fruit, and no signals consistent with those of triterpene components were identified. The crude saponin extract was then washed further by stirring the extract with petrol (500 ml) under reflux for 30 minutes before filtering the suspension through a Buchner funnel under reduced pressure to collect the pale brown / off white solid product. This was broken down to a fine powder in a pestle and mortar and the washing process repeated a further 2 times until the petrol washings were running clear with no visible green colouration. The combined petrol washings were evaporated to obtain only more of the fatty oil. The filtered solid was dried at 50 °C for 16 hours to afford an off-white powder of the crude saponin products (32.56 g, 27 %) which was identical by ^{1}H NMR to that prepared in 6.2 (i).

6.3. "Defatting" of Ivy fruit extract

(i.) A portion of the crude fruit saponin extract (2.50 g) was dissolved in 1-butanol (100 ml) and heated at reflux for 30 minutes at 150 °C. A solid remained, and the mixture was then cooled in an ice bath for 15 minutes causing further solid to precipitate. The solid was filtered through a Buchner funnel, and the filter paper dried in an oven at 50 °C overnight, recovering an off-white solid (1.56 g, 62 %) of the desired TTS extract which showed δ_H (500 MHz; $CDCl_3$): 5.22 (1 H, m), 4.38 (1 H, dd, *J* 33.1 / 7.9 Hz), 4.00 – 3.91 (1 H, m), 3.50 (1 H, t, J 6.6 Hz), 3.29 (1 H, s), 2.77 (1 H, dd, *J* 13.7 / 2.6 Hz), 2.18 (1 H, m), 2.04 (1 H, m), 1.91 (1 H, td, *J* 13.7 / 3.5 Hz), 1.79 (3 H, m), 1.66 (4 H,), 1.55 (4 H, m), 1.44 (3 H, m), 1.28 (4 H, m), 1.20 (2 H, s), 1.14 (1 H, m), 1.08 (3 H, s), 1.05 – 0.92 (3 H, m), 0.90 (3 H, s), 0.87 (3 H, s), 0.84 (3 H, s), 0.79 (2 H, m), 0.75 (1 H, s), 0.72 (3 H, s), 0.70 (2 H, s); ν_{max} ($CHCl_3$) / cm $^{-1}$: 3944.5 s, 3692.3 br, 3054.7 s, 2987.1 s, 2685.69 s, 2521.7 w, 2410.6 w, 2305.8 s, 2126.5 w, 2054.8 w, 1688.6 w, 1605.0 w, 1551.0 w, 1421.9 s, 1267.9; MALDI MS m/z $[M+Na]^+$ 1243.95, 1097.87 (calculated for $[C_{59}H_{96}O_{26}Na]^+$, 1243.61); $[\alpha]_D^{24}$ = 5.41 (0.0112 g in 1 ml $CHCl_3$); m.p. 199-216 °C.

(ii.) Crude fruit saponin extract (65.23 g) was ground to a very fine powder in a pestle and mortar and added to a cellulose extraction thimble and extracted in a Soxhlet apparatus with refluxing petrol for 18 hours. Some green colouration was initially observed in the extraction solvent but by this time the solvent appeared to be running clear. Heating was ceased and the thimble allowed to drain. The contents were then emptied into a foil tray and the solid dried at 50 °C for 6 hours. The dried solid was then ground in a pestle and

mortar and passed through a sieve to recover a fine off-white powder of the defatted saponin extract (62.03 g, 95 % yield) which was identictal by ^{1}H NMR, ^{13}C NMR, IR, MALDI MS and optical rotation to the defatted product in (i) above.

6.4. Small scale acid hydrolysis of TTS extract of *H. helix* fruits

(i.) Crude fruit TTS extract (1.56 g) was dissolved in 5 % hydrochloric acid solution (150 ml) and reacted for 5 hours at 100 °C under reflux. This solution was filtered through a Buchner funnel under reduced pressure and the brown solid product was washed with water (3 x 20 ml). The solid was dissolved in the minimum amount of hot methanol and refluxed at 120 °C for 2 hours. The mixture was then cooled in an ice bath for 15 minutes causing the solid to precipitate. This was collected by rapidly filtering the solution through a Buchner funnel under reduced pressure and washing the solid with ice cold methanol (3 x 10 *m*l). The brown colouration of the solid was observed to have been significantly reduced, though some still remained. The solid was ground to a fine powder with a pestle and mortar and the methanol reflux was repeated as before and the product collected by filtering through a Buchner funnel under reduced pressure to recover a white solid. The solid was again washed with ice cold methanol (3 x 10 ml). On drying, a white solid (0.20 g) was collected and prepared for ^{1}H NMR analysis, the spectra of which appear to indicate that the TTS had not undergone the desired hydrolysis since the ^{1}H NMR spectum still contained signals consistent with the attached sugars present in that of the starting material.

(ii.) Crude fruit TTS extract (2.50 g) was added to a round bottomed flask with 10 % sulphuric acid solution (150 ml) and was heated at 82 °C under a reflux condenser for 1 hour with stirring. After this time, a sample of the whole reaction mixture (~ 5 ml) was collected by pipette and the suspension filtered through a Hirsh funnel to collect a pale brown solid. This was washed with water (2 x 10 ml) before being dried at 50 °C for 3 Hours. This process was repeated every hour for 4 hours to assess the progress of the reaction, at which time the remainder of the reaction mixture was filtered under reduced pressure to recover the remaining solid (2.16 g). All of the samples of solid obtained were analysed by ^{1}H NMR but apart from some H_2O impurities, all spectra appeared indentical to those of the starting material.

(iii.) The fruit TTS extract (10.00 g) was dissolved in 10 % aqueous sulphuric acid (150 ml) and reacted for 65 hours at 85 °C under reflux. This solution was allowed to cool and collected by filtration through a Buchner funnel under reduced pressure. The brown pasty solid residue was neutralised by washing with water (3 x 40 ml). This was dried first on the filter and then in an oven at 50 °C to afford a brown solid (3.81 g). This solid was ground in a pestle and mortar and washed by refluxing with petrol (150 ml) at 80 °C for 2 hours. The mixture was then cooled and the solid collected by filtration through a Buchner funnel under reduced pressure. The solid residue upon drying gave a lighter brown solid which was again ground to a fine powder in a pestle and mortar and the petrol "defatting" step repeated. The light brown solid (2.55 g) recovered was identified as the crude aglycone by ^{1}H NMR analysis as no signals were observed for attached sugars. TLC analysis of this product showed some impurities were still present; one major spot was observed which corresponded to the aglycone product, but a second spot was observed at the same retention time as the fatty acid component removed during the washing. The other small spots were most likely the result of free sugars in the sample. The crude aglycone (2.50 g) was dissolved in the minimum amount of hot ethanol at reflux for 1.5 hours. The mixture was then allowed to cool to room temperature before being transferred to the freezer for 16 hours until the maximum amount of solid was obtained. This was collected by rapidly filtering the solution through a Buchner funnel under reduced pressure and the solid on the filter paper being washed with ice cold ethanol (3 x 10 ml). The brown colouration of the solid was observed to have been somewhat reduced, though some still remained. The solid was first dried on the filter and then in an oven at 50 °C to give a light brown solid (0.87 g, 1.84, mmol, 22 %) of the aglycone **(2)** which showed δ_H (500 MHz; D_5-Pyridine): 5.47 (1 H, t, J 3.2 Hz) , 4.17 (2 H, m), 3.70 (1 H, d, J 10.4 Hz), 3.28 (1 H, dd, J 4.4 / 13.9 Hz), 1.6-2.2 (15 H, m), 1.10-1.42 (15 H, m), 1.04 (3 H, s), 1.03 (3 H, s), 0.98 (3H, s), 0.95 (3 H s), 0.90 (3H s), 0.85 (3H, s); δ_H (500 MHz; $CDCl_3$): 5.18 (1 H, t, *J* 3.5 Hz), 3.49 (2 H, m), 2.73 (1 H, dd, *J* 4.1 / 13.6 Hz), 1.87 (1 H, td, *J* 13.6 / 3.9 Hz), 1.82 – 1.76 (2 H, m), 1.69 –1.56 (1 H, m), 1.47 (6 H, m), 1.41 – 1.15 (5 H, m), 1.14 – 1.08 (1 H, m), 1.04 (3 H, s), 1.00 – 0.94 (1 H, m), 0.89 (1 H, dd, *J* 8.9 / 4.5 Hz), 0.86 (3 H, s), 0.83 (3 H, s), 0.81 (3 H, s), 0.76 (3 H, s), 0.69 (3 H, s); δ_C (125 MHz; $CDCl_3$): 143.8, 122.2, 76.6, 71.7, 49.8, 47.6, 46.3, 45.9, 41.7, 41.6, 41.1, 39.2, 38.1, 36.9, 33.8, 33.0, 32.5, 32.4, 30.6, 27.6, 26.2, 25.8, 23.5, 23.32, 23.0, 18.4, 16.8, 15.6, 11.4; ν_{max} ($CHCl_3$) / cm^{-1}: 3450.2 s, 3300.6 br, 3055.0 s, 2987.0 s, 2939.1 s, 2986.1 w, 2411.3 w, 2359.6 w, 2306.3 s, 1695.2 s, 1422.1 s, 1265.4 s; MALDI

MS m/z $[M+Na]^+$ 495.48 (calculated for $[C_{30}H_{48}O_4Na]^+$, 495.34); m.p. 318-321°C. The spectra appeared identical to those of a pure sample of hederagenin. By TLC this compound gave a single spot identical to that of a commercial standard of hederagenin.

6.5. Larger scale acid hydrolysis of TTS extract of *H. helix* fruits

Fruit TTS extract (100.00 g) was dissolved in 10 % sulphuric acid solution (600 ml) and reacted for 65 hours at 85 °C under reflux. This solution was allowed to cool and collected by filtration through a Buchner funnel under reduced pressure. The brown pasty solid residue was washed with water (3 x 40 ml). This was dried first on the filter and then in an oven at 50 °C to afford a brown solid (48.22 g). This solid was ground in a pestle and mortar and transferred to a clean round bottomed flask and washed by refluxing with petrol (600 ml) at 80 °C for 2 hours. The mixture was then cooled and the solid collected by filtration through a Buchner funnel under reduced pressure and the solid residue washed with further petrol (3 x 100 ml) and dried in an oven at 50 °C to afford a lighter brown solid (37.58 g) of crude aglycone. This was again ground to a fine powder in a pestle and mortar and transferred to a round bottomed flask and dissolved in the minimum amount of hot ethanol at reflux for 1 hour. The mixture was then cooled in an ice bath and then transferred to the freezer for 17 hours to encourage a precipitate to form. This was collected by rapidly filtering the solution through a Buchner funnel under reduced pressure and washed on the filter with ice cold ethanol (3 x 15 ml). The solid was first dried on the filter and then in an oven at 50 °C to give a light brown solid which was retained and the filtrate condensed to half its volume on a rotary evaporator; this was returned to the freezer to precipitate further solid which was collected as previously. The combined solids were dried at 50 °C for 4 hours. The solid appeared somewhat greasy and so was added to a round bottomed flask with petrol (250 ml) and heated under reflux for 1 hour. This was again collected by filtering it under reduced pressure and the solid was dried on the filter. The brown solid was then dissolved in excess hot ethanol and heated under reflux together with activated charcoal (0.50 g) for 1 hour. The mixture was filtered hot to remove the charcoal and the solvent removed *in vacuo* to afford pale brown / off-white solid of the aglycone **(2)** (18.26 g, 15.0 mmol, 18 %) which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.18 (1 H, t, *J* 3.5 Hz), 3.49 (2 H, m), 2.73 (1 H, dd, *J* 4.1 / 13.6 Hz),1.87 (1 H, td, *J* 13.6 / 3.9 Hz), 1.82 – 1.76 (2 H, m), 1.69 –1.56 (1 H, m), 1.47 (6 H, m), 1.41 – 1.15 (5 H, m), 1.14 – 1.08 (1 H, m), 1.04 (3 H, s), 1.00 – 0.94 (1 H, m), 0.89 (1 H, dd, *J* 8.9 / 4.5 Hz), 0.86 (3 H, s), 0.83 (3 H, s), 0.81 (3 H, s), 0.76 (3

H, s), 0.69 (3 H, s); it was also identical by ^{13}C NMR, IR, MALDI MS and m.p. to the product in 6.4 (iii); The NMR spectra and retention time by TLC were consistent with those of a pure commercial standard of hederagenin.

6.6. Attempted extraction of dried, prepared *H. helix* fruit meal with *in situ* hydrolysis

Ivy fruit meal (27.74 g) was extracted in a Soxhlet apparatus using methanol (200 ml) and 5 % hydrochloric acid solution (75 ml) for 16 hours. The solvent was removed *in vacuo* to recover a purple solution and a dark solid residue. The solid was collected by filtering the mixture through a Buchner funnel under reduced pressure and washed with water (3 x 20 ml). The greasy solid residue was dried at 50 °C for 16 hours and the crude product anlaysed by ^1H NMR in D_4-methanol, which showed a great deal of fatty acid to still be present in the sample and signals characteristic of the attached sugars present in the starting material. By TLC, no spot was observed which corresponded to the desired aglycone. The filtrate was extracted with ethyl acetate (3 x 30 ml) and the solvent removed *in vacuo* to afford an oil which was shown by ^1H NMR to contain no signals associated with any TTS components.

6.7. Base hydrolysis of fruit TTS extract

(i.) Crude fruit saponin extract (20.00 g) was added to a round bottomed flask with a solution of sodium hydroxide (80 ml, 2 M) and heated to 70 °C under a reflux condenser for 7 hours. After this time, the mixture was transferred, as a slurry, to a separating funnel and a solution of sodium hydroxide (20 ml, 2 M) used to wash the flask. The solution was acidified to pH 7 with 10 % sulphuric acid solution to afford a precipitate. Further water was added to the mixture to promote settling, before the solution was extracted with chloroform (500 ml). The chloroform phase was drawn off and the solvent evaporated to afford an oil of the free acid. The principal product of the reaction was drawn off as a thick slurry and collected by filtration on a Buchner funnel under reduced pressure. The solid recovered was oven dried at 50 °C overnight. The dried solid was ground in a pestle and mortar and added to a round bottomed flask with dichloromethane (300 ml) and heated under reflux for 1 hour. The solid was collected by vacuum filtration and washed with further dichloromethane (3 x 100 ml) and petrol (2 x 50 ml) to remove any remaining free acid. The dried solid was dissolved in methanol with gentle heating; some solid did not dissolve so this was removed by filtering the solution hot. The

solution was then heated under reflux for 15 minutes with activated charcoal (0.50 g). The mixture was then filtered and the solvent evaporated *in vacuo* to afford a brown / off-white solid (8.89 g, 16.37 mmol, 72 %) of the base hydrolysis product **(4)**, which showed δ_H (500 MHz; D_4-MeOD): 5.28 (1 H, s), 3.51-3.72 (3 H, m), 3.23-3.46 (21 H, m), 2.86 (1 H, dd, *J* 3.8 / 13.3 Hz), 1.28-2.03 (18 H, m), 1.20 (3 H, s), 1.00 (3 H, s), 0.96 (3 H, s), 0.93 (3 H, s), 0.84 (3 H s), 0.73 (3 H s); ν_{max} ($CHCl_3$) / cm^{-1}: 3582.3 br, 3012.3 s, 2923.8 s, 2395.59 s, 1420.5 s, 1263.1 s, 1216.1 s.

(ii.) Crude TTS (25.00 g) was added to a round bottomed flask with a solution of sodium hydroxide (150 ml, 2 M) and heated to 70 °C under reflux for 4 hours. The mixture was then transferred, as a slurry, to a separating funnel and a solution of sodium hydroxide (20 ml, 2 M) used to wash the flask. The solution was acidified to pH 7 with 10 % sulphuric acid solution to afford a precipitate. The mixture was then transferred into centrifuge tubes and the solid collected by centrifugation. The supernatant solution of the free sugars was decanted and the solid residue collected and oven dried at 50 °C for 16 hours. The solid was ground in a pestle and mortar to obtain a brown solid of crude product (23.19 g). A ^{1}H NMR analysis indicated this to be predominantly the base hydrolysed TTS. A portion of this product (7.19 g) was "defatted" by heating under reflux fo 2 hours with petrol (200 ml). The resulting mixture was filtered through a Buchner funnel under reduced pressure to collect the pale brown solid which was washed with further petrol (2 x 50 ml). The dried defatted saponin (6.17 g) was dissolved in hot methanol and heated under reflux with activated charcoal (0.6 g) for 1 hour. The mixture was then filtered hot to remove the charcoal, before the solvent was evaporated *in vacuo* to afford a pale brown / off white solid of the base hydrolysis product **(4)** (5.38 g, 7.76 mmol, 38 %) which was identical by ^{1}H NMR and IR to the product from 5.7 (i).

6.8. Further "defatting" of base hydrolysis product

Base hydrolysis product **(4)** (91.47 g) was ground to a very fine powder in a pestle and mortar and added to a cellulose extraction thimble and extracted in a Soxhlet apparatus with refluxing petrol (500 ml) for 18 hours. Some yellow colouration was initially observed in the extraction solvent but by this time the solvent appeared to be running clear. Heating was ceased and the thimble allowed to drain. The contents were then emptied into a foil tray and the solid dried at 50 °C for 6 hours. The dried solid was then ground in a pestle and mortar and passed through a sieve to recover a fine off white powder of the defatted base hydrolysis product (87.22 g, 95.4 % yield) which showed δ_H

(500 MHz; $CDCl_3$): 5.15 (1 H, t, 3.2 Hz), 4.18 (1 H, d, *J* 6.9 Hz), 3.78 (1 H, dd, *J* 3.4 / 1.7 Hz), 3.70 (1 H, m), 3.65 (1 H, m), 3.60 (1 H, m), 3.50 (1 H, m), 2.71 (1 H, dd, *J* 13.6 / 4.0 Hz), 1.85 (1 H, td, *J* 13.3 / 3.6 Hz), 1.80 – 1.70 (2 H, m), 1.68 – 1.55 (2 H, m), 1.53 – 1.33 (6 H, m), 1.30 – 1.06 (8 H, m), 1.03 (3 H, s), 1.00 – 0.85 (3 H, m), 0.83 (1 H, s), 0.82 (2 H, s), 0.81 (3 H, s), 0.78 (3 H, s), 0.75 (1 H, m), 0.73 (1 H, s), 0.72 – 0.68 (1 H, m), 0.66 (3 H, s), 0.62 (1 H, s), 0.57 (3 H, s); δ_C (125 MHz; D_5-Pyridine): 181.8, 151.0, 146.4, 137.5, 136.7, 124.2, 106.1, 103.3, 82.6, 77.4, 76.5, 75.7, 74.2, 74.0, 71.3, 71.0, 67.4, 65.5, 49.8, 49.3, 48.2, 48.0, 45.1, 43.7, 43.6, 41.4, 40.6, 38.5, 35.8, 34.9, 34.9, 34.5, 32.6, 30.0, 27.8, 25.4, 25.3, 20.2, 19.7, 19.0, 17.7, 15.6; ν_{max} ($CHCl_3$) / cm^{-1}: 3582.3 br, 3012.3 s, 2923.8 s, 2395.59 s, 1420.5 s, 1263.1 s, 1216.1 s; MALDI MS m/z $[M+Na]^+$ 773.65 (calculated for $[C_{41}H_{66}O_{12}Na]^+$, 773.44); $[\alpha]_D^{24}$ = 20.00 (0.0101 g in 1 ml $CHCl_3$); m.p. 235 - 250 °C.

6.9. Preparation of Ivy leaf for extraction

Leaves of the *H. helix* plant were collected and air-dried at room temperature for 18 hours. These leaves were then placed in a 50 °C drying oven for 65 hours to remove the remaining moisture. The dried leaf matter was first broken down by hand before being passed through a hammer mill using a 0.25 mm sieve plate. The crudely milled material was collected and again passed through a hammer mill, this time using a 0.5 mm sieve plate to give a slightly coarse powder. This powder was manually passed through a sieve in order to remove the larger particle size material and collect a very fine powder of the ivy leaves. The coarser material removed in the sieve was broken down in a pestle and mortar until at a sufficient particle size was reached to pass through the sieve and this material was combined with the remainder of the fine green powder. All of this milled dried leaf material was then transferred to dried plastic containers and sealed with screw cap lids and parafilm to minimise exposure to atmospheric moisture until use.

6.10. Soxhlet extraction of dried, prepared *H. helix* leaves

(i.) Milled ivy leaf (131.97 g) was added to a cellulose extraction thimble and extracted in a Soxhlet apparatus with refluxing industrial methylated spirits (2000 ml) for 5 hours, at which point the solvent was running clear. The heating was ceased and the resultant solution evaporated *in vacuo* to attain a green solid containing a dark tarry oil. This mixture was treated with petrol (1000 ml) and stirred for 16 hours, after which time it was

passed through a Buchner funnel under reduced pressure to collect a dark green solid of the very crude leaf saponin (24.75 g). The filtrate was evporated to recover only the viscous green oil, ^{1}H NMR analysis of which showed only signals for similar fatty components to those observed in the extraction of the fruit; no signals consistent with those of triterpene components were identified. The crude saponin extract was then washed further by stirring the extract with petrol (500 ml) under reflux for 30 minutes before filtering the suspension through Buchner funnel under reduced pressure to collect the green solid product. This was broken down to a fine powder in a pestle and mortar and the washing process repeated a further 2 times until the petrol washings were running clear with no visible green colouration. The combined petrol washings were evaporated to obtain only more of the fatty oil. The filtered solid was dried at 50 °C for 16 hours to afford a green powder of the crude saponin products (18.03 g, 15 %).

