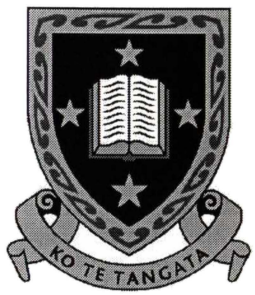
Chemical Aspects of Saponin Bioactivity and Ovine Photosensitization Diseases



THE UNIVERSITY OF
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Abstract

A general review of saponin chemistry, bioactivities and of chemical aspects of saponin-associated hepatogenous photosensitization of ruminants (sheep, goats, and cattle) is presented.

A field trial was undertaken to explore the *genin dose/response hypothesis* that saponins produced by the plant *Narthecium ossifragum* may be the direct cause of the toxicity leading to the photosensitization disease alveld seen in Norwegian lambs grazing *N. ossifragum* containing pastures. The genin content of *N. ossifragum* leaves from 2 control and 2 outbreak pasture areas, in a region of Norway known for periodic livestock outbreaks of alveld, ranged from 4553 to 10879 mg/kg DM. No significant statistical differences in the mean sapogenin content from the 2 control and 2 outbreak pasture areas were found. Total faecal sapogenin levels determined for lambs grazing the 2 control and 2 outbreak areas ranged from 973 to 36314 mg/kg DM. No obvious relationships between faecal sapogenin levels of lambs exhibiting external alveld symptoms and lambs not exhibiting symptoms were identified. The data does not support the hypothesis that saponins are the sole causative agent for the development of alveld disease symptoms in lambs grazing pastures containing *N. ossifragum*.

Genin levels in Scottish collections of *N. ossifragum* plant material, gathered from pasture on which sheep were photosensitized, were determined. Elevated levels of sarsasapogenin and smilagenin containing saponins were found in flower heads (*ca.* 18000 mg/kg DM). Significant levels of free genins were found in plant roots (832-1184 mg/kg DM). The percentage contribution (*ca.* 45-65%) of smilagenin (a 25*R*-genin) in the Scottish samples was typically 2-4 times greater than is the case for Norwegian collections of *N. ossifragum* (typically *ca.* 10-15%).

A field trial involving the daily intraruminal administration of a 1:4 mixture of [2,2,4,4-$^{2}H_4$]sarsasapogenin and [2,2,4,4-$^{2}H_4$]episarsasapogenin to 4 ewes, their twin lambs, and to 3 weaned lambs over a 21 day period was undertaken in Norway. The results showed that the isotope-dilution methodology can be applied under field conditions to estimate the uptake of sapogenins from plant material under conditions characteristically associated with the development of alveld.

Mother ewes, on average, consumed 2-3 times the daily weight of *N. ossifragum* (*ca.* 700-750 g DM/day) consumed by the lambs (*ca.* 200-250 g DM/day). Percentage deuterium ratios, determined using SIM GC-MS protocols, while lower that anticipated, were consistent across the animals involved in the trial (between *ca.* 2-3%). Average *N. ossifragum* intakes, calculated relative to live-weights, were determined for all sheep involved in the trial. No correlation between the quantity of *N. ossifragum* consumed and live-weight (g DM day/kg) and the development of alveld was identified.

The synthesis of sarsasapogenin β-D-galactoside, episarsasapogenin β-D-glucoside, episarsasapogenin β-D-galactoside, betulin 3-(β-D-glucoside) and betulin 3,28-(β-D-diglucoside), was performed via Koenig-Knorr coupling of sarsasapogenin, episarsasapogenin, or betulin with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, or the corresponding galactoside, followed by hydrolysis of the resulting tetra-*O*-acetyl-β-D-glycosides. Structures of intermediates, and product glycosides were established using a combination of ES-MS and one- and two-dimensional NMR spectral data.

The synthesized sarsasapogenin, episarsasapogenin and betulin saponins, 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside), *Yucca schidigera* 70° Brix extract, a methanol conjugate extract of *N. ossifragum* leaves and the active triketone fraction (triketone oil) from East Cape Manuka oil were subjected to a bioactivity evaluation against a range of bacterial and fungal organisms using the agar gel well diffusion technique. A possible structure-activity relationship amongst the synthesized saponins is discussed.

The activity of triketone oil against two fish-pathogenic strains of *Saprolegnia parasitica* was evaluated. The evaluation, performed *in vitro*, required the use of small percentages of water-miscible organic solvents and phosphate buffer solutions to obtain homogenous aqueous solutions. Inhibition of *S. parasitica* growth was seen at triketone oil concentrations of: (i) 150 and 200 ppm using aqueous solutions containing 0.5% and 1.0% pyrrolidinone, each containing 0.5% Tween 80 and (ii) at 150 and 200 ppm, at both pH 7.0 and 7.5 of a 0.25 $molL^{-1}$ phosphate buffer solution. An *in vivo* toxicity trial found that East Cape triketone oil at 200 ppm was acutely toxic to Atlantic salmon fry, with a 50% mortality rate after a 2 h single dose static bath exposure.

Acknowledgements

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The greatest extension of thanks goes to my family: mum and dad, brothers and sister, nieces and nephew, grandma, grandpa, aunties, uncles, cousins.... Their love, support and inspiration defy words. You all believed in me and that has provided more than enough inspiration. Love and thanks to you all. God Bless.

Finally to anyone I have failed to mention, please don't take it personally. Thanks once again.

There's only one sure thing in life and that's doubt...

....I think....

- Goodbye Pork Pie

Table of Contents

List of Figures

Chapter One

Chapter Two

Chapter Three

Chapter Four

Chapter Five

Chapter Seven

List of Tables

Chapter Two

Chapter Three

Chapter Four

Chapter Five

Chapter Six

Chapter Seven

Abbreviations

Σ	sum of
%	percent
α	lower face
β	upper face
δ	chemical shift
^1J	one bond NMR coupling constant
^2J	two bond NMR coupling constant
^3J	three bond NMR coupling constant
-ve	negative
+ve	positive
>	greater than
<	less than
1D	one-dimensional
2D	two-dimensional
3D	three-dimensional
Ac	acetate functional group
AGWD	agar gel well diffusion
Ara	arabinose
a.s.l	above sea level
ax	axial orientation
Betulin-diglu	betulin 3,28-(β-D-diglucoside)
Betulin-glu	betulin 3-(β-D-glucoside)
br	broad
°C	degrees Celsius
ca.	approximately
CC	column chromatography
$CDCl_3$	deutero-chloroform
C_5D_5N	deutero-pyridine
conc	concentration
conj	conjugated
COSY	correlated spectroscopy
CV	coefficient of variation
d	doublet NMR signal
D	dextrorotary
DD	disk diffusion
DEPT	distortionless enhancement by polarization transfer

DM	dry matter
DMSO-D_6	deutero-dimethylsulphoxide
etc	etcetera
e.g.	for example
EI	electron impact ionisation
Episar	episarsasapogenin
Epismil	epismilagenin
eq	equatorial orientation
ES-MS	electrospray-mass spectrometry
et al.	and others
expt	experiment
Fuc	fucose
Furanone-glu	4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside)
Gal	galactose
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
Glu	glucose
HMBC	gradient selected heteronuclear multiple bond correlation
HPLC	high performance liquid chromatography
HR-MS	high resolution mass spectrometry
HSQC	gradient selected heteronuclear single quantum coherence
Hz	hertz
i.e.	that is
J	coupling constant
L	laevorotatory
lit	literature
M^-	molecular ion (negative ion detection)
M^+	molecular ion (positive ion detection)
m	multiplet NMR signal
m.p.	melting point
m/z	mass / charge ratio
Me	methyl
MEA	malt extract agar
MHz	mega hertz
MS	mass spectrometer
MY	mycobiotic agar
NMR	nuclear magnetic resonance

NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser effect spectroscopy
pers commun	personal communication
ppm	parts per million
q	quartet NMR signal
R	absolute stereochemistry at an assymmetric centre defined by the Cahn/Prelog rules
RC	radial chromatography
R_F	response factor
Rha	rhamnose
ROESY	rotating frame Overhauser effect spectroscopy
S	absolute stereochemistry at an assymmetric centre defined by the Cahn/Prelog rules
s	singlet NMR signal
SAB	Sabourands agar
Sar	sarsasapogenin
Sar-CO	sarsasapogenone
SELTOCSY	selected total correlation spectroscopy
SEM	scanning electron microscope
SIM	selected ion mode
Smil	smilagenin
Smil-CO	smilagenone
spp	species
STD	standard deviation
TIC	total ion chromatogram
TLC	thin layer chromatography
TMS	tetramethylsilane
TOCSY	total correlation spectroscopy
t	triplet NMR signal
UV	ultraviolet
V	volts
w.r.t	with respect to
wt	weight
Xyl	xylose

Chapter One

Introduction

1.1 General Introduction

Saponins are a naturally occurring class of high molecular weight glycosides. They are widely distributed throughout the plant kingdom (most commonly in higher plants), and in a few marine organisms. They consist of a sugar moiety (glycone) linked to triterpene, steroid or steroid alkaloid aglycones. A classical property of saponin compounds is their characteristic foaming in aqueous solutions. Indeed, the name saponin comes from the Latin word *sapo* (soap), hence saponin = soap forming compound. Historically, the definition of saponins is based on their surface activity. Many possess detergent properties, show haemolytic activity, piscicidal activity (fish poison), anti-inflammatory activity, impart a bitter taste and have antibacterial properties. However due to the vast range of structures and the numerous exceptions which exist, saponins are now more conveniently defined on the basis of molecular structure, either as triterpene or steroid glycosides. Saponins are notorious for their disparate activities and applications (Waller and Yamasaki, 1996; Renault *et al.*, 2003).

Saponins are constituents of many plant drugs and folk medicines (Hostettmann and Marston, 1995). For example, *Maesa lanceolata* is a shrub or small tree growing in many African countries including Rwanda, where traditional healers include it in medical preparations for a wide range of diseases (Rwangabo, 1993). In a methanol extract of *M. lanceolata,* 6 oleanane-type triterpenoid saponins have been identified (Sindambiwe *et al.*, 1998). There has been great interest shown in the investigation of the pharmacological and biological properties of saponins.

The field of saponin research has undergone remarkable advances since Kofler's 1927 book, *Die Saponine*. Although at the time not a single saponin had been characterized, his

description of the properties and pharmacological activities of saponins was very detailed (Hostettmann and Marston, 1995). By 1987, the structure of over 360 sapogenins and 750 triterpene glycosides had been elucidated (Bader and Hiller, 1987). The rapid progress in this area is the result of the astounding advancement of isolation and structure elucidation techniques. Further advancement over the last 2 decades has benefited from recent research interest in the search for naturally occurring bioactive compounds.

This search is due in part to the increased number of immuno-compromised patients over the last 20 years which has seen a steady rise in the frequency and types of opportunistic fungal infections (De Lucca *et al.*, 2002; Renault *et al.*, 2003; Favel *et al.*, 1994). This situation, in addition to the emergence of fungal pathogens resistant to available antibiotics and the ongoing problems of toxicity and/or adverse drug interactions, has given impetus to the search for safe, novel and effective antifungal compounds. In the field of natural products with antimycotic activity, higher plants still remain largely unexplored compared to microorganisms and marine invertebrates (Favel *et al.*, 1994; Hamburger and Hostettmann, 1991). Saponins fall firmly into the higher plants category, besides their other biological properties, they have been shown to be active against human and plant pathogenic fungi (Renault *et al.*, 2003; Takechi *et al.*, 1991).

1.2 Definitions

Saponins can be divided into 3 major groups or classes depending on the type of genin backbone present: (i) triterpene glycosides, (ii) steroid glycosides and (iii) steroid alkaloid glycosides. Depictions of these 3 genin classes can be seen in Figure 1.1.

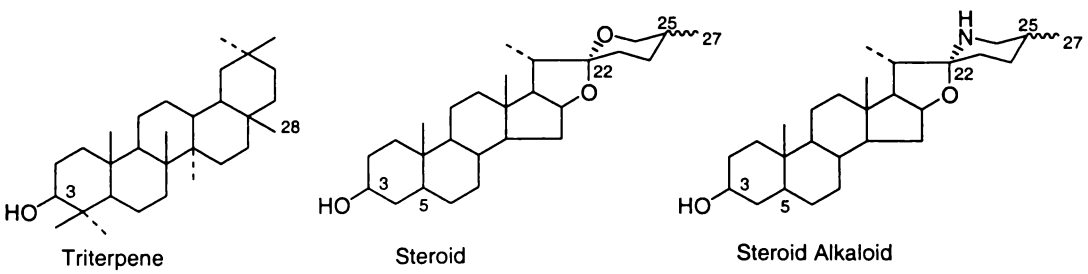


Figure 1.1. Skeletal genin types of the 3 main classes of saponins.

The genins are typically hydroxylated at C-3 and certain methyl groups may be oxidised to hydroxymethyl, aldehyde or carboxyl functionalities. When an acid group is esterified to the genin, the corresponding glycoside is often termed an ester saponin. All saponins have in common the attachment of sugar residues at 1, 2 or more points of the genin backbone. *Monodesmosidic* saponins have a single sugar chain attached at one point of the genin, usually through a glycosidic linkage at C-3. *Bidesmosidic* saponins have 2 sugar chains attached, normally at C-3 and 1 attached through an ester linkage at C-28 (triterpene saponins) or at another glycosidic linkage at C-26 (steroid furostanol saponins). Attachments of 3 sugar chains, termed *tridesmosidic* saponins, exist but are rarely found (Hostettmann and Marston, 1995). Bidesmosidic saponins can easily be converted into monodesmosidic analogues by partial hydrolysis, for example, of the C-28 sugar in triterpene saponins.

The carbohydrate or sugar portion of a saponin may consist of 1 or more glycosyl units. There exist 3 structural possibilities for each attached carbohydrate unit: (i) a single monosaccharide residue, (ii) sugars linked together in a linear chain and (iii) sugar residues linked in a branched chain. Often saponins from a particular plant differ only in the number of glycosyl residues in the saponin, and in their point(s) of attachment. Glycosyl units commonly encountered in saponins include glucose, galactose, rhamnose, xylose, arabinose and fucose. Glucose, arabinose, glucuronic acid and xylose are the monosaccharides most frequently directly attached to the genin (Hostettmann and Marston, 1995; Wilkins *et al.*, 1996; Deng, 1999). Configurations of the interglycosidic linkages are denoted by α- and β- and the glycosyl residues can be in either pyranosyl or furanosyl forms. Arabinose and rhamnose in the sugar chains are generally in the L-form and linked α-glycosidically while other monosaccharides are generally in the D-form and are β-glycosidically linked.

1.2.1 Genin or Aglycone Classes

1.2.1.1 Triterpenes

Pentacyclic triterpenes can be divided into 3 main classes, depending on whether they have a β-amyrin, α-amyrin or lupeol skeleton (Figure 1.2). The most frequently occurring triterpene saponins are those belonging to the β-amyrin type (>50%). Important structural characteristics of the triterpene classes are: (i) unsaturation at C-12(13),

(ii) functionalization of 1 or more of the C-28, C-29 or C-30 methyl groups and (iii) multiple polyhydroxylation possibilities at C-2, C-7, C-11, C-15, C-16, C-19, etc. Through esterification or lactonization, formation of an additional ring is possible. Other classes of triterpenes can be obtained via minor modifications of these skeletons.

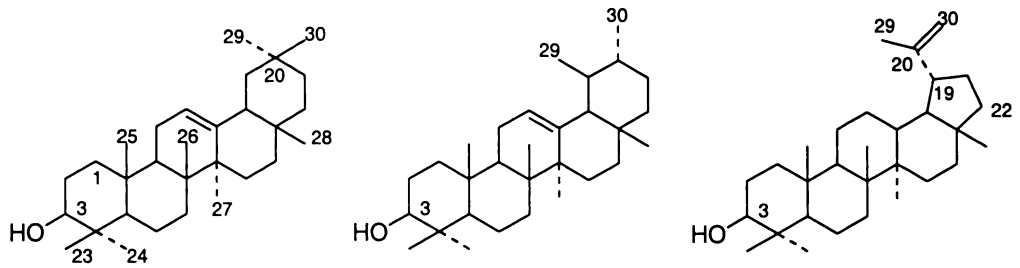


Figure 1.2. Three commonly encountered triterpene genin classes.

Another class of triterpenes are the dammaranes which are tetracyclic triterpenes. Pentacyclic and tetracyclic triterpenes are visualized in Figure 1.3. X-ray crystallographic studies show a relatively planar conformation of the triterpene ring system (Roques *et al.*, 1978). As can be seen in the pentacyclic (β-amyrin type) example of Figure 1.3, rings A/B, B/C and C/D are generally *trans* linked, while rings D/E are *cis* linked.

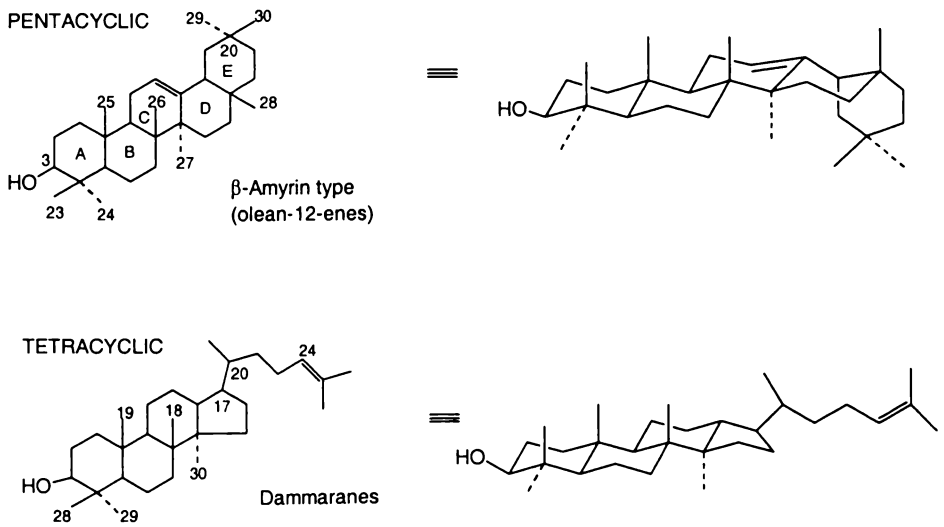


Figure 1.3. 3D configurations of 2 representative groups of pentacyclic and tetracyclic triterpenes.

1.2.1.2 Steroids

Steroidal saponins are typically 27-carbon spirostanes (Figure 1.1) with the same configuration for ring skeletal carbons, other than at C-5 (5α- or 5β-) and/or at C-25 (*R* or *S* forms). Steroidal genins are usually hydroxylated at C-3. There is sometimes a C-5(6) double bond present, for example in diosgenin and yamogenin. In all cases the C-18 and C-19 angular methyl groups are β-orientated and the C-21 methyl group has the α-configuration.

Tigogenin and sarsasapogenin are typical examples of 25*R*-5α- and 25*S*-5β-sapogenins respectively (see Figure 1.4). The absolute configuration of the equatorially orientated C-25 methyl group in tigogenin, is *R*, whilst the C-25 axially orientated C-25 methyl group in sarsasapogenin, is *S*. A 5α-proton results in a *trans* configuration across the ring A/B junction and an equatorial orientation for the 3β-OH group, for example in tigogenin. A 5β-proton results in a *cis* configuration across the A/B junction and an axial orientation for the 3β-OH group (with respect to ring A), as is the case for sarsasapogenin.

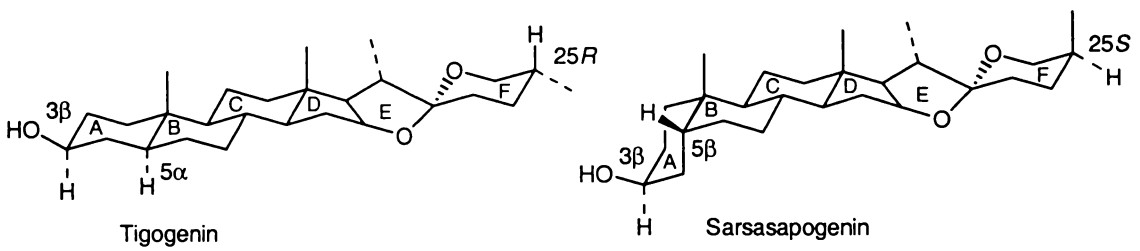


Figure 1.4. 3D structures of the steroidal genins tigogenin and sarsasapogenin.

There are over 100 steroidal sapogenins known (Hostettmann and Marston, 1995). Steroidal sapogenins are typically hexacyclic (rings A-F, spirostanol type), or pentacyclic (rings A-E, furostanol type) (Figure 1.5). Furostanol sapogenins are open ring F analogues of spirostanol sapogenins. They often have a C-26 hydroxyl group and the glycosides of the furostanol type have the sugar moiety attached at C-26. Upon hydrolysis, furostanol 26-glycosides afford the corresponding closed ring F spirostanol isomers (Munday *et al.*, 1993; Wilkins *et al.*, 1996; Meagher *et al.*, 1996).

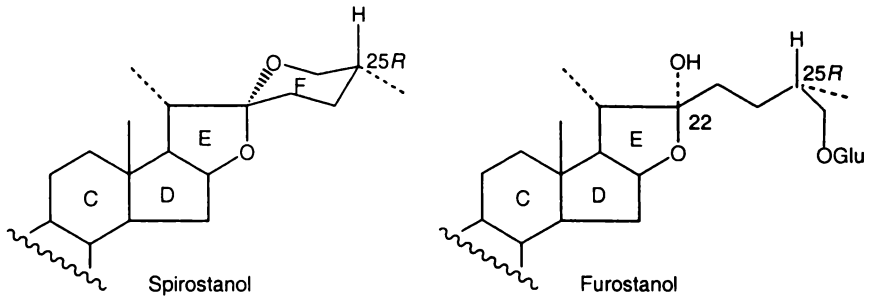


Figure 1.5. Partial structures of spirostanol and furostanol saponins.

1.2.1.3 Steroid Alkaloids

There are 2 classes of steroid alkaloid sapogenin – the spirosolanes and the solanidanes, wherein the nitrogen atom can be either secondary (spirosolane type) or tertiary (solanidane type) (Figure 1.6). The spirosolanes are the aza-analogues of the spirostanes and can exist in either the 22*R*,25*R* (e.g. solasodine) or the 22*S*,25*S* (e.g. tomatidine) configurations. The nitrogen atom in the solanidanes belongs to two ring systems simultaneously. The solanidanes have a 22*R*,25*S* configuration, with the C-25 methyl group in an equatorial position (Hostettmann and Marston, 1995).

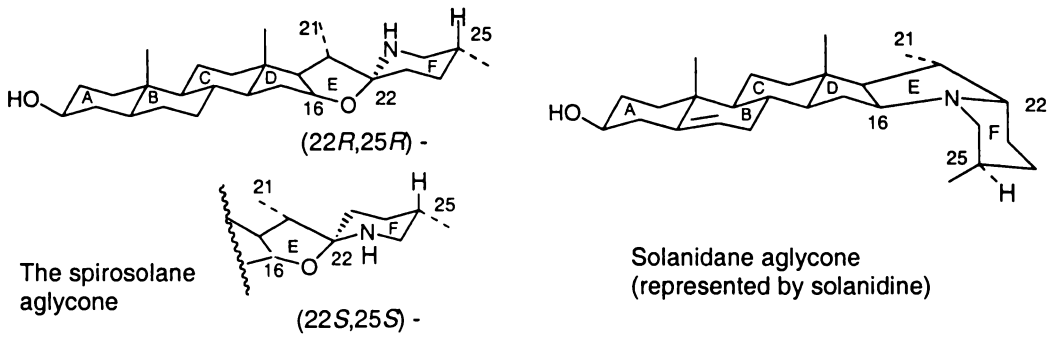


Figure 1.6. Structures of the 2 classes of steroid alkaloid sapogenin.

1.3 General Biosynthesis

The biosynthesis of steroids and triterpenes has been reviewed by various authors including Heftmann, 1968; Takeda, 1972 and Hostettmann and Marston, 1995. A brief outline of the biosynthesis of triterpenes and steroids is shown in Figure 1.7. All are constructed from 6 isoprene units and have a common biosynthetic origin. They are derived from squalene, presumably via ring opening of squalene-2,3-epoxide, followed by a concerted cyclization.

Oxidative cleavage (loss) of 3 methyl groups from a C_{30} intermediate affords steroidal genins which have a 27-carbon backbone, while triterpenes retain all of the 30 carbon atoms. Formation of cholesterol via the cyclization of squalene-2,3-epoxide is shown in Figure 1.7.

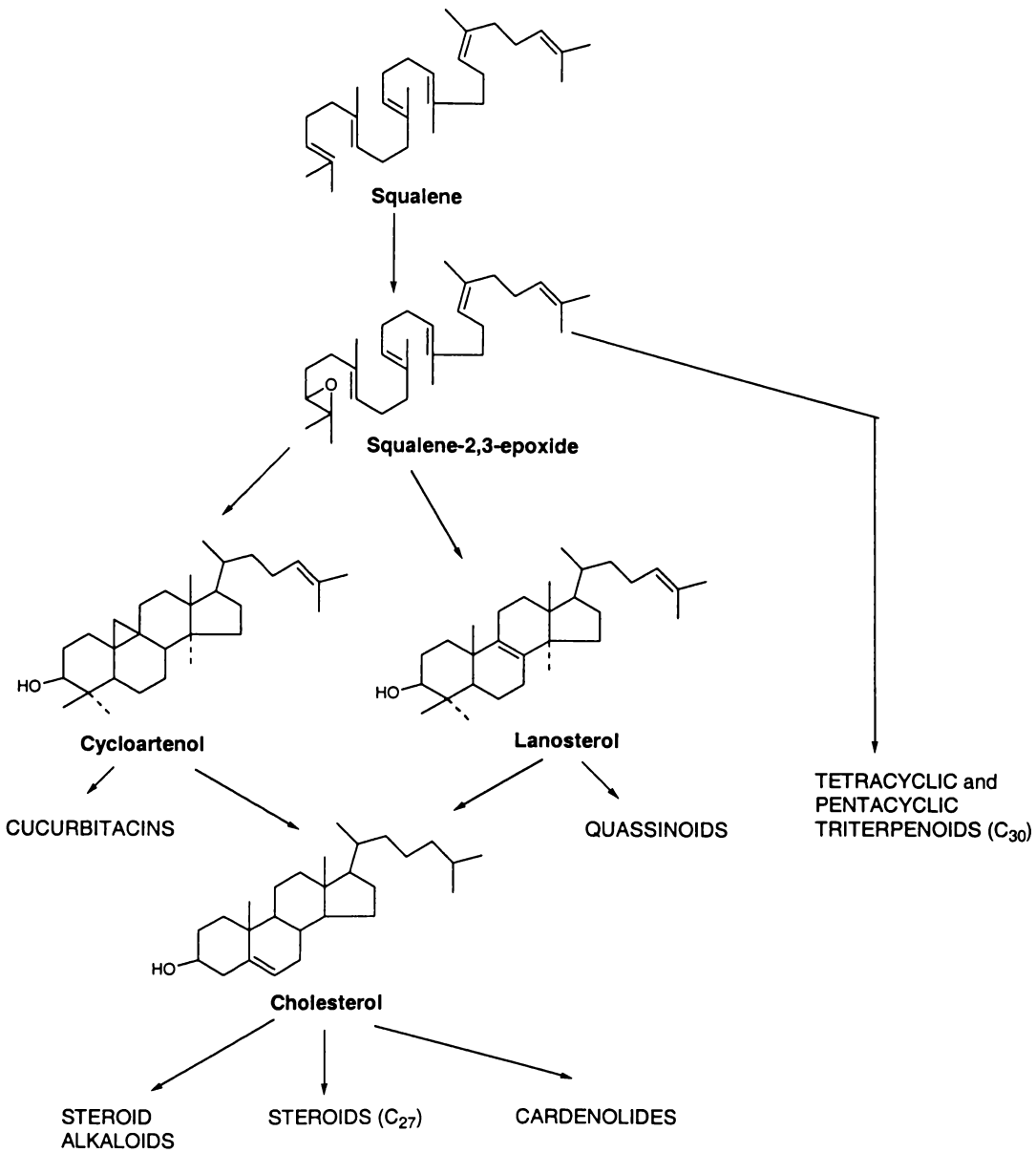
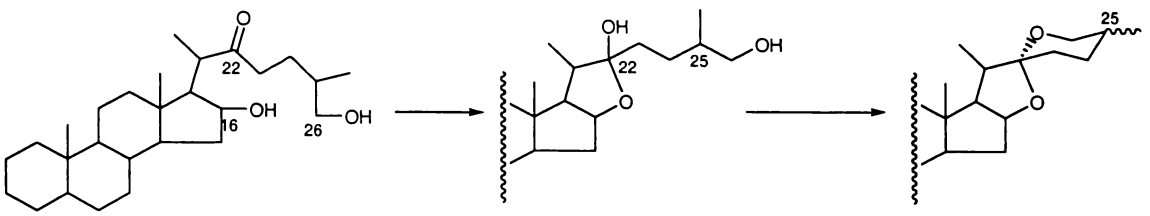


Figure 1.7. Biosynthesis of triterpenes and steroids.

Tschesche and Hulpke (1966) demonstrated that cholesterol is a precursor of spirostanols by treating *Digitalis lanata* leaves with ^{14}C-labelled cholesterol-β-D-glucoside and showing that radioactive tigogenin and gitogenin were produced. One possibility is that furostanol and spirostanol steroid genins are biosynthetically derived from a 16,26-dihydroxycholestan-22-one (Figure 1.8).



16,26-Dihydroxycholestan-22-one (hypothetical intermediate) Furostanol type Spirostanol type

Figure 1.8. Biosynthesis of spirostanols and furostanols from 16,26-dihydroxycholestan-22-one.

Some plants are able to incorporate a nitrogen atom (from arginine) into the side chain of the steroidal moiety, giving rise to the steroidal alkaloid nucleus (Kaneko *et al.*, 1976).

1.4 Occurrence and Distribution

Saponins are extremely widely distributed throughout the plant kingdom. By 1927, 427 saponin-containing plants were listed (Kofler, 1927). Over 90 plant families are now known to contain saponins (Hostettmann and Marston, 1995). Gubanov *et al.* (1970) conducted a systematic investigation of 1730 Central Asian plant species and found 76% of the families contained saponins.

Saponins occur in the *Liliaceae* family in *Yucca*, *Trillium*, *Chlorogalum*, *Smilax*, *Agave*, *Nolina*, *Agapanthus*, *Ruscus* and *Narthecium*; in the *Amaryllidaceae* family in *Manfredda*; in the *Dioscoreaceae* family in *Dioscorea*; in the *Scrophulariaceae* family in *Digitalis*; in the *Solanaceae* family in *Solanum*, *Lycopersicon* and *Cestrum* (Miller, 1973; Skerman and Riveros, 1990; Hostettmann and Marston, 1995). In the *Poaceae* family in *Brachiaria* and

Panicum (Skerman and Riveros, 1990), and in the *Zygophillaceae* family in *Tribulus* (Kellerman and Coetzer, 1984). The list above is indicative only and not intended to be exhaustive.

Saponins are found in a variety of plants, many of which are human foods, including, soybeans, potatoes, garlic, sugar beet, kidney beans, chick peas, spinach, oats, tomatoes, peppers, onions and beans (Renault *et al.*, 2003; Siddhuraju *et al.*, 2001; Birk and Peri, 1980; Oakenfull, 1981; Price *et al.*, 1987). Saponins are present in numerous forage species, for example, *Narthecium ossifragum*, *Tribulus terristris* and *Brachiaria decumbens* (Loader *et al.*, 2003; Wilkins *et al.*, 1994; Miles *et al.*, 1994b; Wilkins *et al.*, 1996) and in a few marine organisms, for example, sea cucumbers and starfish, and have been reviewed (Habermehl and Krebs, 1990; D'Auria *et al.*, 1993; Minale *et al.*, 1993). Finally, saponins are found in numerous plant species utilized for traditional and herbal remedies (Hostettmann and Marston, 1995; Siddhuraju *et al.*, 2001; Waller and Yamasaki, 1996; Sindambiwe *et al.*, 1998; Abel-Kader *et al.*, 2001).

The saponin content of plants depends on a number of factors, including, age, physiological state, climatic conditions and geographical location. Saponin distribution throughout the plant itself also varies considerably. For example, oleanolic acid saponins with a C-3 glucuronyl group are found in the flowers, while saponins with a glucosyl group at C-3 are found in the roots of the garden marigold *Carlendula officinalis* (Vidal-Ollivier *et al.*, 1989).

1.4.1 Triterpenes

By far the most abundant class of saponins are the triterpene glycosides. The majority have either oleanane (β-amyrin) or dammarane skeletons, with ursanes, hopanes, lanostanes or lupanes assuming a secondary abundance with respect to distribution. Usually 1 sugar residue is attached at C-3 and frequently a second is esterified to the carboxyl group at C-17 of the genin skeleton. Some dammarane and lanostane glycosides have a second or even a third glycosidic residue. By far the largest single group of known triterpene glycosides is the bidesmosides, probably due to their abundance in individual plants and widespread distribution.

The first tridesmosidic triterpene saponin, a 9,19-cyclolanostane (cycloartane) derivative substituted glycosidically at C-3, C-6 and C-25, was isolated from the roots of Korean *Astragalus membranaceus* (Katagawa *et al.*, 1983). Quinoside A is a rare example of a tridesmosidic olean-12-ene saponin ester. Sugar units are attached at positions C-3 (Glu), C-23 (Glu) and C-28 (Ara^3-Glu) of hederagenin (Meyer *et al.*, 1990).

Sometimes isolation of the genuine genin skeleton from the parent saponin is difficult, due to rearrangement products being formed during extraction and/or hydrolysis procedures. A well known problem when isolating saponins containing the 13β,28-oxido-oleanane structure is the tendency for acid catalyzed ring-opening to afford the corresponding 12-en-28-ols (Figure 1.9).

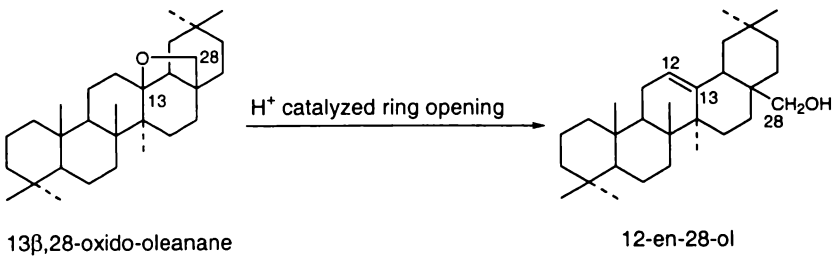


Figure 1.9. Example of possible artefact formation via acid catalyzed ring-opening.

1.4.2 Steroids

Of the 2 major classes of steroidal saponins, the largest group is the spirostanol glycosides, which comprise a spirostanol genin skeleton with a sugar residue generally attached at C-3. The furostanol glycosides have a second sugar residue at position C-26 and hence, are bidesmosidic. Spirostanol glycosides are frequently found in the seeds, roots or plant bulbs, whereas furostanol glycosides are generally located in the assimilatory parts.

Spirostanol and 26-glucosyl furostanol saponins frequently co-occur in plants (Miles *et al.*, 1993; Munday *et al.*, 1993; Wilkins *et al.*, 1996; Deng, 1999). 26-Glucosyl furostan-22-ols can be difficult to isolate. Reasons include the C-26 glucosyl group being readily cleaved by enzymes present in the plant. Enzymatic cleavage (hydrolysis) of the 26-glucosyl group, followed by elimination of a water molecule affords the corresponding spirostanol saponin. Furthermore, the 22-hydroxyl group of furostanols is prone to methyl ether formation when methanol is used as the extraction or chromatography solvent. Boiling extracts with aqueous acetone usually regenerates the 22-OH group. Ethanol is not as prone to ether formation as is the case for methanol.

Mono- to bidesmosidic saponin ratios from plants can depend on the extraction procedure. For example, water extraction of *Phytolacca dodecandra* berries yields larger amounts of the active monodesmosides, whereas methanol extraction yields more of the inactive bidesmosides (Domon and Hostettmann, 1984). This trend may be due to the inactivation of hydrolytic plant enzymes, by methanol, which cleave ester-linked sugar residues during water extraction procedures.

The occurrence of sulphonated steroid glycosides is rare in the plant kingdom. A ruscogenin glycoside (Figure 1.10), from the tubers of *Ophiopogon planiscapus* is one of the few examples known (Watanabe *et al.*, 1983).

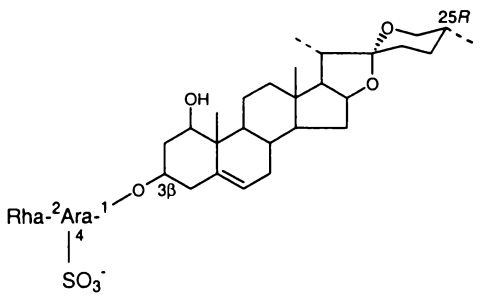


Figure 1.10. A sulphonated ruscogenin glycoside from the tubers of *O. planiscapus*.

1.4.3 Steroid Alkaloids

Steroid alkaloid glycoside numbers are very limited when compared to the triterpene and steroid glycosides. The largest source of their distribution is the Solanaceae family, which includes many agricultural crops important to humans, including, the potato, tomato, capsicum and eggplant. Glycoalkaloids are generally found in all parts of the plant with regions of high metabolic activity, for example, flowers, unripe berries, young leaves and sprouts, having the highest concentration. With respect to the Solanaceae family, in the fruits, there is usually a gradual metabolization to nitrogen-free constituents during ripening, so that when mature they contain almost exclusively non-toxic neutral saponins. Steroid alkaloids are no longer present in ripe tomatoes, being metabolized to products other than neutral saponins. Contrary to this, unripe green tomatoes are rich in α-tomatine (Hostettmann and Marston, 1995).

1.5 Pharmacological and Biological Properties

As mentioned, many saponins share haemolytic, piscicidal, mollusicidal, cholesterol complexation and foaming abilities. However, the exceptions are far too numerous to characterize saponins purely on their physical or biological properties. Generalizations about saponin properties are hazardous. Although some properties are well defined, other effects are difficult to evaluate. In some instances, responses are only observed when experimental animals are delivered high doses. With this in mind, the purely classical 'general' properties of saponins are described below.

1.5.1 General Properties

The general properties of saponins rely mainly on their tensioactive nature. The amphiphilic nature (being both hydrophobic and hydrophilic) of saponins dominates their physical properties in solution. They are strongly surface active, act as emulsifying agents, form stable foams and form micelles in much the same way as detergents.

Saponins from a variety of sources have been shown to have stable foam formation, biological cholesterol reduction, haemolytic, bitterness and plant growth-inhibitory properties, antibiotic, antifungal, antibacterial, antiviral, antioxidant, anti-inflammatory, antihepatotoxic, antiallergic, antifertility, antifeedant, spermicidal, anticarcinogenic, anticoagulant, antitumour, cytotoxic, cardiovascular, antiulcer, sedative, analgesic, antimalarial, antiseptic and antidysenteric activities. They are used in cosmetics, sweeteners, herbs, soaps and shampoos, medicines, food supplements and as foaming agents in beverages. The above list is indicative only and not intended to be exhaustive.

Bidesmosidic saponins either lack or exhibit the properties of the corresponding monodesmosides, and are generally described as weak or non-biologically active compounds (Favel *et al.*, 1994; Mahato and Nandi, 1991). An exception to this is surface activity, which is more pronounced in bidesmosides and increases with length and branching of the sugar moiety (Hostettmann and Marston, 1995). However the generally less active bidesmosidic compounds can easily be converted into their corresponding monodesmosidic saponins by hydrolysis.

Details of selected properties are expanded below.

1.5.1.1 Stable Foams

The most characteristic of saponins properties is their ability to foam in aqueous solution and has been known for centuries. Saponin containing plants that have been employed as soaps for hundreds of years include: *Saponaria officinalis* (soapwort), *Chlorogalum pomeridianum* (soaproot), *Quillaja saponaria* (soapbark), *Sapindus sapononaria* (soapberry) and *Sapindus mukurossi* (soapnut). This phenomenon has been exploited as a test for the presence of saponins (Steiner and Holtzem, 1955). Caution should however be exercised when applying this test as not all saponins impart aqueous foaming properties and other natural products are also capable of creating foams. The mechanism of foam formation is still uncertain. It is not clear, for example, whether the amphiphilic character of saponins is entirely responsible (Hostettmann and Marston, 1995).

1.5.1.2 Cholesterol Complexation

Many saponins have the ability to form complexes (aggregates) with cholesterol, a property discovered by Windaus (1909). As such, saponins are often promoted as cholesterol reducing agents. Saponins are also able to form complexes with amyl alcohol, terpene alcohols, phenols, thiophenes, ergosterol and lecithin (Hostettmann and Marston, 1995). *Yucca schidigera* saponins appeared to bind ammonia when tested in an *in vitro* rumen fermentation system (Makker *et al.*, 1998).

1.5.1.3 Biological Cholesterol Reduction

Many studies have shown a decrease in cholesterol levels in blood and body tissue of test animals when saponins are introduced into their diets. For example, Cheeke (1971) showed that the presence of triterpene-glycosides in hen feed decreased cholesterol in blood and body tissues. Ivanov *et al.* (1987) showed semi-synthetic mono- and bidesmosidic betulin glycosides led to the reduction of cholesterol in cats with experimentally induced hypercholesterolaemia. The monodesmosidic betulin glycosides showed the greatest activity.

It is well documented that cholesterol-saponin complexes in the digestive tract result in the inability of the complex to be absorbed, more so than the saponins having a direct effect on cholesterol metabolism. For example, bile acids, made from cholesterol in the liver and gall bladder, pass into the small intestines mixing with water to aid digestion (Davenport, 1978; Malinow *et al.*, 1977). As this mixture enters the colon, water is absorbed back into the body carrying with it the bile acids and cholesterol. Saponins complex/bind with the bile acids making them unavailable for reabsorption since they are not able to penetrate the intestinal walls. The saponin complexes pass into the colon and are excreted in faecal material. This forces the production of more bile. To produce more bile, the liver must remove endogenous cholesterol from the blood. This theory is supported through a number of studies. For example, Oakenfull and Topping (1983) showed that in the blood of laboratory animals, saponins form complexes with plasma lipids. Topping *et al.* (1978) observed that a 1% saponin concentration in the diet of rats decreased plasma cholesterol levels and increased bile production.

Fenugreek (*Trigonella foenum-graecum*) seed contains several spirostanol and furostanol steroidal saponins, which include the aglycones diosgenin, yamogenin, gitogenin, neogitogenin, tigogenin, neotigogenin, yuccagenin, smilagenin and sarsasapogenin (Taylor *et al.*, 1997; Petit *et al.*, 1995; Mahato *et al.*, 1982; Knight, 1977). Extracts of fenugreek seed possess hypocholesterolemic properties and are responsible for lowering cholesterol levels in dogs. The parent saponins and resulting aglycones (from hydrolysis and metabolism in the digestive tract) are believed to be implicated in the inhibition of cholesterol absorption, decreased cholesterol liver concentrations and increased liver bile conversion into cholesterol (Sauvaire *et al.*, 1991).

1.5.1.4 Haemolytic Activity

Kobert first reported *in vitro* haemolysis of blood by saponins in 1887. Haemolytic activity, the sensitivity of red blood cells to saponins and the ability of saponins to disrupt erythrocyte integrity has been used as a detection device for decades.

The *European Pharmacopoeia* uses as a unit the quantity of 1 mL of ox blood diluted 1:50, which is totally haemolyzed by 1 g of test substance. As a standard, the saponin mixture from the roots of *Gyposhila paniculata* has by definition a haemolytic index (HI) of 30 000.

$$HI = 30\,000 \times a/b$$

where:

a is the quantity of std saponin (g) required for complete haemolysis of blood

b is the quantity of test saponin (g) required for complete haemolysis of blood

Saponins are capable of destroying (lysing) the erythrocyte (red blood cell) membranes, causing haemoglobin leakage. This phenomenon involves a reduction in surface tension between the aqueous and lipid phases of erythrocyte membranes resulting in lipid emulsion and their subsequent departure from the membrane. Na^+ ions and water enter the cell through these holes and K^+ ions depart. This ion flux continues until the membrane integrity is lost, rupture occurs and haemoglobin is shed into the plasma.

There is great variation in the haemolytic activity of saponins. Activity depends greatly on the structure and number of glycoside residues and on the presence of polar substituents on the aglycone (particularly rings D and E). With some exceptions, monodesmosidic steroid and triterpene saponins are strongly haemolytic. Bidesmosidic furostanol and triterpene saponins are generally only weakly active. Ester containing saponins are often strongly haemolytic. Saponins containing polyhydroxylated genins are generally less active. Exceptions exist; for example, bidesmosidic steroidal saponins from *Convallaria* species have both sugar residues attached in the ring A and B regions, unlike most bidesmosidic saponins. They therefore have characteristic monodesmosidic properties and possess haemolytic activity.

With respect to the carbohydrate chains, generally haemolysis increases with branching, and increases with increasing chain length, although this has been challenged (Favel *et al.*, 1994). Furthermore, there is no correlation between the haemolytic index and the pharmacological properties of saponins or their capability to reduce surface activity (Segal *et al.*, 1974).

It has been suggested that the complexation of biological membrane cholesterol is likely to account for the fungistatic, haemolytic, and molluscicidal activities of saponins (Takaeshi *et al.*, 1991; Kintia *et al.*, 1996). A haemolysis kinetics investigation found that steroidal saponins reached their haemolytic activity maximum before the triterpene saponins, most likely due to the higher affinity of steroidal genins for erythrocytes. Furthermore, haemolysis was significantly reduced when a small amount of cholesterol was incubated with the saponins (Takechi *et al.*, 1992). This is consistent with the hypothesis of haemolytic saponins targeting cholesterol on erythrocyte membranes.

1.5.1.5 Bitterness

Many saponins impart bitterness and are renowned for this property. The partially acetylated soya saponins give rise to the bitter and sharp taste of soybeans (Shiraiwa *et al.*, 1991). Furostanol glycosides are responsible for the bitterness of asparagus (Kawano *et al.*, 1975). It is common to discard the bottom cut of asparagus (which has a higher saponin level) during processing. Nevertheless the odd exception exists; for example, glycyrrhizin, the principal triterpene saponin of liquorice is 50 times sweeter than sucrose (Hostettman and Marston, 1995).

1.5.2 Pharmacological and Biological Properties

The biological activity connected with steroid and triterpene saponins is long and extensive. Many properties, for example, piscicidal, fungicidal and mollusicidal effects have been known for many years. Others are continually being uncovered. Biological and pharmacological properties are generally structure dependent.

1.5.2.1 Antimicrobial Activity

Saponin function in plants is a topic of debate, and often, satisfactory explanations as to why some plant species possess very high levels (up to 30%) of saponins do not exist. As there is often an increase in saponin levels of a plant under microbial attack, one theory is that they are protection/defence agents against pathogenic attack (Défago, 1977; Papadopoulou *et al.*, 1999; Osbourn, 2003). Given the milieu of microorganisms in soil, where plants not only live, but thrive, it is not surprising that plants produce a large variety of antimicrobial compounds. Spices have been used for centuries to enhance flavour and to retard microbial growth. CAY-1, a monodesmosidic steroidal saponin from cayenne pepper (*Capsicum frutescens*), has potent fungicidal properties against a number of fungal pathogens, including *Candida albicans* and *Pneumocystis carinii* (De Lucca *et al.*, 2002).

Bidesmosidic saponins are thought to be antimicrobial transport systems (Hostettmann and Marston, 1995), since plant wounding often leads to their transportation from organs not at risk (e.g. leaves) to those parts under attack (e.g. roots, seeds) from microorganisms. Once at the invasion site, released plant enzymes transform these into their biologically active monodesmosidic forms and the saponins can provide defence against the attacking organism(s) (Tschesche and Wulff, 1972). Indeed, bidesmosidic furostanol saponins (void of antibiotic activity) are found mainly in leaves, whereas their monodesmosidic spirostanol counterparts (active forms) are often found in seeds and roots (Voigt and Hiller, 1987). An example can be seen in *Hedera helix*, where ivy leaves contain an enzyme which cleaves the ester-linked sugar residue of the bidesmoside, hederasaponin C (hederagenin-3-Ara^2-Rha, 28-Glu^6-Glu^4-Rha), which as an antimicrobial agent is virtually inactive, to yield the highly antibiotic α-hederin (hederagenin-3-Ara^2-Rha) (Tschesche, 1971; Schlösser, 1973). Saponins have also been found to inhibit mould growth, and their presence in the shoots and bark of trees reinforces the view they help protect the plant from attack (Milgate and Roberts, 1995).