(ii.) Milled dried Ivy leaf (140.30 g) was added to a cellulose extraction thimble and extracted in a Soxhlet apparatus with refluxing ethanol (2500 ml) for 6 hours, at which point the solvent was running clear. The heating was ceased and the apparatus allowed to cool, the thimble was then removed and the Soxhlet recharged by inserting a new thimble containing fresh ivy leaf meal (139.78 g) before recommencing the heating for a further 6 hours. After this time the above process was repeated, recharging the extraction with new material a further 4 times until a total of 788.07 g of leaf meal had been extracted. The resultant solution was a dark green colour and had a rather thick consistency. The ethanol was evaporated *in vacuo* to obtain a green solid containing a dark green tar / oil. This mixture was stirred with petrol (1000 ml) for 16 hours, after which time it was passed through a Buchner funnel under reduced pressure to collect a dark green solid of the very crude leaf saponin. The filtrate was evporated to recover only the viscous green oil, ^{1}H NMR analysis of which showed only signals corresponding to the fatty components and none consistent with those of triterpene components. The crude saponin extract was then washed further by stirring the extract with petrol (500 ml) under reflux for 30 minutes before filtering the suspension through a Buchner funnel under reduced pressure to collect the green solid product. This was broken down to a fine powder in a pestle and mortar and the washing process repeated until the petrol washings were running clear with no visible green colouration. The combined petrol washings were evaporated to obtain only more of the fatty oil. The filtered solid was dried at 50 °C for 16 hours to afford a green powder of the crude saponin products (133.98 g, 17 %).

6.11. Defatting of Ivy leaf extract

Crude leaf saponin extract (57.80 g) was ground to a very fine powder in a pestle and mortar and added to a cellulose extraction thimble and extracted in a Soxhlet apparatus with refluxing petrol for 18 hours. Some green colouration was initially observed in the extraction solvent but by this time the solvent appeared to be running clear. Heating was ceased and the thimble allowed to drain. The contents were then emptied into a foil tray and the solid dried at 50 °C for 6 hours. The dried solid was then ground in a pestle and mortar and passed through a sieve to recover a fine green powder of the defatted saponin extract containing both monodesmosidic and bidesmosidic saponins (54.17 g, 94 %) which showed δ_H (500 MHz; $CDCl_3$): 6.80 (1 H, dd, *J* 3.6, 2.2 Hz), 5.25 (1 H, m), 4.33 (1 H, t, *J* 4.3 Hz), 3.95 (1 H, dd, *J* 10.8 / 8.0 Hz), 3.91 – 3.77 (3 H, m), 3.75 – 3.65 (4 H, m), 3.18 (1 H, s), 2.77 (1 H, dd, *J* 18.5 / 5.2 Hz), 2.23 (1 H, m), 2.12 (1 H, s), 1.97 (1 H, s), 1.86 – 1.75 (3 H, m), 1.52 – 1.44 (2 H, m), 1.44 (1 H, s), 1.29 – 1.21 (9 H, m), 1.19 (6 H, s), 1.17 (1 H, s), 1.16 (2 H, s), 1.14 (1 H, s), 1.08 (3 H, m), 0.87 (1 H, d, *J* 6.7 Hz), 0.83 (1 H, s), 0.83 (1 H, s), 0.82 (1 H, s), 0.81 (1 H, s), 0.80 (3 H, s), 0.80 (1 H, s), 0.79 (3 H, s), 0.78 (1 H, s), 0.78 (1 H, s), 0.77 (1 H, s), 0.77 (1 H, s), 0.75 (1 H, d, *J* 7.6 Hz), 0.72 (1 H, s), 0.62 (1 H, s); ν_{max} ($CHCl_3$) / cm^{-1}: 3686.8 br, 3020.3 s, 2410.1 w, 2305.8 s, 1608.2 w, 1559.7 w, 1423.2 w, 1265.1 w, 1215.8 s; MALDI MS m/z $[M+Na]^+$ 773.18 (calculated for α-hederin (**4**) $[C_{41}H_{66}O_{12}Na]^+$, 773.18), 1243.20 (calculated for hederacoside C (**3**) $C_{59}H_{96}O_{26}Na+1$, 1243.61), $[\alpha]_D^{24}$ = 10.7 (0.0112 g in 1 ml $CHCl_3$); m.p. 168-175 °C.

6.12. Acid hydrolysis of TTS extract of *H. helix* leaf

Leaf TTS extract (5.00 g) was dissolved in 10 % aqueous sulphuric acid (150 ml) and reacted for 70 hours at 95 °C under reflux. This solution was allowed to cool and collected by filtration through a Buchner funnel under reduced pressure. The dark green pasty solid residue was washed with water (3 x 40 ml) to remove any remaining acid. This residue was dried, first on the filter and then in an oven at 50 °C to afford a green solid (3.81 g). This solid was ground to a very fine powder in a pestle and mortar and added to a cellulose extraction thimble and extracted in a Soxhlet apparatus with refluxing petrol for 18 hours. A dark green colouration was initially observed in the extraction solvent but by the end of this period the solvent appeared to be running clear. Heating was ceased and the thimble allowed to drain. The contents were then emptied

into a foil tray and the solid dried at 50 °C for 6 hours. The fine green / brown powder (0.52 g, 1.10 mmol, 26 %) of the aglycone **(2)** which showed δ_H (500 MHz; $CDCl_3$): 5.29 (1 H, t, *J* 3.5 Hz), 3.73 (1 H, d, *J* 10.3 Hz), 3.65 (1 H, m), 3.43 (1 H, d, *J* 10.3 Hz), 2.83 (1 H, dd, *J* 14.4 / 3.4 Hz), 1.99 (1 H, td, *J* 13.5 / 3.9 Hz), 1.93 – 1.86 (2 H, m), 1.83 – 1.69 (6 H, m), 1.68 – 1.48 (5 H, m), 1.46 – 1.30 (8 H, m), 1.26 (8 H, s), 1.20 (1 H, s) 1.14 (3 H, s), 1.00 (1 H, s), 0.97 (2 H, s), 0.94 (3 H, s), 0.91 (3 H, s), 0.90 (3 H, s), 0.87 (3 H, s), 0.78 (3 H, s); δ_C (125 MHz; D_5-Pyridine): 180.6, 145.3, 123.1, 78.6, 73.9, 68.4, 56.3, 49.1, 48.6, 47.1, 46.9, 43.4, 42.7, 42.5, 40.2, 39.9, 39.3, 37.9, 37.7, 34.7, 33.7, 33.7, 33.4, 31.4, 30.5, 29.2, 28.8, 28.2, 26.6, 24.3, 24.2, 24.1, 19.1, 18.0, 17.9, 17.0, 16.4, 16.0, 13.6.; ν_{max} ($CHCl_3$) / cm^{-1}: 3450.0 s, 3300.5 br, 3054.9 s, 2987.0 s, 2939.0 s, 2986.1 w, 2411.3 w, 2359.5 w, 2306.3 s, 1695.2 s, 1422.1 s, 1265.4 s; MALDI MS m/z $[M+Na]^+$ 495.52 (calculated for $[C_{30}H_{48}O_4Na]^+$, 495.34); m.p. 288-295 °C. The spectra appeared identical to those of a pure sample of commercial hederagenin. By TLC this compound gave a single spot identical to that of a commercial standard of hederagenin and to the corresponding aglycone product obtained from the fruit extract.

6.13. Base hydrolysis of leaf TTS extract

Crude leaf TTS (25.00 g) was added to a round bottomed flask with a solution of sodium hydroxide (150 ml, 2 M) and heated to 70 °C under a reflux condenser for 4 hours. After this time, the mixture was transferred, as a slurry, to a separating funnel and a solution of sodium hydroxide (20 ml, 2 M) used to wash the flask. The solution was acidified to pH 7 with 10 % sulphuric acid solution to afford a green precipitate. The mixture was then transferred into centrifuge tubes and the solid collected by centrifugation. The supernatant solution of the free sugars was decanted and the solid residue collected and oven dried at 50 ° C for 16 hours. The solid was ground in a pestle and mortar to obtain a green solid of crude product (20.32 g). A crude ^1H NMR indicated this to be predominantly the base hydrolysed TTS. The crude product was ground in a pestle and mortar before adding it to a cellulose extraction thimble and it was extracted in a soxhlet apparatus with refluxing petrol (500 ml) for 14 hours. The thimble was removed from the apparatus and dried in an oven at 50 ° C for 16 hours to afford a fine powder of the green product **(5)** (19.38 g, 77.5 %) which gave a single spot by TLC and showed δ_H (500 MHz; D_4-MeOD): 5.03 (1 H, s), 4.35 (1 H, d, *J* 6.9 Hz), 3.69 (1 H, s), 3.63 (2 H, dd, *J* 13.5 / 6.9 Hz), 3.58 – 3.54 (1 H, m), 3.50 (2 H, m), 3.45 – 3.37 (2 H, m), 3.34 – 3.23 (2 H, m), 3.22 – 3.12 (2 H, m), 2.64 (1 H, dd, *J* 13.7 / 4.0 Hz), 2.03 (1 H, t, *J* 7.5 Hz), 1.95 (1 H, s), 1.86

– 1.75 (2 H, m), 1.68 (3 H, m), 1.61 – 1.47 (4 H, m), 1.36 (8 H, m), 1.12 (3 H, m), 1.08 (4 H, s), 1.03 (3 H, s), 0.97 (3 H, s), 0.93 – 0.81 (3 H, m), 0.77 (3 H, s), 0.73 (3 H, s), 0.70 (3 H, s), 0.61 (3 H, s), 0.49 (3 H, s); δ_C (125 MHz; $CDCl_3$): 181.8, 152.3, 146.3, 137.6, 136.8, 124.1, 106.0, 103.3, 83.1, 77.4, 76.5, 75.7, 74.1, 74.0, 71.4, 71.0, 67.4, 65.5, 49.8, 49.3, 48.2, 48.0, 45.1, 43.7, 43.6, 41.4, 40.6, 38.5, 35.8, 34.9, 34.9, 34.5, 32.6, 30.0, 27.8, 25.4, 25.3, 20.2, 19.7, 19.0, 17.7, 15.6; ν_{max} ($CHCl_3$) / cm^{-1}: 3582.3 br, 3012.3 s, 2923.8 s, 2395.6 s, 1420.5 s, 1263.2 s, 1216.1 s; MALDI MS m/z $[M+Na]^+$ 773.10 (calculated for α- hederin $[C_{41}H_{66}O_{12}Na]^+$, 773.44); $[\alpha]_D^{24}$ = 22.01 (0.0102 g in 1 ml $CHCl_3$); m.p. 184-190 °C.

6.14. Preparation of methyl ester of hederagenin

(i.) Defatted fruit aglycone **(2)** (0.01 g, 0.02 mmol) was dissolved in ether (10 ml) with continuous stirring. To this mixture an ethereal solution of diazomethane (2 ml) was added and the flask sealed with a stopper and nescofilm. The mixture was stirred for 70 hours, then the stirring was ceased and the solvent and excess diazomethane were evaporated under reduced pressure to afford, as an off white solid, the methyl ester **(15)** (0.01 g, 0.02 mmol, 97 %) which gave only a single spot by TLC at a different retention time to the starting material. This showed δ_H (500 MHz; $CDCl_3$): 5.31 (1 H, s), 3.74 (1 H, d, *J* 10.4 Hz), 3.63 (3 H, s), 3.44 (1 H, d, *J* 10.4 Hz), 2.88 (1 H, dd, *J* 4.4 / 13.9 Hz), 1.80-1.98 (4 H, m), 1.43-1.65 (12 H, m), 1.26 (3 H, s), 1.13 (3 H, s), 0.96 (3 H, s), 0.93 (3 H, s), 0.90 (3 H s), 0.73 (3 H s); m.p. 200-210 °C.

(ii.) Defatted fruit aglycone **(2)** (0.30 g, 0.63 mmol) was reacted in methanol (30 ml) and sulphuric acid (0.03 ml) under reflux with constant stirring for 18 hours. On cooling, diethyl ether (100 ml) was added to the mixture before neutralising it to pH 7 by the addition of an aq. $NaHCO_3$. The aqueous layer was drawn off, the organic layer dried over anhydrous magnesium sulphate, filtered and the solvent removed *in vacuo* to afford an off-white solid (0.08 g). The ^1H NMR analysis of this showed only starting material to be present, no signal was present representing the methyl ester and TLC analysis showed only a single spot with the same retention time as the starting material.

(iii.) Defatted aglycone **(2)** (0.20 g, 0.42 mmol) was added to a round bottomed flask with methyl iodide (0.09 g, 0.63 mmol) in acetonitrile (25 ml) in the presence of potassium carbonate (0.02 g) and stirred for 16 hours at 50 – 60 ° C under a reflux condenser. After this time the mixture was filtered under reduced pressure and the solid washed with

further acetonitrile (30 ml). The filtrate was then evaporated *in vacuo* to afford an off white solid which was purified by column chromatography on a short silica column and petrol / ether (5:2) as solvent to give the desired aglycone methyl ester **(15)** (0.09 g, 0.17 mmol, 41 %) as a white solid which gave a single spot by TLC and showed ^{1}H NMR data identical to that in 6.14 (i) above; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3055.1 s, 2987.0 s, 2939.1 s, 2986.1 w, 2411.3 w, 2359.6 w, 2306.3 s, 1695.2 s, 1422.1 s, 1290 s, 1265.4 s; MALDI MS m/z $[M+Na]^+$ 509.99 (calculated for $[C_{31}H_{50}O_4Na]^+$ 509.37); $[\alpha]_D^{23}$ = 40.61 (0.0109 g in 1 ml $CHCl_3$); m.p. 205-208 °C.

(iv.) Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in cold ethyl acetate (100 ml) and stirred with DCC (0.48 g, 2.33 mmol) and N-hydroxysuccinimide (0.27 g, 2.33 mmol) until a white precipitate of DCHU was formed, after which an excess of methanol was added (10 ml) and the mixture was stirred for 18 hours. The mixture was then filtered under reduced pressure and the residue washed with further ethyl acetate (3 x 30 ml) and methanol (3 x 30 ml) in order to recover any remaining triterpene product. The solvent was then evaporated to afford an off-white solid (1.36 g). This residue was purified by column chromatography on a silica column with petrol / ethyl acetate (1:1) as solvent; however, other than the DCHU impurity only one major triterpene fraction was recovered as a white solid (0.95 g). TLC showed this to have the same retention time as the aglycone starting material; 1 H NMR analysis gave a spectrum consistent with the starting material and no 3 H singlet signal was observed for the methyl ester of the desired product. The column was washed with ethyl acetate and then with methanol but none of the washings contained any of the desired product.

6.15. Attempted preparation of ethyl ester of hederagenin

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in cold ethyl acetate (100 ml) and stirred with DCC (0.48 g, 2.33 mmol) and N-hydroxysuccinimide (0.27 g, 2.33 mmol) until a white precipitate of DCHU was formed, after which, an excess of ethanol was added (10 ml) and the mixture allowed to stir for 18 hours. The mixture was then filtered under reduced pressure to remove the white precipitate and the residue washed with further ethyl acetate (3 x 30 ml) and ethanol (3 x 30 ml) in order to recover any remaining triterpene product. The solvent was then evaporated to afford a yellow / off-white solid residue (1.20 g). TLC analysis of the crude product showed only 1 major spot at a retention time identical to that of the starting material. ^{1}H NMR analysis of this crude

product showed signals consistent only with the unreacted aglycone starting material and those of the remaining DCHU impurity, indicating that the reaction was unsuccessful. No signals were observed for the desired ethyl ester of the product.

6.16. Epoxidation of hederagenin double bond

(i.) Defatted aglycone **(2)** (0.20 g, 0.42 mmol) was added to a round bottomed flask with dichloromethane (10 ml) and sodium hydrogen carbonate (0.10 g, 1.26 mmol). MCPBA (0.10 g, 0.63 mmol) was added at 0 ° C to this solution and allowed to reach room temperature before being stirred for 16 hours. After this time no change was observed by TLC so the mixture was transferred to a separating funnel with further dichloromethane (20 ml) and washed with sodium bicarbonate solution (3 x 20 ml). The combined organic washings were dried, filtered and the solvent evaporated to a white solid residue (0.27 g). This was observed by crude ^{1}H NMR analysis to contain predominantly MCPBA so it was purified by column chromatography using petrol / ether (5:2) as solvent to afford only unreacted starting material and MCPBA, no fraction was obtained containing the epoxide or any corresponding product.

(ii.) Defatted aglycone **(2)** (0.25 g, 0.52 mmol) was added to a round bottomed flask with dichloromethane (20 ml) and sodium hydrogen carbonate (0.10 g, 1.26 mmol). MCPBA (0.90 g, 5.20 mmol) was added at 0 ° C to this solution and the solution allowed to reach room temperature before being stirred for 16 hours. After this time no change was observed by TLC so the mixture was transferred to a separating funnel with further dichloromethane (40 ml) and washed with sodium bicarbonate solution (3 x 20 ml). The combined organic phases were dried, filtered and the solvent evaporated to afford a white solid residue (0.55 g). This was purified by column chromatography using petrol / ether (5:2) as solvent to afford only unreacted starting material and MCPBA; once again no fraction was obtained containing the epoxide or any corresponding product.

(iii.) Defatted aglycone **(2)** (0.51 g, 1.08 mmol) was treated with a freshly prepared solution of DMDO in acetone (100 ml). The reaction mixture was sealed with a septum and allowed to stir at room temperature for 84 hours, by which time the initially cloudy solution had dissolved completely to give a clear, colourless solution. The acetone solvent was removed *in vacuo* to a clumpy wet solid. This solid was dissolved in chloroform (15 ml) and dried with anhydrous magnesium sulphate, filtered and the chloroform evaporated to afford a white solid reside. This solid was dissolved in the minimum amount of ethyl acetate and treated with an excess of ice cold petrol to cause a cloudy white precipitate to

form. This was collected by filtering through a Buchner funnel under reduced pressure and dried at 50 °C to attain a white crystalline solid of a 1:1 mixture (as determined by the integration of signals in the proton NMR spectum) of the the enone product **(66a)** and a lactone product **(66b)** (0.50 g, 1.03 mmol, 95 %) which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.70 (1 H, s), 4.80 (2 H m), 3.93 (2 H, m) 3.70 (1 H, d, *J* 11.7 Hz), 3.69 (1 H, d, *J* 11.3 Hz), 3.10 (1 H, m), 3.00 (1 H, dd, *J* 13.4 / 5.6 Hz), 2.86 – 2.61 (3 H, m), 2.59 – 2.52 (1 H, m), 2.47 (1 H, s), 2.44 – 2.22 (3 H, m), 2.10 (1 H, s), 2.08 – 1.96 (5 H, m), 1.94 – 1.85 (4 H, m), 1.82 – 1.48 (25 H, m), 1.40 (3 H,s), 1.39 (1 H, s), 1.34 (1 H, s), 1.33 (3 H, s), 1.31 (3 H, s), 1.28 (1 H, s), 1.23 (2 H, s), 1.21 (1 H, s), 1.16 (1 H, s), 1.14 (1 H, s), 1.11 (3 H, s), 1.02 (3 H, d, *J* = 1.8 Hz), 1.00 (2 H, s), 0.98 (1 H, s), 0.95 (3 H, d, *J* = 4.8 Hz), 0.91 (3 H, s); δ_C (125 MHz; $CDCl_3$): 184.0, 179.8, 150.0, 114.1, 78.6, 78.3, 78.2, 77.6, 76.4, 76.2, 76.2, 76.2, 76.1, 66.8, 66.6, 60.8, 52.9, 51.2, 48.8, 48.7, 44.7, 43.8, 42.3, 39.5, 36.1, 35.2, 34.1, 33.3, 33.2, 31.6, 30.9, 30.7, 28.0, 27.5, 23.9, 23.4, 22.9, 21.2, 18.6, 18.5, 18.5, 16.7, 16.1; MALDI MS m/z $[M+Na]^+$ 509.53 (calculated for $[C_{30}H_{46}O_5Na]^+$ 509.70); $[\alpha]_D^{20}$ = 57.29 in chloroform (0.0109 g in 1 ml $CHCl_3$); m.p. 150-152 °C.

6.17. Attempted bromination of hederagenin double bond

(i.) Defatted aglycone **(2)** (0.25 g, 0.53 mmol) in dichloromethane (20 ml) was treated with bromine (0.03 ml, 0.53 mmol) and the mixture stirred for 48 hours. After this time there was no obvious discolouration of the orange / brown solution so the stirring was ceased and the solvent evaporated to afford a sticky brown solid residue. This crude residue was purified by column chromatography on a short silica column using petrol / ethyl acetate (1:1) as solvent to recover one major fraction as a white solid (0.20 g) which gave a retention time by TLC identical to that of the starting material. ^1H NMR analysis of this gave signals consistent with the aglycone starting material, including one corresponding to the unreacted olefinic proton; no signals consistent with the desired bromide were observed.