Antifungal Activity

Numerous examples of saponin antifungal activity against a range of fungal strains exist in the literature. Plant pathogens, particularly fungi, are responsible for yield reductions in food and crops throughout the world (Carpinella *et al.*, 2003). Although losses may be controlled by using resistant cultivars, crop rotation or increasing sanitary practices, fungicides are also required to maximize crop yields (Knight *et al.*, 1997). Fungicides greatly contribute to the quality of foods and human health by controlling many of the fungi that produce mycotoxins (Dmello *et al.*, 1999) or by interfering with their biosynthesis (Hasan, 1999). Despite their benefits, the generation of toxic residues and development of resistant pathogens can be attributed, in part, to the use of synthetic fungicides (Knight *et al.*, 1997; Hall, 1984; Wilson *et al.*, 1997). Therefore there is a continuing need for development and discovery of new fungi-toxic chemicals/compounds that preserve the environment and allow for efficient fungi control and improved crop and food quality (Carpinella *et al.*, 2003).

In a study of the saponin fraction (monodesmosidic steroidal saponins) of Mohave Yucca (*Y. schidigera*) potent growth-inhibitory activities against certain food-deteriorating yeasts, film-forming yeasts and dermatophytic yeasts and fungi were found (Miyakoshi *et al.*, 2000). As a result of this study, Mohave extract and its saponin fraction are now on sale in Japan as an anti-deteriorating agent for extending the shelf life of food products containing cooked beans and rice, pickled vegetables, processed fish meat and fermented seasonings. Mohave extract is used commercially in the United States and is recognized as a 'safe food adjuvant' by the U.S Food and Drug Administration (FDA) (Miyakoshi *et al.*, 2000).

Another area of interest is in human pathogenic fungi. Frequent use and abuse of primary medical antifungal agents, such as fluconazole and amphotericin B, has been accompanied by development of resistance in fungi (Moraes *et al.*, 2003; Renault *et al.*, 2003). This situation, combined with the ongoing problems of toxicity and/or adverse drug interactions, has given momentum to the search for safe, novel and effective antifungal compounds. Amphotericin B, for example, known as the 'gold standard', is limited because of its infusion-related reactions and nephrotoxicity (Renault *et al.*, 2003; Warnock, 1998; Alexander and Perfect, 2000).

Some examples of saponins that demonstrate activity against human and plant pathogenic fungi are: α-hederin (Favel *et al.*, 1994); CAY-1, from cayenne pepper (*C. frutescens*) (De Lucca *et al.*, 2002; Renault *et al.*, 2003); a triterpenoidal saponin mixture from *Maesa lanceolata* (Sindambiwe *et al.*, 1998); steroidal glycosides from *Tribulus terrestris* (Bedir *et al.*, 2002); 5 steroidal saponins isolated from *Solanum chrysotrichum* leaves (Zamilpa *et al.*, 2002); phytolaccoside B (3-*O*-β-D-xylopyranosyl-phytolaccagenin), isolated from Argentinean *Phytolacca tetramera* (Escalante *et al.*, 2002); etc.

Antibacterial Activity

The majority of saponins have virtually no antibacterial activity. Some are known to be active against Gram-positive bacteria, but virtually none show effect on Gram-negative bacteria. 21 of 24 saponins and saponin-like compounds, isolated from starfish and brittle stars originating from 3 different seas, were tested *in vitro* against bacteria and cell cultures. Gram-positive *Staphylococcus aureus* was affected by the polyhydroxylated steroidal glycosides, polyhydroxylated sterols and disulphonated sterols. However, all 21 compounds were inactive against Gram-negative *Escherichia coli* (Andersson *et al.*, 1989).

1.5.2.2 Piscicidal Activity

The piscicidal activity of saponins has long been known. Over the centuries plant toxicity towards fish has been exploited as a means of harvesting fish from streams and ponds. For example, in regions of the Pacific, sea cucumbers, known to contain large amounts of triterpene and steroidal saponins (Andersson *et al.*, 1989; Hostettmann and Marston, 1995) have traditionally been mashed and chopped and used to poison fish in tidal pools (Burnell and Apsimon, 1983). Death from saponin poisoning arises from damage to the gill capillaries of fish. Apart from being the respiratory organs of fish, gills also regulate ion balance and osmotic pressure.

Some plants, which have been used as fish poisons include: *Entada phaseoloides* Merrill (Gila bean) (Siddhuraju *et al.*, 2001); *Swartzia madagascariensis* (Leguminosae); *Sapindus saponaria* (Sapindaceae); *Cyclamen* species (Primulaceae) and *Securinega virosa* (Euphorbiaceae) (Hostettmann and Marston, 1995).

Piscicidal saponins, found in marine organisms, are thought to also function as predator repellents. Extracts and pure saponins from starfish have been observed to repel other marine organisms (Halstead *et al.*, 1978), cause abnormalities in the early development of sea urchin larvae (Ruggieri and Nigrelli, 1974) and have an inhibiting effect on spawning (Ikegami *et al.*, 1967). Defence secretions of the sole (*Paradaschirus* species), which contain steroidal glycosides, is effective at repelling sharks as well as being toxic to fish (Hostettmann and Marston, 1995).

1.5.2.3 Molluscicidal Activity

Molluscicidal properties of saponins are a well known and documented phenomenon, although the exact mechanism of action is not entirely certain (Hostettmann and Marston, 1995). Archibald made the first report of molluscicidal activity in 1933, and noted that extracts of the fruits of *Balanites aegyptiaca*, known to be piscicidal, also killed *Bulinus* snails and the cercsariae of schistosomes. In 1982 tri- and tetraglycosides of yamogenin were found to be responsible for this activity (Liu and Nakanishi, 1982). So far only monodesmosidic saponins have been shown to exhibit noticeable molluscicidal activity (Domon and Hostettmann, 1984). 5 triterpenoid saponins isolated from *Dizygotheca elegantissima* were tested against *Biomphalaria alexandrina* snails, the intermediate host of *Schistosoma mansoni* in Egypt. The monodesmosidic saponins showed strong molluscicidal activity, whereas the 4 bidesmosidic saponins were inactive up to 50 ppm (Abdel-Gawad *et al.*, 1997). Bidesmosidic saponins require conversion to their corresponding monodesmosides before activity is observed. An example can be seen in 2 bidesmosidic oleanane saponins from *Guaiacum officinale*. Inactive against the *Biomphalaria glabrata* snail at 10 ppm, the corresponding monodesmosidic saponins, produced via basic hydrolysis, were active at 2.5 and 5 ppm respectively (De Almeida Alves *et al.*, 1996).

Snail toxicity is generating great interest for the control of schistosomiasis, a tropical parasite disease affecting approximately 250 million people worldwide. Snails (of the *Biomphalaria*, *Bulinus* and *Oncomelania* genera) are directly involved in disease transmission as they operate as intermediate hosts for the miracidial state of the life cycle of the parasite (Mott, 1987). Several plants effective at killing these water-borne snails have already reached field trial status in areas of high occurrence where it is too expensive for use of synthetic controls (Hostettmann, 1989).

One of the most promising plants for schistosomiasis control is the African species *Swartzia madagascariensis*. Each tree can possess around 30 kg of pods. The activity of a rhamnosylglucuronide of oleanolic acid, contained in *S. madagascariensis,* approaches those of synthetic molluscicides (Borel and Hostettmann, 1987). Water extracts of *S. madagascariensis* bore significant toxic effects, down to 100 ppm of the ground pods, against *Biomphalaria glabrata* and *Bulinus globosus* snails. Tanzanian field trials showed densities of *B. globosus* dropped to zero 1 week after a single dose of water extracts were added to ponds at concentrations no less than 100 ppm. The saponins were also found to have half-lives in the field of 12-24 h. Rapid biodegradation is important in minimizing secondary effects (Suter *et al.*, 1986; Hostettmann and Marston, 1995).

As with other saponin activities, sugar residue characteristics influence molluscicidal potency. Saponins of oleanolic acid or hederagenin are the most active of the molluscicidal saponins (Marston and Hostettmann, 1985).

1.5.2.4 Insecticidal and Antifeedant Activity

Steroidal alkaloid saponins are particularly well known as insect antifeedants. Compounds with well-established activities include α-tomatine, α-solanine and α-chaconine (Figure 1.11). For example, α-tomatine is effective against the larvae of potato beetles (Schreiber, 1968). α-Solanine and α-chaconine have effect against the potato leafhopper (Jadhav *et al.*, 1981), with α-chaconine known to inhibit insect acetyl-cholinesterase of German cockroaches, *Aedes* mosquitoes, houseflies, and cotton leaf beetles (Wierenga and Hollingworth, 1992). There is evidence that the action of glycosyl-steroidal alkaloids as resistance factors against insects or as antifungal agents may be attributable to their membrane destabilizing properties. A 1:1 mixture of α-solanine and α-chaconine resulted in pronounced synergistic effects in membrane-disruption tests (Roddick *et al.*, 1988).

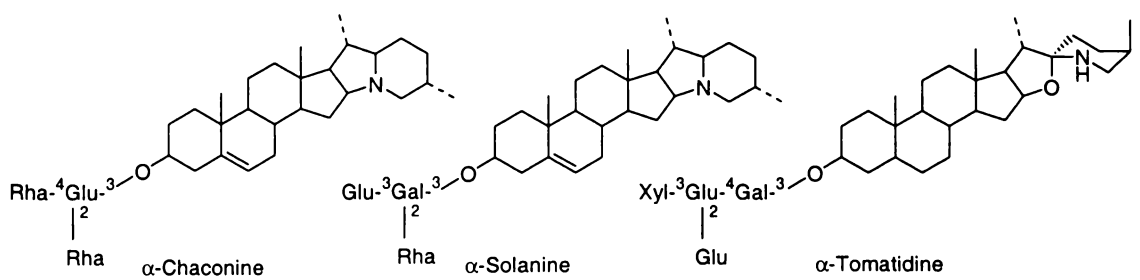


Figure 1.11. Structures of the saponins α-chaconine, α-solanine and α-tomatidine.

Saponins have been found to be strong termite repellents and wood resistance to termite infestation has been attributed to saponin constituents. Both mono- and bidesmosidic saponins repel termites, possibly due to enzyme presence in the termites themselves which convert the bidesmosides into monodesmosides (Tschesche *et al.*, 1970; Tschesche, 1971). *Reticulitermes flavipes*, which contains steroidal saponins, is a natural termite antifeedant and in laboratory trials showed termite mortality at concentrations of 0.5% saponin in their feedant (Tschesche *et al.*, 1970). Two triterpene saponin containing plants, *Ternstroemia japonica* and *Kalopanax septemlobus* also show termite protection and repellent properties (Kondo *et al.*, 1963).

Some other saponins that exhibit insecticidal and antifeedant properties, i.e. against caterpillar and moth larvae etc, are; solamargine, solasonine, aginoside (Harmatha *et al.*, 1987), diosgenin and yamogenin glycosides from *Balanites roxburghii* (Jain and Tripathi, 1991), peduncloside, rotungenoside (Nakatani *et al.*, 1989) and glycosides of medicagenic acid (Pracros, 1988).

1.5.2.5 Toxicity

Toxicity is an extremely important issue, due to the widespread occurrence of saponins in human foods. However very little data exists to date with respect to toxicity by saponins towards humans (Hostettmann and Marston, 1995; Rao and Gurfinkel, 2000). Occasionally there are instances where ingestion of a saponin-containing plant(s) can be fatal, but such occurrences are extremely rare. Mortality cases relating to the consumption of *Agrostemma githago* have been recorded (Gessner, 1974). Signs of saponin intoxication include abundant salivation, vomiting, diarrhoea, appetite loss and manifestations of paralysis.

Oral toxicity of saponins towards warm-blooded animals is relatively low when compared to direct intravenous effects. The generally low-risk oral ingestion can be explained by the poor absorption saponins undergo in the body and enzyme deactivation of the saponins via full hydrolysis, reconjugation and excretion. Long term saponin consumption by humans seem to have little or no detrimental effects, enforced by the lack of negative effects observed after continued intake of saponins, for example, from edible plants or from ginseng (Hostettmann and Marston, 1995; Baldwin *et al.*, 1986; Yamamoto and Uemura, 1980). Over-consumption however can have ill effects. For example, prolonged excessive

intake of liquorice (which contains glycyrrhizin and a number of other saponins) has been known to produce hypertension, flaccid quadriplegia, hypocalcaemia, fulminate congestive heart failure and hyperprolactinaemia with amenorrhoea (Spinks and Fenwick, 1990).

Alfalfa saponins have been fed to rats at a dietary level of 1% for 6 months in order to gauge their safety for human consumption. No ill effects were noticed; however a beneficial reduction in serum cholesterol was observed (Malinow *et al.*, 1981).

Parenteral (especially intravenous) injection can have much greater and injurious effects. For example, an oral application in mice with the glycoalkaloids α-solanine, α-chaconine and α-tomatine had no reaction up to 1000 ppm, whereas intraperitoneal LD_{50} values were 42 ppm, 28 ppm and 34 ppm respectively (Nishie *et al.*, 1971). By comparison, hydrogen cyanide (HCN) LD_{50} values in mice for oral, inhalation and intravenous exposures are 3.7 ppm, 323 ppm and 1 ppm respectively.

LD_{50} values can vary considerably, for example holothurin A, from sea cucumbers, has a reported intravenous LD_{50} value in mice as low as 0.75 ppm (Habermehl and Kerbs, 1990). Once in the bloodstream, liver damage, red cell haemolysis, respiratory failure, coma and convulsions can result (Martindale, 1982). Fatal doses of *Agrostemma* saponins lead to liver necroses and bleeding of intestinal walls and other vessel walls (Vogel, 1963). Interestingly, no parallel has been observed between toxicity and haemolytic activity of a saponin (Vogel, 1963; Hostettmann and Marston, 1995). Saponins can be highly toxic to fish, molluscs, frogs and other gill-breathing organisms. Death occurs via permeabilization of the respiratory membranes and loss of physiological function.

1.6 Steroidal Saponins in the Pharmaceutical Industry

From early times, saponin-containing plants have had widespread application for the treatment of various ailments – coughs, syphilis, gastric disorders, skin diseases are some examples. The pharmacologically active saponins from many of these applications have now largely been isolated and characterized, with their activities confirming the respective medicinal properties of the plant drugs (Hostettman and Marston, 1995). Synthetic pharmaceuticals have, to a certain extent, replaced a lot of the original saponin-containing preparations but saponins still assume a central and important position. One of the most important is the use of steroids in medicines.

Onken and Onken (1980) reported that greater than 6% of all human prescription medicines were steroid hormones. Of greatest importance are the corticosteroids and especially sex hormones, i.e. oral contraceptives. The majority of steroids utilized in therapy and contraceptives are produced by semi-synthesis from natural products; saponins, phytosterols, cholesterol and bile acids.

The commercial significance of spirostanol saponins began with the discovery by Marker that the aglycone portions could easily be converted to pregnane derivatives and utilized as substrates for steroid hormone synthesis. In 1940, Marker synthesized the female sex hormone progesterone from diosgenin. (Marker *et al.*, 1947; Fieser and Fieser, 1959). In 1949 Marker found that the Mexican plant *Dioscorea barbasco* contained large quantities of diosgenin. Steroid research exploded, when in the same year, Hench and Kendall recognized anti-inflammatory and antirheumatic properties of cortisone (Onken and Onken, 1980).

Diosgenin is the most important sapogenin from an economic standpoint. It is extracted almost entirely from *Dioscorea* species, of which *D. mexicana* and *D. composite* are the 2 most important species. At the end of the 1950's, 80-90% of the total world steroid production stemmed from diosgenin as the starting material. By the early 1970's the figure was 40-45%, due to the use of additional starting materials; for example, hecogenin which accounted for *ca.* 6% of steroid precursors (Blunden *et al.*, 1975).

Solasodine, a nitrogen-containing analogue of diosgenin that occurs in various *Solanum* species, is also assuming a greater role as a source of pregnane derivatives (Franz and

Jatisatienr, 1983). Production of corticosteroids from diosgenin and solasodine, and the essential intermediate partial synthetic steps, are presented in Figure 1.12. The key intermediate is 16-dehydropregnenolone, which can be converted into corticosteroids, pregnanes, androstanes and 19-*nor*-steroids.

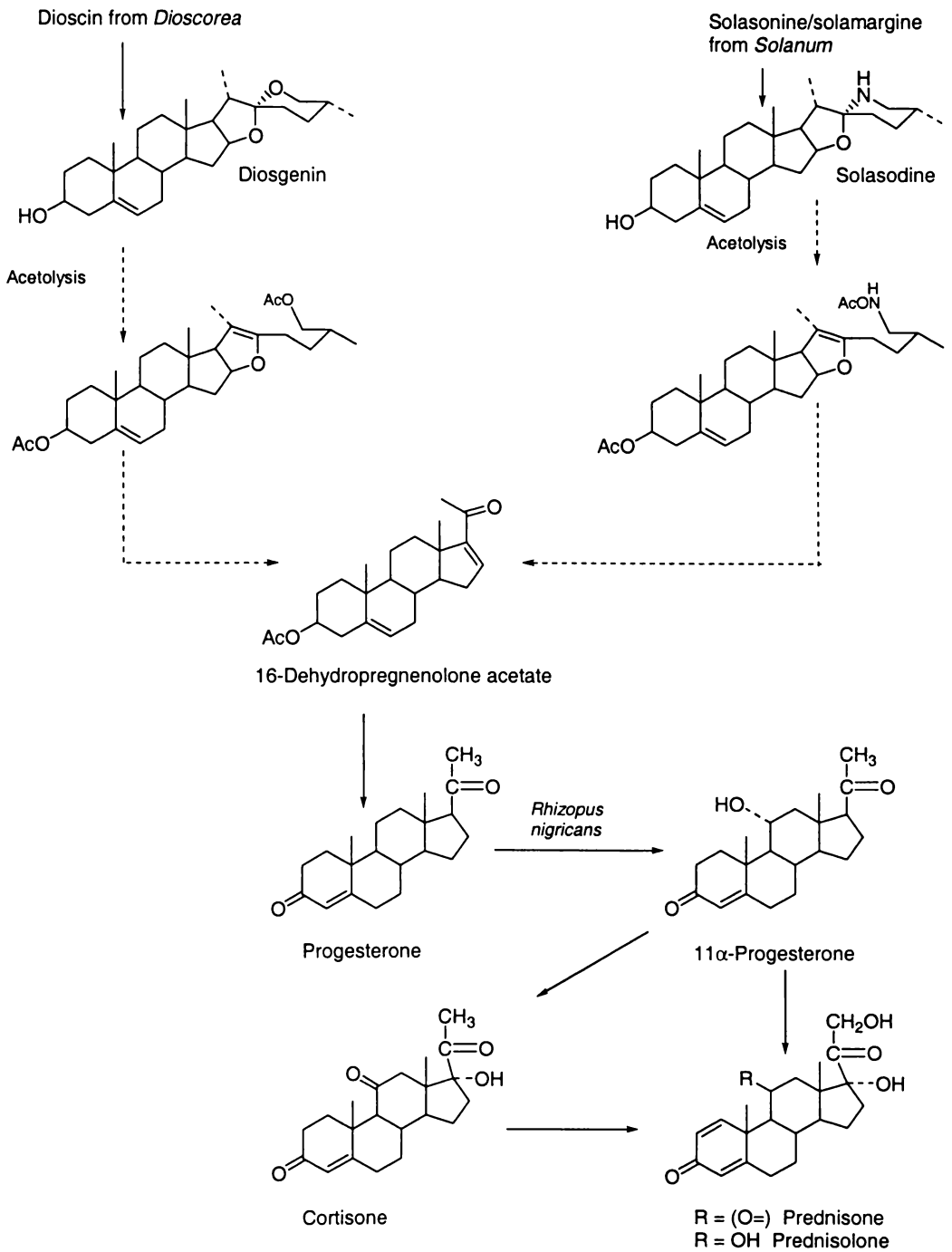


Figure 1.12. Production of corticoids from diosgenin and solasodine (from Hostettmann and Marston, 1995).

1.7 Photosensitization

Saponins are widely distributed amongst forage and agricultural plants, with a variety of these plants implicated as causative agents in hepatogenous photosensitization diseases. At least 10 plants containing steroidal saponins are implicated in the hepatogenous photosensitizations of sheep (Flåøyen, 2000). Hepatogenous photosensitization of animals is both economically important and an animal welfare problem in many parts of the world (Flåøyen *et al.*, 1996). In some years, for example, the photosensitization disease known as alveld, associated with the grazing of *Narthecium ossifragum*, has affected up to 50% of some Norwegian lamb flocks (Flåøyen, 1996). Major photosensitization losses occur in South Africa, where the disease known as geeldikkop (yellow thick head) is associated with the ingestion of *Tribulus terrestris*. In severe outbreaks, geeldikkop has been reported to kill as many as 500,000 South African sheep (Kellerman *et al.*, 1994).

Photosensitization results when a toxin causes liver damage resulting in retention of photosensitizing agents such as phylloerythrin (Clare, 1952; Flåøyen, 1999). Common features of all of the diseases associated with the grazing of saponin-containing plants are their sporadic occurrence, the difficulty in reproducing symptoms during dosing experiments (Flåøyen *et al.*, 1996, Kellerman *et al.*, 1991), and the appearance of calcium salts of episapogenin glucuronides in the liver and in the bile ducts (Miles *et al.*, 1991, 1992a/b, 1993, 1994a/b). Photosensitization outbreaks associated with saponin-containing plants has also been observed in cattle and goats (Lemos *et al.*, 1997; Holland *et al.*, 1991).

Photosensitization diseases of animals are characterised by swelling, sunburn-like symptoms, blistering and lesions on the face, ears and other sunlight exposed areas. The clinical symptoms of all saponin associated photosensitization diseases are similar (Flåøyen and Frøslie, 1997). Generally, only unpigmented animals or animals with unpigmented areas of skin become photosensitized. In serious cases the animals' condition will worsen until death occurs. Often in severe cases, even with intervention, animals are unable to recover and euthanasia is frequently the only option.

1.7.1 Primary and Secondary Photosensitization Diseases

Plant associated photosensitization of livestock have two major classifications and are distinguished by their differing modes of action. Primary photosensitization results from the direct ingestion of a photodynamic agent, absorbed unchanged by the animal. Once in the circulatory system, the toxic agent can absorb sunlight when it reaches the skin. Examples of plants containing primary photosensitizers are St John's Wort (*Hypercum perforatum*), buckwheat (*Fagopyrum esculentum*), and bishop's weed (*Ammi majus*) (Flåøyen and Frøslie, 1997; Familton, 1990).

Secondary or hepatogenous photosensitization occurs when ingested plant toxins/compounds cause liver damage or dysfunction. Damage results in the accumulation of photoactive species such as phylloerythrin in the blood. Phylloerythrin is derived from the ruminal metabolism of chlorophyll. When phylloerythrin reaches the skin sunlight absorption occurs and energy transfer to surrounding tissue(s) causes skin lesions (Familton, 1990).

There are two types of hepatogenous photosensitization:

i) Facial eczema is a hepatogenous photosensitization disease caused primarily by the mycotoxin sporidesmin A, found in the spores of the fungus *Pithomyces chartarum* that grows in pastures.

ii) Induction of photosensitization associated with saponin ingestion. Some examples of saponin associated hepatogenous photosensitization diseases of ruminants include *Brachiaria decumbens* toxicity in Australia, Papua New Guinea, Indonesia, Malaysia, Nigeria, Brazil (Abas-Mazni *et al.*, 1983; Abas-Mazni *et al.*, 1985; Opasina, 1985; Graydon *et al.*, 1991; Abdullah *et al.*, 1992; Smith and Miles, 1993; Low *et al.*, 1993; Lemos *et al.*, 1997), *Narthecium ossifragum* toxicity in Norway (Flåøyen, 1993; Flåøyen *et al.*, 1994), *Nolina texana* toxicity in the USA and northern Mexico (Mathews, 1940; Hershey, 1945; Rankins *et al.*, 1986; Rankins *et al.*, 1993) and *Panicum* toxicity in New Zealand and Australia (Clare, 1952; Holland *et al.*, 1991; Miles *et al.*, 1991).

1.7.2 Biliary Crystals

The accumulation of birefringent crystals in hepatocytes, in and around the bile ducts and related structures is a common finding of photosensitization of sheep involving saponins. Severe liver damage is also evident (Flåøyen and Frøslie, 1997). It is still unclear whether the crystalloid material is the primary causal agent for the liver legions, or if they are a secondary response to an, as yet unknown, primary factor(s). Indications from experimental results suggest that while implicated, ingestion of saponins alone is not sufficient for disease symptoms to be observed (Flåøyen *et al.*, 1991; Flåøyen *et al.*, 1993; also see Chapter 2).