(ii.) Defatted aglycone **(2)** (0.30 g, 0.63 mmol) was dissolved in dichloromethane (35 ml) with stirring and the solution cooled to - 40 °C. At this temperature, the solution was treated with bromine (0.02 ml, 0.31 mmol) by syringe and the mixture allowed to stir for 2 hours with constant cooling to maintain a temperature below - 35 °C. After this time the deep red colouration was unchanged and no reaction was observed by TLC so the

cooling was ceased and the reaction mixture allowed to attain room temperature before being allowed to stir at this temperature for 48 hours. After this time, the colouration once again remained unchanged and so the solvent evaporated to afford a brown solid residue (0.32 g) which gave a retention time by TLC identical to that of the starting material and ^{1}H NMR analysis of this crude product gave signals consistent with the aglycone starting material, including one corresponding to the unreacted olefinic proton, no signals consistent with the desired bromide were observed.

6.18. Attempted reduction of hederagenin at the C-28 position

Defatted aglycone **(2)** (0.20 g, 0.42 mmol) was dissolved in THF (5 ml). Lithium aluminium hydride (0.02 g, 0.63 mmol) was dissolved in dry THF (8 ml) and the hederagenin solution added to it drop-wise. This was then stirred for 16 hours under a nitrogen atmosphere at room temperature. The flask was then cooled in an ice bath and the mixture treated with aq. ammonium chloride (20 ml). Further THF (25 ml) was added and the mixture stirred for 2 minutes. The remaining solid was removed by filtering the solution through a bed of celite. The filter cake was then washed with further THF (3 x 25 ml) to ensure no product remained. The THF was then evaporated to afford a white solid (0.17 g) which on ^{1}H and ^{13}C NMR analysis appeared to be unreacted aglycone starting material and gave only a single spot by TLC at an identical retention time to that of the aglycone.

6.19. Protection of hederagenin hydroxyl groups as acetonide

Defatted aglycone **(2)** (2.50 g, 5.29 mmol) was dissolved in dichloromethane (100 ml) and stirred for 16 hours in the presence of 2,2-dimethoxypropane (1.38 g, 13.23 mmol) and a catalytic amount of p-toluenesulfonic acid (0.05 g, 0.26 mmol). After this time, TLC confirmed the reaction to be complete. The reaction mixture was transferred to a separating funnel with further dichloromethane (50 ml) and washed with sodium bicarbonate solution (3 x 40 ml) and the organic phase collected. This was dried over anhydrous magnesium sulphate, filtered and the solvent evaporated to afford a white solid. The solid was suspended on silica and purified by column chromatography to afford a white solid (1.09 g, 2.13 mmol, 40 %) of the acetonide **(57)** which gave only a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.07 (1 H, t, *J* 3.1 Hz), 3.91 (1 H, q, *J* 7.0 Hz), 3.28 (2 H, m), 3.23 (1 H, d, *J* 10.3 Hz), 2.61 (1 H, dd, *J* 4.1 / 13.9 Hz), 1.67 (1 H, s), 1.51-1.30 (6 H, m), 1.24 (1 H, s), 1.20 (1 H, s), 1.05 -1.02 (5 H, m), 0.94 (3 H, s),

0.83 (3 H, s), 0.75 (3 H, s), 0.72 (3 H, s), 0.96 (3 H s), 0.53 (3 H s); δ_C (125 MHz; $CDCl_3$): 184.1, 171.2, 143. 6, 122.5, 99.0, 77.6, 72.6, 60.4, 51.5, 47.7, 46.5, 45.9, 41.6, 40.9, 39.5, 38.8, 37.3, 36.8, 33.8, 33.1, 32.4, 32.1, 30.7, 29.9, 27.7, 26.0, 23.6, 23.5, 23.2, 22.8, 21.0, 19.4, 17.6, 17.0, 16.5, 14.2, 12.4; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3055.3 s, 2987.5 s, 2952.1 s, 2685.9 w, 2410.8 w, 2305.8 s, 1694.8 s, 1550.9 w, 1421.9 s, 1381.9 w, 1365.9 w, 1266.2; MALDI MS m/z $[M+Na]^+$ 535.53 (calculated for $[C_{33}H_{52}O_4Na]^+$, 535.38); $[\alpha]_D^{22}$ = 63.33 (0.0105 g in 1 ml $CHCl_3$); m.p. 260-266 °C.

6.20. Preparation of methyl ester of acetonide protected hederagenin

(i.) Acetonide protected aglycone **(57)** (0.02 g, 0.04 mmol) was dissolved in ether (10 ml) with continuous stirring. To this mixture an ethereal solution of diazomethane (2 ml) was added and the flask sealed with a stopper and nescofilm. The mixture was stirred for 16 hours, at which time the stirring was ceased and the solvent and excess diazomethane evaporated under reduced pressure to afford a yellow solid (0.02 g, 0.04, 97 %) of the methyl ester **(58)** which gave only a single spot by TLC at a different retention time to the starting material and showed δ_H (500 MHz; $CDCl_3$): 5.31 (1 H, t, *J* 3.8 Hz), 3.55 (3 H, s), 3.33 (4 H, m), 2.79 (1 H, dd, *J* 4.4 / 13.9 Hz), 1.92-1.80 (3 H, m), 1.65-1.42 (9 H, m), 1.38 (3 H, s), 1.34 (3 H, s), 1.29-1.09 (4 H, m), 1.07 (3 H, s), 0.98 (3 H, s), 0.89 (3 H, s), 0.86 (3 H, s), 0.83 (3 H, s), 0.64 (3 H, s); δ_C (125 MHz; $CDCl_3$): 178.0, 149.8, 122.5, 122.4, 99.0, 77.6, 51.6, 47.8, 47.1, 45.9, 45.3, 41.7, 41.6, 41.3, 41.4, 39.3, 38.9, 37.7, 37.1, 36.9, 33.7, 33.5, 33.0, 32.4, 32.0, 30.7, 30.7, 29.9, 27.4, 26.4, 24.7, 24.6, 23.6, 20.1, 18.8, 17.3, 17.3, 15.2,; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3156.0 s, 2985.8 s, 2950.2 s, 2254.1 s, 1794.2 w, 1725.3 s, 1643.8 w, 1469.3 s, 1382.8 s, 1265.0 s, 1216.18 w; MALDI MS m/z $[M+Na]^+$ 549.50 (calculated for $[C_{34}H_{54}O_4Na]^+$, 549.39); m.p. 225-235 °C.

(ii.) Acetonide protected aglycone **(57)** (0.71 g, 1.38 mmol) was added to a round bottomed flask with methyl iodide (0.29 g, 2.07 mmol) in acetonitrile (25 ml) in the presence of potassium carbonate (0.02 g) and stirred for 16 hours at 50 – 60 °C under a reflux condenser. After this time the reaction mixture was filtered under reduced pressure and the solid washed with further acetonitrile (30 ml). The filtrate was then evaporated *in vacuo* to afford an off white solid which was purified by column chromatography on a short silica column and petrol / ether (5:2) as solvent to give the desired acetonide ester **(58)** (0.30 g, 0.57 mmol, 80 %) which gave a single spot by TLC and was identical by 1H

NMR, 13C NMR, and IR to the product produced in 6.20 (ii) above and showed $[\alpha]_D^{21}$ = 61.48 (0.0116 g in 1 ml $CHCl_3$); m.p. 227-233 °C.

6.21. Attempted epoxidation of acetonide protected hederagenin double bond

(i.) The acetonide protected aglycone **(57)** (0.10 g, 0.20 mmol) was added to chloroform (25 ml) and sodium hydrogen carbonate (0.05 g, 0.60 mmol). MCPBA (0.11 g, 0.30 mmol) was added at 0 ° C to this solution and allowed to reach room temperature before being left to stir for 16 hours. After this time no change was observed by TLC so the reaction mixture was transferred to a separating funnel with further dichloromethane (20 ml) and treated with sat. aq. sodium bicarbonate (3 x 20 ml). The combined organic washings were dried, filtered and the solvent evaporated to a white solid residue (0.25 g). This was observed by ^{1}H NMR analysis to contain predominantly MCPBA so it was purified by column chromatography using petrol / ether (5:2) as solvent to afford unreacted starting material as a white solid (0.12 g).

(ii.) The acetonide protected aglycone **(57)** (0.50 g, 0.98 mmol) was treated with a freshly prepared solution of DMDO in acetone (100 ml). The reaction mixture was sealed with a septum and allowed to stir at room temperature for 84 hours, by which time the initially cloudy solution had dissolved completely to give a clear, colourless solution. The acetone solvent was removed *in vacuo* to a clumpy wet solid. This solid was dissolved in chloroform (15 ml) and dried with anhydrous magnesium sulphate, filtered and the chloroform evaporated to afford a white solid (0.51 g). However, upon ^{1}H NMR analysis, this gave a spectrum identical to that of the starting material, including a clear signal for an olefinic proton.

6.22. Attempted bromination of acetonide protected hederagenin double bond

Acetonide protected aglycone **(57)** (0.25 g, 0.50 mmol) was dissolved in dichloromethane (15 ml) with stirring. The resultant solution was treated with bromine (0.03 ml, 0.50 mmol) by syringe and the mixture allowed to stir for 48 hours. After this time there was no obvious discolouration of the orange / brown solution so the stirring was ceased and the solvent evaporated to afford a sticky brown solid residue. This crude residue was purified by column chromatography on a short silica column using petrol /

ethyl acetate (1:1) as solvent to recover one major fraction as a white solid (0.20 g) which gave a retention time by TLC identical to that of the starting material. ^{1}H NMR analysis gave signals consistent with the acetonide starting material, including one corresponding to the unreacted olefinic proton. No signals consistent with the desired bromide were observed.

6.23. Attempted reduction of acetonide protected hederagenin at C-28 position

Acetonide protected aglycone **(57)** (0.20 g, 0.40 mmol) was added to a round bottomed flask and dissolved in THF (5 ml). Lithium aluminium hydride (0.02 g, 0.60 mmol) was dissolved in dry THF (8 ml) and the hederagenin solution added to it drop-wise. This was then stirred for 16 hours under a nitrogen atmosphere at room temperature. The flask was then cooled in an ice bath and the mixture treated with aq. ammonium chloride (20 ml). Further THF (25 ml) was added and the mixture stirred for 2 minutes. The remaining solid was removed by filtering the solution through a bed of celite. The filter cake was then washed with further THF (3 x 25 ml) to ensure no product remained. The THF was then evaporated to afford a white solid (0.19 g) which on ^{1}H NMR analysis appeared to be the unreacted acetonide starting material and gave a single spot by TLC with the same retention time as the acetonide starting material.

6.24. Attempted oxidation of acetonide protected aglycone

(i.) The acetonide protected aglycone **(57)** (0.20 g, 0.40 mmol) was dissolved in glacial acetic acid (5 ml). A solution of chromium (VI) oxide (0.20 g, 0.48) in aqueous acetic acid (70 %, 10 ml) was stirred with cooling until at a temperature of 0 °C.[96] At this temperature, the solution was treated with the prepared acetonide solution in a drop-wise manner, ensuring the temperature was maintained at below 10 °C throughout. Once the addition was complete the mixture was allowed to reach room temperature and stirred for 18 hours. After this time, the initially yellow / orange mixture had developed a dark brown colouration. The mixture was treated with water (60 ml) and transferred to a separating funnel with ether (20 ml). The organic phase was extracted and the aqueous phase re-extracted with ethyl acetate (3 x 25 ml). The combined organic phases were washed with brine (15 ml), dried and the solvent evaporated to a viscous oil residue. This was dissolved in chloroform (5 ml) and placed under a high vacuum to recover a colourless solid (0.17 g). TLC analysis of this showed only a single spot at the same

retention time to the starting material and a crude ^{1}H NMR spectrum identical to that of the unreacted starting material.

(ii.) The acetonide protected aglycone **(57)** (0.20 g, 0.40 mmol) was dissolved in glacial acetic acid (5 ml). A solution of chromium (VI) oxide (0.20 g, 0.60 mmol) in aqueous acetic acid (70 %, 10 ml) was stirred at 0 °C. To this solution PCC (0.13 g, 0.60 mmol) and PDC (0.20 g, 0.60 mmol) was also added.[153] At 0 °C, the solution was treated with the prepared acetonide solution in a drop-wise manner, ensuring the temperature was maintained at below 10 °C throughout. Once the addition was complete the mixture was allowed to reach room temperature and stirred for 18 hours. The reaction mixture was worked up as in 6.24 (i) above to recover a brown solid residue (0.22 g). TLC analysis of this residue showed only a single spot at the same retention time to the starting material and a crude ^{1}H NMR spectrum showed signals consistent with that of the unreacted starting material.

6.25. Protection of hederagenin hydroxyl groups as a benzylidene acetal

Defatted aglycone **(2)** (0.25 g, 0.53 mmol) was dissolved in acetonitrile (15 ml) and benzaldehyde dimethyl acetal (0.40 g, 2.64 mmol) was added with stirring along with p-toluene sulfonic acid (0.03 g, 0.16 mmol). The reaction was stirred at room temperature for 24 hours at which point no further change was observed by TLC. The solution was then extracted with chloroform (75 ml) and washed with water (50 ml). The aqueous layer was then re-extracted with further chloroform (2 x 30 ml). The combined organic phases were dried, filtered and the solvent evaporated to afford a pale brown solid (0.23 g) which showed one major spot by TLC. The solid was suspended on silica and purified on a short silica column, eluting with petrol / ether (5:2) to afford a white solid (0.18 g, 0.32 mmol, 61.0 %), the benzylidene acetal protected aglycone **(59)** which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 8.15 – 8.11 (2 H, m), 7.63 (1 H, m), 7.54 – 7.45 (2 H, m), 7.36 (1 H, m), 5.55 (1 H, s), 5.32 (1 H, t, *J* 3.4 Hz), 3.95 (1 H, d, *J* 10.4 Hz), 3.47 (2 H, d, *J* 9.9 Hz), 2.88 (1 H, dd, *J* 13.6 / 4.1 Hz), 2.02 (2 H, td, *J* 13.7 / 4.2 Hz), 1.96 – 1.88 (2 H, m), 1.86 – 1.71 (6 H, m), 1.65 (7 H, m), 1.53 – 1.43 (3 H, m), 1.42 – 1.34 (3 H, m), 1.33 – 1.23 (6 H, m), 1.20 (3 H, s), 1.19 (3 H, s), 1.14 – 1.04 (4 H, m), 1.00 (3 H, s), 0.96 (3 H, s), 0.94 (3 H, s), 0.80 (3 H, s); δ_C (125 MHz; $CDCl_3$): 192.4, 183.6, 143.6, 138.6, 136.4, 134.5, 133.7, 130.2, 128.9, 128.5, 128.3, 122.5, 102.7, 86.2, 78.8, 51.6, 47.7, 46.5, 45.9, 41.7, 41.0, 39.5, 38.9, 37.3, 36.7, 33.8, 33.1, 32.4, 32.2, 30.7, 27.7, 26.0, 23.6, 23.4, 23.3, 22.9, 17.8, 17.0, 16.5, 13.4; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3692.4 w, 3155.2

s, 3052.3 s, 2984.9 s, 2951.9 s, 2684.6 w, 2253.8 s, 1793.7 w, 1725.3 s, 1695.5 s, 1603.9 w, 1585.0 w, 1468.7 s, 1418.9 w, 1381.2 s, 1318.7 w, 1265.0 s, 1216.4 w; MALDI MS m/z $[M+Na]^+$ 583.55 (calculated for $[C_{37}H_{52}O_4Na]^+$, 583.38; $[\alpha]_D^{21}$ = 29.83 (0.0108 g in 1 ml $CHCl_3$); m.p. 263-267 °C.

6.26. Preparation of methyl ester of benzylidene acetal protected hederagenin

Benzylidene acetal protected aglycone **(59)** (0.20 g, 0.36 mmol) was added to a round bottomed flask with methyl iodide (0.08 g, 0.53 mmol) in acetonitrile (20 ml) in the presence of potassium carbonate (0.02 g) and stirred for 16 hours at 50 – 60 °C under a reflux condenser. The reaction mixture was then filtered under reduced pressure and the solid washed with further acetonitrile (30 ml). The filtrate was then evaporated *in vacuo* to afford a yellow / off white solid residue which was purified by column chromatography on a short silica column and petrol / ether (5:2) as solvent to give the desired methyl ester **(60)** (0.03 g, 0.05 mmol, 14 %) which gave a single spot by TLC at a different retention time to that of the product and showed δ_H (500 MHz; $CDCl_3$): 8.15 – 8.11 (2 H, m), 7.63 (1 H, m), 7.54 – 7.45 (2 H, m), 7.36 (1 H, m), 5.55 (1 H, s), 5.32 (1 H, t, *J* 3.4 Hz), 3.95 (1 H, d, *J* 10.4 Hz), 3.47 (2 H, d, *J* 9.9 Hz), 2.88 (1 H, dd, *J* 13.6 / 4.1 Hz), 2.02 (2 H, td, *J* 13.7 / 4.2 Hz), 1.96 – 1.88 (2 H, m), 1.86 – 1.71 (6 H, m), 1.65 (7 H, m), 1.53 – 1.43 (3 H, m), 1.47 (3 H, s), 1.44 (3 H, s), 1.42 – 1.34 (3 H, m), 1.33 – 1.23 (6 H, m), 1.20 (3 H, s), 1.19 (3 H, s), 1.14 – 1.04 (4 H, m), 1.00 (3 H, s), 0.96 (3 H, s), 0.94 (3 H, s), 0.80 (3 H, s); δ_C (125 MHz; $CDCl_3$): 192.4, 183.6, 143.6, 138.6, 136.4, 134.5, 133.7, 130.2, 128.9, 128.5, 128.3, 122.5, 102.7, 86.2, 78.8, 51.6, 47.7, 46.5, 45.9, 41.7, 41.0, 39.5, 38.9, 37.3, 36.7, 33.8, 33.1, 32.4, 32.2, 30.7, 27.7, 26.0, 23.6, 23.4, 23.3, 22.9, 17.8, 17.4, 17.2, 17.0, 16.5, 13.4; ν_{max} ($CHCl_3$) / cm^{-1}: 3692.4 w, 3155.2 s, 3052.3 s, 2984.9 s, 2951.9 s, 2684.6 w, 2253.8 s, 1793.7 w, 1725.3 s, 1695.5 s, 1603.9 w, 1585.0 w, 1468.7 s, 1418.9 w, 1381.2 s, 1318.7 w, 1265.0 s, 1216.4 w; MALDI MS m/z $[M+Na]^+$ 597.63 (calculated for $[C_{38}H_{54}O_4Na]^+$, 597.41); $[\alpha]_D^{22}$ = 68.21 (0.0111 g in 1 ml $CHCl_3$); m.p. 233-235 °C.

6.27. Attempted reduction of benzylidene acetal protected hederagenin at C-28 position

Benzylidene protected aglycone **(59)** (0.25 g, 0.45 mmol) was dissolved in THF (5 ml). Lithium aluminium hydride (0.02 g, 0.53 mmol) was dissolved in dry THF (8 ml) and the hederagenin solution added to it drop-wise. This was then stirred for 16 hours under a

nitrogen atmosphere at room temperature. The flask was then cooled in an ice bath and the mixture treated with aq. ammonium chloride (20 ml). Further THF (25 ml) was added and the mixture stirred for 2 minutes. The remaining solid was removed by filtering the solution through a bed of celite. The filter cake was then washed with further THF (3 x 25 ml) to ensure no product remained. The THF was then evaporated to afford a white solid (0.25 g) which on ^{1}H NMR analysis appeared to be the unreacted starting material, gave a single spot by TLC with the same retention time as the starting material and no signals were observed corresponding to the reduced product.

6.28. Attempted partial deprotection of benzylidene acetal protected aglycone

Benzylidene acetal protected aglycone **(59)** (0.50 g, 1.10 mmol) and triethylsilane (0.62 g, 5.29 mmol) were dissolved in dichloromethane (10 ml) and cooled to 0 °C. Triflouroacetic acid (0.60 g, 5.29 mmol) was added drop-wise to this stirred solution and the temperature maintained at 0 °C throughout. When the addition was complete, the reaction was allowed to warm to room temperature and stirred for a further 5 hours. By this time the initial orange / pink colouration of the solution was observed to have faded to a yellow colour and no further change was observed by TLC. The mixture was transferred to a separating funnel with further dichloromethane (10 ml) and sat. aq. sodium bicarbonate (20 ml). The organic phase was collected and the aqueous phase re-extracted with further dichloromethane (2 x 10 ml). The combined organic phases were dried, filtered and the solvent evaporated to afford a viscous orange coloured oil (0.60 g) showing several spots by TLC. This residue was purified by column chromatography to afford two main fractions. The first as a white solid (0.35 g) gave signals by ^{1}H NMR consistent with those of unreacted starting material **(59)**. The second fraction (0.17 g) as a white solid gave signals consistent with the unprotected aglycone **(2)**. No other triterpene fraction was identified and none of the desired product was recovered.

6.29. Protection of hederagenin hydroxyl groups as 4-methoxy-benzylidene acetal

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in dry DMF (10 ml) and 4-methoxybenzaldehyde dimethyl acetal (0.42 g, 0.40 ml, 2.33 mmol) was added to it with stirring along with a catalytic amount of p-toluene sulfonic acid (0.20 g, 0.21 mmol). The reaction was stirred at room temperature under a nitrogen atmosphere for 48 hours at which point no further change was observed by TLC. The solution was then quenched with water (30 ml) and extracted with ethyl acetate (3 x 30 ml). A white solid was

observed at the boundary of the phases so this was collected by gravity filtration to recover a white solid residue (0.75 g) which gave a single spot by TLC and appeared by ^{1}H NMR to be consistent with the unreacted aglycone starting material. The combined organic phases were dried, filtered and the solvent evaporated to afford a white solid (0.30 g) which showed two major spots by TLC. The solid was suspended on silica and purified by column chromatography on a short silica column, eluting with petrol / ethyl acetate (1:1) to afford a first fraction as a white solid (0.15 g, 0.25 mmol, 12 %) of the methoxybenzylidene acetal protected aglycone **(61)** which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 7.44 (2 H, d, *J* 8.7 Hz), 6.89 (1 H, d, *J* 8.7 Hz), 5.50 (1 H, s), 5.30 (1 H, t, *J* 3.4 Hz), 3.93 (1 H, d, *J* 10.4 Hz), 3.80 (3 H, s), 3.53 – 3.42 (1 H, m), 2.84 (1 H, dd, *J* 13.7 / 4.1 Hz), 2.00 (1 H, td, *J* 13.4 / 3.8 Hz), 1.94 – 1.89 (2 H, m), 1.76 (5 H, tdd, *J* 16.8 / 13.4 / 3.7 Hz), 1.68 – 1.56 (6 H, m), 1.50 – 1.42 (2 H, m), 1.41 – 1.32 (4 H, m), 1.19 (3 H, s), 1.17 (3 H, s), 1.12 – 1.03 (3 H, m), 1.01 (3 H, s), 0.94 (3 H, s), 0.92 (3 H, s), 0.87 (3 H, m), 0.77 (3 H, s); δ_C (125 MHz; $CDCl_3$): 183.0, 160.0, 143.6, 131.3, 127.6, 122.5, 113.7, 102.5, 86.1, 78.8, 55.3, 51.6, 47.7, 46.5, 45.9, 41.6, 41.0, 39.5, 38.8, 37.3, 36.6, 33.8, 33.1, 32.4, 32.2, 30.7, 29.7, 27.7, 26.0, 23.6, 23.4, 23.3, 22.9, 17.8, 17.1, 16.5, 13.4; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3692.4 w, 3155.2 s, 3052.3 s, 2984.9 s, 2951.9 s, 2684.6 w, 2253.8 s, 1793.7 w, 1725.3 s, 1695.5 s, 1652.0 s, 1603.9 w, 1585.0 w, 1468.7 s, 1418.9 w, 1381.2 s, 1318.7 w, 1265.0 s, 1216.4 w; MALDI MS m/z $[M+Na]^+$ 613.59 (calculated for $[C_{38}H_{54}O_5Na]^+$, 613.38; $[\alpha]_D^{20} = 27.08$ (0.0104 g in 1 ml $CHCl_3$); m.p. 282-288 °C. The second fraction collected gave a white solid (0.15 g) which had an identical retention time by TLC to that of the starting material and gave ^{1}H NMR signals identical to the unreacted aglycone.

6.30. Attempted partial deprotection of methoxybenzylidene acetal protected aglycone

Methoxybenzylidene acetal protected aglycone **(61)** (0.10 g, 0.17 mmol) in dichloromethane (20 ml) was cooled to – 20 °C and treated with DIBAL [1 M] (0.66 ml, 0.66 mmol). This mixture was stirred for 4 hours, maintaining a temperature below – 20 °C throughout. After this time the reaction was quenched with water (20 ml) and extracted with dichloromethane (3 x 20 ml). The combined organic extracts were dried, filtered and the solvent evaporated to afford a white solid residue (0.18 g). ^{1}H NMR analysis of this crude solid showed only signals consistent with the fully deprotected

aglycone, together with signals in the region between 7 – 8 ppm consistent with the aromatic fragment removed during this deprotection. No signals were observed consistent with the desired mono substituted product. By TLC the only major spot observed was that at the same retention time as the unprotected aglycone material (**2**).

6.31. Attempted hydrogenation of hederagenin double bond

(i.) Defatted aglycone **(2)** (0.20 g, 0.42 mmol) was dissolved in ethyl acetate (35 ml). Palladium on charcoal (1 %, 1.00 g) was added to a two necked flask with ethyl acetate (20 ml) and the side neck sealed with a rubber septum. The flask was then connected to the hydrogenation apparatus and treated with hydrogen gas. Once the catalyst was fully saturated the hederagenin solution was added to the stirred solution by syringe. The mixture was left under hydrogen gas to react for 20 hours until no more hydrogen appeared to be absorbed, then removed from the hydrogenator and the contents filtered through a bed of celite. The filtrate was evaporated to an off-white solid (0.18 g); crude ^{1}H NMR analysis of this still showed a signal in the olefinic region indicating that the reaction was unsuccessful. The hydrogenation was repeated; however the solid recovered still gave a spectrum identical to the starting material and a single spot by TLC at the same retention time as the aglycone.

(ii.) Defatted aglycone **(2)** (0.50 g) was dissolved in THF / methanol 2:1 (10 ml) and cooled to 10 °C and dipotassium azodicarboxylate (0.20 g) added. The solution, under nitrogen, was then treated with acetic acid/THF [2:1] (6 ml) in portions over 2 hours before being allowed to attain room temperature and stirred for 2 hours. After this time the reaction mixture had become pale so further dipotassium diazocarboxylate (0.20 g) was added and the mixture once again treated with acetic acid / THF [2:1] (4 ml) in portions over the course of 2 hours and then stirred at room temperature for 24 hours. After this time the yellow reaction mixture was washed with sat.aq. ammonium chloride (20 ml) and extracted with ether (30 ml). The ethereal phase was collected, dried and the solvent evaporated to afford a white solid (0.44 g) which by ^{1}H NMR gave a spectrum identical to that of the starting material.

6.32. Attempted hydrogenation of double bond in TTS extract under high pressure

Defatted TTS extract (5.00 g) was dissolved in water (150 ml) and to this solution was added palladium on charcoal (1 %, 2.00 g). The suspension was transferred into a high pressure reaction vessel, and the remaining solid washed in with further water (15 ml).

The air in the vessel was evacuated under high vacuum before hydrogen was added into it until a pressure of 10 bar was achieved. The mixture was stirred at this pressure for 4 hours with heating at 40 °C by a water jacket. After this time the pressure was released and the hydrogen evacuated from the chamber. A sample of the reaction mixture (1.5 ml) was collected by pipette and the catalyst removed from it by filtering it through a bed of celite under reduced pressure. The filtrate was extracted with ethyl acetate (3 x 5 ml) and these combined phases dried, filtered and the solvent evaporated to afford a white solid residue. A crude ^{1}H NMR analysis of this still showed a clear signal in the olefinic region for a proton consistent with the double bond. As such the hydrogenation procedure was repeated for a further 5 hours in the same manner described previously. After this time the reaction mixture was allowed to cool before it was drained from the reaction vessel and washed out with water (40 ml). The aqueous suspension was filtered through a bed of celite under reduced pressure to remove the catalyst. The filtrate was extracted with ethyl acetate (3 x 150 ml) and the combined organic phases were dried, filtered and evaporated to afford a white solid residue (4.88 g) but once again, this showed signals by ^{1}H NMR consistent with the TTS starting material.

6.33. Attempted hydrogenation of acetonide protected hederagenin double bond

The acetonide protected aglycone **(57)** (0.20 g, 0.40 mmol) was dissolved in ethyl acetate (20 ml). Palladium on charcoal (1 %, 1.00 g) was added to a two necked flask with ethyl acetate (20 ml) and the side neck sealed with a rubber septum. The flask was then connected to the hydrogenation apparatus and treated with hydrogen gas. Once the catalyst was fully saturated, the acetonide solution was added to the stirred solution by syringe. The septum was sealed with nescofilm and the mixture left under hydrogen gas to react for 20 hours until no more hydrogen appeared to be absorbed. Once this was complete the flask was removed from the hydrogenator and the contents filtered through a bed of celite to remove the catalyst. The filtrate was evaporated to an off-white solid (0.19 g). ^{1}H NMR analysis of this still showed a signal in the olefinic region indicating that the reaction was unsuccessful. The hydrogenation process was repeated again; however the solid recovered (0.17 g) still gave a spectrum identical to the starting material.

6.34. Preparation of methyl ester of base hydrolysis product

(i.) A sample of the fruit base hydrolysis product (0.10 g, 0.13 mmol) was dissolved in ether (10 ml) with continuous stirring. An ethereal solution of diazomethane (2 ml) was added and the flask sealed with a stopper and nescofilm. The mixture was stirred for 70 hours, at which time the stirring was ceased and the solvent and excess diazomethane evaporated under reduced pressure to afford an yellow / white solid of the methyl ester **(56)** (0.10 g, 0.13 mmol, 98 %) which showed δ_H (500 MHz; $CDCl_3$): 5.11 (1 H, t, J 3.05 Hz), 3.61 (1 H, s), 3.55-3.38 (8 H, m), 3.38 (1 H, d, *J* 7.3), 2.78 (1 H, dd, *J* 6.38 / 9.80 Hz), 2.10-0.91 (26 H, m), 0.85 (3 H, s), 0.83 (3 H, s), 0.81 (3 H, s), 0.78 (3 H, d, *J* 5.35 Hz), 0.64 (3 H, s), 0.61 (3 H s); ν_{max} ($CHCl_3$) / cm $^{-1}$: 3580.5 br, 3012.4 s, 2923.8 s, 2395.6 s, 1420.5 s, 1272.6 s, 1263.1 s, 1216.1 s; m.p. 183-185 °C.

(ii.) Fruit base hydrolysis product (0.20 g, 0.26 mmol) was heated under reflux for 16 hours with methanol (50 ml) and a few drops of sodium hydroxide solution. The reaction was allowed to cool and extracted with ether (25 ml). The ethereal phase was dried and the solvent evaporated to afford a solid, predominantly starting material.

(iii.) Fruit base hydrolysis product (0.20 g, 0.26 mmol) was stirred with methyl iodide (0.05 g, 0.39 mmol) in acetonitrile (20 ml) in the presence of potassium carbonate (0.02 g) for 16 hours at 50 – 60 °C under reflux. The reaction mixture was then filtered under reduced pressure and the solid washed with further acetonitrile (30 ml). The filtrate was evaporated *in vacuo* to afford a yellow / off white solid residue which was purified by column chromatography on a silica column and petrol / ether (1:1) to recover only one major triterpene fraction. This gave a spot by TLC with the same retention time as the starting material and by ^{1}H NMR no signals were present corresponding to the desired methyl ester.

6.35. Attempted transesterification of hederagenin

(i.) A solution of sodium methoxide was prepared by dissolving sodium metal (0.01 g) in methanol (40 ml). The defatted TTS (1.00 g) was added to the methoxide solution and allowed to stir for 62 hours, when the initial green colouration had changed to brown. The mixture was treated with ether (30 ml) and water (20 ml) and a few drops 10 % sulphuric acid to neutralise the base. The ether phase was collected and the aqueous phase re-extracted with further ether (2 x 20 ml), the combined organic phases were dried and evaporated to afford an off white as the solid (0.30 g) which by TLC analysis gave a spot at the same retention time as TTS starting material. In addition to this, ^{1}H NMR

analysis showed signals corresponding to the attached sugars of the TTS saponin and none corresponding to the methylated product.

(ii.) A solution of sodium ethoxide was prepared by dissolving sodium metal (0.01 g) in ethanol (40 ml). The defatted TTS (1.00 g) was added to the ethoxide solution and allowed to stir for 62 hours, when the initial green colouration had changed to brown. This was worked up as in part (i) above to afford an off white solid (0.65 g) which by TLC analysis gave a spot at the same retention time to a sample of the TTS starting material. In addition to this, crude ^{1}H NMR analysis showed signals corresponding to the attached sugars of the TTS saponin and none corresponding to the ethylated product.

6.36. Attempted preparation of heptanoyl ester of acetonide protected hederagenin

Acetonide protected aglycone **(57)** (0.20 g, 0.39 mmol) was dissolved in anhydrous toluene (3 ml) with stirring under a nitrogen atmosphere. Oxalyl chloride (68 µl, 0.78 mmol) was added to this solution together with a catalytic amount of anhydrous DMF (5 µl, 0.04 mmol). This mixture was stirred for 2 hours, after which the solvent was evaporated under high vacuum to afford a sticky solid. This was dissolved in dry ether (3 ml) and the solvent evaporated under high vacuum to draw off any remaining DMF or toluene. This process was repeated with further ether (2 x 3 ml) until a yellow solid was obtained of the acid chloride. The solid was dissolved in anhydrous THF (2 ml). In a separate flask a solution of heptanol (0.17 ml, 1.17 mmol) was prepared in anhydrous THF (3 ml) and anhydrous pyridine (0.2 ml), together with DMAP (0.05 g). This solution was stirred under nitrogen at 0 °C and treated with the prepared acid chloride solution in portions so as to maintain the temperature below 5 °C. The reaction mixture was allowed to warm to room temperature, when a white precipitate was observed to form, stirred at for 16 hours, then quenched with sat. aq. sodium bicarbonate (20 ml) and extracted with ether (3 x 30 ml). The combined ethereal phases were dried and the solvent evaporated to an orange oil residue (0.29 g), ^{1}H NMR analysis of which showed it to contain a substantial amount of heptanol and pyridine. The residue was purified by column chromatography on a short silica column with petrol / ether (5:2). Other than the excess heptanol only one major fraction was collected as an off-white solid (0.15 g) which gave an identical retention time by TLC to the starting material and gave ^{1}H and ^{13}C NMR spectra showing the same signals as those of the starting material.

6.37. Attempted preparation of heptanoyl ester of benzylidene acetal protected hederagenin

Benzylidene acetal protected aglycone **(59)** (0.20 g, 0.36 mmol) was dissolved in anhydrous toluene (3 ml) with stirring under a nitrogen atmosphere. Oxalyl chloride (62 µl, 0.71 mmol) was added together with a catalytic amount of anhydrous DMF (5 µl, 0.04 mmol). This mixture was stirred for 2 hours, then the solvent was evaporated under high vacuum to afford a sticky solid residue. This was dissolved in dry ether (3 ml) and the solvent evaporated under high vacuum to draw off any remaining DMF or toluene. This was repeated with further ether (2 x 3 ml) until a yellow solid was obtained of the acid chloride. The solid was dissolved in anhydrous THF (2 ml). A solution of heptanol (0.20 ml, 1.08 mmol) in anhydrous THF (3 ml) and anhydrous pyridine (0.2 ml), together with DMAP (0.05 g) was stirred under nitrogen and cooled to 0 °C and treated with the prepared acid chloride solution in portions so as to maintain a temperature below 5 °C. The mixture was allowed to warm to room temperature, when a white precipitate formed, stirred for 16 hours, then quenched with sat. aq. sodium bicarbonate (20 ml) and extracted with ether (3 x 30 ml). The combined ethereal phases were dried and the solvent evaporated to an orange oil (0.25 g), the ^{1}H NMR of which showed a substantial amount of heptanol and pyridine. The residue was purified by column chromatography on a short silica column with petrol / ether (5:2). Other than the excess heptanol only one major fraction was collected as an off-white solid (0.11 g) which gave an identical retention time by TLC to the benzylidene acetal starting material and gave ^{1}H and ^{13}C NMR spectra showing the same signals as those of the starting material.

6.38. Attempted preparation of behenyl ester of acetonide protected hederagenin

Acetonide protected aglycone **(57)** (0.20 g, 0.39 mmol) was dissolved in anhydrous toluene (3 ml) with stirring under a nitrogen atmosphere. Oxalyl chloride (68 µl, 0.78 mmol) was added to this solution together with a catalytic amount of anhydrous DMF (5 µl, 0.04 mmol). This mixture was stirred for 2 hours, after which the solvent was evaporated under high vacuum to afford a sticky solid residue. The residue was dissolved in dry ether (3 ml) and the solvent evaporated under high vacuum to draw off any remaining DMF or toluene. This process was repeated with further ether (2 x 3 ml) until a yellow solid residue was obtained of the acid chloride. The solid was dissolved in anhydrous THF (2 ml). In a separate flask a solution of behenic alcohol (0.38 g, 1.17 mmol) was prepared in anhydrous THF (3 ml) and anhydrous pyridine (0.2 ml), together

with DMAP (0.05 g). This alcohol solution was stirred under nitrogen and cooled to 0 °C and treated with the prepared acid chloride solution in a portion-wise manner so as to maintain a temperature below 5 °C. Once added the cooling bath was removed and the reaction mixture allowed to warm to room temperature, as this occurred a white precipitate was observed to form. The mixture was allowed to stir at room temperature for 16 hours, then quenched with sat. aq. sodium bicarbonate (20 ml) and extracted with ether (3 x 30 ml). The combined ethereal phases were dried, filtered and the solvent evaporated to a white solid residue (0.52 g), ^{1}H NMR analysis of which showed it to consist largely of behenic alcohol. The residue was purified by column chromatography on a short silica column with petrol / ether (5:2). Separation from the behenyl alcohol was not good but only one major fraction was collected as an off-white solid (0.23 g) which gave an identical retention time by TLC to the aglycone starting material and gave ^{1}H and ^{13}C NMR spectra showing the same signals as those of the starting material (together with some consistent with a small quantity of the unresolved free behenyl alcohol).

6.39. Esterification of hederagenin with acetic anhydride

(i.) Acetic anhydride (1 equivalent)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with acetic anhydride (0.20 ml, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time no solid was observed to remain in the orange solution. The mixture was extracted between water (30 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford a white solid (1.13 g). This was purified by column chromatography with petrol / ethyl acetate (1:1) to afford the two main fractions. The first fraction, a white solid (0.04 g, 0.09 mmol, 4 %),was the bis substituted product **(9)**, which gave one spot by TLC and is characterised fully in 5.39 (ii). The second fraction was a (2:3) mixture of both the secondary **(10)** and primary **(11)** mono substituted products as a white solid (0.31 g, 0.60 mmol, 28 %) which appeared as one spot by TLC and showed the common signals δ_H (500 MHz; $CDCl_3$): 5.29 (1 H, t, *J* 3.8 Hz), 2.82 (1 H, dd, *J* 3.8 / 13.6 Hz), 2.11 (2 H, s),

2.09 (0.5 H, s), 2.08 (1 H, s), 2.05 (0.5 H, s), 1.98 (1 H, dt, *J* 3.8 / 16.4 Hz), 1.89 (2 H, m), 1.85 – 1.69 (3 H, m), 1.67 - 1.51 (8 H, m), 1.46 - 1.34 (3 H, m), 1.31 – 1.19 (4 H, m), 1.17 (0.6 H, m), 1.14 (0.4 H, s), 1.13 (3H, s), 1.09 (0.6 H, m) 1.07 (1H, m), 0.98 (2 H, m), 0.95 (3 H, s), 0.91 (3 H, s), 0.91 (3 H, s), 0.78 (2 H, s), 0.76 (3 H, s), 0.67 (1 H, s) as well as signals for the secondary substituted product only at δ_H (500 MHz; $CDCl_3$): 4.89 (0.4 H, dd, *J* 4.7 / 12.0 Hz), 3.38 (0.4 H, d, *J* 12.3 Hz), 2.91 (0.4 H, d, *J* 12.3 Hz) and for the primary substituted product only at δ_H (500 MHz; $CDCl_3$): 4.19 (0.6 H, d, *J* 11.4 Hz), 3.82 (0.6 H, d, *J* 11.4 Hz), 3.43 (0.6 H, t, *J* 8.5 Hz); δ_C (125 MHz; $CDCl_3$): 183.3, 172.4, 171.5, 143.8, 143.6, 122.5, 122.4, 74.7, 72.7, 67.5, 64.5, 50.9, 48.2, 47.8, 47.5, 46.7, 46.5, 46.5, 45.9, 42.3, 42.0, 41.7, 41.6, 41.0, 40.9, 39.3, 39.3, 38.2, 38.0, 36.9, 36.8, 33.8, 33.1, 32.4, 32.3, 32.2, 30.7, 27.6, 26.1, 26.0, 25.9, 23.6, 23.4, 23.4, 22.9, 22.9, 21.2, 21.0, 18.2, 17.6, 17.1, 17.1, 16.0, 15.8, 14.2, 12.8, 12.0; ν_{max} ($CHCl_3$) / cm^{-1}: 3055 s, 2952 s, 2686 s, 2306 s, 1696 s, 1465 s, 1422 s, 1386 s; MALDI MS m/z $[M+Na]^+$ 537.38 (calculated for $[C_{32}H_{50}O_5Na]^+$, 537.35) for both isomers; $[\alpha]_D^{20} = 45.43$ (0.0105 g in 1 ml $CHCl_3$); m.p. 173-180 ° C.