Until 1991, the composition of biliary crystals was not known. Holland *et al.* (1991) initially identified a genin, obtained via the hydrolysis of biliary crystalloid material, as epismilagenin on the basis of ^{1}H and ^{13}C NMR spectroscopic analyses. Subsequently Miles *et al.* (1992a, 1994b) identified the biliary crystals as insoluble, mainly calcium, salts of the β-D-glucuronides of epismilagenin and/or episarsasapogenin.

A GC-MS procedure for the analysis of saponins from plant and animal samples by Wilkins *et al.* (1994) revealed that although hepatogenous photosensitization of sheep was associated with five plant species from three different families (*N. ossifragum, P. dichotomiflorum, P. schinzii, T. terrestris* and *P. miliaceum*), the biliary crystals from all affected sheep were insoluble calcium salts of epismilagenin and/or episarsasapogenin β-D-glucuronides (Figure 1.13) (Wilkins *et al.*, 1994; Miles *et al.*, 1993; Miles *et al.*, 1994a/b).

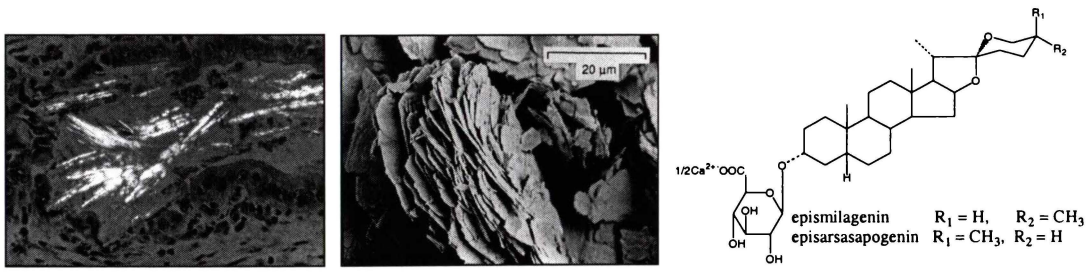


Figure 1.13. SEM micrographs of biliary crystals from the liver of a photosensitized sheep. Structures of the calcium salts of epismilagenin and episarsasapogenin β-D-glucuronides are shown.

1.7.3 Causative Plants Implicated in Photosensitization Diseases

Plants implicated in the development of ovine photosensitization diseases include *Agave lecheguilla*, *B. decumbens*, *N. ossifragum*, *N. texana*, *P. coloratum*, *P. dichotomiflorum*, *P. miliaceum*, *P. schinzii*, *P. virgatum* and *T. terrestris*.

A. lecheguilla

A. lecheguilla (Agavaceae) grows thickly over a wide area of southwestern Texas, USA. It is reported to cause the photosensitization of sheep and goats. (Mathews, 1937; Wall *et al.*, 1962). Smilagenin is reported to be the major genin constituent of this plant (Wall *et al.*, 1962; Camp *et al.*, 1988).

B. decumbens

B. decumbens (Panicoideae) is native to Africa, but is now widespread in tropical and subtropical countries. Diosgenin and yamogenin are the major genin constituents present in *B. decumbens* (Smith and Miles, 1993; Loader, 2001). Photosensitization diseases of sheep, cattle and goats grazing *B. decumbens* have been reported to occur in Australia, Malaysia, Indonesia, Nigeria, Papua New Guinea and Brazil (Flåøyen and Frøslie, 1997; Abas-Mazni *et al.*, 1985; Opasina, 1985; Grayden *et al.*, 1991; Low *et al.*, 1993).

N. ossifragum

N. ossifragum (Liliaceae) (Figure 1.14) is confined to the northern hemisphere and is found in Scandinavia, the British Isles, the Netherlands, Belgium, north western Germany, western and central France, northern Spain, and eastern Portugal (Summerfield, 1974). Photosensitization of sheep grazing this plant has been reported in Norway, the Faroe Islands, and the British Isles (Ford, 1964; Flåøyen *et al.*, 1994). In Norway the disease is called alveld, which translated literally means "elf-fire" (Flåøyen *et al.*, 1994).

Photosensitization normally occurs only in lambs and rarely in adult sheep. Certain flocks can become affected in some years, but in other years almost no lambs from the same flocks are affected (Flåøyen, 1996; Flåøyen, 1993). More cases are seen in cold and rainy summers than in warm and dry summers.

Figure 1.14. Norwegian *N. ossifragum.*

The two major genins present in *N. ossifragum* saponins are sarsasapogenin and smilagenin (Stabursvik, 1954; Wilkins *et al.*, 1994; Flåøyen and Wilkins, 1997). Structures of the dominant saponin constituents have been defined (Ceh and Hauge, 1981; Deng, 1999). Dosing of large amounts of *N. ossifragum* to lambs has been shown to induce photosensitization, but dosing of lesser amounts over 3 weeks was not found to cause liver dysfunction (Abdelkader *et al.*, 1984; Ender, 1955; Flåøyen *et al.*, 1991). Insoluble salts of episarsasapogenin β-D-glucuronide and epismilagenin β-D-glucuronide have been found in bile from sheep suffering alveld (Miles *et al.,* 1993). Bile crystals containing conjugated sapogenins, from dose induced photosensitized lambs are very difficult to isolate.

Hepatoxic fungi have been linked to the etiology of this disease. The fungus *Cladosporium magnusianum*, which is unique to *N. ossifragum*, is apparently not toxic to sheep. However, there is the possibility that in some years as a response to *C. magnusianum* infection, the plant may produce phytoalexins that might be hepatoxic to sheep (Flåøyen and Frøslie, 1997; Flåøyen, 1993).

N. ossifragum has also been reported to induce nephrotoxicity in Norwegian cattle (Malone *et al.*, 1992; Flåøyen *et al.*, 1995a/b), sheep (Flåøyen *et al.*, 1995c), goats (Flåøyen *et al.*, 1997a), moose, red deer and reindeer (Flåøyen *et al.*, 1997b). It is known that 3-methoxyfuran-2(5*H*)-one, a non-steroidal compound, is responsible for the nephrotoxicity of *N. ossifragum* ((Langseth *et al.*, 1999).

N. texana

N. texana (Agavaceae) is found over a wide area of southwestern USA and northern Mexico. This plant has been reported to be highly poisonous to sheep and goats (Mathews, 1940; Rankins *et al.*, 1993; Hershey, 1945). This poisoning is locally referred to as "swellhead". Deng (1999) has identified the principal genin constituents of hydrolysed saponins from USA sourced *N. texana* as ruscogenin, neoruscogenin and $\Delta^{25(27)}$-ruscogenin. He also isolated 3 monodesmosidic $\Delta^{25(27)}$-ruscogenin saponins from this plant material.

Panicum species

Panicum grasses (Poaceae) are widely distributed from the tropics to warm temperate regions (Dahlgren *et al.*, 1985). *P. coloratum* (kleingrass) originates from South Africa, where it is known to cause the photosensitization disease dikoor. It is now associated with photosensitization outbreaks in the USA and Australia (Kellerman and Coetzer, 1984; Bridges *et al.*, 1987; Regnault, 1990). The principal genins are diosgenin and yamogenin (Patamalai *et al.*, 1990).

P. dichotomiflorum (smooth witch grass) is known to cause photosensitization of sheep, cattle and goats in New Zealand (Holland *et al.*, 1991; Miles *et al.*, 1991). Diosgenin has been identified as the principal genin (Miles *et al.*, 1991), whilst biliary crystals from photosensitized sheep were identified as the calcium salt of epismilagenin β-D-glucuronide (Holland *et al.*, 1991; Miles *et al.*, 1991; Miles *et al.*, 1992b).

P. miliaceum (broom corn millet) has also been implicated in photosensitization of sheep in New Zealand and Australia (Holland *et al.*, 1991; Miles *et al.*, 1991; Everist, 1974). The major genin constituents are diosgenin and yamogenin (Miles *et al.*, 1993). Deng (1999) has identified two saponins from *P. miliaceum*. In southeastern Australia *P. schinzii* (sweet grass) can be a major component of summer pastures, provided rainfall is adequate. Photosensitization of sheep has been reported in Australia (Lancaster *et al.*, 1991; Miles *et al.*, 1992a/b). Diosgenin has been identified as the only genin constituent (Miles *et al.*, 1992a). Bile crystals from *P. schinzii* intoxicated sheep have been identified as the calcium salt of epismilagenin β-D-glucuronide (Miles *et al.*, 1992b).

P. virgatum (switchgrass) has been associated with photosensitization of sheep in the USA (Puoli *et al.*, 1992). Diosgenin and yamogenin have been reported as the major genin constituents (Bernt, 1997; Lee *et al.*, 2001). Deng (1999) reported quantitative levels of free and conjugated sapogenins from 13 USA collections of *P. virgatum* and identified the dominant saponins.

T. terrestris

T. terrestris (Zygophillaceae) is native to Europe and Africa, and is now widespread in many parts of the world. In South Africa it is extensively grazed in the Karoo, a semi arid region covering over one-third of the country. Photosensitization outbreaks have been reported in Australia (Glastonbury *et al.*, 1984), USA (Camp *et al.*, 1988), Argentina (Tapia *et al.*, 1994), and Iran (Amjadi *et al.*, 1977).

South African *T. terrestris* contains several steroidal sapogenins derived from diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin, neogitogenin, ruscogenin, and neoruscogenin saponins (Miles *et al.*, 1994b; Wilkins *et al.*, 1996).

1.7.4 Ovine Saponin Metabolism

Metabolism and absorption of ingested saponins and sapogenins are implicated directly or indirectly with the development of hepatogenous photosensitization disease symptoms. Some sapogenins are considered to be lithogenic (crystal forming), whilst others are not lithogenic (Miles *et al.*, 1994b; Wilkins *et al.*, 1996). Diosgenin, yamogenin, smilagenin, sarsasapogenin, epismilagenin and episarsasapogenin are considered to be capable of contributing to bile crystal formation. Miles *et al.* (1993, 1994b) have proposed that ovine ruminal metabolism of diosgenin, yamogenin, smilagenin and sarsasapogenin saponins proceeds to afford lithogenic epismilagenin and episarsasapogenin glucuronide conjugates. In contrast, tigogenin, neotigogenin and dihydroxylated sapogenins appear not to be lithogenic.

A pathway for the ovine metabolism of diosgenin saponins based on results obtained for sheep affected by grazing *P. dichotomiflorum* has been proposed by Miles *et al.* (1992b) (Figure 1.15). Metabolism of yamogenin (25*S*-isomers of diosgenin) is envisaged to proceed via the same pathway to afford the corresponding episarsasapogenin analogues (Miles *et al.*, 1993). The metabolism of ingested saponins to epismilagenin and episarsasapogenin conjugates is supported by the results of dosing experiments (Miles *et al.*, 1994b; Meagher, 1996; Flåøyen and Wilkins, 1997). It has been noted that 25*R*-saponins appear to be 2-6 times more bio-accumulative than 25*S*-saponins (Deng, 1999; Wilkins, *pers commun*).

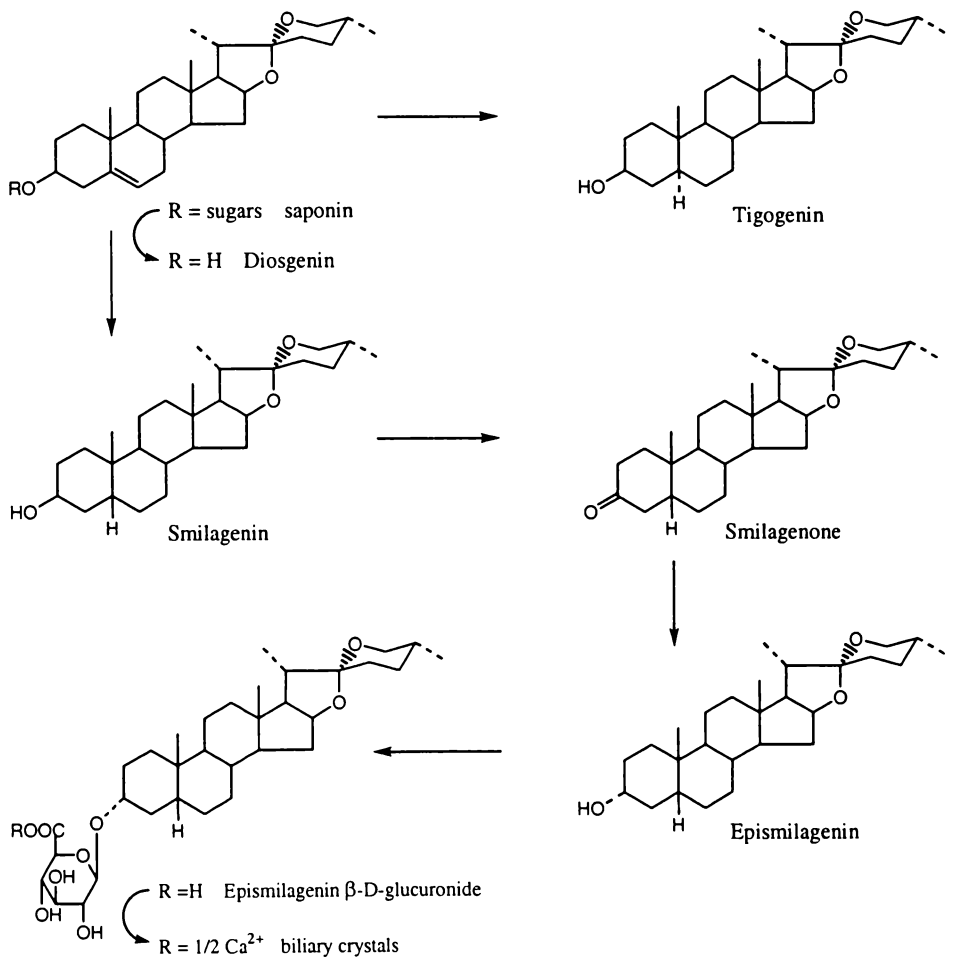


Figure 1.15. Proposed pathway for the ruminal metabolism of diosgenin-containing saponins to the calcium salt of epismilagenin β-D-glucuronide (from Miles *et al.*, 1992b).

1.8 Aims of the Present Investigation

The following areas of research are reported in this thesis:

Photosensitization Related Research

To better understand the pathogenesis of alveld (daily intakes, periodic outbreaks etc), the results from 3 *N. ossifragum* and alveld related projects are reported.

(i) An investigation was directed towards an assessment of a *genin dose/response hypothesis*. The hypothesis that saponins produced by *N. ossifragum* may be the direct cause of the toxicity leading to the hepatogenous photosensitivity disease alveld seen in Norwegian lambs was evaluated by determining the total genin levels (mg/kg dry matter (DM)) from lamb faecal samples and samples of *N. ossifragum* leaves, collected from 2 alveld outbreak areas and 2 control areas in a region of Norway known for periodic livestock outbreaks of alveld.

(ii) An investigation into sapogenin levels in Scottish collections of *N. ossifragum* plant material, gathered from pasture on which sheep were photosensitized was performed. It was envisaged this investigation would add to the sapogenin and saponin knowledge of *N. ossifragum* and the likely role they and the plant play in the photosensitization of livestock.

(iii) A field trial involving the administration of a daily dose of a deuterated sapogenin standard (a 1:4 mixture of [2,2,4,4-$^{2}H_4$]sarsasapogenin and [2,2,4,4-$^{2}H_4$]episarsasapogenin) to 4 ewes, their twin lambs, and to 3 weaned lambs over a 21 day period was undertaken in Norway. The expectation was that it would be possible to calculate typical daily intakes of natural pasture saponins/sapogenins under conditions characteristically associated with the development of alveld.

Saponin Bioactivity Directed Research

An investigation into some bioactive properties of a series of synthetically prepared saponin compounds was undertaken. The research involved the following:

(i) The synthesis and characterization of 5 steroidal and triterpenoidal saponin compounds. Namely: sarsasapogenin β-D-galactoside, episarsasapogenin β-D-glucoside, episarsasapogenin β-D-galactoside, betulin 3-(β-D-glucoside) and betulin 3,28-(β-D-diglucoside).

(ii) The bioactivity evaluation of these synthesized saponins, as well as sarsasapogenin β-D-glucoside, a furanone glucoside (isolated from Norwegian *N. ossifragum* leaves) and of 3 natural plant extracts, was undertaken against a range of bacterial and fungal organisms at the National Veterinary Institute (NVI), Oslo, Norway, and at the University of Canterbury, New Zealand. It was envisaged that this would lead to the identification of some structure-activity relationships amongst the synthesized saponins.

Saprolegnia parasitica

The active fraction from East Coast Manuka oil (triketone oil) was subjected to a preliminary screening and evaluation of its activity against 2 fish-pathogenic strains of an invasive fish fungus, *S. parasitica*. The evaluation was performed using an *in vitro* testing method developed at the NVI, Oslo, Norway. The toxicity of triketone oil against Atlantic salmon fry was evaluated using an *in vivo* testing protocol.

Chapter Two

Alveld Outbreak Study

2.1 Introduction

Bog asphodel (*Narthecium ossifragum*), a member of the lily family, is suspected of causing alveld, a hepatogenous photosensitivity disease that occurs in domestic lambs (*Ovis aries* L.) in parts of Norway (Flåøyen, 2000). Outbreaks of this disease among grazing sheep are of great economic importance in Norway, which annually has a total of *ca.* 2.2 million sheep on summer grazing fields. In some years alveld can affect up to 50% of some Norwegian lamb flocks (Flåøyen, 1996). *N. ossifragum* has a fairly wide distribution along the coast of Norway (Hultén, 1971), and alveld outbreaks are sporadically reported from many areas. Outbreaks and severe disease problems vary widely both in time and geographical location, and uncertainty characterizes accounts of the aetiology of the disease. It has long been claimed that some *N. ossifragum* pastures are safe to graze, while other pastures in the same general area tend to be highly toxic (Ender, 1955; Ulvund, 1984; Mysterud, 2001).

Steroidal saponins of *N. ossifragum* have been suggested to be causative agents in the development of the characteristic liver lesions, which lead to the retention of the photosensitizing agent phylloerythrin (Ender, 1955; Adelkader *et al.*, 1984; Flåøyen, 2000). Hepatogenous photosensitizations similar to alveld have been reported in sheep grazing at least 10 plant species containing steroidal saponins (Flåøyen, 2000). Common features of all diseases associated with the grazing of saponin-containing plants are their sporadic occurrence, the difficulty of reproducing symptoms during dosing experiments, the observation that the plants seem to be only occasionally toxic to grazing animals (Flåøyen, 1996), and the appearance of calcium salts of episapogenin conjugates in the liver and in the bile ducts (Holland *et al.*, 1991; Miles *et al.*, 1991, 1993, 1994a).

An improved understanding of the aetiology of alveld requires clarification of the role of saponins in the development of the disease. Whether the saponins are the sole cause of these diseases is still uncertain (Flåøyen, 2000). Two hypotheses may be formulated. The *dose/response hypothesis* contends that the saponin content of *N. ossifragum* is the sole causal agent for the liver lesions resulting in phylloerythrin retention. On the other hand, the *other factor hypothesis* contends that saponins alone are not the sole cause of disease, and that some other agent(s) are involved. It has been suggested that these agent(s) might include mycotoxins produced by *N. ossifragum*-associated fungi (Aas and Ulvund, 1989; di Menna *et al.*, 1992; Flåøyen *et al.*, 2000).

The investigations reported herein were directed towards an assessment of the *genin dose/response hypothesis* as revealed by sapogenin data determined for lambs grazing natural *N. ossifragum* containing pastures. Tenets of the *dose/response hypothesis* which were investigated were:

(i) That the level of saponins in *N. ossifragum* leaves might be higher in outbreak areas than in leaf samples from control areas.

(ii) That faeces from lambs grazing in outbreak areas might have higher average total sapogenin levels than for lambs grazing in control areas.

2.2 Methodology

2.2.1 Grazing Range Description

The grazing area was part of a *ca.* 80 km^2 local grazing range between Valsøybotn and Bæverdalen in the municipalities of Halsa and Surnadal in Møre og Romsdal county, western Norway (Figure 2.2). The region is mountainous and lies near the coast. Elevations are between 100 and 978 m a.s.l. Climate in the region varies from predominantly cool oceanic to slightly continental in the valleys, with humid to slightly humid conditions and cool summers (Nordisk Ministerråd, 1984). Average January (winter) and July (summer) temperatures at the nearest weather stations (Tingvoll-Hanem, No. 64550) are -1.6°C and 13.6°C, respectively. Average annual precipitation is 1621 mm (Halsafjord II, No. 64460).

Birch and pine trees occur in mixed stands in the valleys and lower slopes. At higher attitudes birch trees dominate up to the climatic tree line. Understorey vegetation in some areas is a mixture of shrubs such as bilberry (*Vaccinum myrtillus*), heather (*Calluna vulgaris*) and bog whortleberry (*Vaccinum uliginosum*), and in others a luxuriant mixture of forbs and ferns (*Dryopteris* spp.). From 400-450 m a.s.l. the birch forest disappears, and vegetation varies from lichen (*Cladonia* spp.) on dry exposed ridges, to hairgrass (*Deschampsia flexuosa*) and mat-grass (*Nardus stricta*) in semi-dry areas, to purple moor-grass (*Molinia caerulea*) and bog asphodel (*N. ossifragum*) in moist areas. Bogs, particularly those with seeping water formed in slopes (In Norwegian: "bakkemyrer") and moist vegetation communities are common over most of the area. Over the whole area, both above and below the tree line, *N. ossifragum* is a common species with high abundance in vegetation communities, dominating in some. Some photographs of the grazing area are presented in Figure 2.1.

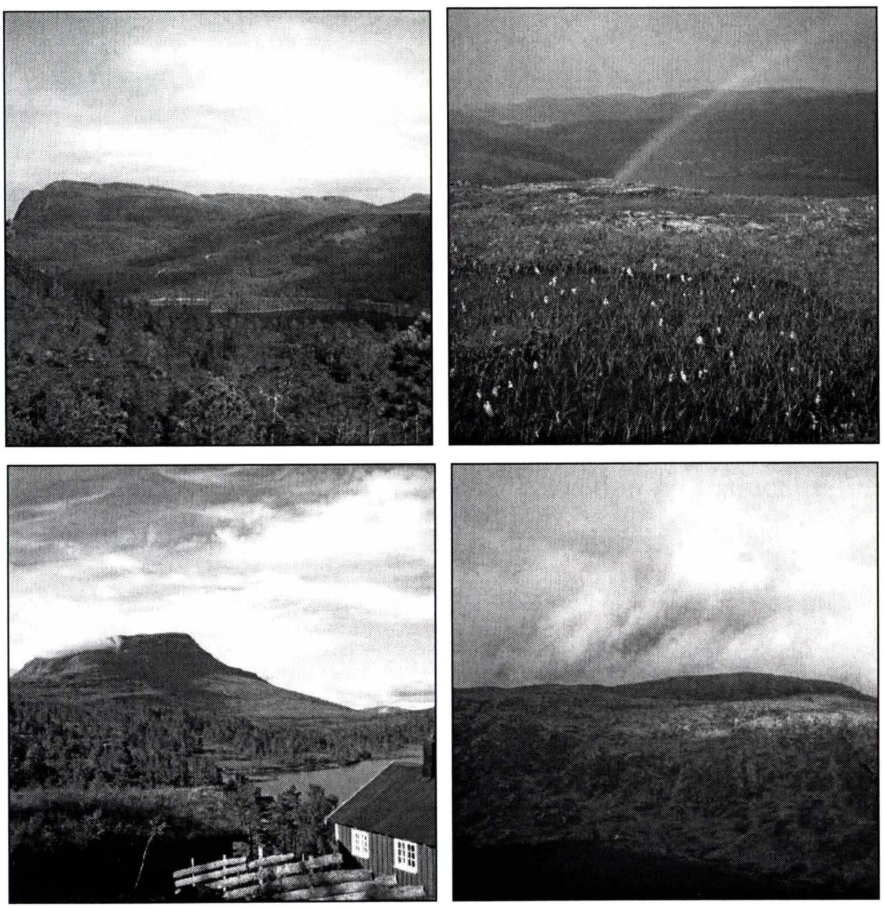


Figure 2.1. Some photographs of the mountainous grazing range area between Valsøybotn and Bæverdalen, western Norway.