(ii.) Acetic anhydride (10 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with acetic anhydride (2.00 ml, 21.20 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time no solid was observed to remain in the orange solution. The mixture was extracted between water (30 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford an orange solid (1.14 g). This was purified by column chromatography with petrol / ethyl acetate (1:1) to afford the two main fractions. The first fraction, a colourless crystalline solid (0.71 g, 1.28 mmol, 60 %), was the bis substituted product **(9)**, which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.29 (1 H, t, *J* 3.5 Hz), 4.80 (1 H, dd, *J* 7.3 / 11.3 Hz), 3.88 (1 H, dd, *J* 4.8 / 11.4 Hz), 3.70 (1 H, d, *J* 11.4 Hz), 2.83 (1 H, dd, *J* 4.1 / 13.6 Hz), 2.18 (2 H, s), 2.08 (3 H, s), 2.03 (3 H, s), 1.99 - 1.86 (3 H, s), 1.82 – 1.54 (12 H, m), 1.44 – 1.16 (9 H, m), 1.13 (3 H, s), 1.10 – 1.04 (2 H, m), 0.98 (3 H, s), 0.96 (3 H, s), 0.94 (3 H, s), 0.92 (3 H, s), 0.84 (3 H, s), 0.76 (3 H, s); δ_C (125 MHz; $CDCl_3$):

206.9, 182.8, 171.0, 170.7, 143.6, 122.5, 74.5, 65.4, 47.9, 47.7, 46.5, 45.8, 41.6, 41.0, 40.5, 39.3, 37.7, 36.8, 33.8, 33.1, 32.4, 32.3, 30.9, 30.7, 27.6, 25.8, 23.6, 23.4, 23.0, 22.9, 21.2, 20.9, 17.9, 17.1, 15.8, 14.2, 13.1; ν_{max} ($CHCl_3$) / cm^{-1}: 2924 s, 2726 s, 1749 s, 1697 s, 1460 s, 1377 s; MALDI MS m/z $[M+Na]^+$ 579.30 (calculated for $[C_{34}H_{52}O_6Na]^+$, 579.37); $[\alpha]_D^{19}$ = 63.08 (0.0108 g in 1 ml $CHCl_3$); m.p. 154-157 °C. The second fraction was a 2:3 mixture of both the secondary **(10)** and primary **(11)** mono substituted products as a colourless solid (0.12 g, 0.23 mmol, 11 %) which appeared as one spot by TLC and was identical to the mixture of products in 5.39 (ii) by ^1H NMR, ^{13}C NMR,, IR, and MALDI MS and showed m.p. 172-178 ° C. The column was then washed with methanol to recover further mono substituted products in a ratio of 5:2 (primary and secondary) together with an excess of the acetic anhydride.

6.40. Esterification of hederagenin with butyric anhydride

(i.) Butyric anhydride (1 equivalent)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with butyric anhydride (0.03 g, 0.35 ml, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time no solid was observed to remain in the orange solution. The mixture was extracted between water (30 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford a white solid (1.05 g). This was purified by column chromatography with petrol / ethyl acetate (5:1) to afford three main fractions. The first fraction was a white solid (0.33 g, 0.61 mmol, 29 %) of a mixture of both the secondary **(73)** and primary **(74)** mono substituted products in a ratio of 2:3, which appeared as a single spot by TLC and showed the common signals δ_H (500 MHz; $CDCl_3$): 5.26 (1 H, t, *J* 4.7 Hz), 2.81 (1 H, dd, *J* 13.6 Hz), 2.31 (4 H, p, *J* 7.3 Hz), 1.96 (2 H, m), 1.87 (2 H, m), 1.79 – 1.51 (17 H, m), 1.45 – 1.20 (10 H, m), 1.13 (1 H, s), 1.11 (2 H, s), 1.05 (4 H, m), 0.96 (3 H, s), 0.94 (2 H, s), 0.94 (3 H, s), 0.91 (3 H, s), 0.89 (3 H, s), 0.77 (1 H, s), 0.75 (2 H, s), 0.74 (3 H, s), 0.61 (2 H, s) as well as signals for the secondary substituted product only at δ_H (500 MHz; $CDCl_3$): 4.87 (0.4 H, dd, *J* 4.4 / 12.0 Hz), 3.36 (0.4 H, d, *J* 12.6 Hz), 2.89 (0.4 H, d, *J* 12.6 Hz) and for the primary substituted product only at δ_H (500 MHz; $CDCl_3$): 4.16 (0.6 H, d,

J 11.5 Hz), 3.82 (0.6 H, d, *J* 11.5 Hz), 3.43 (0.6 H, t, *J* 8.8 Hz); δ_C (125 MHz; $CDCl_3$): 183.3, 178.41, 175.0, 174.0, 171.2, 143.5, 122.6, 72.7, 67.0, 60.4, 48.1, 47.9, 46.5, 45.9, 42.4, 42.1, 41.6, 41.0, 39.3, 38.3, 36.9, 36.8, 36.6, 36.5, 35.7, 33.8, 33.1, 32.4, 30.7, 27.6, 26.0, 25.8, 23.6, 23.4, 22.9, 21.1, 18.7, 18.2, 17.0, 15.8, 14.2, 13.8, 13.7, 13.6, 12.9, 12.0; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3156 s, 2985 s, 1794 s, 1701 s, 1468 s, 1383 s; MALDI MS m/z $[M+Na]^+$ 565.52 (calculated for $[C_{33}H_{52}O_5Na]^+$, 565.40); $[\alpha]_D^{22}$ = 38.75 (0.0094 g in 1 ml $CHCl_3$); m.p. 116-120 ° C. The second fraction which on evaporation was collected as a white solid (0.67 g, 1.23 mmol, 58 %) appeared to be only the primary substituted mono butyrate **(74)** which showed δ_H (500 MHz; $CDCl_3$): 5.58 (1 H, t, *J* 3.5 Hz), 3.74 (1H, d, *J* 10.4 Hz), 3.65 (1 H, t, *J* 7.0 Hz), 3.44 (1 H, d, *J* 10.4 Hz), 2.83 (1 H, dd, *J* 4.4 / 13.6 Hz), 2.35 (1 H, t, *J* Hz), 1.96 (2 H, m), 1.89 (2 H, m), 1.80 – 1.50 (17 H, m), 1.45 – 1.30 (9 H, m), 1.27 (5 H, t, *J* 7.3 Hz), 1.14 (3 H, s), 0.97 (3 H, s), 0.94 (3 H, s), 0.91 (3 H, s), 0.90 (3 H, s), 0.88 (1 H, s), 0.87 (2 H, s), 0.78 (3 H, s); ν_{max} ($CHCl_3$) / cm $^{-1}$: 3156 s, 2985 s, 1794 s, 1701 s, 1468 s, 1383 s; MALDI MS m/z $[M+Na]^+$ 565.50 (calculated for $[C_{33}H_{52}O_5Na]^+$, 565.40); m.p. 115-118 ° C.

(ii.) Butyric anhydride (10 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with butyric anhydride (0.34 g, 3.46 ml, 21.20 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time no solid was observed to remain in the orange solution. The mixture was extracted between water (30 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford an orange oil (2.48 g). This was purified by column chromatography with petrol / ethyl acetate (5:1) but only one fraction was collected which appeared to be that of the butyric anhydride; however a crude ^{1}H NMR analysis indicated that this fraction contained both butyric anhydride and bis-substituted product. The column was washed with methanol and the silica washed with water but no other products were identified. The mixture collected from the column was therefore purified by flash distillation of the remaining butyric anhydride to afford a colourless oil reside of the bis-substituted butyrate product **(72)** (0.42 g, 0.68 mmol, 32 %) which appeared as a single spot by TLC and showed δ_H

(500 MHz; $CDCl_3$): 5.31 (1 H, t, *J* 3.5 Hz), 4.80 (1 H, dd, *J* 4.4 / 11.4 Hz), 3.93 (1 H, dd, *J* 4.8 / 11.4 Hz), 3.64 (1 H, d, *J* 11.5 Hz), 2.82 (1 H, dd, *J* 4.4 / 13.9 Hz), 2.43 (2 H, t, *J* 7.3 Hz), 2.31 (2H, t, *J* 7.3 Hz), 2.25 (2 H, t, *J* 7.3 Hz), 2.10 - 1.99 (1 H, m), 1.95 - 1.85 (2 H, m), 1.84 – 1.52 (17 H, m), 1.43 – 1.18 (9 H, m), 1.14 (3 H, s), 1.12-1.03 (2 H, m), 0.99 (2 H, s), 0.98 (3 H, s), 0.97 (3 H, s), 0.96 (3 H, s), 0.94 (2 H, s), 0.93 (3 H, s), 0.92 (4 H, s), 0.84 (3 H, s), 0.82 (1 H, s), 0.80 (1 H, s); δ_C (125 MHz; $CDCl_3$): 183.5, 173.4, 173.1, 171.1, 143.5, 122.5, 74.2, 65.0, 60.4, 47.7, 47.7, 45.8, 41.6, 41.0, 40.6, 39.3, 37.8, 36.8, 36.6, 36.4, 33.8, 33.0, 32.4, 32.2, 30.7, 27.6, 25.7, 23.6, 23.4, 22.9, 22.9, 21.0, 18.6, 18.5, 17.9, 17.1, 15.8, 14.2, 13.8, 13.6, 13.1.; ν_{max} ($CHCl_3$) / cm^{-1}: 2938 s, 2872 s, 1735 s, 1691 s, 1459 s, 1384 s; MALDI MS m/z $[M+Na]^+$ 635.59 (calculated for $[C_{36}H_{56}O_6Na]^+$, 635.44); $[\alpha]_D^{22}$ = 60.83 (0.0110 g in 1 ml $CHCl_3$); m.p. 105-115 ° C.

(iii.) Butyric anhydride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with butyric anhydride (0.74 g, 0.76 ml, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time no solid was observed to remain in the orange solution. The mixture was extracted between water (30 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 50 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford a sticky white solid (1.32 g). This was purified by column chromatography to recover the bis butyrate **(72)** as a colourless oil (0.13 g, 0.22 mmol, 10 %) which gave a single spot by TLC and was identical by ^1H NMR, ^{13}C NMR, IR, MALDI MS, and optical rotation as the product from part (ii) above and showed m.p. 104-110 ° C. A further fraction was recovered which appeared to contain a mixture of the mono substituted products was recovered (0.32 g) but this could not be separated from the excess of butyric anhydride and requires further purification. The column was washed with methanol and on evaporation afforded an oil residue (0.62 g) which appeared by ^1H NMR analysis to be predominantly the excess butyric acid.

6.41. Esterification of hederagenin with oleoyl chloride

(i.) Oleoyl Chloride (1 equivalent)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with oleoyl chloride (0.70 ml, 0.64 g, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 65 hours, after which time no solid was observed to remain in the orange solution. The mixture was treated with water (50 ml) and transferred to a separating funnel with ethyl acetate (50 ml). The aqueous phase was acidified to pH 2 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford a pasty brown solid residue (1.74 g). This was purified by column chromatography with petrol / ethyl acetate (5:1) to afford the three main fractions. The first fraction was a colourless oil (0.09 g) and gave an identical ^{1}H NMR spectrum to that of the oleoyl chloride and had the same retention time by TLC. The second fraction was a colourless viscous oil (0.20 g, 0.20 mmol, 9 %) of the bis-substituted product **(86)**, which gave only a single spot by TLC analysis and appeared identical by ^{1}H NMR, ^{13}C NMR, IR MALDI MS, optical rotation and m.p. to the product fully characterised in part (ii) below. The third fraction, collected as a colourless solid, was a 1:1 mixture of both the secondary **(87)** and primary **(88)** mono substituted products (0.59 g, 0.78 mmol, 37 %) which appeared as two spots running together at very close retention times and showed the common signals δ_H (500 MHz; $CDCl_3$): 4.51 (1 H, dd, *J* 7.3, 2.2 Hz), 2.35 (3 H, t, *J* 7.5 Hz), 2.18 (1 H, s), 2.00 (5 H, m), 1.90 (2 H, t, *J* 7.2 Hz), 1.84 – 1.74 (3H, m), 1.73 – 1.49 (21 H, m), 1.32 (18 H, s), 1.28 (12 H, s), 1.27 (6 H, s), 1.23 (3 H, s), 1.17 (2 H, s), 1.14 (3 H, s), 1.11 (1 H, s), 1.08 (2 H, s), 1.06 (3 H, s), 0.98 (1 H, s), 0.96 (5 H, s), 0.94 (4 H, s), 0.91 (3 H, s), 0.90 (2 H, s), 0.89 (2 H, s), 0.88 (1 H, s), 0.80 (2 H, s), 0.78 (2 H, s), 0.78 (2 H, s), 0.77 (3 H, s), 0.67 (1 H, s) as well as signals for the secondary substituted product only at δ_H (500 MHz; $CDCl_3$): 5.30 (1 H, t, *J* 3.6 Hz), 4.89 (0.5 H, dd, 4.5 / 12.5), 3.37 (0.5 H, d, *J* 12.5 Hz), 2.93 (0.5 H, dd, *J* 3.8 / 13.9 Hz), 2.89 (0.5 H, d, *J* 12.5 Hz) and for the primary substituted product only at δ_H (500 MHz; $CDCl_3$): 5.35 (2 H, t, *J* 3.6 Hz), 4.21 (1 H, d, *J* 11.5 Hz), 3.82 (1 H, d, *J* 11.5 Hz), 3.44 – 3.40 (1 H, t, *J* 11.5), 2.84 (1 H, dd, *J* 3.8 / 13.9 Hz); δ_C (125 MHz; $CDCl_3$): 183.8, 174.3, 143.7, 130.0, 129.7, 122.5, 122.4, 81.1, 72.2, 64.4, 50.5, 50.1, 47.8, 47.5, 46.5, 46.1, 46.1, 45.8, 45.6, 42.9, 42.8, 42.5, 42.3, 42.1, 41.9, 41.7, 41.5, 41.0, 40.6, 39.3, 39.0, 38.8, 38.2,

38.0, 37.8, 37.6, 36.9, 36.7, 34.6, 33.8, 33.7, 33.1, 33.0, 32.6, 32.4, 32.3, 32.2, 31.9, 31.8, 30.7, 30.7, 29.8, 29.7, 29.7, 29.5, 29.5, 29.3, 29.3, 29.2, 29.1, 29.1, 27.9, 27.6, 27.2, 27.2, 26.0, 25.9, 25.2, 25.1, 24.9, 24.7, 23.6, 23.4, 22.7, 18.8, 18.1, 17.6, 17.4, 17.3, 17.1, 16.0, 15.8, 14.1, 12.8, 12.0; ν_{max} ($CHCl_3$) / cm $^{-1}$: 2928 s, 2856 s, 2400 s, 1725 s, 1522 s, 1475 s, 1423 s, 1017 s; MALDI MS m/z $[M+Na]^+$ 758.54 (calculated for $[C_{48}H_{80}O_5Na]^+$, 758.58); $[\alpha]_D^{22}$ = 36.81 (0.0107 g in 1 ml $CHCl_3$); m.p. 83-86 ° C.

(ii.) Oleoyl Chloride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with oleoyl chloride (1.54 ml, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 65 hours, after which time no solid was observed to remain in the orange solution. The mixture was treated with water (50 ml) and transferred to a separating funnel with ethyl acetate (50 ml). The aqueous phase was acidified to pH 2 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford a brown oil residue of crude product (0.81 g). This residue was purified by column chromatography with petrol / ethyl acetate (1:1) to afford the two desired main fractions. The first fraction was a colourless oil (0.57 g, 0.56 mmol, 26 %) of the bis-substituted product **(86)**, which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.46 – 5.33 (4H, m), 5.29 (1 H, d, *J* 3.5 Hz), 4.80 (1 H, dd, *J* 4.7 / 11.4 Hz), 3.91 (1 H d, *J* 11.6 Hz), 3.67 (1H, s), 3.63 (1 H, d, *J* 11.6 Hz), 2.83 (1 H, dd, *J* 3.7 / 13.6Hz), 2.37 – 2.30 (3 H, m), 2.27 (2 H, dd, *J* = 6.0 / 13.7Hz), 2.18 (1H, s), 2.02 (3 H, s), 2.01 (3 H, s), 1.91 (2H, m), 1.82 – 1.69 (3 H, m), 1.61 (13 H, m), 1.47 – 1.21 (60 H, m), 1.13 (3 H, s), 1.08 (2 H, s), 0.98 (2 H, s), 0.96 (1 H, s), 0.94 (3 H, s), 0.91 (3 H, s), 0.90 (3 H, s), 0.89 (3 H, s), 0.87 (2 H, s), 0.84 (3 H, s), 0.80 (1 H, s), 0.76 (3 H, s); δ_C (125 MHz; $CDCl_3$): 183.3, 173.6, 143.6, 130.0, 129.8, 129.7, 122.5, 74.2, 65.2, 51.4, 47.7, 46.5, 45.8, 41.6, 41.0, 40.6, 39.3, 37.8, 36.8, 34.7, 34.5, 33.8, 33.1, 32.4, 32.3, 31.9, 30.7, 29.8, 29.7, 29.5, 29.3, 29.3, 29.2, 29.1, 27.7, 27.2, 27.2, 25.8, 25.2, 25.1, 23.6, 23.4, 23.0, 22.7, 17.9, 17.1, 15.8, 14.1, 13.2; ν_{max} ($CHCl_3$) / cm $^{-1}$: 2933 s, 2858 s, 2401 s, 1726 s, 1521 s, 1468 s, 1423 s, 1013 s; MALDI MS m/z $[M+Na]^+$ 1023.90 (calculated for $[C_{66}H_{113}O_6Na]^+$, 1024.85); $[\alpha]_D^{22}$ = 32.59 (0.0103 g in 1 ml $CHCl_3$). The second fraction was a 1:1 mixture

of both the secondary **(87)** and primary **(88)** mono substituted products as a colourless solid (0.15 g, 0.20, mmol, 9 %) which appeared by TLC as two spots running together at very close retention times and was identical by ^{1}H NMR, ^{13}C NMR, IR MALDI MS, optical rotation and m.p. to the products in part (i) above.

6.42. Esterification of hederagenin with succinic anhydride

(i.) Succinic anhydride (10 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) together with succinic anhydride (2.12 g, 21.20 mmol) and DMAP (0.20 g, 1.63 mmol) were dissolved in anhydrous pyridine (20 ml). The mixture was stirred at room temperature under a nitrogen atmosphere for 48 hours. After this time the mixture was heated under reflux at 60 °C for 3 hours and then allowed to cool to ambient temperature. The mixture was then poured into 20 % sulphuric acid (50 ml), cooled in an ice bath and stirred for 10 minutes to ensure the crude solid product had been completely precipitated. This solid was then collected by filtering under reduced pressure through a Buchner funnel. The solid was washed on the filter with further 20 % sulphuric acid (3 x 50 ml) to ensure no pyridine remained before it was oven dried at 50 °C for 16 hours to afford a brown solid (2.30 g). Crude ^{1}H NMR analysis of this showed that the bis-substituted product (**77**) alone had been formed. However, due to the high polarity of this product, attempts to separate a portion (0.50 g) of it from the excess succinic acid by column chromatography proved unsuccessful. Therefore, another portion of the crude product (0.10 g) was treated with diazomethane and stirred for 15 minutes until no more effervescence was observed. The solvent was evaporated and the solid residue purified by column chromatography on a short silica column with petrol / ethyl acetate (1:1) to afford two main fractions. The first, an off white solid (0.04 g) was the methyl ester product of the bis-succinate **(80)** which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.29 (1 H, t, *J* 3.5 Hz), 4.78 (1 H, dd, *J* 11.5 / 4.9 Hz), 4.13 (1 H, q, *J* 7.1 Hz), 3.90 (1 H, d, *J* 11.6 Hz), 3.72 (1 H, d, *J* 6.4 Hz), 3.70 (3 H, s), 3.68 (3 H, s), 3.63 (3 H, s), 2.87 (1 H, dd, *J* 13.8 / 4.1 Hz), 2.70 – 2.57 (8 H, m), 2.05 (1 H, s), 1.98 (1 H, td, *J* 14.0 / 4.1 Hz), 1.93 – 1.85 (2 H, m), 1.76 – 1.58 (9 H, m), 1.58 – 1.49 (2 H, m), 1.44 – 1.30 (5 H, m), 1.26 (2 H, t, *J* 7.2 Hz), 1.23 – 1.16 (3 H, m), 1.14 (3 H, s), 1.11 – 0.99 (4 H, m), 0.97 (3 H, s), 0.93 (3 H, s), 0.90 (3 H, s), 0.89 – 0.85 (2 H, m), 0.83 (3 H, s), 0.73 (3 H, s); δ_C (125 MHz; $CDCl_3$): 177.3, 171.7, 171.6, 170.9, 170.6, 142.9, 121.1, 73.8, 64.4, 59.4, 50.8, 50.8, 50.5, 46.8, 46.6, 45.7, 44.8, 40.6, 40.3, 39.7, 38.3, 36.7, 35.8, 32.8, 32.1, 31.3, 31.2, 29.7, 28.5, 28.3, 28.0, 27.9, 26.6, 24.8,

22.6, 22.4, 22.0, 21.9, 21.6, 20.0, 18.4, 16.9, 15.8, 14.8, 13.2, 12.1, 0.01. A second fraction was recovered as a white solid (0.01 g) which appeared by TLC and ^{1}H NMR to correspond to the methyl ester product of the aglycone starting material (**15**). The column was washed with methanol, but no further fractions were observed. Another portion of the prepared crude succinate product (1.00 g) was dissolved in the minimum quantity of ethyl acetate with heating under reflux. Once dissolved, the solution was treated with an excess of petrol until the solution became cloudy. This cloudy suspension was then placed in a freezer for 2 hours to encourage further precipitate to crash out of the ice cold solution. The precipitate was collected in a Buchner funnel under reduced pressure and dried in an oven at 50 °C for 6 hours to afford a white powder (0.27 g, 0.40 mmol, 44 %) of the bis-substituted product (**77**) only, which appeared as one spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.28 (1 H, t *J* 3.5 Hz), 4.82 (1 H, dd, *J* 11.6 / 4.9 Hz), 3.94 (1 H, d, *J* 11.6 Hz), 3.69 (1 H, d, *J* 11.7 Hz), 2.82 (1 H, d, *J* 12.9 Hz), 2.75 – 2.50 (7 H, m), 1.99 (1 H, td, *J* 14.7 / 4.2 Hz), 1.90 (2 H, m), 1.84 – 1.68 (2 H, m), 1.66 – 1.54 (5 H, m), 1.51 (1 H, s), 1.35 (4 H, m), 1.32 – 1.16 (5 H, m), 1.13 (3 H, s), 1.08 (1 H, m), 0.98 (3 H, s), 0.94 (3 H, s), 0.91 (3 H, s), 0.90 (1 H, s), 0.88 (s, 1H), 0.87 (1 H, s), 0.85 (1 H, s), 0.83 (3 H, s), 0.74 (3 H, s); δ_C (125 MHz; $CDCl_3$): 131.3, 130.7, 129.3, 128.8, 98.9, 98.8, 67.8, 67.7, 63.8, 63.1, 63.0, 62.4, 62.3, 49.2, 46.6, 37.0, 37.0, 36.9, 36.8, 36.8, 33.8, 33.7, 33.7, 32.7, 32.6, 32.3, 32.3, 32.2, 32.2, 30.8, 30.7, 30.3, 30.1, 29.9, 29.9, 29.9, 29.7, 29.6, 29.5, 29.4, 29.4, 29.2, 29.1, 29.1, 29.0, 28.5, 27.0, 27.0, 26.9, 26.8, 26.2, 25.7, 25.6, 25.5, 25.5, 25.2, 24.8, 24.7, 24.4, 24.2, 20.2, 19.7, 19.7, 19.6, 19.6; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3752 w, 3691 w, 3055 s, 2987 s, 2686 w, 2522 w, 2411 w, 2306 s, 2126 w, 2054 w, 1731 s, 1723.s, 1605 w, 1551 w, 1422 s, 1266 s, 1217 w; MALDI MS m/z $[M+Na]^+$ 695.46 (calculated for $[C_{38}H_{56}O_{10}Na]^+$, 695.38); $[\alpha]_D^{22}$ = 46.87 (0.0099 g in 1 ml $CHCl_3$); m.p. 145-153. The mother liquor was evaporated to recover a pale brown solid residue which appeared by ^{1}H NMR to contain unreacted aglycone and succinic anhydride.

(ii.) Succinic anhydride (1 equivalent)

Defatted aglycone (**2**) (1.00 g, 2.12 mmol) together with succinic anhydride (0.21 g, 2.12 mmol) and DMAP (0.20 g, 1.63 mmol) were dissolved in anhydrous pyridine (20 ml). The mixture was stirred at room temperature under a nitrogen atmosphere for 48 hours. The mixture was then heated under reflux at 60 °C for 3 hours and then allowed to cool to ambient temperature. The mixture was then poured into 20 % sulphuric acid (50 ml), cooled in an ice bath and stirred for 10 minutes to ensure the crude solid product had been

completely precipitated. This solid was then collected by filtering the suspension under reduced pressure through a Buchner funnel. The solid was washed on the filter with further 20 % sulphuric acid (3 x 50 ml) to ensure no pyridine remained before it was oven dried at 50 °C for 16 hours to afford a brown solid (2.21 g). Crude ^{1}H NMR analysis of this confirmed that both forms of the mono substituted product were present in a ratio of 1:1. Since the polarity of the succinate products made it difficult to separate from the excess succinic anhydride by column chromatography, a portion of this solid (100 mg) was treated with diazomethane to protect the acid groups as the corresponding methyl esters and this was purified by column chromatography using petrol / ethyl acetate (1:1) as solvent to recover a mixture of the methyl esters of the primary **(81)** and secondary **(82)** substituted products which appeared as a single spot by TLC and showed the common signals δ_H (500 MHz; $CDCl_3$): 5.28 (1 H, t, *J* 3.2 Hz), 3.97 (0.5 H, s), 3.70 (0.5 H, d, *J* 3.5 Hz), 3.63 (0.5 H, d, *J* 1.3 Hz), 2.86 (1 H, dd, J 13.6 Hz), 2.65 (4 H, m), 2.10 (1 H, s), 2.02 – 1.76 (4 H, m), 1.72-1.49 (10 H, m), 1.46 – 1.25 (5 H, m), 1.23 - 1.15 (2 H, m), 1.14 (3 H, s), 1.10 – 0.99 (3 H, m), 0.97 (2 H, s), 0.95 (2 H, s), 0.93 (3 H, d, J 2.2 Hz), 0.90 (3 H, d, J 1.6 Hz), 0.78 (2 H, s), 0.73 (3 H, s), 0.68 (1 H, s) as well as those for the primary substituted product only δ_H (500 MHz; $CDCl_3$): 3.41 (0.5 H, t, J 8.8 Hz), 4.23 (0.25 H, d, *J* 11.4 Hz), 3.84 (0.25 Hz, d, *J* 11.4 Hz) and those for the secondary substituted product only δ_H (500 MHz; $CDCl_3$): 4.91 (0.25 H, dd, *J* 4.8 / 12.3 Hz), 3.37 (0.5 H, d, J 12.6 Hz), 2.96 (0.5 H, d, J 12.6 Hz); δ_C (125 MHz; $CDCl_3$): 178.3, 178.3, 173.4, 172.7, 127.6, 144.0, 143.9, 122.3, 122.1, 75.2, 72.4, 67.4, 64.4, 58.5, 51.9, 51.9, 51.5, 51.5, 48.1, 47.8, 47.5, 46.7, 46.7, 45.9, 42.4, 42.1, 41.7, 41.6, 41.3, 39.3, 39.3, 38.2, 38.0, 36.9, 36.7, 33.9, 33.1, 32.4, 32.3, 32.3, 30.7, 29.4, 29.3, 29.0, 28.9, 27.7, 26.1, 26.0, 25.9, 23.6, 23.4, 23.4, 23.3, 23.1; ν_{max} ($CHCl_3$) / cm^{-1}: 3755 w, 3691 w, 3050 s, 2986 s, 2686 w, 2411 w, 2306 s, 1731 s, 1605 w, 1552 w, 1422 s, 1266 s. Another portion of the prepared crude succinate product (1.00 g) was dissolved in the minimum quantity of ethyl acetate with heating under reflux. Once dissolved, the solution was treated with an excess of petrol. Very little precipitate was observed so the solution was placed in a freezer for 4 hours to encourage further precipitate to crash out of the ice cold solution. After this time the solution was filtered through a Buchner funnel under reduced pressure while still cold but only a small trace of solid residue could be collected. It appeared that no bis-substituted product **(77)** was obtained, and that the mono-succinate does not precipitate by the same means. The mother liquor was evaporated to recover a brown solid (0.98 g) of the crude product.

(iii.) Succinic anhydride (1 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with succinic anhydride (0.21 g, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours. The mixture was transferred to a separating funnel with water (30 ml) and ethyl acetate (30 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected. The boundary between the two phases was unclear so brine (40 ml) was added to encourage separation. On separation a white precipitate was observed in the aqueous phase, this was filtered and dried at 50 °C for 6 hours to afford a white powder (0.23 g) which by ^{1}H NMR analysis was identical to the starting material. The aqueous phase was then extracted again with further ethyl acetate (2 x 50 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford a white solid residue (0.62 g). This residue was purified by column chromatography with petrol / ethyl acetate (1:1) and 2 % acetic acid but only one fraction was obtained as a white solid (0.68 g) and ^{1}H NMR analysis of this showed only signals corresponding to the unreacted aglycone starting material **(2)**. The column was washed with methanol but no further products were obtained.

(iv.) Succinic anhydride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) together with succinic anhydride (0.42 g, 4.66 mmol) and DMAP (0.20 g, 1.63 g) were dissolved in anhydrous pyridine (20 ml) and stirred at room temperature under a nitrogen atmosphere for 48 hours. The reaction mixture was then heated under reflux at 60 °C for 3 hours and then allowed to cool to ambient temperature. The mixture was then poured into 20 % sulphuric acid (50 ml), cooled in an ice bath and stirred for 10 minutes to ensure the crude solid product had been completely precipitated. This solid was collected by filtering the suspension under reduced pressure. The solid was washed on the filter with further 20 % sulphuric acid (3 x 20 ml) to ensure no pyridine remained before it was oven dried at 50 °C for 18 hours to afford a brown solid (1.75 g). A portion of this solid (0.10 g) was treated with diazomethane to protect the acid groups as the corresponding methyl esters and this was purified by column chromatography using petrol / ethyl acetate (1:1) as solvent to recover the methyl ester of the bis substituted product **(77)** only as a white solid (0.04 g) which appeared as a single spot by TLC and was identical by ^{1}H and ^{13}C NMR to the methyl ester prepared in (i) above. Another portion of the prepared crude succinate product (1.00

g) was dissolved in the minimum quantity of ethyl acetate with heating under reflux. Once dissolved, the solution was treated with an excess of petrol until the solution became cloudy. This cloudy suspension was then placed in a freezer for 2 hours. The precipitate was collected in a Buchner funnel under reduced pressure and dried in an oven at 50 °C for 6 hours to afford an off-white powder (0.50 g, 0.74 mmol, 61 %) of the bis-substituted product only **(77)**, which gave a single spot by TLC and was identical by ^{1}H NMR, ^{13}C NMR, IR, MALDI MS, optical rotation and m.p. to the bis product prepared in part (i). The mother liquor was evaporated to recover a pale brown solid residue (0.50 g) which appeared by ^{1}H NMR to contain unreacted aglycone and succinic anhydride.

(v.) Succinic anhydride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and treated with succinic anhydride (0.42 g, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, then transferred to a separating funnel with water (40 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected. The aqueous phase contained a precipitate at the phase boundary so this was filtered to recover a white solid on drying (0.18 g) which by ^{1}H NMR analysis was identical to the starting material. The aqueous phase was then extracted with further ethyl acetate (2 x 50 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford a white solid residue (0.76 g). This residue was purified by column chromatography with petrol / ethyl acetate (1:1) and 10 % acetic acid but only one fraction was obtained as a white solid (0.68 g) and ^{1}H NMR analysis of this showed only signals corresponding to the starting material. The column was washed with methanol but no further products were obtained.

6.43. Esterification of hederagenin with glutaric anhydride

(i.) Glutaric anhydride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) together with glutaric anhydride (0.53 g, 4.66 mmol) and DMAP (0.20 g) were dissolved in anhydrous pyridine (15 ml) and this mixture was stirred at room temperature under a nitrogen atmosphere for 50 hours at room temperature. After this time, the reaction mixture was heated at 70 °C under reflux for 3 hours and then allowed to reach room temperature. Once cooled the mixture was poured into 20 % sulphuric acid (100 ml) to precipitate a pale brown solid and cooled in an ice bath for 15 minutes to ensure precipitation was complete. The suspension was

filtered under reduced pressure and the solid washed on the filter with further acid solution (3 x 20 ml). The recovered solid was dried first on the filter, and then at 50 °C for 12 hours to give a brown solid of crude product (0.82 g). This was dissolved in the minimum amount of hot ethyl acetate before being cooled in an ice bath to 5 °C. At this temperature a large excess of cold petrol was added to afford a white precipitate. The precipitate was collected by filtering the suspension under reduced pressure to recover a white solid (0.20 g, 0.29 mmol, 13 %) of the bis-substituted product **(79)**, which showed gave only one spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 8.65 (1H, s), 8.36 (1 H, s), 7.84 (1 H, s), 5.29 (1 H, s), 4.85 – 4.68 (1 H, m), 3.84 (1 H, d, *J* 11.3 Hz), 3.77 (1 H, d, *J* 11.7 Hz), 2.83 (1 H, dd, *J* 10.2 / 4.5 Hz), 2.55 – 2.26 (8 H, m), 2.10 – 1.84 (8 H, m), 1.84 – 1.67 (4 H, m), 1.67 – 1.50 (6 H, m), 1.48 – 1.34 (4 H, m), 1.32 – 1.18 (4 H, m), 1.13 (3 H, s), 1.12-1.05 (3 H, m), 0.98 (2 H, s), 0.94 (3 H, m), 0.92 (3 H, s), 0.88 (1 H, s), 0.86 (2 H, s), 0.85 (3 H, s), 0.84 (1 H, s), 0.75 (3 H, s); ν_{max} ($CHCl_3$) / cm^{-1}: 3687 w 3019 s, 2400 s, 1711 s, 1524 s, 1476 s, 1422 w, 1216 s; MALDI MS m/z $[M+Na]^+$ 723.55 (calculated for $[C_{40}H_{62}O_{10}Na]^+$, 723.41); $[\alpha]_D^{20}$ = 41.79 (0.0114 g in 1 ml $CHCl_3$); m.p. 160-165 °C. The mother liquor of the precipitation was evaporated to recover a pale brown solid residue (1.45 g), the ^1H NMR and TLC of which showed it to be composed of the unreacted aglycone and glutaric anhydride starting materials.

(ii.) Glutaric anhydride (10 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) together with glutaric anhydride (2.38 g, 21.2 mmol) and DMAP (0.20 g) were dissolved in anhydrous pyridine (15 ml) and stirred at room temperature under a nitrogen atmosphere for 50 hours. After this time, the reaction mixture was heated at 70 °C under reflux for 3 hours and then allowed to reach room temperature. The mixture was poured into 20 % sulphuric acid (100 ml) to precipitate a pale brown solid and cooled in an ice bath for 15 minutes to ensure precipitation was complete. The suspension was filtered under reduced pressure and the solid washed on the filter with further acid solution (3 x 20 ml). The recovered solid was dried first on the filter, and then at 50 °C for 12 hours to give a brown solid of crude product (1.91 g). This was dissolved in the minimum amount of hot ethyl acetate before being cooled in an ice bath to 5 °C. At this temperature a large excess of cold petrol was added to afford a white precipitate. The precipitate was collected by filtering under reduced pressure to recover a white solid (0.42 g, 0.60 mmol, 27 %) of the bis-substituted product **(79)**, which appeared as a single spot by TLC analysis and was identical to the product from part (i) above. The

mother liquor of the precipitation was evaporated to recover a pale brown solid residue (1.45 g), the ^{1}H NMR and TLC of which showed it to be composed of the unreacted aglycone and glutaric anhydride.

6.44. Esterification of hederagenin with maleic anhydride

(i.) Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with maleic anhydride (0.46 g, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, and then extracted between water (40 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected. The aqueous phase was then extracted again with ethyl acetate (3 x 50 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford a white solid (1.27 g). This was purified by column chromatography with petrol / ethyl acetate (1:1) and 10 % acetic acid in order to remove the excess maleic anhydride, but only one major fraction was obtained and ^{1}H NMR analysis showed only signals corresponding to the unreacted aglycone starting material (0.73 g). The column was washed with methanol but no further product was obtained only a solid residue of maleic anhydride (0.46 g). A white solid was observed in the aqueous phase following the extraction so this was collected by gravity filtration and dried at 50 °C for 6 hours to obtain a white solid (0.05 g), however, this again showed only signals corresponding to the unreacted aglycone.

(ii.) Defatted aglycone **(2)** (1.00 g, 2.12 mmol) together with maleic anhydride (1.76 g, 21.20 mmol) and DMAP (0.20 g, 1.63 mmol) were dissolved in anhydrous pyridine (20 ml) and stirred at room temperature under a nitrogen atmosphere for 48 hours. The mixture was then heated under reflux at 60 °C for 3 hours and allowed to cool to ambient temperature, then poured into 20 % sulphuric acid (50 ml), cooled in an ice bath and stirred for 10 minutes to ensure the crude solid product had been completely precipitated. This solid was collected by filtering the suspension under reduced pressure. The solid was washed on the filter with further 20 % sulphuric acid (3 x 50 ml) to ensure no pyridine remained before it was oven dried at 50 °C for 16 hours to afford a brown solid (2.14 g). This was dissolved in the minimum amount of hot ethyl acetate before being cooled in an ice bath to 5 °C. At this temperature a large excess of cold petrol was added to afford a white precipitate. The precipitate was collected by filtering the suspension under reduced pressure to recover a white solid (0.56 g, 0.84 mmol, 40 %) of the bis-substituted product

(78), which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 6.25 – 6.15 (1 H, m), 5.20 (1 H, t, *J* 3.2 Hz), 4.97 – 4.87 (1 H, m), 3.94 (1 H, d, *J* 11.3 Hz), 3.08 (1 H, d, *J* 12.2 Hz), 2.81 – 2.68 (1 H, m), 2.12 (1 H, s), 1.98 (1 H, s), 1.89 (1 H, td, *J* 13.7 / 3.9 Hz), 1.80 (2 H, m), 1.71 – 1.62 (2 H, m), 1.61 – 1.43 (8 H, m), 1.40 – 1.17 (6 H, m), 1.13 (1H, m), 1.08 (1 H, s), 1.06 (1 H, s), 1.05 (1 H, s), 1.03 – 0.96 (2 H, m), 0.91 (1 H, s), 0.87 2 H, s), 0.85 (3 H, s), 0.83 (3 H, s), 0.78 (3 H, s), 0.71 (3 H, s), 0.64 (1 H, s); δ_C (125 MHz; $CDCl_3$): 143.8, 122.2, 78.8, 55.2, 49.5, 48.4, 47.6, 46.3, 45.9, 41.7, 41.2, 39.2, 38.6, 38.4, 36.9, 33.8, 32.9, 32.7, 32.5, 30.6, 27.9, 27.6, 26.7, 25.7, 23.4, 23.3, 23.0, 18.2, 16.7, 15.4, 15.2; ν_{max} ($CHCl_3$) / cm^{-1}: 3684 w 3020 s, 2928 w, 2400 w, 2361 s, 1732 w, 1602 w, 1522 s, 1476 s, 1424 s, 1216 s; MALDI MS m/z $[M+Na]^+$ 691.40 (calculated for $[C_{38}H_{52}O_{10}Na]^+$, 691.36); $[\alpha]_D^{20}$ = 49.72 (0.0106 g in 1 ml $CHCl_3$); m.p. 150-158 °C. The mother liquor of the precipitation was evaporated to recover a pale brown solid residue (1.45 g), the ^1H NMR and TLC of which showed it to be composed of the unreacted aglycone and glutaric anhydride starting materials.

6.45. Attempted Esterification of hederagenin with succinimide

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (20 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) with succinimide (0.46 g, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours. The mixture was extracted between water (40 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected. The aqueous phase was then extracted again with further ethyl acetate (3 x 50 ml). An insoluble solid remained in the aqueous phase and was collected by gravity filtration. Upon drying gave a white powder (0.60 g) of unreacted starting material. The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford a white solid residue (0.56 g). This residue was purified by column chromatography with petrol / ethyl acetate (1:1) but only one major triterpene fraction was recovered (0.43 g) which gave a retention time by TLC identical to that of the aglycone and gave ^1H NMR signals identical to the starting material.