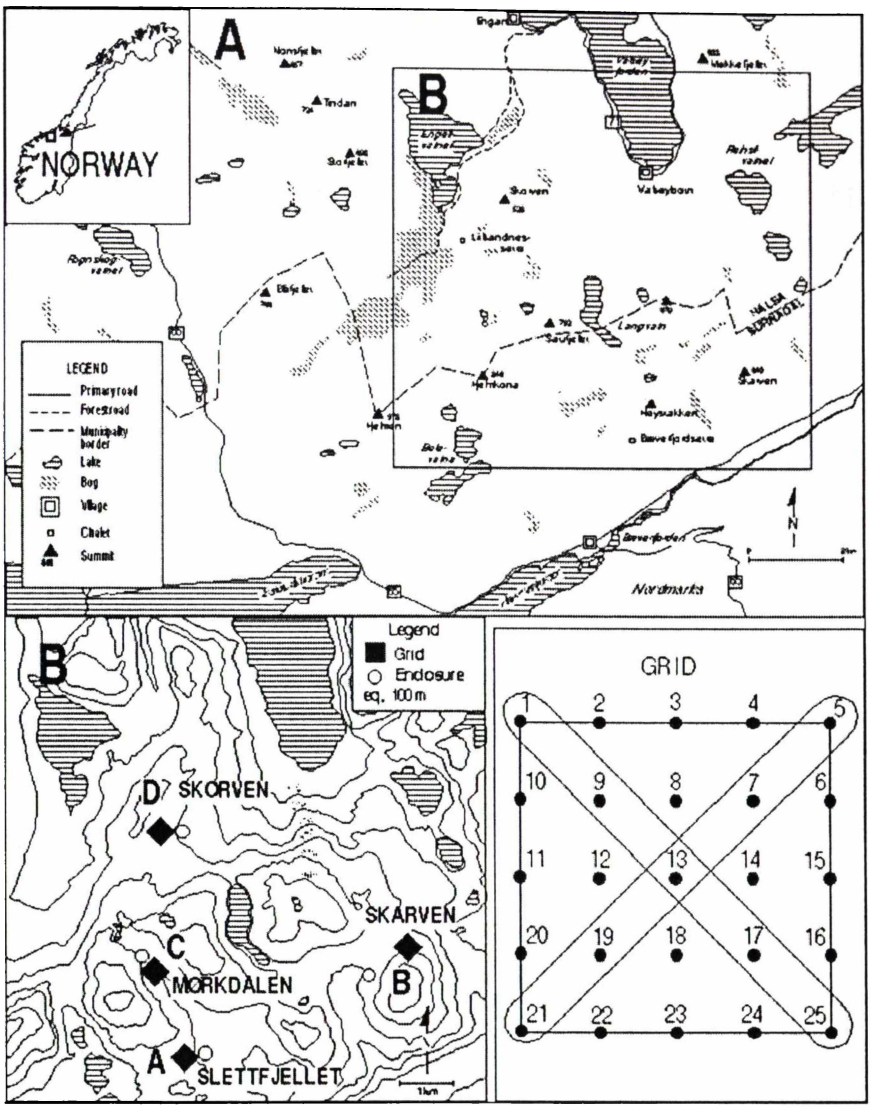


Figure 2.2. A. Map of the Halsa/Surnadal district, western Norway. **B.** Map enlargement of grid and enclosure areas. Also shown is the plant collection grid structure (bottom left); only samples from the 9 framed areas were used in the analyses.

2.2.2 Flock Descriptions

6 flocks of sheep (mothers and lambs), of which 374 lambs from a total of 380 were radio-collared (98.4% coverage) with silent mortality transmitters ("Televilt"-TXD-25-Mo), grazed among other sheep in the Valsøybotn and Bæverdalen areas (Figure 2.2) from mid June until the end of September 2001. Other sampling details are given below.

Slettfjellet Control Pasture (area A)

The sample consisted of 18 lambs, all of dala breed except for 4 lambs, which were dala/spæl hybrids. Weight at sampling varied from 14-34 kg (average 21.9 kg). 16 lambs bore mortality collars. None exhibited alveld symptoms. Post sampling history: all lambs survived and were in good health when gathered in the autumn. Weights varied between 28-49 kg.

Skarven Outbreak Pasture (area B)

The sample consisted of 19 spæl breed lambs. Weight at sampling varied from 14-26 kg (mean 18.9 kg). All lambs bore mortality collars. 3 lambs (B1017, B1018, and B1039) showed alveld symptoms. Post sampling history: the 3 affected lambs survived. 1 lamb (B1022) was chased to death by a dog (August 16th), another (B1032) died in a drowning accident and when found on August 17th showed external symptoms of alveld. 17 lambs survived and were in good health when gathered in the autumn. Weights varied between 27-45 kg.

Mørkdalen Control Pasture (area C)

The sample consisted of 22 dala breed lambs. Weight at sampling varied from 15-34 kg (mean 27.9 kg). All lambs bore mortality collars. None showed external symptoms of alveld. Post sampling history: all lambs survived and were in good health when gathered in the autumn. Weights varied between 27-56 kg.

Skorven Outbreak Pasture (area D)

The sample consisted of 22 lambs, all of dala breed except for 1 hybrid dala/suffolk lamb. Weight at sampling ranged from 14-34 kg (mean 27.3 kg). 20 lambs bore mortality collars. 3 lambs (D1018, D1026, and D1125) exhibited external symptoms of alveld. Post sampling history: the 3 affected lambs all died. The remaining 19 lambs survived and were in good health when gathered in the autumn. Weights varied between 27-51 kg.

2.2.3 Plant Collection

200 × 200 m^2 collection grids with 25 stations, 50 m apart, (Figure 2.2) were established in each of the *N. ossifragum* dominated control and outbreak pasture areas and 20-40 g of *N ossifragum leaves* were hand-picked from each of the 25 grid stations. No flowering *N. ossifragum* plants were observed in the collection grids and/or in the control and outbreak pasture areas.

2.2.4 Sample Storage and Transportation

Plant and faecal samples were placed in plastic bags and frozen at -20°C on the day of collection. The samples were transported in a refrigerated bag to the Department of Biology, University of Oslo, refrozen, and later transferred to the National Veterinary Institute, Oslo, where saponin hydrolyses and sapogenin extractions were performed. All samples were stored at –20°C prior to extraction. After extraction, hydrolysis, solvent evaporation, and drying under vacuum, sapogenin extracts were transported (air freight) to the University of Waikato, Hamilton, New Zealand, where samples were acetylated and GC-MS analyses where performed.

2.2.5 Plant Total Genin Analyses

Representative sub-sample portions of the collected plant material, originating from the 9 diagonal elements of each of the area A-D sampling grids (grid stations 1, 5, 7, 9, 13, 17, 19, 21 and 25, Figure 2.2), were freeze-dried for 24 h. Accurately weighed sub-samples (*ca.* 0.20-0.25 g) of the freeze-dried plant material were extracted with a SoxTech HT 1043 Extraction Unit (Tecator, Sweden) apparatus for 4 h with MeOH (50 mL). The MeOH extracts were concentrated to *ca.* 10 mL on the SoxTech extractor and transferred, with MeOH washing (3 × 3 mL), to boiling tubes and blown down to dryness under a stream of warm nitrogen. 0.5 $molL^{-1}$ HCl (15 mL) was added to the boiling tubes, which were heated in a water bath for 90 min at 85-90°C. After cooling, hydrolysate solutions were extracted with CH_2Cl_2 (4 × 4 mL) and filtered through a short alumina column (*ca.* 3 cm) packed in a Pasteur pipette. The combined extracts were evaporated to dryness under a stream of warm

nitrogen, acetylated at room temperature for 16 h using pyridine-acetic anhydride (1:1) (0.5 mL) and analyzed using the GC-MS protocol described below (Section 2.2.7).

2.2.6 Faeces Total Genin Analyses

Portions of the frozen faecal samples were freeze-dried for 24 h. Accurately weighed sub-samples (0.2-0.4 g) of the freeze-dried faeces material were extracted with a SoxTech apparatus for 4 h with MeOH (50 mL). The MeOH extracts were concentrated, hydrolyzed, and acetylated as described above for *N. ossifragum* extracts (Section 2.2.5).

2.2.7 GC-MS Analyses and Peak Identification

Quantitative GC-MS analyses of the acetylated extracts were performed using selected ion mode (SIM) protocols as previously reported (Flåøyen and Wilkins, 1997; Wilkins *et al.*, 1994; Loader *et al.*, 2003). These protocols, when applied to acetylated steroidal sapogenin samples, utilizes the intense *m/z* 139 ion, known to arise via ring E cleavage, which is typically the base peak in the mass spectrum of steroidal sapogenins lacking a ring E or F substituent group (Figure 2.3). Sarsasapogenin propionate was added as the internal standard to all the acetylated extracts. Sapogenin acetate peaks were identified by comparison of retention times of authentic standards. All calculations were performed using purpose-written Excel spreadsheets.

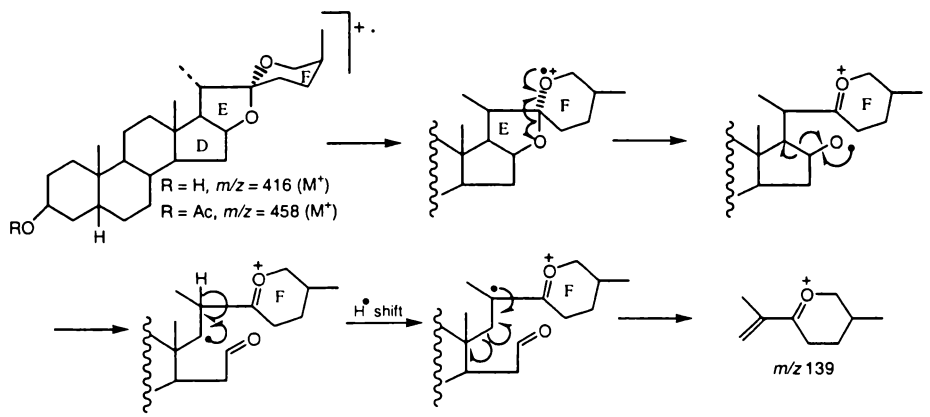


Figure 2.3. Postulated origin of the *m/z* 139 ion of steroidal sapogenins (Blunden *et al.*, 1980).

2.2.8 Statistical Methods

Statistical analyses were conducted by Norwegian collaborators; Ivar Mysterud (1. Amanuensis) who initiated this investigation and Professor Ørnulf Borgan (Department of Biology and The Institute of Mathematics respectively, University of Oslo, Norway). Differences between sapogenin levels found at each of the areas were tested using the Wilcoxon Rank Sum test (Norman and Streiner, 2000).

2.3 Results and Discussion

The Valsøybotn and Bæverdalen areas, in the municipalities of Halsa and Surnadal in Møre og Romsdal county, western Norway, have for several decades been utilized as a summer grazing areas by local sheep farmers. It is well known that *N. ossifragum,* which has high concentrations of minerals such as Na, Co and Cu, is a palatable and preferred food plant for sheep (Stabursvik, 1959; Staaland and Nedkvitne, 1998). Alveld is the most common mortality factor amongst lambs grazing the Valsøybotn and Bæverdalen pastures (Mysterud *et al.*, 2000; Mysterud, 2001). Some pastures in the Valsøybotn and Bæverdalen areas are considered to be more prone to alveld outbreaks than others (Mysterud, *pers commun*). This consideration is consistent with proposals from others that some *N. ossifragum* pastures are safe to graze, while other pastures in the same general area tend to be periodically highly toxic (Ender, 1955; Ulvund, 1984; Mysterud, 2001).

By the use of mortality transmitters on grazing lambs, combined with daily flock observations in risk areas, it was possible to immediately identify and locate outbreak areas. As soon as an outbreak was verified, generally through the finding of several dead lambs and/or sightings of lambs showing external symptoms of alveld, flocks in the outbreak area and an adjacent control area were gathered and faecal samples were collected from the rectums of the sampled lambs together with samples of *N. ossifragum* leaves from the outbreak and control pastures. The sapogenin content of these samples, after hydrolysis of parent saponins, were determined.

Alveld broke out amongst lambs grazing the Skarven outbreak pasture (Figure 2.2, area B) in late June. The first sighting of a lamb showing external symptoms of alveld was made June 22nd and the first radio-collared lamb death from alveld occurred on June 28th. On July 8th, after sightings of several other alveld affected lambs, the sheep were driven into an enclosure (Figure 2.4). 19 of the lambs were weighed and rectal faecal samples were collected. External symptoms of photosensitization amongst the sampled lambs were noted. After sampling all sheep were released simultaneously. Later in the day the same procedure was applied to a sample of 18 lambs in the Slettfjellet control area (Figure 2.2, area A), 4.6 km west-southwest of the Skarven outbreak area.

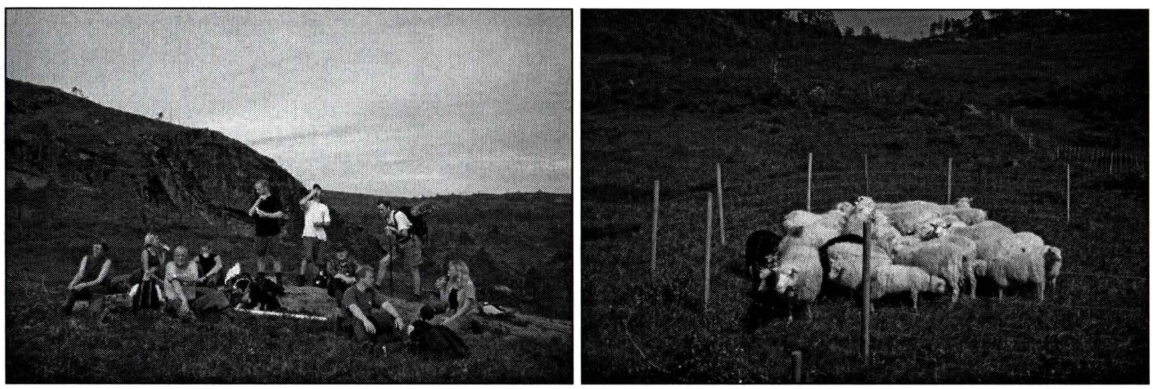


Figure 2.4. Left: People involved in the collection of plant and animal samples, local farmers and collaborators. Right: A temporary enclosure in which the sheep were herded.

The first sighting of lambs showing external symptoms of alveld amongst lambs grazing the Skorven pasture (Figure 2.2, area D) occurred on July 8th. Further sightings of lambs showing external alveld symptoms occurred on July 14th. The sampling procedures described above were also applied at the Skorven outbreak area (Figure 2.2, area D) and at the Mørkdalen control area (Figure 2.2, area C), located 2.5 km south of the Skorven area. The lambs investigated in this study developed alveld relatively early in the summer grazing season. It is known that lambs grazing Valsøybotn and Bæverdalen pastures can develop alveld much later in the grazing season than reported here (Flåøyen, 1990; Mysterud *et al.*, 2000; Mysterud, 2001).

2.3.1 Analytical Methodology

Since only knowledge of total plant and faecal genin levels was required in this investigation, rather than a more detailed knowledge of free and conjugated genin levels, freeze-dried plant and faeces material were extracted with MeOH to yield a combined free (sapogenins) and conjugate (saponins) genin extract, i.e. a total free and conjugated genin extract. Previous investigations have shown that saponins (conjugated sapogenins) are the dominant constituents of *N. ossifragum* extracts whereas sapogenins are the dominant constituents of faecal extracts (Loader *et al.*, 2003; Flåøyen and Wilkins, 1997; Wilkins *et al.*, 1994). Episarsasapogenin, sarsasapogenin, epismilagenin, smilagenin, smilagenone and sarsasapogenone were the only sapogenins found in the faecal extracts.

2.3.2 Genin Content of *N. ossifragum* Leaves

The mean levels of smilagenin and sarsasapogenin (mg/kg DM) determined after hydrolysis of saponins present in *N. ossifragum* leaf samples collected from the 2 control and the 2 outbreak areas, are presented in Table 2.1. Except for one grid station, plot site B1, all samples had total sapogenin contents in the range 5000-11000 mg/kg DM plant material. In all cases, sarsasapogenin was the dominant sapogenin, typically accounting for 89-91% of the genin content of each extract.

Table 2.1. Smilagenin and sarsasapogenin levels (mg/kg DM) found in *N. ossifragum* leaves from areas A to D in the Halsa/Surnadal district, collected on July 8th (area B), July 9th (area A) and July 15th (areas C and D), 2001, respectively.

Sample	Smil	Sar	Total	% Smil	% Sar
area A					
Plot A1	880	7843	8723	10.1	89.9
Plot A5	701	8638	9339	7.5	92.5
Plot A7	761	6893	7654	9.9	90.1
Plot A9	825	8229	9054	9.1	90.9
Plot A13	762	8039	8801	8.7	91.3
Plot A17	1133	9340	10473	10.8	89.2
Plot A19	967	8803	9770	9.9	90.1
Plot A21	900	8067	8967	10.0	90.0
Plot A25	830	7883	8713	9.5	90.5
Mean	862	8193	9055	9.5	90.5
STD	130	692	780	1.0	1.0
% CV	15.1	8.5	8.6	10.2	1.1
area B					
Plot B1	862	3691	4553	18.9	81.1
Plot B5	740	7989	8729	8.5	91.5
Plot B7	1070	8550	9620	11.1	88.9
Plot B9	786	8495	9281	8.5	91.5
Plot B13	1046	9833	10879	9.6	90.4
Plot B17	1189	9586	10775	11.0	89.0
Plot B19	984	9493	10477	9.4	90.6
Plot B21	989	9544	10533	9.4	90.6
Plot B25	501	5578	6079	8.2	91.8
Mean	907	8084	8992	10.1	89.9
STD	208	2104	2238	3.3	3.3
% CV	23.0	26.0	24.9	32.9	3.7
area C					
Plot C1	609	4908	5517	11.0	89.0
Plot C5	693	6125	6818	10.2	89.8
Plot C7	591	6517	7108	8.3	91.7
Plot C9	689	7018	7707	8.9	91.1
Plot C13	557	4983	5540	10.1	89.9
Plot C17	512	5397	5909	8.7	91.3
Plot C19	662	6322	6984	9.5	90.5
Plot C21	512	6411	6923	7.4	92.6
Plot C25	668	7018	7686	8.7	91.3
Mean	610	6078	6688	9.1	90.9
STD	72	803	842	0.0	0.0
% CV	11.8	13.2	12.6	12.1	1.2
area D					
Plot D1	704	6416	7120	9.9	90.1
Plot D5	633	4880	5513	11.5	88.5
Plot D7	752	5426	6178	12.2	87.8
Plot D9	835	7411	8246	10.1	89.9
Plot D13	777	7611	8388	9.3	90.7
Plot D17	609	5673	6282	9.7	90.3
Plot D19	646	4698	5344	12.1	87.9
Plot D21	1117	6038	7155	15.6	84.4
Plot D25	762	6135	6897	11.0	89.0
Mean	759	6032	6791	11.2	88.8
STD	153	1010	1077	0.0	0.0
% CV	20.2	16.7	15.9	17.4	2.2

Smil = smilagenin (mg/kg DM); Sar = sarsasapogenin (mg/kg DM); Total = total genin (mg/kg DM); % Smil = percentage smilagenin of total genin; % Sar = percentage sarsasapogenin of total genin; STD = standard deviation; % CV = percent coefficient of variation. Values <100 rounded to 1 d.p.

The mean genin level (9055 mg/kg DM) determined for *N. ossifragum* leaves from the Skarven outbreak pasture (area B) was statistically similar to that determined for the Slettfjellet control pasture (area A) (mean level 8992 mg/kg DM). Similarly, the mean genin level (6688 mg/kg DM) for the Skorven outbreak pasture (area D) was statistically similar to the mean genin level (6791 mg/kg DM) determined for the Mørkdalen control pasture (area C). Figure 2.5 illustrates the mean genin levels from the four *N. ossifragum* plot sites A to D.

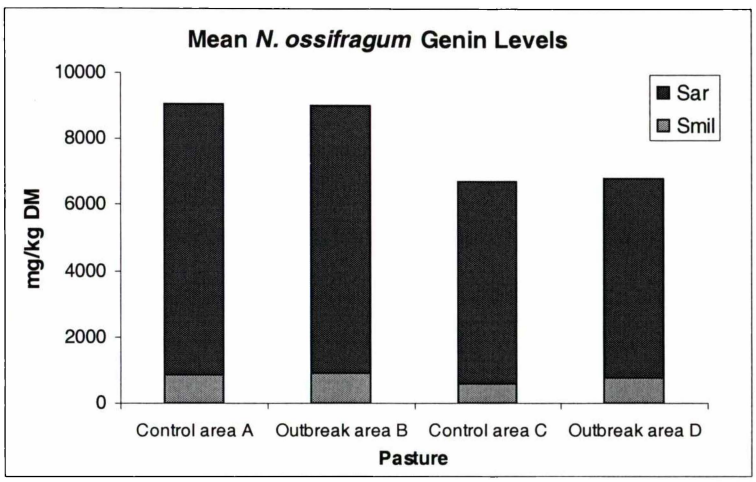


Figure 2.5. Mean sarsasapogenin and smilagenin levels (mg/kg DM) from each of the *N. ossifragum* plot sites A to D.

The mean genin levels determined for the Skarven outbreak pasture (area B) and Slettfjellet control pasture (area A) areas were however significantly higher than the mean sapogenin levels determined for Skorven outbreak (area D) and Mørkdalen control (area C) pastures respectively (Wilcoxon rank sum test, P-values: 0.1% for the Slettfjellet (area A) vs Mørkdalen (area C) pastures; 0.1% for Slettfjellet (area A) vs Skorven (area D) pastures; 2.7% for Skarven (area B) vs Mørkdalen (area C) pastures; 3.4% for Skarven (area B) vs Skorven (area D) pastures.

No obvious geographical or physical reason (e.g. slope, exposure, height, precipitation etc) for this difference was apparent. It is possible the lesser levels found at the Mørkdalen and Skorven pastures (areas C and D respectively) may be associated with the phenology, i.e.

the seasonal plant and vegetation community development in these areas. The sapogenin levels reported in Table 2.1 can be compared with the mean level of 2155 mg/kg DM previously determined for *N. ossifragum* leaves collected during June-August 1997 from other areas in Møre og Romsdal county (Flåøyen *et al.*, 2000). On the other hand sapogenin levels comparable to those reported in Table 2.1 have recently been found in samples from Jøsokdalen (80-90 m a.s.l.) on Gurskøy in Møre og Romsdal county (Ryste, 2001). The detailed and systematic information required to understand the ecological factors which may influence temporal and spatial genin variations in *N. ossifragum* leaves is not yet available (Mysterud, 2001).

2.3.3 Slettfjellet (Control) and Skarven (Outbreak) Pastures

At the time of sampling, faeces from 18 of the 19 lambs grazing the Skarven outbreak pasture, including the 3 lambs which showed external alveld symptoms, and 16 of the 18 Slettfjellet control pasture lambs were found to possess total sapogenin levels (after hydrolysis of conjugated sapogenins) in the range 5000-36500 mg/kg DM. A complete tabulation of the levels of sapogenins found in faecal material recovered from the rectums of the Slettfjellet control pasture (area A) and Skarven outbreak pasture (area B) lambs is given in Table 2.2.

Levels less than 5000 mg/kg DM can be interpreted as indicating that, at least in the preceding 4 day (96 h) period, the lambs had not consumed a significant quantity of *N. ossifragum* plants. Flåøyen *et al.* (2001) have previously shown that the daily consumption of *N. ossifragum* plant materials leads to the presence of a consistent level (steady-state) of sapogenins in faeces collected *ca.* 96 h and onwards after the commencement of a dosing experiment.

Table 2.2. Individual and total levels of genins (mg/kg DM) found in lamb faecal samples from Slettfjellet control pasture (area A) and Skarven outbreak pasture (area B) in the Halsa/Surnadal study area July 8th 2001.

Sheep	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Total
area A							
A1001	97	822	632	1547	5446	7307	15853
A1003	136	1170	1068	2048	8692	9228	22342
A1007	194	1946	1516	2751	14156	15751	36314
A1008	118	1016	1210	2811	12164	14560	31878
A1009	110	944	715	1548	6789	7863	17969
A1015	107	696	773	2243	6304	9003	19126
A1024	87	622	281	2049	2243	11933	17213
A1025	78	720	517	1377	5488	6534	14714
A1030	80	886	546	2576	6164	13787	24040
A1031	70	498	192	1415	1278	7644	11097
A1037	143	974	729	2279	6378	10717	21220
A1038	81	680	691	961	5754	3626	11793
A1052	120	795	701	3490	5818	14340	25264
A1054	117	1435	1485	2905	14704	13965	34611
A1055	83	519	205	0	1526	261	2594
A1056	44	338	348	1029	3527	5849	11135
A1059	74	375	228	499	1217	1545	3939
A1060	79	373	189	2616	1004	8383	12644
Mean	101	823	668	1897	6036	9016	18541
STD	34	403	421	918	4197	4520	9491
% CV	34	49	63	48	70	50	51
area B							
B1001	73	502	150	1139	1044	5177	8085
B1002	73	513	193	1199	1959	7186	11123
B1007	81	408	138	1271	793	3976	6668
B1008	51	372	113	1124	801	4828	7288
B1009	57	499	783	1496	9552	8345	20731
B1011	175	1356	674	1745	7882	9747	21579
B1013	122	962	563	1846	7155	10996	21643
B1015	74	513	296	1858	2293	8384	13418
B1016	67	687	471	119	6018	1156	8518
B1017*	128	843	364	1338	3345	5996	12015
B1018*	135	1154	583	1822	6126	10779	20600
B1021	169	1171	872	1627	9592	7216	20647
B1022	72	625	523	1988	7000	10008	20217
B1023	97	841	195	1768	1760	10133	14794
B1026	145	1068	938	2611	9604	14652	29019
B1027	118	764	135	1666	1141	7792	11616
B1032	95	606	161	1925	1306	9076	13169
B1038	64	536	165	0	1641	249	2654
B1039*	175	1330	442	815	4832	3235	10830
Mean	104	776	408	1440	4413	7312	14453
STD	41	314	270	631	3303	3607	6796
% CV	40	40	66	44	75	49	47

* indicates external alveld symptoms at time of sample collection; Smil-CO = smilagenone (mg/kg DM); Sar-CO = sarsasapogenone (mg/kg DM); Smil = smilagenin (mg/kg DM); Epismil = epismilagenin (mg/kg DM); Sar = sarsasapogenin (mg/kg DM); Episar = episarsasapogenin (mg/kg DM); Total = total genin (mg/kg DM); % Smil = percentage smilagenin of total genin; % Sar = percentage sarsasapogenin of total genin; STD = standard deviation; % CV = percent coefficient of variation. Values <100 rounded to 2 s.f.

The faecal sapogenin levels (12015, 20600 and 10830 mg/kg DM) determined for samples from the rectums of the 3 Skarven outbreak pasture lambs (B1017, B1018 and B1039) which showed external alveld symptoms were comparable with the widely varying sapogenin levels determined for other lambs grazing the Skarven outbreak and Slettfjellet control pastures (Figure 2.6) excluding those lambs which analytical data showed had recently consumed very little *N. ossifragum* plant material.

No obvious relationship between total sapogenin levels in rectum faecal samples and external alveld symptoms was apparent amongst lambs grazing the Skarven outbreak and Slettfjellet control pastures. Amongst lambs with faecal sapogenin levels greater than 5000 mg/kg DM, the mean sapogenin level determined for 16 lambs grazing the Skarven outbreak pasture (15109 mg/kg DM) was appreciably lower than that determined for 18 lambs grazing the Slettfjellet control pasture (20451 mg/ kg DM). This variation may however be attributable to the varying incidence of *N. ossifragum* plants in and/or the edibility of other grazing plants in the respective pastures.

2.3.4 Mørkdalen (Control) and Skorven (Outbreak) Pastures

6 days after the detection of external alveld symptoms in lambs grazing the Skarven outbreak pasture (area B) external alveld symptoms were also detected among 3 lambs (lambs D1018, D1026 and D1125) grazing the Skorven outbreak pasture (area D). All of these lambs later died during the grazing season. No external alveld symptoms were apparent amongst the lambs grazing the Mørkdalen control pasture (area C), both at the time of sampling or later in the grazing season. A complete tabulation of the levels of sapogenins found in faecal material recovered from the rectums of the Mørkdalen control pasture (area C) and Skorven outbreak pasture (area D) lambs is given in Table 2.3.

Table 2.3. Individual and total levels of genins (mg/kg DM) found in lamb faecal samples from Mørkdalen control pasture (area C) and Skorven outbreak pasture (area D) in the Halsa/Surnadal study area July 14th 2001.

Sheep	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Total
area C							
C1005	118	851	105	0	558	360	1992
C1009	105	638	134	301	769	1179	3126
C1010	126	1043	1071	1697	8732	6450	19119
C1018	88	751	317	1576	2406	7363	12502
C1019	108	689	306	2004	2372	7583	13062
C1021	96	516	133	1865	685	6575	9871
C1022	58	283	72	78	283	283	1058
C1028	78	396	61	0	321	116	973
C1029	62	404	149	1000	970	3992	6578
C1034	1039	1131	997	950	9249	4882	18248
C1035	166	1517	946	3623	7887	17600	31739
C1063	103	641	192	2221	1710	8573	13440
C1064	68	575	297	1938	1844	10002	14724
C1074	108	717	674	2206	4740	10401	18846
C1075	115	923	327	2728	2396	13777	20265
C1079	103	691	176	199	1513	972	3654
C1080	98	702	226	2183	1795	10191	15195
C1081	0	1410	639	1423	10308	11409	25189
C1082	112	617	756	3375	4887	11599	21346
C1083	140	730	339	1576	2199	5027	10011
C1090	77	665	906	1879	6930	8494	18951
C1091	144	860	187	1943	1167	7772	12073
Mean	142	761	410	1580	3351	7027	13271
STD	203	302	334	1025	3202	4681	8216
% CV	144	40	81	65	96	67	62
area D							
D1001	242	2633	581	4231	4112	14830	26630
D1002	143	1143	987	882	7664	3860	14677
D1005	192	1890	1542	1259	13158	7272	25312
D1006	158	1456	1316	2447	10402	12311	28090
D1015	184	1654	1235	1635	10521	7551	22780
D1016	208	2560	1353	1257	9568	5964	20910
D1018*	159	963	1781	2046	12470	9352	26770
D1026*	20	99	95	177	473	551	1414
D1035	100	786	673	1475	8587	9725	21346
D1036	138	1176	1072	1549	13327	10705	27966
D1037	198	2626	1477	2509	12728	13674	33213
D1038	109	1223	1431	2041	12244	11222	28270
D1046	169	1223	917	2658	6885	12546	24399
D1050	197	1045	1566	4347	10178	16842	34175
D1065	189	1174	1526	2662	12241	12579	30371
D1090	162	1081	1902	2907	15340	12464	33857
D1091	141	1181	562	3665	5102	17808	28460
D1094	105	752	898	3107	7165	12129	24156
D1095	336	2956	1227	2332	10106	10991	27948
D1097	193	1793	719	1350	5767	6496	16318
D1116	181	1496	1748	3370	12012	14714	33522
Mean	168	1472	1172	2281	9526	10647	25266
STD	61	719	469	1088	3645	4240	7616
% CV	37	49	40	48	38	40	30

* indicates external alveld symptoms at time of sample collection; Smil-CO = smilagenone (mg/kg DM); Sar-CO = sarsasapogenone (mg/kg DM); Smil = smilagenin (mg/kg DM); Epismil = epismilagenin (mg/kg DM); Sar = sarsasapogenin (mg/kg DM); Episar = episarsasapogenin (mg/kg DM); Total = total genin (mg/kg DM); % Smil = percentage smilagenin of total genin; % Sar = percentage sarsasapogenin of total genin; STD = standard deviation; % CV = percent coefficient of variation. Values <100 rounded to 2 s.f.

No faecal material was present for collection in the rectum of lamb D1125 at the time of sampling. The sapogenin levels determined for faecal samples recovered from the rectums of lambs D1018 and D1026 were 26700 and 1414 mg/kg DM respectively. The very low faecal sapogenin level determined for lamb D1026 shows that during the past 96 h this lamb had consumed little *N. ossifragum* plant material, possibly as consequence of its ill-health and/or the development of an adverse reaction to its consumption. Although Ryste (2001) in his analyses of *N. ossifragum* saponin intakes suggested that lambs that did not develop the disease might have experienced a subclinical reaction and then reduced *N. ossifragum* intake the following days, there is no definitive evidence that this might be the case. Lamb D1018 which exhibited external alveld symptoms at the time of sampling shows genin levels comparable to sapogenin levels determined for other lambs grazing the Skorven outbreak pastures (Figure 2.6). Faecal genin levels for lambs grazing the Mørkdalen control pasture (area C) whose total genin levels were greater than 5000 mg/kg DM are presented in Figure 2.6.

No relationship between total sapogenin levels in rectum faecal samples and external alveld symptoms was apparent amongst lambs grazing the Skorven outbreak pasture. Faecal sapogenin levels for lambs grazing the Skorven pasture, which did not show external symptoms of alveld, were in the range 14500-34500 mg/kg DM (average level 26459 mg/kg DM). On the other hand a lesser average faecal sapogenin level (16539 mg/kg DM) was determined for lambs grazing the Mørkdalen control pasture, excluding 4 lambs, which appeared to have consumed little *N. ossifragum* during the past 96 h. This finding, which contrasts with our finding (above) that the mean sapogenin level for faecal material from grazing the Slettfjellet control pasture (20451 mg/ kg DM) was significantly greater than that determined for lambs grazing the Skarven outbreak pasture (15109 mg/kg DM), may however be attributable to the varying incidence of *N. ossifragum* plants and/or the edibility of other grazing plants in the respective pastures.

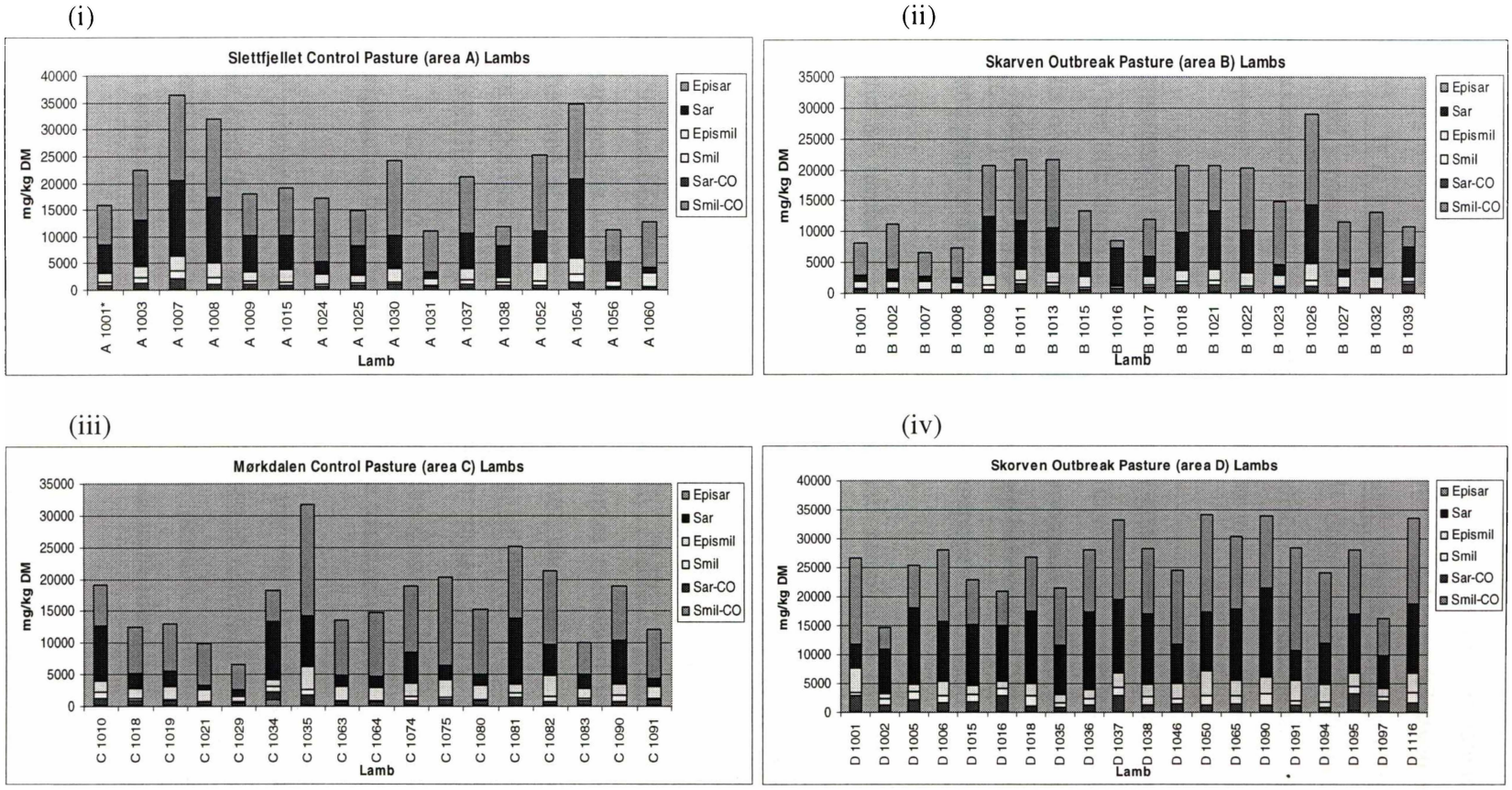


Figure 2.6. Sapogenin levels (mg/kg DM) determined for faecal material recovered from the rectum of lambs grazing; (i): Slettfjellet control pasture (area A), (ii): Skarven outbreak pasture (area B), (iii): Mørkdalen control pasture (area C) and (iv): Skorven outbreak pasture (area D).

2.4 General Discussion

The results presented (Tables 2.2, 2.3 and Figure 2.6) are consistent with results from previous dosing trials carried out under controlled conditions (Flåøyen *et al.*, 2001; Loader *et al.*, 2003). It is apparent that under natural summer grazing conditions, sapogenins (smilagenin and sarsasapogenin) are also partly converted to episapogenins (epismilagenin and episarsasapogenin) via the intermediate keto-sapogenins (smilagenone and sarsasapogenone), during passage through the digestive tracts of the sampled lambs.

While the conversion of sapogenins to episapogenins is implicated in the deposition in the liver and in the bile ducts of the birefringent crystalloid material, identified as mainly the calcium salts of epismilagenin and episarsasapogenin glucuronides (Holland *et al.*, 1991, Miles *et al.*, 1991, 1993, 1994a), no relationship between the observation of external alveld symptoms and plant or faecal sapogenin or episapogenin concentrations was observed.

Seven of the 81 lambs (8.6%) monitored and sampled in this investigation developed symptoms of alveld. Given the occurrence of similar elevated levels of sapogenins (determined after hydrolysis of parent saponins) in *N. ossifragum* leaves from the Skarven outbreak and Slettfjellet control pastures (mean levels of 8992 and 9055 mg/kg DM respectively) (Table 2.1), and the Skorven outbreak and Mørkdalen control pastures (mean levels of 6791 and 6688 mg/kg DM respectively) (Table 2.1), and coupled with the variable levels of sapogenins identified in faecal samples recovered from the rectums of lambs which at the time of sampling did, and did not, show external symptoms of alveld, it is unlikely that saponins alone were the sole cause of alveld. Outbreaks of alveld have always occurred sporadically (Flåøyen *et al.*, 2000, Ryste, 2001). It seems obvious that *N. ossifragum* is involved in the aetiology of the disease, but it cannot be confirmed that saponins originating from the plant are the sole toxic principles.

The results presented in this chapter support the view that *factors other than total genin levels alone contribute to the patho-physiological reactions that characterise alveld.* To better understand the pathogenesis of alveld (periodic outbreaks, with increased risk of outbreak in some areas) the other factor(s) needs to be identified.

2.5 Conclusions

The principal conclusions from the investigation reported in this chapter are:

(i) The sapogenin content of *N. ossifragum* leaves from the 2 control and 2 outbreak pasture areas ranged from 4553 to 10879 mg/kg DM. No significant statistical differences in the mean sapogenin content from the 2 control and 2 outbreak pasture areas were found.

(ii) Total faecal sapogenin levels determined for lambs grazing the 2 control and 2 outbreak areas ranged from 973 to 36314 mg/kg DM. No obvious relationships between faecal sapogenin levels of lambs exhibiting external alveld symptoms and lambs not exhibiting symptoms could be drawn.

(iii) The *N. ossifragum* and faecal sapogenin data does not support the hypothesis that saponins are the sole causative agent for the development of alveld disease symptoms in lambs grazing *N. ossifragum* containing pastures.

2.6 Acknowledgements

The investigation reported in this chapter, supported by the Fund for Small Stock and Poultry Enterprise and by the Møre og Romsdal County, Norway, was initiated by Ivar Mysterud (1. Amanuensis), Department of Biology, University of Oslo, Norway and administered by the county agronomist Eivind Ryste. Special thanks go to Tor Stokke and Anne Botten Stokke who willingly and without any compensation let their sheep graze in the Skarven Mountain area during the project. Thanks goes to members of Valsøyfjord and Åsskard grazing units for their hospitality and help during field sampling; cand. scient. Sverre Hasvold, cand. scient. Gry Koller, graduate students Arild Jakobsen, Sigrid Nortvedt and Mette Vang for assistance with fieldwork. Dr Arne Flåøyen, National Veterinary Institute, Oslo, for veterinary advice and Professor Ørnulf Borgan, Institute of Mathematics, University of Oslo for statistical advice.

Chapter Three

Scottish *Narthecium ossifragum*

3.1 Introduction

Narthecium ossifragum (bog asphodel) is a saponin containing plant implicated in the hepatogenous photosensitization of sheep (Flåøyen, 1999) in Northern Europe (Section 1.10). Most reported outbreaks of *N. ossifragum* associated photosensitization of sheep originate from Norway where the disease is known as alveld (Ender, 1955; Ceh and Hauge, 1981; Abdelkader *et al.*, 1984; Flåøyen, 1999). There are also reports however of *N. ossifragum* associated photosensitization of sheep grazing *N. ossifragum* containing pastures in the Faroe Islands (Flåøyen *et al.*, 1995), Scotland and the North of England (Ford, 1964).

While there is some knowledge of the levels of saponins (steroidal sapogenin glycosides) and steroidal sapogenins in Norwegian collections of *N. ossifragum* (see Chapters 2 and 4), there is no information concerning the levels of steroidal sapogenins in Scottish collections of *N. ossifragum.*

During the ISOPP6, (International Symposium of Poisonous Plants No.6, Scotland, July 2001), delegates visited the Scottish Agricultural Council's (SAC) Auchtertyre Farm and were introduced to high country farming practices on the property. During the visit delegates viewed several photosensitized sheep (see Figure 3.1) that had recently been removed from a pasture which included appreciable quantities of *N. ossifragum*, some of which were flowering. It was noted that on some occasions 5% of sheep of the 2500 sheep on the SAC farm may become photosensitized.

Figure 3.1. Two recently photosensitized sheep removed from *N. ossifragum* pastures on the SAC Auchtertyre farm, July 2001.

It was of interest to ascertain if *N. ossifragum* in the pasture had elevated levels of saponins and sapogenins which may have been involved in the photosensitization of the sheep. Also of interest was to look at the levels of both free sapogenins and conjugated sapogenins (saponins) present in various parts of the *N. ossifragum* collected, for example, the leaves, stems, flowers and roots. It was envisaged this information would help add to the sapogenin and saponin knowledge of *N. ossifragum* and the likely role they and the plant play in the photosensitization of livestock.

This chapter reports the levels of free and conjugated sapogenins (saponins) found in *N. ossifragum* plants collected from Auchtertyre Farm, Strathfillian, West Perthshire, Scotland, during the ISOPP6 field trip.

3.2 Methodology

3.2.1 Plant Collection and Storage

Plant material (leaf, stems, flower heads and roots) were collected on August 8th 2001 from 2 sites on Auchtertyre Farm, Strathfillian, West Perthshire, Scotland (Figure 3.2), adjacent to the access road leading to the West Highland Railway Line bridge, *ca.* 100 and 150 m above the farm workshop area (sites 1 and 2, young, recently grazed plants) and from a

hill-side slope *ca.* 500 m above the railway bridge (site 3, mature plants that had not been grazed). Plant samples were frozen within 6 h of collection, and maintained at freezer temperature, other than for the period (10 h) they were transported to the National Veterinary Institute, Oslo, Norway, where they were refrozen and freeze-dried. Dirt was removed from root samples by careful washing with distilled water, and towelled dry with tissue paper prior to freeze drying.

Figure 3.2. Hillside grazing pasture typical of the Auchtertyre Farm. Insert: Pasture close-up of flowering *N. ossifragum.*

3.2.2 Free and Conjugate Plant Genin Analyses

Accurately weighed sub-samples (*ca.* 0.20-0.25 g) of the freeze-dried plant material were extracted with a SoxTech system HT 1043 Extraction Unit (Tecator, Sweden) apparatus for 3 h with CH_2Cl_2 (50 mL) to afford extracts containing free sapogenins. The extracts were blown down to dryness under a stream of warm nitrogen and acetylated at room temperature for *ca.* 16 h using pyridine-acetic anhydride (1:1) (0.5 mL). After overnight drying of the extraction thimbles, they were extracted with MeOH (50 mL) to afford extracts containing conjugated sapogenins (saponins). MeOH extracts were concentrated to *ca.* 10 mL on the SoxTech extractor and transferred, with MeOH washing (3 × 3 mL), to boiling tubes and blown down to dryness under a stream of warm nitrogen. 0.5 $molL^{-1}$ HCl (15 mL) was added to the boiling tubes which were heated in a water bath

for 90 min at 85-90°C. After cooling, hydrolysate solutions were extracted with CH_2Cl_2 (4 × 4 mL) and filtered through a short alumina column (*ca.* 3 cm) packed in a Pasteur pipette. The combined extracts were evaporated to dryness under a stream of warm nitrogen. Extracts were acetylated at room temperature for 16 h using pyridine-acetic anhydride (1:1) (0.5 mL) and all extracts (free and conjugate) were analyzed using the SIM GC-MS protocol described below.

3.2.3 GC-MS Analyses and Peak Identification

Quantitative GC-MS analyses of the acetylated extracts were performed using selected ion mode (SIM) protocols as previously reported (Flåøyen & Wilkins, 1997; Wilkins *et al.*, 1994; Loader *et al.*, 2003). These protocols, when applied to acetylated steroidal sapogenin samples utilizes the intense *m/z* 139 ion which is typically the base peak in the mass spectrum of steroidal sapogenins lacking a ring E or F substituent group (see Chapter 2, Figure 2.3).

Sarsasapogenin propionate was added as the internal standard to all the acetylated extracts. Sapogenin acetate peaks were identified by comparison of retention times of authentic standards. All calculations were performed using purpose-written Excel spreadsheets.

3.3 Results and Discussion

3.3.1 Genin Content of *N. ossifragum* Plant Material

The dry matter (DM) content and levels of free and conjugated sapogenins determined for the freeze-dried plant materials are presented in Table 3.1. Confirmation of the presence of smilagenin and sarsasapogenin acetates (25*R*- and 25*S*-epimers respectively of the same sapogenin; Figure 3.3) in the acetylated extracts was established by comparison of total ion chromatogram mode mass spectra *m/z* 255/315, *m/z* 269/329 and *m/z* 284/344 ion ratios (Wilkins *et al.*, 1994) and retention time data for the acetylated sapogenins and for authentic specimens of smilagenin and sarsasapogenin acetates.

Table 3.1. % Dry matter (DM) and free and conjugated sapogenin levels (mg/kg DM) determined for *N. ossifragum* plant samples.

Site/Sample	Extract	% DM[a]	Smil	Sar	Total
site 1 (young plants)					
leaf (0-15 cm)	free	34.3	tr	tr	tr
	conj		1050	993	2040
flower heads	free	75.4	3	55	58
	conj		8140	10250	18380
stems	free	52.7	tr	tr	0
	conj		26	28	54
roots	free	54.1	145	687	832
	conj		44	167	211
site 2 (young plants)					
leaf (0-15 cm)	free	44.6	tr	tr	tr
	conj		2790	1830	4620
site 3 (mature plants)					
leaf (0-10 cm)	free	30.4	tr	tr	tr
	conj		75	36	111
leaf (10-20 cm)	free	30.8	tr	tr	tr
	conj		2120	1450	3570
leaf (20-30 cm)	free	40.2	tr	tr	tr
	conj		1870	1410	3280
leaf (bulk)	free	32.3	6	12	18
	conj		1260	1030	2290
stems	free	36.0	12	20	32
	conj		23	24	47
flower heads	free	57.5	24	32	56
+ stems	conj		42	30	72
roots	free	62.0	341	841	1180
	conj		14	54	68

Smil = smilagenin (mg/kg DM); Sar = sarsasapogenin (mg/kg DM); Total = total genins (mg/kg DM); tr = trace amount.