6.46. Esterification of hederagenin with trimethyl acetyl chloride

(i.) Trimethyl acetyl chloride (1 equivalent)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (20 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with trimethyl acetyl chloride (0.26 ml, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 60 hours, after which time no solid was observed to remain in the orange solution. The mixture was treated with water (50 ml) and transferred to a separating funnel with ethyl acetate (3 x 30 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford an orange solid reside (0.92 g). This was purified by column chromatography with petrol / ethyl acetate (5:1) to afford the two main fractions. The first fraction was a colourless solid (0.30 g, 0.47 mmol, 22 %) of the bis-substituted product **(89)**, which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.31 (1 H, s), 4.18 (1 H d, *J* 11.5 Hz), 3.81 (1 H, d, *J* 11.5 Hz), 3.39 (1 H, dd, *J* 7.4 / 8.5 Hz), 2.83 (1 H, dd, *J* 4.3 /12.9), 2.18 (1 H. s), 2.10 (1 H, s), 2.05 (1 H, s), 1.99 (1 H, td, *J* 4.0 / 13.3 Hz), 1.94 – 1.86 (1 H, td, *J* 4.0 / 13.3), 1.82 – 1.70 (3H, m), 1.69 – 1.51 (9 H, m), 1.51 – 1.29 (5 H, m), 1.28 (1 H, s), 1.27 (3 H, s), 1.26 (3 H, s), 1.24 (3 H, s), 1.23 (3 H, s), 1.21 (3 H, s), 1.18 - 1.13 (2 H, m), 1.11-1.01 (2 H, m), 0.99 (2 H, s), 0.98 (1 H, s), 0.96 (2 H, s), 0.93 (2 H, s), 0.92 (1 H, s), 0.91 (1 H, s), 0.90 (1 H, s) 0.83 (1 H, s), 0.79 (1 H, s), 0.77 (2 H, s), 0.68 (1 H, s); δ_C (125 MHz; $CDCl_3$): 122.6, 77.3, 77.0, 76.8, 72.5, 68.1, 66.2, 66.2, 48.0, 46.5, 45.9, 42.3, 41.7, 41.1, 39.2, 38.5, 36.8, 33.8, 33.1, 32.3, 30.7, 27.6, 27.3, 27.0, 26.0, 25.7, 23.5, 18.1, 17.0, 15.8, 11.9; ν_{max} ($CHCl_3$) / cm^{-1}: 3069 s, 2940 s, 2357 s, 1698 s, 1490 s, 1383 s, 1214 s, 1022 s; MALDI MS m/z $[M+Na]^+$ 663.67 (calculated for $[C_{38}H_{64}O_6Na]^+$, 663.46); $[\alpha]_D^{22}$ = 48.53 (0.0115 g in 1 ml $CHCl_3$); m.p. 106-109 °C. The second fraction collected, a colourless solid, was a single compound of only the primary mono-substituted product **(91)** (0.08 g, 0.14 mmol, 7 %) which gave only one spot by TLC analysis and showed δ_H (500 MHz; $CDCl_3$): 5.28 (1 H, t, *J* 3.5 Hz), 4.16 (1 H, d, *J* 11.5 Hz), 3.80 (1 H, d, *J* 11.5 Hz), 3.45 – 3.33 (1 H, m), 2.82 (1 H, dd, *J* 4.1 / 13.7 Hz), 2.04 (1 H, s), 2.01 – 1.92 (1 H, s), 1.88 (2 H, td, *J* 10.8 / 5.0 Hz), 1.75 (2 H, ddd, *J* 26.9 / 13.6 / 4.0 Hz), 1.69 – 1.52 (7 H, m), 1.51 – 1.29 (5 H, m), 1.27 (1 H, s), 1.26 (1 H, s), 1.24 (1 H, d, *J* 2.0 Hz), 1.22 (10 H, s), 1.20 (1 H, s), 1.15 (1 H, s), 1.12 (3 H, s), 1.10 – 1.03 (1 H, m), 0.95 (3 H, s), 0.92 (3 H, s), 0.89 (3 H, s), 0.85 (1 H, s), 0.76 (6 H, s); δ_C (125 MHz; $CDCl_3$): 184.6, 184.1, 178.7, 171.3, 143.5, 122.5, 72.5, 66.2, 60.4, 48.0, 47.9, 46.6, 45.8, 42.3, 41.6, 41.0, 39.2, 39.0, 38.5, 38.5, 36.8, 33.8, 33.1,

32.4, 32.3, 30.7, 27.6, 27.3, 27.0, 26.0, 25.7, 23.5, 23.4, 22.8, 21.0, 18.1, 17.0, 15.8, 14.2, 11.9; ν_{max} ($CHCl_3$) / cm^{-1}: 3020 s, 2935 s, 2400 s, 1698 s, 1522 s, 1478 s, 1424 s, 1215 s, 1018 s; MALDI MS m/z $[M+Na]^+$ 579.81 (calculated for $[C_{34}H_{56}O_5Na]^+$, 579.35); $[\alpha]_D^{22}$ = 40.69 (0.0102 g in 1 ml $CHCl_3$); m.p. 145-152 °C. The column was then washed first with ethyl acetate, then with methanol and the resultant solution evaporated to afford an orange solid reside (0.40 g) of what appeared to contain predominantly unreacted starting material.

(ii.) Trimethyl acetyl chloride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (20 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with trimethyl acetyl chloride (0.57 ml, 0.57 g, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 60 hours, after which time no solid was observed to remain in the orange solution. The mixture was treated with water (50 ml) and transferred to a separating funnel with ethyl acetate (3 x 30 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford an orange solid reside (1.24 g). This was purified by column chromatography with petrol / ethyl acetate (5:1) to afford the two main fractions. The first fraction was a colourless solid (0.16 g, 0.25 mmol, 12 %) of the bis-substituted product **(89)**, which gave a single spot by TLC and was identical by ^1H, ^{13}C, IR, MALDI MS, optical rotation and m.p to the bis product in part (i) above. The second fraction, a colourless solid, was the primary mono-substituted product **(91)** only (0.55 g, 0.99 mmol, 47 %) which appeared as only one spot by TLC and was identical by ^1H, ^{13}C, IR, MALDI MS, optical rotation and m.p. to the mono product obtained in (i). The column was then washed first with ethyl acetate, then with methanol and the resultant solution evaporated to a white solid (0.43 g) of what appeared to contain predominantly unreacted starting material.

6.47. Esterification of hederagenin with isobutyryl chloride

(i.) Isobutyryl chloride (1 equivalent)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (20 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with isobutyryl chloride (0.23 ml, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 60 hours, after which time no solid was observed to remain in the orange solution. The mixture was treated with water (50 ml) and transferred to a separating

funnel with ethyl acetate (3 x 30 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford a white solid (1.07 g). This was purified by column chromatography with petrol / ethyl acetate (1:1) to afford the two main fractions. The first fraction was a colourless solid (0.08 g, 0.13 mmol, 6 %) of the bis-substituted product **(92)**, which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.29 (1 H, t, *J* 3.4 Hz), 4.87 (1 H, dd, *J* 4.6 / 12.1Hz), 4.18 (1 H, d, *J* 11.5 Hz), 3.84 (1 H, d, *J* 11.5 Hz), 3.41 (1 H, dd, *J* 8.7 / 7.2 Hz), 2.83 (1 H, dd, *J* 3.3 / 13.5 Hz), 2.60 (1 H, dtd, *J* 14.0 / 7.0 / 3.5 Hz), 2.39 – 2.08 (1 H, m), 1.94 (2 H, tt, *J* 13.5 / 4.0 Hz), 1.90 (2 H, ddd, *J* 11.5 / 7.8 / 4.0 Hz), 1.82 – 1.68 (2 H, m), 1.67 – 1.49 (9 H, m), 1.49 – 1.23 (9 H, m), 1.21 (4 H, s), 1.20 (4 H, s), 1.19 – 1.15 (2 H, m), 1.14 (1 H, s), 1.13 (1 H, s), 1.07 (1 H, s), 0.99 (1 H, s), 0.96 (2 H, s), 0.94 (3 H, s), 0.92 (1 H, s), 0.91 (1 H, s), 0.90 (1 H, s), 0.78 (1 H, s), 0.77 (3 H, s), 0.76 (1 H, s), 0.68 (1 H, s); δ_C (125 MHz; $CDCl_3$): 183.4, 182.2, 177.3, 143.5, 122.6, 79.1, 72.6, 66.5, 55.2, 48.0, 47.9, 47.6, 46.6, 45.9, 42.2, 41.6, 41.1, 39.3, 38.4, 37.1, 36.9, 34.3, 33.8, 33.7, 33.1, 32.4, 30.7, 28.1, 27.6, 26.0, 25.9, 25.7, 23.6, 23.4, 22.9, 19.1, 19.1, 18.8, 18.2, 17.0, 15.8, 15.5, 15.3, 11.9; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3156 s, 2941 s, 2354 s, 1794 s, 1700 s, 1470 s, 1385 s, 1216 s, 1165 s, 1096 s; MALDI MS m/z $[M+Na]^+$ 635.65 (calculated for $[C_{36}H_{60}O_6Na]^+$, 635.43); $[\alpha]_D^{22}$ = 42.13 (0.0102 g in 1 ml $CHCl_3$); m.p. 131-132 °C. The second fraction, a colourless solid, was the primary mono-substituted product **(94)** only (0.16 g, 0.29 mmol, 14 %) which gave one spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.29 (1 H, t, *J* 6.4 Hz), 4.17 (0.8 H, d, *J* 11.5 Hz), 3.84 (0.8 H, d, *J* 11.5 Hz), 3.41 (0.8 H, t, *J* 11.5 Hz), 3.23 (1 H, dd, *J* 4.2 / 11.5 Hz), 2.83 (1 H, dd, *J* 3.2 / 13.6 Hz), 2.65 – 2.54 (3 H, m), 2.05 (2 H, s), 2.03 – 1.93 (1 H, m), 1.92 – 1.85 (1 H, m), 1.82 – 1.70 (2 H, m), 1.68 – 1.52 (8 H, m), 1.50 – 1.30 (5 H, m), 1.26 (3 H, t, *J* 7.1 Hz), 1.21 (8 H, s), 1.20 (8 H, s), 1.16 (2 H, dd, *J* 6.9 / 12.8 Hz), 1.13 (2 H, s), 1.11 – 0.97 (4 H, m), 0.95 (3 H, s), 0.93 (3 H, s), 0.90 (3 H, s), 0.77 (3 H, s), 0.76 (2 H, s), 0.67 (1 H, s); δ_C (125 MHz; $CDCl_3$): 184.1, 183.0, 177.3, 143.5, 122.6, 72.6, 66.5, 60.4, 48.0, 47.9, 46.6, 45.9, 42.2, 41.6, 41.0, 39.3, 39.3, 38.4, 36.9, 34.3, 33.8, 33.8, 33.1, 32.4, 32.4, 32.3, 30.7, 27.6, 26.0, 25.7, 23.6, 23.4, 22.9, 21.1, 19.1, 19.1, 18.8, 18.2, 16. 9, 15.8, 14.2, 11.9; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3156 s, 2986 s, 2560 s, 1794 s, 1643 s, 1602 s, 1561 s, 1469 s, 1383 s, 1298 s, 1216 s, 1096 s; MALDI MS m/z $[M+Na]^+$ 565.71 (calculated for $[C_{33}H_{54}O_5Na]^+$, 565.39); $[\alpha]_D^{22}$ = 30.61 in chloroform (0.0110 g in 1ml $CHCl_3$); m.p. 270-280 °C (decomposition). The column was then washed first with ethyl acetate, then with methanol and the resultant solution evaporated

to afford a white solid reside (0.68 g) of what appeared to contain predominantly unreacted starting material.

(ii.) Isobutyryl chloride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (20 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with isobutyryl chloride (0.49 ml, 0.50 g, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 62 hours, after which time no solid was observed to remain in the solution. The mixture was treated with water (80 ml) and transferred to a separating funnel with ethyl acetate 80 ml) and brine (20 ml) added to encourage separation. The organic phase was collected and the aqueous phase further extracted with ethyl acetate (100 ml x 2). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford an orange (2.10 g). This was purified by column chromatography with petrol / ethyl acetate (1:1) to afford the two main fractions. The first fraction, a colourless solid (0.13 g, 0.21 mmol, 10 %), was the bis-substituted product **(92)**, which gave only a single spot by TLC and was identical by ^{1}H, ^{13}C, IR, MALDI MS, optical rotation and m.p. to the product from (i) above. The second fraction, a colourless solid, was a 1:4 mixture of the secondary **(93)** and primary **(94)** mono-substituted products (0.16 g, 0.29 mmol, 14 %) which gave two spots by TLC at very similar retention times and showed the common signals δ_H (500 MHz; $CDCl_3$): 5.29 (1 H, t, *J* 6.4 Hz), 3.23 (1 H, dd, *J* 4.2 / 11.5 Hz), 2.83 (1 H, dd, *J* 3.2 / 13.6 Hz), 2.65 – 2.54 (3 H, m), 2.05 (2 H, s), 2.03 – 1.93 (1 H, m), 1.92 – 1.85 (1 H, m), 1.82 – 1.70 (2 H, m), 1.68 – 1.52 (8 H, m), 1.50 – 1.30 (5 H, m), 1.26 (3 H, t, *J* 7.1 Hz), 1.21 (8 H, s), 1.20 (8 H, s), 1.16 (2 H, dd, *J* 6.9 / 12.8 Hz), 1.13 (2 H, s), 1.11 – 0.97 (4 H, m), 0.95 (3 H, s), 0.93 (3 H, s), 0.90 (3 H, s), 0.77 (3 H, s), 0.76 (2 H, s), 0.67 (1 H, s) as well as signals for the secondary substituted product only at δ_H (500 MHz; $CDCl_3$): 4.87 (0.2 H, dd, *J* 4.4 / 12.2 Hz), 3.39 (0.2 H, d, 12.5), 2.87 (0.2 H, d, 12.5); and for the primary substituted product only at δ_H (500 MHz; $CDCl_3$): 4.17 (0.8 H, d, *J* 11.5 Hz), 3.84 (0.8 H, d, *J* 11.5 Hz), 3.41 (0.8 H, t, *J* 11.5 Hz); δ_C (125 MHz; $CDCl_3$): 184.1, 183.0, 177.3, 143.5, 122.6, 72.6, 66.5, 60.4, 48.0, 47.9, 46.6, 45.9, 42.2, 41.6, 41.0, 39.3, 39.3, 38.4, 36.9, 34.3, 33.8, 33.8, 33.1, 32.4, 32.4, 32.3, 30.7, 27.6, 26.0, 25.7, 23.6, 23.4, 22.9, 21.1, 19.1, 19.1, 18.8, 18.2, 16. 9, 15.8, 14.2, 11.9; ν_{max} ($CHCl_3$) / cm^{-1}: 3156 s, 2986 s, 2560 s, 1794 s, 1643 s, 1602 s, 1561 s, 1469 s, 1383 s, 1298 s, 1216 s, 1096 s; MALDI MS m/z $[M+Na]^+$ 565.71 (calculated for $[C_{33}H_{54}O_5Na]^+$, 565.39). The column was then washed first with ethyl

acetate, then with methanol and the resultant solution evaporated to a white solid (0.17 g) of what appeared to contain predominantly unreacted starting material.

6.48. Esterification of hederagenin with benzoic anhydride

(i.) Benzoic anhydride (1 equivalent)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (20 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with benzoic anhydride (0.47 g, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 62 hours. After this time, the mixture was treated with water (50 ml) and transferred to a separating funnel with ethyl acetate (40 ml). Incomplete separation was observed, with an insoluble white suspension present. This suspension was filtered by gravity to obtain a white solid (0.53 g) of what was observed by ^{1}H NMR to be unreacted aglycone. The filtered phases were returned to the separating funnel and the organic phase drawn off. The aqueous phase was re-extracted with further ethyl acetate (2 x 30 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford a white solid reside (1.32 g). This crude product was purified by column chromatography on a short silica column using petrol / ethyl acetate (1:1) as solvent to afford two major fractions. The first fraction was a colourless solid (0.10 g, 0.15 mmol, 7 %) of the bis-substituted product **(12)**, which appeared as a single spot by TLC and was identical to the product fully characterised in part (ii) below. The second fraction, a colourless solid, was the primary mono-substituted product **(76)** (0.15 g, 0.26 mmol, 12 %) which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 8.11 (4 H, d, *J* 7.1 Hz), 8.04 (1 H, d, *J* 7.2 Hz), 7.67 – 7.59 (2 H, m), 7.52 – 7.45 (5 H, m), 7.27 (8 H, s), 5.40 (0.5 H, *J* 3.3 Hz), 5.30 (1 H, t, *J* 3.4 Hz), 4.36 (1 H, t, *J* 7.1 Hz), 3.75 (1 H, d, *J* 2.3 Hz), 3.66 (1 H, ddd, *J* 14.5 / 7.9 / 5.0 Hz), 3.45 (1 H, d, *J* 10.4 Hz), 2.94 (1 H, d, *J* 9.8 Hz), 2.85 (1 H, dd, *J* 3.4 / 14.9), 2.51 (1 H, t, *J* 8.2 Hz), 2.28 (2 H, dt, *J* 19.9 / 7.6 Hz), 2.12 (1 H, S), 2.05 – 1.86 (7 H, m), 1.86 – 1.69 (8 H, m), 1.67 – 1.54 (12 H, m), 1.50 – 1.30 (10 H, m), 1.27 (2 H, s), 1.26 (1 H, s), 1.26 (1 H, s), 1.25 (2 H, s), 1.24 (1 H, s), 1.18 (2 H, s), 1.14 (4 H, s), 1.12 - 0.99 (4 H, m), 0.97 (1 H, s), 0.96 (3 H, s), 0.94 (3 H, s), 0.92 (3 H, s), 0.90 (3 H, s), 0.86 (1 H, s), 0.78 (2 H, s); ν_{max} ($CHCl_3$) / cm $^{-1}$: 3019 s, 2952 s, 2672 s, 2548 s, 2401 s, 1790 s, 1695 s, 1605 s, 1522 s, 1417 s, 1288 s, 1216 s; MALDI MS m/z $[M+Na]^+$ 599.60 (calculated for $[C_{36}H_{52}O_4Na]^+$, 599.38); $[\alpha]_D^{22}$ = 32.01 (0.0096 g in 1 ml $CHCl_3$); m.p. 269-273 °C. The column was then washed first with ethyl acetate,

then with methanol and the resultant solution evaporated to a white solid (0.10 g) of what appeared to contain further unreacted starting material.

(ii.) Benzoic anhydride (2.2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (20 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with benzoic anhydride (1.05 g, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 62 hours. The mixture was then treated with water (50 ml) and transferred to a separating funnel with ethyl acetate (30 ml). Incomplete separation was observed, with an insoluble white suspension present. This suspension was filtered by gravity to obtain a white solid (0.14 g) of what was observed by ^{1}H NMR to be unreacted aglycone. The filtered phases were returned to the separating funnel and the organic phase drawn off. The aqueous phase was re-extracted with further ethyl acetate (2 x 30 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford a white solid (0.65 g). This was dissolved in the minimum amount of hot ethyl acetate before being cooled in an ice bath to 5 °C. At this temperature a large excess of cold petrol was added to afford a white precipitate. The precipitate was collected by filtering the suspension under reduced pressure to recover a white solid (0.38 g, 0.56 mmol, 26 %) of the bis-substituted product **(12)**, which appeared as a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 7.86 (1 H, d, *J* 7.7 Hz), 7.80 (4 H, dd, *J* 18.2 / 7.4 Hz), 7.36 (2 H, dt, *J* 24.0, 8.8 Hz), 7.23 (5 H, dt, *J* 23.8 / 12.1 Hz), 5.07 (1 H, t, *J* 3.1), 3.81 (1 H, dt, *J* 24.1 / 12.2 Hz), 3.51 (1 H, d, *J* 10.3 Hz), 3.42 (1 H, t, *J* 7.8 Hz), 3.21 (1 H, d, *J* 10.3 Hz), 2.60 (1 H, dd, *J* 13.8 / 3.1 Hz), 1.66 (10 H, m), 1.36 (15 H, m), 1.18 – 0.94 (20 H, m), 0.91 (8 H, s), 0.89 – 0.80 (4 H, s), 0.73 (5 H, s), 0.71 (6 H, s), 0.68 (3 H, s), 0.67 (3 H, s), 0.63 (3 H, s), 0.55 (2 H, s); δ_C (125 MHz; $CDCl_3$): 171.0, 162.4, 148.8, 143.6, 143.0, 137.0, 134.5, 134.2, 133.4, 130.4, 129.9, 129.6, 129.4, 129.1, 128.8, 128.5, 124.2, 122.5, 76.9, 72.0, 71.9, 49.8, 48.5, 48.1, 47.6, 46.6, 45.9, 45.8, 41.9, 41.8, 41.8, 41.7, 41.3, 41.1, 39.4, 39.3, 38.2, 38.1, 36.9, 33.8, 33.7, 33.1, 33.00, 32.5, 32.5, 31.6, 30.7, 27.7, 26.7, 26.0, 25.8, 23.6, 23.6, 23.5, 23.4, 23.3, 23.0, 18.5, 17.1, 16.9, 15.7, 15.7, 11.4, 11.4; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3020 s, 2950 s, 2675 s , 2401 s, 1790 s, 1695 s, 1585 s, 1494 s, 1416 s, 1319 s, 1216 s; MALDI MS m/z $[M+Na]^+$ 703.52 (calculated for $[C_{42}H_{56}O_5Na]^+$, 703.40); $[\alpha]_D^{22}$ = 44.83 °; M.P. 204-208 °C. The mother liquor was evaporated to afford a white solid residue (0.60 g) which appeared by crude ^{1}H NMR analysis to be benzoic acid.