[a] DM percentages reflect partial drying of collected plant material before the freeze-drying process.

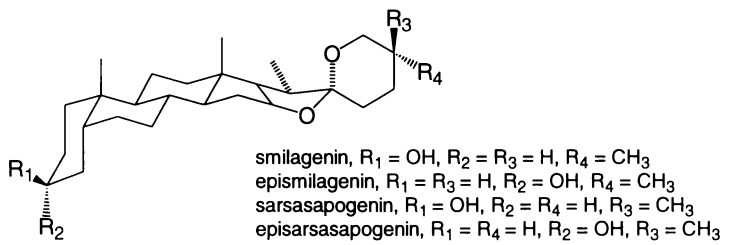


Figure 3.3. Chemical structures of smilagenin, epismilagenin, sarsasapogenin and episarsasapogenin.

No seasonal variation studies on sapogenin/saponin levels exist on Norwegian or Scottish *N. ossifragum*. Recent research has shown that the *N. ossifragum* contribution to pasture plants in grazing rangelands, and the levels of saponins in *N. ossifragum* plants when grazed may be highly variable (Ryste, 2001). Since plant material was collected from only 2 sites on a single Scottish farm and at a single time of year, caution must be exercised when comparing the characteristics of Norwegian and Scottish collections of *N. ossifragum*. It has been proposed however, that saponin levels in *N. ossifragum*, and other saponin containing plants implicated in the photosensitization of livestock, are elevated around the time of and during the outbreaks of photosensitization (Flåøyen *et al.*, 2004). It is known that weather stress (drought, temperature, frost etc) can increase toxin concentrations in many plants (Ralphs, 2002).

The total sapogenin levels reported here in the Scottish *N. ossifragum* pasture, collected in early August 2001, are discernibly lower when compared to total genin levels found in Norwegian *N. ossifragum* leaves from pastures in the Halsa/Surnadal area in Western Norway collected in early-mid July 2001 (Chapter 2). Both the Scottish and Norwegian pastures where the respective plant material was collected contained sheep which exhibited external symptoms of photosensitization. However due to the lack of correlated topographical and environmental data, caution must be exercised when comparing data from the 2 respective sites.

The *ca.* 45-65% contribution of smilagenin to total sapogenins determined for the Scottish collections of *N. ossifragum* (Table 3.1) can be compared to the typical *ca.* 10-15% contribution of smilagenin to total sapogenins in Norwegian collections (Flåøyen *et al.*, 2001; Loader *et al.*, 2003; see Chapters 2 and 4). Miles *et al.* (1991) have suggested that, following the ovine metabolism of sapogenins to episapogenins, epismilagenin (25*R*-epimer) may be more lithogenic (crystal-forming) than episarsasapogenin (25*S*-epimer) (Figure 3.3), hence possibly more damaging to livestock and increasing the risk of the onset of photosensitization symptoms. Deng (1999) also noted that 25*R*-saponins appeared to be 2-6 times more lithogenic than 25*S*-saponins. Again it must be noted that due to the lack of seasonal sapogenin/saponin variation data, conclusions and comparisons must be approached with caution.

One intriguing aspect of the results was the detection of significantly higher levels of free sapogenins, compared to conjugated sapogenins, in the roots of the plants from sites 1 and 3 (832 and 1184 mg/kg DM respectively). It is tempting to speculate that sapogenins are initially synthesized in the roots and following glycosylation, they are transported around the plant, for example to the leaf tips and flower heads, where they may possibly act as antifungal agents (Flåøyen *et al.*, 2004). More detailed studies are however required to validate this hypothesis.

3.3.2 Comparison with *Nolina texana*

A further intriguing aspect of the data was the detection of a substantial level of conjugated sapogenins (saponins) in the flower heads of young plants from site 1 (*ca.* 18000 mg/kg DM). This data can be compared to the free and conjugate genin levels that Deng (1999) reported for leaves, stems and blossoms of *N. texana*. Although Deng (1999) found only moderate levels of free and conjugate genins in the samples he examined, the greatest levels were found in the blossoms. The stems had only low levels of conjugated sapogenins and the leaves contained no sapogenin material at all.

The buds, blossoms and seeds of *N. texana* have been reported to be highly poisonous to sheep and goats (Tunnicliff, 1929; Mathews, 1937; Mathews, 1940; Hershey, 1945; Rankins *et al.*, 1993) with suggestions (Mathews (1937, 1940; Rankins *et al.*, 1993) that saponins present in flower stalks and seed pods of sacahuiste plants (*N. microcarpa, N. texana*) may contribute to toxicosis when ingested by livestock. It is interesting to note, in respect of Mathews' (1940) observation, that toxicity outbreaks have been reported for sheep grazing both leaves and blossoms of *Nolina* species, whereas no toxicity reports for cattle, which mainly graze the leaves, have appeared.

Although the leaves of the collected Scottish *N. ossifragum* did not have highly elevated sapogenin levels when pooled with the very high sapogenin levels found in the flowers, the combination may have lead to sheep grazing the SAC pasture ingesting a substantial level of lithogenic sarsasapogenin and smilagenin saponins, during the flowering season period.

3.4 Conclusions

The principal conclusions from the investigation reported in this chapter are:

(i) The presence in Scottish *N. ossifragum* samples, from a pasture on which sheep were photosensitized, of elevated levels of conjugated steroidal sapogenins has been established.

(ii) Total levels of free and conjugated steroidal sapogenins found in Scottish *N. ossifragum* leaf samples are comparable to those present in summer collections of Norwegian *N. ossifragum.*

(iii) Highly elevated levels of sapogenins were found in the flowers of Scottish *N. ossifragum* (*ca.* 18000 mg/kg DM).

(iv) Significant levels of free sapogenins were found in the roots of the plants from sites 1 and 3 (832 and 1184 mg/kg DM respectively).

(v) The percentage contribution of smilagenin (a 25*R*-genin) in the Scottish samples was typically 2-4 times greater than is the case for Norwegian collections of *N. ossifragum.*

3.5 Acknowledgements

Special thanks to Prof. Alistair Wilkins (University of Waikato), Dr Arne Flåøyen (National Veterinary Institute (NVI), Oslo) and Dr Ivar Mysterud (Biology Department, University of Oslo) for collection of *N. ossifragum* plant material and to Dr Arne Flåøyen for his hospitality and collaboration at the NVI.

Chapter Four

Isotope-Dilution Methodology
- Field Trial -

4.1 Introduction

Hepatogenous photosensitization of animals grazing plants containing steroidal saponins is both an animal welfare problem and economically important in many parts of the world (Flåøyen *et al.*, 1996). At least 10 steroidal saponin-containing plants have been implicated in hepatogenous photosensitization diseases of sheep grazing these plants (Flåøyen, 2000). Features common to these diseases are their sporadic occurrence, the difficulty of reproducing disease symptoms during dosing experiments (Flåøyen *et al.*, 1996; Kellerman *et al.*, 1991) and the accumulation of mainly calcium salts of episapogenin conjugates in the liver and bile ducts (Holland *et al.*, 1991; Miles *et al.*, 1991, 1992a/b, 1993, 1994a/b)

Narthecium ossifragum (bog asphodel) is a saponin-containing plant associated with sheep hepatogenous photosensitization in Norway, the British Isles and the Faroe Islands (Flåøyen *et al.*, 1996; Flåøyen, 1999, 2000). Reproduction of typical photosensitization symptoms in sheep has been performed by dosing with crude saponin extracts from *N. ossifragum*, but the administered doses, which at least at the time of the experiments, were believed to have been unrealistically large and far beyond those that probably could have been ingested by grazing (Ender, 1955; Abdelkader *et al.*, 1984). In a study where doses of *N. ossifragum* plant material were administered daily to lambs for 21 days, toxicity and liver damage did not occur (Flåøyen *et al.*, 1991). At the time, the *N. ossifragum* doses administered (20 g wet wt/kg live-weight) were thought to be comparatively large with respect to intakes thought to be experienced by lambs under natural conditions. However, based on results presented in this chapter, it is now believed that the amount of *N. ossifragum* dosed daily to the lambs in the 1991 study was low compared to natural intakes

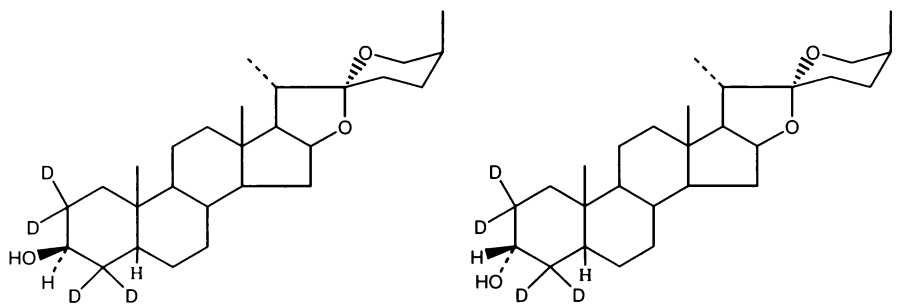
that can be experienced on some pastures. An attempt to reproduce the typical lesions in the liver by dosing with pure sarsasapogenin and diosgenin also failed (Flåøyen *et al.*, 1993). These attempts to reproduce alveld experimentally have been troubled by the lack of knowledge concerning the level of steroidal saponins ingested by sheep during natural toxicity outbreaks (Flåøyen *et al.*, 1991).

One step in the clarification of this process is to measure the intake of steroidal saponins in sheep under normal grazing conditions and the doses necessary to induce toxic responses, i.e. photosensitization symptoms. Flåøyen *et al.* (2001) proposed that by dosing sheep once daily with a known amount of a deuterated sapogenin, that were additionally fed *N. ossifragum* and determining the ratio of deuterated to non-deuterated (natural) sapogenins in faeces, an estimate of the amount of sapogenin originating from the ingested plant material could be accomplished. To succeed, this approach requires the deuterated substrate to retain a high percentage of its incorporated deuterium atoms during dosing and subsequent analytical procedures. A presupposition to this approach is the ratio of deuterated to non-deuterated sapogenins in faeces remains reasonably constant and is not sensitive to changes in the time of collection, or the time of dosing and ingestion of saponins and sapogenins.

Flåøyen *et al.* (2001) attempted to exploit this approach using 20,23,23-$^{2}H_3$-sarsasapogenin, but failed since the 20- and 23-deuterium atoms were lost (exchanged with ^{1}H atoms) during ruminal metabolism and/or the chemical analytical procedures. Similarly, we reported (Loader *et al.*, 2003) the retention of only 1 or 2 of the 4, initially present, deuterium atoms when 2,2,4,4-$^{2}H_4$-sarsasapogenone was dosed to a single sheep. In contrast a 1:4 mixture of 2,2,4,4-$^{2}H_4$-sarsasapogenin and 2,2,4,4-$^{2}H_4$-episarsasapogenin, administered to a single sheep, showed retention of >94% of the introduced deuterium atoms in sapogenins recovered from faeces samples (Loader *et al.*, 2003). A copy of the Loader *et al.* (2003) paper is presented in Appendix V. An important observation from the dosing trial conducted by Flåøyen *et al.* (2001) was that *ca.* 96 h after the commencement of the trial, in which the sheep were additionally fed *N. ossifragum* as well as being dosed 20,23,23-$^{2}H_3$-sarsasapogenin, essentially 'steady state levels' of sapogenins were present in the faeces collected at varying times throughout the trial period.

An account of the validation dosing trial calculated on a mass basis has been reported (Loader, 2001) where 3 sheep were dosed with 72 g DM of *N. ossifragum* plant material and a 1:4 mixture of 2,2,4,4-$^2H_4$-sarsasapogenin and 2,2,4,4-$^2H_4$-episarsasapogenin once daily for 14 days. Subsequent re-calculation of the results using the % deuterium ion ratio (isotope-dilution) approach has now been submitted for publication (in *Vet. Res. Commun.*). The text of the submitted paper is included in Appendix VI. The results obtained validate the isotope-dilution methodology and demonstrate that it can be applied under field conditions to estimate the uptake of plant sapogenins where only a rectum sample, collected once daily would be available and the total daily amount of faecal material can not be ascertained.

Reported here are results concerning the chemical aspects of a field dosing trial where 4 ewes (mothers) and 11 lambs (4 sets of twins and 3 weaned lambs) were dosed once daily with a 1:4 mixture of 2,2,4,4-$^2H_4$-sarsasapogenin and 2,2,4,4-$^2H_4$-episarsasapogenin (Figure 4.1) and were allowed to graze a natural pasture containing *N. ossifragum*. Results obtained from the dosing experiments are the subject of report in two separate theses. Evrind Ryste, at the time a Masters candidate in the Department of Husbandry, Agricultural University of Norway, Ås, Norway, was responsible for the daily dosing of the ewes and lambs, collection of faecal material, dispatch of samples for chemical analyses, and the veterinary aspects of the trial. Veterinary aspects of the dosing experiments and estimates of the daily consumption of *N. ossifragum* (based on the chemical data presented below) expressed as 'grams DM per day per kilogram live-weight of animal' have been reported by Ryste (2001) in his thesis.



2,2,4,4-$^2H_4$-sarsasapogenin 2,2,4,4-$^2H_4$-episarsasapogenin

Figure 4.1. Structures of 2,2,4,4-$^2H_4$-sarsasapogenin and 2,2,4,4-$^2H_4$-episarsasapogenin.

4.2 Methodology

4.2.1 Trial Period

The sheep trial was conducted between June 5th to 26th, 2000, in the Jøsok Valley, Gurskøya, Møre og Romsdal County, Norway. Ethical consent for the trial was obtained by Dr Arne Flåøyen, National Veterinary Institute, Oslo, Norway. The fenced off grazing area used in the trial was a north facing 2.5 hectare pasture, 80-90 m a.s.l. The vegetation was typical for the west coast of Norway. *Calluna vulgaris* (common heather) together with *N. ossifragum* were the dominating plants. The trial was conducted before the *N. ossifragum* started blossoming. The lower part of the grazing area was wet and moss covered.

4.2.2 Animal Description

Involved in the trial were 4 ewes with un-weaned twin lambs (total 8 lambs, born between 08/04/00 and 03/05/00) and 3 weaned lambs that were bottle-fed since birth (born between 11/04/00 and 15/04/00). All sheep and lambs were Norwegian White Sheep breed. The bottle-fed lambs were off the milk before the trial started and the sheep were kept on cultivated pastures before commencement of the trial. All animals were dosed with anthelmintica (parasite treatment) (Panacur®) 1 day before the trial begun.

4.2.3 Dosing Trial

Registrations started at 9.00 am every day throughout the experimental period, the sheep were mustered, faecal samples collected and all sheep were dosed via intraruminal intubations with the deuterated sapogenin mixture (1:4 mixture of 2,2,4,4-$^2H_4$-sarsasapogenin and 2,2,4,4-$^2H_4$-episarsasapogenin). The deuterated sapogenin was dissolved in 50 mL of a 1:4 mixture of ethanol and water. The tube was rinsed twice with water after dosing but prior to its removal from the rumen to ensure that all material was dosed. The ewes received 80 mg per day of deuterated sapogenin and the lambs 40 mg per day, every day from day 1 to 21.

Faecal samples (5-10 g) were collected daily from the rectum of all animals on days 1 to 22. The faecal samples were frozen at -20°C. It was not possible to get faecal samples from some animals on some days (no faeces in the rectum). All animals were weighed on days 1 and 20, and all lambs were in addition weighted on day 10.

Blood samples (10 mL) were taken from the jugular vein every day from day 1 to 22. Blood samples were not analyzed for saponin and sapogenin content. Information relating to the blood samples and results from the blood analysis have been reported (Scheie *et al.*, 2003).

4.2.4 Plant Material

A minimum of 25 g *N. ossifragum* was collected from 5 sites on the pasture on each of days 1, 10 and 20 of the dosing trial. Leaves only were collected since the flower stems had not started to grow. All leaves were collected within a 1 m diameter at each site, and the same sites were sampled on the 3 collection days. The plant material was kept frozen at -20°C until transported frozen on an overnight service to the National Veterinary Institute, Oslo.

Samples of freeze-dried leaf from each site and day (0.20-0.30 g) were extracted with MeOH (50 mL) for 4 h using a SoxTech system HT 1043 Extraction Unit (Tecator, Sweden) apparatus, which yielded a total sapogenin extract (i.e. free and conjugate sapogenins). The resulting extracts were evaporated, dried and hydrolyzed at 90-95°C for 90 min using 0.5 $molL^{-1}$ HCl (10 mL) to cleave glycosyl (and other) conjugated residues. The resulting hydrolysate solutions were extracted with CH_2Cl_2 (3 × 5 mL), filtered through a short alumina column (*ca.* 3 cm) packed into a Pasteur pipette, evaporated to dryness under a stream of warm nitrogen and acetylated for *ca.* 16 h with a 1:1 mixture of acetic anhydride:pyridine (0.5 mL).

4.2.5 Faecal Samples

All faecal samples collected were freeze-dried and had their dry matter (DM) content determined.

4.2.6 Total Genin Analyses

Freeze-dried faecal samples (0.5-0.7 g) were SoxTech extracted for 4 h with MeOH (50 mL). The extracts were concentrated to dryness and the resulting residues hydrolyzed at 90-95°C for 90 min using 0.5 $molL^{-1}$ HCl (10 mL). The resulting hydrolysate solutions were extracted with CH_2Cl_2 (3 × 5 mL), filtered through a short alumina column (*ca.* 3 cm) packed into a Pasteur pipette, evaporated to dryness under a stream of warm nitrogen and acetylated as described above for the plant samples.

4.2.7 Free and Conjugate Genin Analyses

On days 6, 13 and 20, freeze-dried faecal samples (0.5-0.7 g) were sequentially extracted with 50 mL CH_2Cl_2 (free genins) followed by 50 mL MeOH (conjugated genins) using a SoxTech apparatus. The CH_2Cl_2 extract was evaporated to dryness and acetylated for *ca.* 16 h with a 1:1 mixture of acetic anhydride:pyridine. The MeOH extract was subjected to the hydrolysis procedure described above and acetylated for *ca.* 16 h with a 1:1 mixture of acetic anhydride:pyridine (0.5 mL).

4.2.8 GC-MS Analyses

Acetylated samples were freeze-dried and forwarded to the University of Waikato, New Zealand, for determination of their genin content by SIM GC-MS. Sarsasapogenin propionate was added as the internal standard to all of the acetylated extracts.

Quantitative analyses of sapogenin levels were performed using the SIM GC-MS protocol previously reported (Wilkins *et al.*, 1994; Flåøyen and Wilkins, 1997; Loader *et al.*, 2003). This protocol when applied to acetylated sapogenin samples utilizes the intense *m/z* 139 ion which is typically the base peak in the mass spectrum of a steroidal sapogenin lacking a ring E or F substituent group and arises from cleavage across ring E. See Chapter 2, Figure 2.3 (Blunden *et al.*, 1980; Wilkins *et al.*, 1994).

Sapogenin acetate peaks were identified by comparison of the retention times of authentic standards. The percentage deuterium (% D) content of sapogenins found in faecal samples was determined according to Loader *et al.* (2003) which utilizes the *m/z* 315-320 ion cluster. A detailed account of the methodology utilised in this investigation has been

reported in a Masters thesis (Loader, 2001). A copy of the paper reporting the development and evaluation of the methodology (Loader *et al.*, 2003) is included in Appendix V.

4.3 Results and Discussion

4.3.1 Genin Content of *N. ossifragum* Leaves

The mean dry matter (DM) content of the collected plant material was determined to be 18.3%. The levels of smilagenin (25*R*-genin), sarsasapogenin (25*S*-genin) and total genins (mg/kg DM) found in the *N. ossifragum* sub-samples are presented in Table 4.1 The mean total genin content was 7310 mg/kg DM (STD = 966 mg/kg DM). The mean percentage contribution of sarsasapogenin (25*S*-genin) to total genins was found to be 91%, i.e. 6656 mg/kg DM.

Table 4.1. Levels of smilagenin, sarsasapogenin and total genins (mg/kg DM) found in collected *N. ossifragum* material from the 5 collection sites.

Day	Site	Smilagenin	Sarsasapogenin	Total	% Sarsasapogenin
1	1	714	6553	7268	90
	2	669	6458	7127	91
	3	480	5884	6364	92
	4	563	6119	6682	92
	5	554	5308	5862	91
	Mean Day 1	596	6064	6661	
10	1	664	5152	5816	89
	2	671	6205	6876	90
	3	811	6774	7585	89
	4	663	7975	8638	92
	5	910	7834	8744	90
	Mean Day 10	744	6788	7532	
20	1	704	6882	7586	91
	2	654	7367	8021	92
	3	591	7335	7925	93
	4	515	6011	6526	92
	5	649	7984	8633	92
	Mean Day 20	623	7115	7738	
	Mean	654	6656	7310	91
	STD	110	908	966	0.0
	% CV	17	14	13	1.4

4.3.2 Alveld Development

No mother ewes developed alveld symptoms during the dosing trial. 5 of the lambs involved in the trial did however exhibit physical signs of alveld and were subsequently euthanised as follows:

- Lamb-67 showed mild signs of photosensitization on day 5. The signs were not present on days 6-9. Symptoms reoccurred on day 10 and the lamb was euthanised on day 11.
- Lamb-54 showed signs of photosensitization on day 13 and was killed on the same day.
- Lamb-121 showed signs of photosensitization on day 15 and was killed on the same day.
- Lamb-128 showed signs of photosensitization on day 16 and was killed on the same day.
- Lamb-27 showed signs of photosensitization on day 18 and was killed on the same day.

Gastrointestinal tract samples (rumen, omasum, abomasum, duodenum, jejunum, ileum, caeceum, colon and rectum) of euthanised lambs were analyzed for free and conjugated genins. They were not subjected to deuterium ion ratio analyses, and are not reported in this thesis. However, a significant post-mortem observation of the euthanised lambs was the amount of *N. ossifragum* plant material present in their rumens. The rumen content consisted predominantly of *N. ossifragum* material, which could indicate that *N. ossifragum* was the dominant plant ingested in the days preceding death (Wilkins, *pers commun*).

4.3.3 Levels of Genins in Faeces

Representative dosing trial results are presented below for a mother ewe and her twin lambs, namely ewe-8012, lamb-26 and lamb-27. Lamb-26 did not develop alveld symptoms whereas lamb-27 developed alveld symptoms on day 18 and was euthanised on day 18. Tabled data for remaining ewes and lambs involved in the trial can be found in Appendix I.

4.3.4 Absolute Genin Levels

The total levels of free and conjugated natural and deuterated smilagenone, sarsasapogenone, smilagenin, epismilagenin, sarsasapogenin and episarsasapogenin genins (mg/kg DM) found in faeces of ewe-8012, lamb-26 and lamb-27, on days 1 to 22 are presented below in Table 4.2 and Figure 4.2. On days 6, 13 and 20 analyses were run for both free and conjugated faecal genin levels (mg/kg DM) , whereas on the remaining days only total genin analyses were undertaken. Reproducibility and accuracy of the extraction and GC-MS analysis procedure was demonstrated. For example, triplicate sample extractions and analyses for lamb-26, day 16 faeces showed a coefficient of variation (CV) of 2.6%, whilst duplicate GC-MS injections for the ewe-952, day 21 and 22 samples, afforded genin levels which agreed to within ± 3.4% and ± 1.4% respectively.

Table 4.2. Total genin levels (mg/kg DM) found in ewe-8012, lamb-26 and lamb-27 faeces sampled on days 1 to 22. Lamb-27 developed alveld.

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar[a]	Episar	Sub T	Total
Ewe-8012								
1	-	-	15	-	316	133		464
2	-	72	32	-	450	669		1222
3	-	-	-	-	-	-		-
4	105	1252	1145	-	22785	8102		33389
5	52	641	662	-	14589	6525		22469
6 free	69	754	577	-	13190	6503	21092	
6 conj	-	34	13	-	222	299	567	21659
7	69	632	623	-	11995	5364		18683
8	58	485	354	-	7414	4170		12482
9	89	860	272	-	5522	3678		10421
10	42	434	277	-	6290	4346		11389
11	73	564	503	-	9623	5277		16040
12	78	869	1181	-	22550	7247		31925
13 free	130	1477	1513	-	28190	9611	40922	
13 conj	-	24	11	-	196	222	454	41376
14	126	1411	1368	-	25298	8310		36514
15	162	1688	1107	-	19768	7663		30387
16	75	817	692	-	13092	7038		21715
17	61	642	339	-	7744	6744		15529
18	129	1362	446	-	9182	6637		17756
19	92	1383	883	-	19308	9893		31558
20 free	81	1555	909	-	24264	10546	37354	
20 conj	-	26	10	-	173	251	460	37814
21	75	1141	944	-	20858	8399		31417
22	120	1415	1027	-	21208	8200		31970

cont...