6.49. Preparation of excess behenic acid chloride and subsequent esterification with hederagenin

Behenic acid (0.80 g, 2.12 mmol) was dissolved in anhydrous toluene (6 ml) with continuous stirring under a nitrogen atmosphere. To this solution, oxalyl chloride (0.41 ml, 2.12 mmol) was added together with a catalytic amount of DMF (0.18 ml, 0.11 mmol) and the mixture was stirred for 3 hours. After this time the solvent was evaporated under high vacuum to afford a crude solid residue. The residue was dissolved in dry ether (4 ml) and the solvent evaporated immediately under high vacuum until a cleaner white solid residue was obtained. This process was repeated with further dry ether (2 x 4 ml) until a clean white solid was obtained without any solvent present. The resultant solid of behenic acid chloride was dissolved in dry THF (5 ml). In a separate dry flask a solution of defatted aglycone **(2)** (0.50 g, 1.06 mmol) was prepared in THF (15 ml), together with pyridine (0.50 ml, 6.40 mmol) and DMAP (0.01 g, 0.11 mmol) added as a catalyst. This solution was stirred under nitrogen for 10 minutes and cooled to 0 °C in an ice bath. The prepared behenic acid chloride solution was taken up by syringe and added to the flask drop-wise, maintaining a temperature below 5 °C throughout the addition. Once the addition was complete the flask was removed from the cooling bath and allowed to reach room temperature, at which point a white precipitate was observed to form. The reaction mixture was stirred at room temperature under nitrogen for 62 hours to ensure reaction was complete, then water (50 ml) was added and it was transferred to a separating funnel with ethyl acetate (50 ml). Incomplete separation was observed, so brine (30 ml) was added to encourage separation. The organic phase was collected and the aqueous phase re-extracted with further ethyl acetate (3 x 30 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford an orange solid reside (1.59 g). This was purified by column chromatography on a short silica column using petrol / ethyl acetate (1:1) as solvent to afford three fractions. The first, recovered as a white solid (0.20 g) gave a spot by TLC which corresponded to the unreacted excess of behenic acid. The second fraction was evaporated to recover a white solid (0.38 g, 0.31 mmol, 16 %) of the bis-substituted product **(96)**, which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 8.10 (1 H, s), 8.06 (1 H, d, *J* 0.6 Hz), 5.29 (1 H, t, *J* 3.2 Hz), 4.96 (1 H, dd, *J* 4.8 / 11.5Hz), 4.01 (1 H, d, *J* 11.5 Hz), 3.82 (1 H, d, *J* 11.5 Hz), 2.83 (1 H, dd, *J* 3.9 / 13.5 Hz), 2.35 (5 H, t, *J* 7.5 Hz), 2.05 (3 H, s), 2.04 – 1.95 (1 H, m), 1.93 – 1.84 (3 H, m), 1.83 – 1.70 (4 H, m), 1.69 – 1.51 (14 H, m), 1.42 (4 H, d, *J* 5.0 Hz), 1.38 – 1.20 (2 H, m), 1.14 (3 H, s), 1.08 (2 H, s), 0.99 (3 H, s), 0.94 (3 H, s), 0.92 (3 H, s),

0.90 (3 H, s), 0.89 (3 H, s), 0.88 (3 H, s), 0.87 (2 H, s), 0.86 (1 H, s); δ_C (125 MHz; $CDCl_3$): 178.7, 160.8, 160.7, 143.6, 122.3, 77.6, 74.3, 64.5, 60.4, 47.6, 47.6, 46.5, 45.8, 41.7, 41.0, 40.5, 39.3, 37.6, 36.8, 33.8, 33.1, 32.4, 32.2, 31.9, 30.7, 29.7, 3.7, 29.7, 29.6, 29.5, 29.4, 29.3, 29.1, 27.6, 25.9, 24.7, 23.6, 23.0, 22.9, 22.7, 21.1, 17.9, 16.9, 15.8, 14.2, 14.1, 13.1; ν_{max} ($CHCl_3$) / cm $^{-1}$: 3019 s, 2927 s, 2854 s , 2400 s, 1708 s, 1522 s, 1468 s, 1423 s, 1214 s; MALDI MS m/z $[M+Na]^+$ 1139.57 (calculated for $[C_{74}H_{144}O_6Na]^+$, 1139.99); $[\alpha]_D^{21}$ = 41.03 (0.0107 g in 1 ml $CHCl_3$); m.p. 69-71 °C. The final fraction was recovered as a white solid (0.15 g) which showed signals by ^{1}H NMR corresponding to the unreacted hederagenin and subsequent column washings showed no indication of further product.

6.50. Preparation of excess linoleic acid chloride and subsequent esterification with hederagenin

Linoleic acid (0.59 g, 2.12 mmol) was dissolved in anhydrous toluene (5 ml) with continuous stirring under a nitrogen atmosphere. To this solution, oxalyl chloride (0.26 ml, 2.12 mmol) was added together with a catalytic amount of DMF (0.18 ml, 0.11 mmol) and the mixture was stirred for 3 hours. After this time the solvent was evaporated under high vacuum to afford a crude sticky solid. This was dissolved in dry ether (5 ml) and the solvent evaporated immediately under high vacuum to remove any remaining toluene and DMF. This process was repeated with further dry ether (2 x 4 ml) until a clean amorphous solid was obtained without any solvent present. The resultant solid of linoleic acid chloride was dissolved in dry THF (5 ml). In a separate dry flask a solution of defatted aglycone **(2)** (0.50 g, 1.06 mmol) was prepared in THF (15 ml), together with pyridine (0.50 ml, 6.40 mmol) and DMAP (0.01 g, 0.11 mmol) added as a catalyst. This solution was stirred under nitrogen for 10 minutes and cooled to 0 °C in an ice bath. Once at this temperature, the prepared linoleic acid chloride solution was taken up by syringe and added to the flask drop-wise, maintaining a temperature below 5 °C throughout the addition. Once the addition was complete the flask was removed from the cooling bath and allowed to attain room temperature, at which point a white precipitate was observed to form. The mixture was then allowed to stir at room temperature under nitrogen for 60 hours to ensure reaction was complete, at which point water (50 ml) was added and transferred to a separating funnel with ethyl acetate (50 ml). The organic phase was collected and the aqueous phase re-extracted with further ethyl acetate (3 x 30

ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford an orange solid (0.82 g). This was purified by column chromatography on a silica column using petrol / ethyl acetate (1:1) as solvent to afford three fractions. The first, recovered as a colourless oil (0.46 g) gave a spot by TLC which corresponded to the unreacted excess of linoleic acid. The second fraction was evaporated to recover a pasty white solid (0.29 g, 0.29 mmol, 27 %) of the bis-substituted product **(95)**, which gave one spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.38 – 5.28 (5 H, m), 5.27 – 5.24 (1H, m), 3.70 (1 H, d, *J* 10.4 Hz), 3.61 (1 H, dd, *J* 13.4 / 6.4 Hz), 3.40 (1 H, d, *J* 10.3 Hz), 2.97 (1 H, s), 2.91 (1 H, s), 2.79 (1 H, dd, *J* 12.6 / 3.6 Hz), 2.74 (2 H, t, *J* 6.6 Hz), 2.31 (4 H, t, *J* 7.5 Hz), 2.29 – 2.23 (1 H, m), 2.19 – 2.12 (1 H, m), 2.06 (1 H, s), 2.05 – 1.98 (6 H, m), 1.90 (4 H, m), 1.75 – 1.42 (44 H, m), 1.38 – 1.25 (36 H, m), 1.22 (6 H s), 1.10 (3 H, s), 0.92 (2 H, s), 0.89 (3 H, s), 0.87 (3 H, s), 0.86 (3 H, s), 0.84 (3 H, s), 0.74 (3 H, s); δ_C (125 MHz; $CDCl_3$): 179.1, 133.9, 131.1, 127.6, 127.4, 86.8, 86.7, 77.3, 77.0, 76.8, 76.5, 46.6, 45.9, 41.0, 39.3, 36.7, 33.7, 33.0, 33.0, 32.7, 32.7, 32.6, 32.5, 32.4, 32.3, 32.2, 31.7, 31.7, 31.6, 31.4, 31.4, 30.7, 29.7, 29.6, 29.4, 29.4, 29.3, 29.2, 28.8, 28.7, 28.7, 28.6, 27.8, 27.7, 26.0, 25.2, 25.1, 25.0, 24.9, 24.9, 24.8, 24.6, 24.6, 23.6, 23.5, 23.4, 23.4, 23.3, 23.0, 23.0, 22.9, 22.9, 22.9, 22.5, 22.5, 16.8, 14.0, 14.0, 13.9, 12.0; ν_{max} ($CHCl_3$) / cm^{-1}: 3157 s, 2935 s, 2840 s , 2360 s, 2254 s, 1794 s, 1647 w, 1558 w, 1471 s, 1383 s, 1166 w, 1096 s; $[\alpha]_D^{21}$ = 75.00 (0.0098 g in 1 ml $CHCl_3$); m.p. 168-172 °C. The final fraction was recovered as a white solid (0.05 g) which showed signals by ^1H NMR corresponding to the unreacted hederagenin starting material and subsequent column washings showed no indication of further product.

6.51. Esterification of hederagenin with palmitoyl chloride

(i.) Palmitoyl chloride (10 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with palmitoyl chloride 6.41 ml, 5.80 g, 21.20 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time a white precipitate was observed to have formed. The mixture was transferred to a separating funnel and quenched with water (30 ml) and ethyl acetate (30 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted with further ethyl acetate (2 x 30 ml). The combined organic phases were

washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford a white solid (6.88 g). Although ^{1}H NMR analysis of this crude product indicated that bis-substituted product was present, attempts to purify this residue by column chromatography proved unsuccessful and on each occasion the triterpene components could not be separated from the excess palmitic acid residue present.

(ii.) Palmitoyl chloride (1 equivalent)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with palmitoyl chloride 0.64 ml, 0.58 g, 2.12 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time a white precipitate was observed to have formed. The mixture was transferred to a separating funnel with water (30 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford a yellow / off-white solid residue (1.14 g). This residue was purified by column chromatography with petrol / ethyl acetate (5:1) to afford the two main fractions. The first fraction was a pasty white solid (0.02 g, 0.02 mmol, 1 %) of the bis-substituted product **(83)**, which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 5.28 (1 H, t, *J* 4.1 Hz), 4.79 (1 H, dd, *J* 4.4 / 11.7 Hz), 3.90 (1 H, d, *J* 11.7 Hz), 3.62 (1 Hz, d, *J* 11.7 Hz), 2.82 (1 H, dd, *J* 13.6 Hz), 2.31 (2 H, t, *J* 7.6 Hz), 2.26 (2 H, t, *J* 7.6 Hz), 1.93 – 1.62 (3 H, m), 1.61 (9 H, m), 1.41 – 1.35 (3 H, m), 1.22 (1 H, m), 1.25 (54 H, s), 1.12 (3 H, s), 0.97 (3 H, s), 0.93 (3 H, s), 0.91 (3 H, s), 0.89 (2 H, s), 0.88 (3 H, s), 0.87 (2 H, s), 0.82 (3 H, s), 0.76 (3 H, s); δ_C (125 MHz; $CDCl_3$): 184.1, 173.6, 173.3, 143.6, 122.5, 74.2, 65.0, 51.4, 47.7, 46.5, 45.8, 41.5, 41.0, 40.6, 39.3, 37.8, 36.8, 34.7, 34.6, 34.1, 34.0, 33.8, 33.1, 32.4, 32.2, 31.9, 30.7, 29.7, 29.7, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.3, 29.3, 29.1, 29.1, 27.6, 25.9, 25.2, 25.1, 25.0, 24.7, 23.6, 23.4, 22.9, 22.8, 22.7, 17.9, 17.1, 15.8, 14.1, 13.2 ν_{max} (mull) / cm^{-1}: 2953 s, 2853 s, 1742 s, 1699 s; MALDI MS m/z $[M+Na]^+$ 972.04 (calculated for $[C_{62}H_{110}O_6Na]^+$, 972.82); $[\alpha]_D^{20}$ = 35.60 (0.0100 g in 1 ml $CHCl_3$); m.p. 25-29 °C. The second and third fractions were a 2:3 mixture of both the primary **(84)** and secondary **(85)** mono substituted products as a white solid (0.32 g, 0.46 mmol, 22 %) which showed the common signals δ_H (500 MHz;

$CDCl_3$): 5.26 (1 H, t, J 4.1 Hz), 2.82 (1 H, dd, *J* 13.6 Hz), 2.32 (3 H, p, *J* 6.9 Hz), 1.97 (1 H, m), 1.88 (2 H, m), 1.83 – 1.52 (13 H, m), 1.46 – 1.30 (4 H, m), 1.29 (8 H, s), 1.25 (25 H, s), 1.14 (2 H, s), 1.12 (2 H, s), 0.97 (2 H, s), 0.95 (2 H, s), 0.93 (3 H, s), 0.90 (3 H, s), 0.89 (1 H, s), 0.88 (2 H, s), 0.86 (1 H, s), 0.76 (2 H, s), 0.75 (3 H, s) as well as the signals for the secondary substituted product only at δ_H (500 MHz; $CDCl_3$): 4.87 (0.4 H, dd, *J* 4.7 / 12.3 Hz), 3.37 (0.4 H, d, *J* 12.3 Hz), 2.89 (0.4 H), d, J 12.6 Hz) and for the primary substituted product at δ_H (500 MHz; $CDCl_3$): 4.17 (0.6 H, d, J 11.5 Hz), 3.83 (0.6 Hz, d, J 11.5 Hz), 3.42 (0.6 H, t, J 8.5 Hz). ν_{max} (mull) / cm^{-1}: 2924 s, 2853 s, 2360 s, 1731 s, 1674 s, 1466 s, 1386 s; MALDI MS m/z $[M+Na]^+$ 715.12 (calculated for $[C_{46}H_{79}O_5Na]^+$, 734.58); $[\alpha]_D^{22}$ = 14.55 (0.0109 g in 1 ml $CHCl_3$); m.p. 70-72 °C. The column was washed with methanol and an additional fraction was collected which appeared to contain unreacted starting material and palmitoyl chloride (0.20 g).

(iii.) Palmitoyl chloride (2 equivalents)

Defatted aglycone **(2)** (1.00 g, 2.12 mmol) was dissolved in anhydrous THF (25 ml) and anhydrous pyridine (0.86 ml, 10.60 mmol) and then treated with palmitoyl chloride 1.41 ml, 1.28 g, 4.66 mmol). This mixture was stirred at room temperature under a nitrogen atmosphere for 70 hours, after which time a white precipitate was observed to have formed. The mixture was transferred to a separating funnel with water (30 ml) and ethyl acetate (50 ml). The aqueous phase was acidified to pH 1 by addition of 10 % sulphuric acid and the organic phase was collected and the aqueous phase was extracted again with further ethyl acetate (2 x 30 ml). The combined organic phases were washed with sodium bicarbonate solution (3 x 15 ml), dried with magnesium sulphate and the solvent evaporated to afford an off-white solid residue (2.12 g). This residue was purified by column chromatography with petrol / ethyl acetate (5:1). The first major fraction, as a white solid (0.63 g, 1.04 mmol, 49 %) of the bis-substituted product **(83)**, appeared as a single spot by TLC analysis. It was identical by ^1H NMR, ^{13}C, IR and MALDI MS and showed m.p. 27-30 °C. The next fraction appeared to be a mixture containing further bis-substituted product (0.15 g, 0.25 mmol, 12 %), a 1:1 mixture of the mono-substituted products (0.07 g, 0.10 mmol, 5 %) as well as palmitic acid (0.44 g). The final fraction was more 1:1 mixture of the mono-substituted products (0.05 g, 0.07 mmol, 3 %) together with palmitic acid (0.22 g).

6.52. Protection of β hydroxyl stearic acid as TBDMS ether

Imidazole (1.70 g, 24.96 mmol) was added to a stirred solution of 12-hydroxystearic acid (3.00 g, 9.98 mmol) in dry DMF (50 ml) at room temperature. This mixture was cooled to 0 °C and t-butyldimethylsilyl chloride (1.96 g, 43.60 mmol) was added, after which time the reaction mixture was heated to 45 °C with stirring for 20 hours after which time TLC showed the reaction was complete. The DMF was removed by flash distillation and the residue treated with water (60 ml) and the product extracted with dichloromethane (3 x 50 ml). The combined organic layers were washed with water (60 ml), dried, filtered and evaporated to afford a colourless solid (1.67 g). This was dissolved in THF (20 ml), water (2 ml) and methanol (2 ml). To this, was added potassium carbonate (0.40 g) and the reaction mixture stirred at 45 °C overnight. The heating was ceased and the mixture treated with water (20 ml) and transferred to a separating funnel with dichloromethane (40 ml) and saturated brine solution (20 ml). The organic phase was collected and the aqueous phase re-extracted with further dichloromethane (2 x 30 ml). The combined organic phases were dried, filtered and evaporated to a yellow solid residue. This crude product was purified by column chromatography on a silica column with petrol / ethyl acetate (5:2) as solvent to afford colourless semisolid of the TBDMS protected product **(97)** as the major fraction (1.15 g, 2.45 mmol, 25 % yield) which gave a single spot by TLC and showed δ_H (500 MHz; $CDCl_3$): 4.92 – 4.83 (1 H, m), 3.61 (2 H, p, *J* 5.7 Hz), 2.34 (4 H, t, *J* 7.5 Hz), 2.28 (2 H, t, *J* 7.4 Hz), 2.05 (2 H, s), 1.63 (7 H, dt, *J* = 14.9, 7.5 Hz), 1.51 (4 H, d, *J* 4.4 Hz), 1.40 (5 H, m), 1.26 (28 H, s), 0.89 (25 H, s), 0.04 (10 H, s); ν_{max} ($CHCl_3$) / cm^{-1}: 3020.4, 2930.7, 2857.3, 2400.8, 1710.1, 1521.2, 1470.8, 1423.6, 1215.6;

6.53. Preparation of 12-hydroxystearic acid TBDMS ester of hederagenin

TBDMS protected 12-hydroxystearic acid **(97)**(0.94 g, 2.01 mmol) was dissolved in anhydrous toluene (6 ml) with continuous stirring under a nitrogen atmosphere. To this solution, oxalyl chloride (0.41 ml, 2.12 mmol) was added together with a catalytic amount of DMF (0.18 ml, 0.11 mmol) and the mixture was stirred for 3 hours. After this time the solvent was evaporated under high vacuum to afford a crude solid residue. This was dissolved in dry ether (4 ml) and the solvent evaporated immediately under high vacuum until the yellow colour had diminished. This process was repeated with further dry ether (2 x 4 ml) until a clean white solid was obtained without any solvent present. The resultant solid of acid chloride was dissolved in dry THF (5 ml). In a separate dry

flask a solution of defatted aglycone **(2)** (0.50 g, 1.06 mmol) was prepared in THF (15 ml), together with pyridine (0.50 ml, 6.40 mmol) and DMAP (0.01 g, 0.11 mmol) added as a catalyst. This solution was stirred under nitrogen for 10 minutes and cooled to 0 °C in an ice bath. Once at this temperature, the prepared acid chloride solution was taken up by syringe and added to the flask drop-wise, maintaining a temperature below 5 °C throughout the addition. Once the addition was complete the flask was removed from the cooling bath and allowed to reach room temperature, at which point a white precipitate was observed to form. The reaction mixture was then stirred at room temperature under nitrogen for 62 hours to ensure reaction was complete, at which point water (50 ml) was added and transferred to a separating funnel with ethyl acetate (50 ml). The organic phase was collected and the aqueous phase re-extracted with further ethyl acetate (3 x 30 ml). The combined organic phases were dried with magnesium sulphate and the solvent evaporated to afford an orange oil (1.93 g). This was purified by column chromatography on a short silica column using petrol / ethyl acetate (1:1) as solvent to afford three fractions. The first, recovered as a colourless oil (0.67 g) gave a spot by TLC which corresponded to the unreacted excess of protected 12-hydroxystearic acid. The second fraction was evaporated to recover an orange oil (0.41 g, 0.30 mmol, 15 %) of the bis-substituted product **(98)**, which showed δ_H (500 MHz; $CDCl_3$): 8.15 – 7.96 (1 H, m), 5.30 (5 H, s), 5.00 – 4.91 (1 H, m), 4.89 – 4.83 (1 H, m), 4.00 (1 H, d, *J* 11.7 Hz), 3.82 (1 H, t, *J* 7.5 Hz), 3.65 – 3.55 (1 H, m), 2.99 (1 H, s), 2.97 (1 H, s), 2.89 (1 H, s), 2.83 (1 H, dd, *J* 13.3 / 4.6 Hz), 2.31 (5 H, dt, *J* 32.1 / 7.5 Hz), 2.12 – 1.84 (4 H, m), 1.85 – 1.56 (16 H, m), 1.50 (3 H, m), 1.40 (9 H, m), 1.26 (65 H, m), 1.13 (5 H, m), 1.11 – 1.05 (2 H, m), 0.99 (4 H, m), 0.93 (3 H, s), 0.92 (3 H, s), 0.90 (14 H, s), 0.87 (3 H, s), 0.80 (1 H, s), 0.76 (2 H, s), 0.04 (7 H, s); δ_C (125 MHz; $CDCl_3$): 183.5, 178.9, 160.8, 160.7, 145.5, 143.7, 127.0, 123.5, 122.4, 122.3, 74.3, 74.1, 72.4, 64.4, 57.5, 53.4, 37.2, 33.1, 33.0, 32.7, 32.4, 32.2, 31.9, 31.8, 31.2, 30.7, 30.6, 29.9, 29.7, 29.7, 29.6, 29.6, 29.5, 29.5, 29.4, 29.4, 29.4, 29.3, 29.3, 29.2, 29.1, 27.6, 27.4, 26.0, 25.9, 25.3, 25.3, 25.2, 24.7, 23.6, 23.4, 23.4, 23.0, 22.9, 22.7, 22.6, 22.6, 18.2, 17.9, 16.9, 15.8, 15.8, 14.1, 14.1, 13.1, 13.1. The column was washed with methanol but no further fractions could be recovered.

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