Table 4.2 cont...

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar[a]	Episar	Sub T	Total
Lamb-26								
1	-	-	-	-	28	36		64
2	-	20	-	-	47	138		204
3	-	103	16	111	224	900		1354
4	40	392	4	-	651	651		1740
5	29	174	55	-	974	1596		2828
6 free	25	318	80	38	1029	3380	4865	
6 conj	-	20	7	-	105	234	369	5234
7	-	551	524	-	10004	7075		18153
8	81	1054	720	-	14889	7488		24232
9	110	1242	736	-	15201	7095		24384
10	43	720	356	-	8166	7280		16564
11	61	750	391	-	8011	7699		16912
12	128	1180	688	-	12619	8322		22936
13 free	77	1217	823	-	17254	11954		
13 conj	-	-	-	-	-	-		31324
14	120	1481	886	-	18405	13131		34023
15	80	1146	438	-	10153	11409		23226
16	110	1231	746	-	14210	11094		27391
17	73	1048	508	-	10921	11925		24476
18	134	1277	605	-	12286	10436		24739
19	72	1096	689	-	15373	11765		28993
20 free	59	945	574	-	14204	14533		
20 conj	-	-	-	-	-	-		30315
21	67	882	548	-	13076	12732		27304
22	54	647	521	-	11196	11732		24151
Lamb-27								
1	-	-	-	-	-	-		-
2	-	-	-	-	31	134		164
3	-	15	-	-	76	252		343
4	13	120	14	139	231	808		1324
5	78	831	359	-	7123	6223		14614
6 free	69	1211	933	-	20650	10046	32909	
6 conj	3	28	12	-	210	439	693	33602
7	76	1028	645	-	14921	10886		27556
8	68	879	461	-	10409	7857		19674
9	167	1722	150	-	12458	8017		22513
10	124	1314	963	-	19545	11113		33059
11	147	1424	1151	-	20586	11988		35297
12	163	2294	1206	-	22498	11243		37404
13 free	144	1778	1193	-	24037	13818	40970	
13 conj	-	18	10	-	144	233	404	41374
14	145	1823	1305	-	25238	13325		41836
15	179	2050	926	-	19250	10979		33383
16	133	1488	953	-	18418	12111		33102
17	101	1231	674	-	13428	10818		26252
18	162	1596	320	-	5875	6837		14790

[a] contains *ca.* 10% contribution of epismilagenin, unless otherwise indicated; Smil-CO = smilagenone (mg/kg DM); Sar-CO = sarsasapogenone (mg/kg DM); Smil = smilagenin (mg/kg DM); Epismil = epismilagenin (mg/kg DM); Sar = sarsasapogenin (mg/kg DM); Episar = episarsasapogenin (mg/kg DM); free = free genins (mg/kg DM); conj = conjugated genins (mg/kg DM); Sub T = genin sub total; Total = total free and conjugated genins (mg/kg DM). Values <100 rounded to 2 s.f.

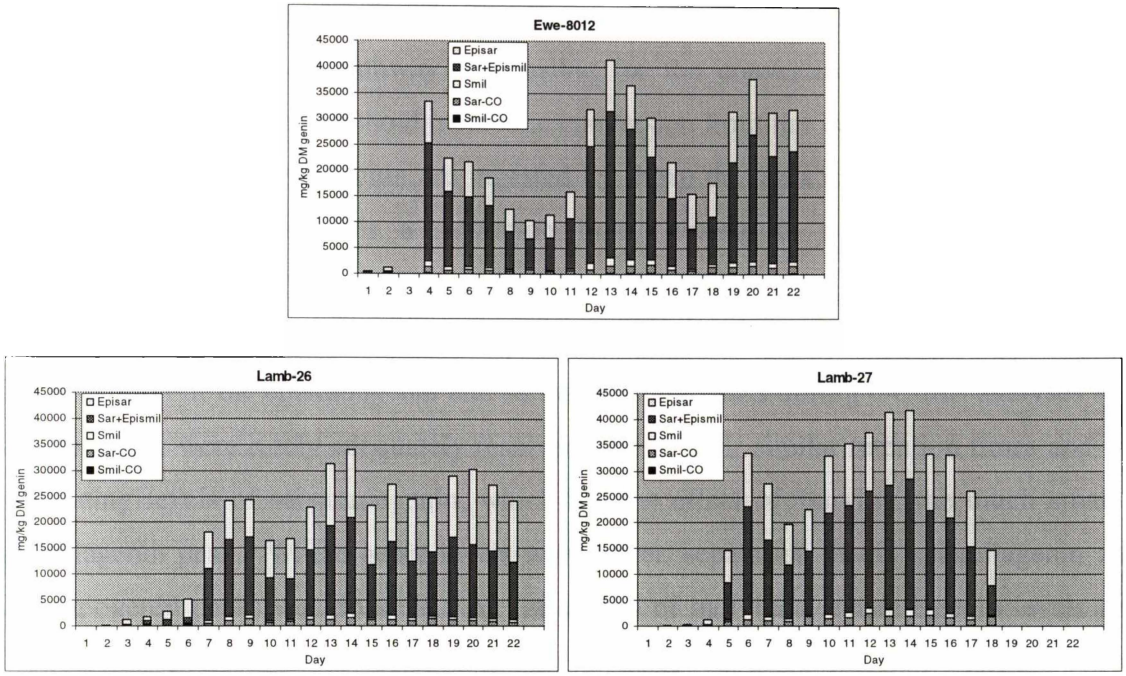


Figure 4.2. Sapogenin levels (mg/kg DM) in faeces for ewe-8012, lamb-26 and lamb-27 on days 1 to 22.

The mother ewes in the trial typically took 4-5 days for the total amount of genin in faeces to reach 'steady state levels', consistent with the results of Flåøyen *et al.* (2001) and Loader *et al.* (2003), whereas the lambs took 5-7 days to obtain steady state genin levels. This trend was consistent across all ewes and lambs involved in the trial. The reason for this is not obvious, however it can be speculated that the lambs took longer to recognise *N. ossifragum* as an acceptable forage plant than was the case for the older ewes.

An interesting observation was that sarsasapogenin was, on average, the major genin constituent found in faecal samples on day 4 onwards. From previous findings (Flåøyen and Wilkins, 1997; Flåøyen *et al.*, 2001; Loader *et al.*, 2003) episarsasapogenin, the ruminal metabolism product of sarsasapogenin (Miles *et al.*, 1993, 1994b), has been the dominant genin found in faecal samples from sheep grazing and/or ingesting *N. ossifragum* plant material. It has been shown (Flåøyen and Wilkins, 1997; Flåøyen *et al.*, 2001; Loader *et al.*, 2003) that when the level of ingested sarsasapogenin is low, the major genin detected in faeces is episarsasapogenin, indicating that the majority of the ingested sarsasapogenin has been ruminally metabolized to episarsasapogenin. It can be seen from the data in

Figure 4.2 that the level of episarsasapogenin remains relatively constant despite the widely varying total levels of genins found. This may, at least in part, be attributable to saturation of the sheep's metabolic pathway responsible for the conversion of sarsasapogenin to episarsasapogenin, i.e. a threshold metabolic conversion limit has been hit. Thus, as higher concentrations of sarsasapogenin are ingested by sheep the metabolic conversion pathway to episarsasapogenin reaches a saturation limit, leading to a higher percentage of sarsasapogenin in faeces.

The resolution of the epismilagenin and sarsasapogenin peaks during GC-MS analyses was such that they were rarely adequately resolved, due to their similar retention times and the dominating levels of sarsasapogenin, which has the effect of 'burying' the much smaller epismilagenin peak. Where genin levels were lower, separation of the epismilagenin and sarsasapogenin peaks was possible. An estimation of individual epismilagenin levels can however be performed by comparing the average ratio of the two ketone peaks, smilagenone and sarsasapogenone (the intermediates whereby sarsasapogenin and smilagenin are metabolized to their corresponding episapogenins). The ketone ratio is indicative of the ratio expected of smilagenin to sarsasapogenin and of epismilagenin to episarsasapogenin. It was estimated that the average ratio of smilagenone to sarsasapogenone was *ca.* 10%. In principle a correction factor could be applied to the level of sarsasapogenin in the overlapping epismilagenin/sarsasapogenin peak and epismilagenin levels can be obtained. Correction factors were not applied, since total genin levels were of interest, rather than a detailed analysis of individual components.

Low levels of sarsasapogenone and smilagenone were detected in the vast majority of the extracts (excepting day 1 samples). This is consistent with results from previous trials (Flåøyen and Wilkins, 1997; Flåøyen *et al.*, 2001; Loader *et al.*, 2003). It has been shown by Loader *et al.* (2003) that ovine metabolism of sapogenins does not result in the epimerization of 25*S*-genins (sarsasapogenin and episarsasapogenin) to 25*R*-genins (smilagenin and epismilagenin).

4.3.5 Deuterium Levels

Sapogenin levels were determined using previously reported methodology (Loader *et al.*, 2003). Ion profiling of the *m/z* 315-320 ion cluster determined for acetylated sarsasapogenin and episarsasapogenin (25*S*-genins) allowed the level of deuterium present in sapogenin material recovered from faecal samples to be calculated. The level of non-deuterated sarsasapogenin and episarsasapogenin was considered to correspond to the sum of the *m/z* 315 and 316 ion intensities, the latter arising predominantly from the ^{13}C isotope of the *m/z* 315 ion (Loader *et al.*, 2003). The amount of deuterated sarsasapogenin and episarsasapogenin contributing to the acetylated sarsasapogenin and episarsasapogenin peaks was assessed as the sum of the *m/z* 317-320 ion intensities of the total *m/z* 315-320 ion intensity. This approach assumes that the contribution from non-deuterated *m/z* 315 ion $^{13}C_2$ isotope species (i.e. *m/z* 317) to the total *m/z* 317 ion intensity is small and that the *m/z* 317 ion intensity arises primarily from dosed deuterated sarsasapogenin and episarsasapogenin containing two incorporated deuterium atoms.

Deuterated smilagenin and epismilagenin acetates (25*R*-genins) were not detected, which is consistent with earlier findings (Loader *et al.*, 2003). As discussed above (Section 4.3.4) the sarsasapogenin peak, unless otherwise indicated, contains an estimated 10% contribution from the 25*R*-genin, epismilagenin, due to unresolved and superimposed GC-MS peaks. This has the effect of raising the level of non-deuterated 25*S*-genins by *ca.* 5% overall, therefore lowering the % ratio of deuterated 25*S*-genins present in the sarsasapogenin and episarsasapogenin peaks. In turn, the lower % deuterium ratio would lead to slight overestimation of the amount of *N. ossifragum* plant material consumed daily by the ewes and lambs calculated using Equations (1) and (2) below (Section 4.3.6).

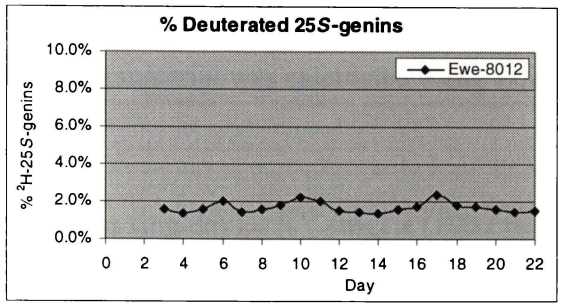
However, deuterium ion ratio analyses do not include the 25*S*-genin ketone peak of sarsasapogenone. Sarsasapogenone in faeces samples was routinely found to be *ca.* 10% of the sarsasapogenin genin levels. To a reasonable level of precision, the effect which epismilagenin exhibits to raise the level of non-deuterated 25*S*-genins (thus lowering the % deuterium ratio) is counterbalanced by the amount of sarsasapogenone that is not included in the ion profiling analyses. Overall, the effect on the non-deuterated 25*S*-genin level is small and no correction factors were applied to the ion profiling data.

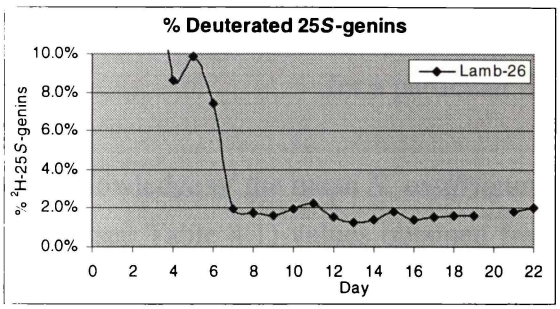
Percentage ratio contributions of deuterated 25*S*-genins to non-deuterated 25*S*-genins in faecal samples for ewe-8012, lamb-26 and lamb-27 are presented in Table 4.3 and Figure 4.3. Day 6, 13 and 20 percentage deuterium ratios are determined for the free sapogenin faecal extracts alone, not for the total (free and conjugate) sapogenin faecal extract as for the remaining days. Conjugate sapogenin levels found in faeces samples on days 6, 13 and 20 were too low to provide reliable deuterium ion ratio analyses.

Table 4.3. Percentage ratio of deuterated 25*S*-genins to non-deuterated 25*S*-genins in faecal samples for ewe-8012, lamb-26 and lamb-27.

Day	% Ratio $^{2}H_{n}$-25*S*-genins:25*S*-genins		
	Ewe-8012	Lamb-26	Lamb-27
3	1.5	19.1	59.2
4	1.4	8.6	8.6
5	1.6	9.9	2.3
6	2.0	7.4	2.8
7	1.5	1.9	2.2
8	1.6	1.7	2.0
9	1.8	1.6	2.1
10	2.2	2.0	1.9
11	2.0	2.2	1.9
12	1.5	1.5	1.3
13	1.5	1.3	1.3
14	1.4	1.4	1.4
15	1.6	1.8	1.5
16	1.7	1.4	1.4
17	2.4	1.5	1.4
18	1.8	1.6	1.9
19	1.7	1.6	
20	1.6	-	
21	1.4	1.8	
22	1.5	2.0	
Mean[a]	1.7	2.5	1.8

[a] mean calculated for day 5 onwards once steady state genin levels achieved; % Ratio $^{2}H_{n}$-25*S*-genins:25*S*-genins = percentage ratio of deuterated 25S-genins to non-deuterated 25*S*-genins, = $\sum$(*m/z* 317-320) / $\sum$(*m/z* 315-320), i.e. % deuterium ratio.





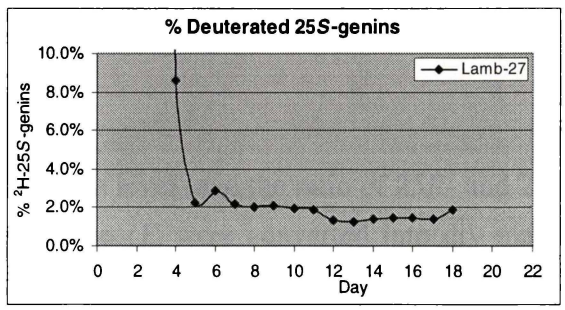


Figure 4.3. Percentage ratio of deuterated 25*S*-genins to non-deuterated 25*S*-genins in faecal samples for ewe-8012, lamb-26 and lamb-27 during the dosing trial period.

Percentage deuterium ratios from day 5 onwards were remarkably consistent for all ewes and lambs involved in the trial, with the occasional exception where some lambs took until day 6 or 7 to obtain steady state genin levels. A consistent observation for all animals is that the % deuterium ratio in faeces is initially elevated before a steady state genin level is achieved (Figure 4.3). This indicates that the dosed deuterated genins (which are in the free genin form and were dosed on day 1 prior to the animals being immediately released onto the grazing pasture) are initially passed through the sheep's metabolism faster than the natural genin material from *N. ossifragum*. This is reasonable, since the genins from ingested *N. ossifragum*, which are primarily conjugated genins, must be digested and extracted from the plant matrix before being the subject of metabolism and excretion into faeces, thus slowing their initial appearance in faecal matter. Whereas the free deuterated genins, which are unbound with respect to plant matrixes, would not be subjected to these initial processes.

4.3.6 Calculation of *N. ossifragum* Genin Content in Faecal Material

The amount of 25*S*-genins (mg/day), derived from consumed *N. ossifragum* material, present each day in sheep faecal material was calculated using Equation (1).

Equation (1):

Faecal 25*S*-genins (mg/day) = [(Y (mg) × (100 / % D ratio)] – Y (mg)

where: Y is the amount of deuterated material dosed daily to sheep
% D ratio is the percentage faecal 25*S*-genin deuterium ratio for a particular day

With knowledge of the mean *N. ossifragum* sapogenin level (and the ratio of 25*R*- and 25*S*-genins, see Table 4.1) values obtained from Equation (1) were converted into dry matter amounts of *N. ossifragum* plant material consumed each day (g/day DM) according to Equation (2).

Equation (2):

wt consumed (g DM/day) = [F (mg/day) / M (mg/kg DM)] × 1000

where: wt consumed is the dry matter weight of *N. ossifragum* plant material eaten by the sheep daily
F is the amount of faecal 25*S*-genins derived from *N. ossifragum*, calculated from Equation (1)
M is the mean level of 25*S*-genins in the *N. ossifragum* plant leaves consumed, i.e. 6656 mg/kg DM (see Table 4.1)
1000 is a multiplier to convert a level of mg DM/day to a level of g DM/day

With respect to Equation (2); dividing the amount of faecal 25*S*-genins (mg/day) derived from *N. ossifragum* [Equation (1)] by the mean *N. ossifragum* 25*S*-genin content (mg/kg DM), 6656 mg/kg DM (Table 4.1), an estimate is afforded of the daily dry matter (g) of *N. ossifragum* plant material consumed by each sheep (Table 4.4).

4.3.7 Calculated Daily *N. ossifragum* Intake

Calculated daily intakes of *N. ossifragum* plant material (DM/day) for ewe-8012, lamb-26 and lamb-27 are presented in Table 4.4.

Table 4.4. Daily % deuterated 25*S*-genins, faecal 25*S*-genins from *N. ossifragum* and calculated *N. ossifragum* plant material consumed for ewe-8012, lamb-26 and lamb-27 during the dosing trial period using Equations (1) and (2).

Day		% deuterated 25*S*-genins	Feacal 25*S*-genins from *N. oss* (mg/day)[a]	Calculated consumed *N. oss* (g DM/day)[b]
Ewe-8012				
3		1.5	5087	764
4		1.4	5669	852
5		1.6	4985	749
6		2.0	3859	580
7		1.5	5410	813
8		1.6	4951	744
9		1.8	4325	650
10		2.2	3485	524
11		2.0	3958	595
12		1.5	5293	795
13		1.5	5392	810
14		1.4	5733	861
15		1.6	5037	757
16		1.7	4499	676
17		2.4	3279	493
18		1.8	4351	654
19		1.7	4611	693
20		1.6	5008	752
21		1.4	5558	835
22		1.5	5246	788
	Mean[c]	1.7	4721	709
	STD	0.0	721	108
	% CV	17	15	15
Lamb-26				
3		19.1	169	25
4		8.6	426	64
5		9.9	364	55
6		7.4	497	75
7		1.9	2019	303
8		1.7	2249	338
9		1.6	2407	362
10		2.0	1999	300
11		2.2	1757	264
12		1.5	2555	384
13		1.3	3108	467
14		1.4	2752	413
15		1.8	2197	330
16		1.4	2856	429

cont...

Table 4.4 cont...

Day		% deuterated 25*S*-genins	Feacal 25*S*-genins from *N. oss* (mg/day)[a]	Calculated consumed *N. oss* (g DM/day)[b]
Lamb-26				
17		1.5	2571	386
18		1.6	2482	373
19		1.6	2443	367
20		-	-	-
21		1.8	2126	319
22		2.0	1928	290
	Mean[c]	2.5	2136	321
	STD	2.4	731	110
	% CV	94	34	34
Lamb-27				
3		59.2	28	4
4		8.6	426	64
5		2.3	1720	258
6		2.8	1364	205
7		2.2	1809	272
8		2.0	1949	293
9		2.1	1890	284
10		1.9	2034	306
11		1.9	2052	308
12		1.3	2962	445
13		1.3	3117	468
14		1.4	2771	416
15		1.5	2693	405
16		1.4	2720	409
17		1.4	2833	426
18		1.9	2112	317
	Mean[c]	1.8	2287	344
	STD	0.0	544	82
	% CV	25	24	24

[a] calculated using Equation (1); [b] calculated using Equation (2); [c] mean, STD and % CV calculated for day 5 data onwards. *N. oss* = *Narthecium ossifragum*.

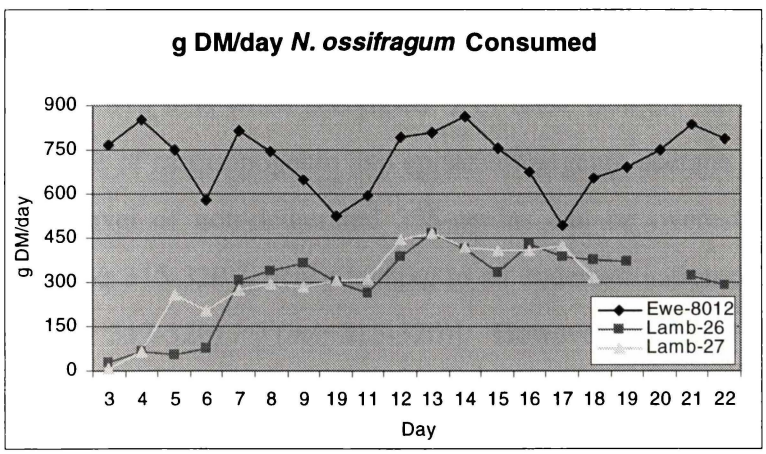


Figure 4.4. Calculated *N. ossifragum* plant material consumed (g DM/day) for ewe-8012, lamb-26 and lamb-27 during the dosing trial period using Equation (2).

Tabled data of daily % deuterated 25*S*-genins, faecal 25*S*-genins from *N. ossifragum* and calculated *N. ossifragum* plant material consumed for remaining ewes and lambs involved in the trial are presented in Appendix I.

A consistent trend, (illustrated for ewe-8012, lamb-26 and lamb-27, Figure 4.4), was that adult ewes consumed 2-3 times the amount, on average, of *N. ossifragum* material per day than was the case for lambs (g DM/day). On average the ewes consumed *ca.* 700-750 g DM/day of *N. ossifragum*, compared to *ca.* 200-250 g DM/day for the lambs involved in the trial. These daily levels of *N. ossifragum* consumption were higher than anticipated when the trial was designed, subsequently, lower than desired % deuterium ratio levels were found in faeces of *ca.* 2-3%. It was hoped that % deuterium ratios in the vicinity of 10-15% would have been achieved.

An advantage of larger % deuterium ratios is that a small change in % deuterium contributions has a modest effect when calculating total sapogenin levels, compared to the situation of an equivalent change in small % deuterium ratios, which can manifest in larger effects on the calculated total sapogenin levels. Hence, larger % deuterium ratios aid, to a greater extent, the buffering of possible interfering *m/z* 315-320 ion currents from dihydro-β-sitosterol isomers (Loader *et al.*, 2003). β-Sitosterol is a constituent of many plants grazed by animals and affords, via ruminal metabolism, 3 isomeric dihydroanalogues which exhibit significant *m/z* 315 ions when acetylated. 2 of these isomers have similar GC-MS retention times to those of sarsasapogenin and episarsasapogenin acetates. If GC-MS peak overlap occurs, the level of non-deuterated 25*S*-genins can be overestimated, [taken = $\sum$(*m/z* 315-316) / $\sum$(*m/z* 315-320)], hence leading to an underestimation of deuterated 25*S*-genins [taken = $\sum$(*m/z* 317-320) / $\sum$(*m/z* 315-320)]. However, provided care is exercised when adjusting GC-MS acquisition parameters, peak overlap can be minimized and interference greatly reduced (Loader *et al.*, 2003).

Due to the lower than anticipated % deuterium ratio levels found in faeces, it was apparent that in some cases the *m/z* 315 ion $^{13}C_2$ isotope contribution to the total *m/z* 317 ion intensity was significant, outweighing the *m/z* 317 ion contribution from deuterated material containing two deuterium atoms. This has the effect of overestimating the % deuterium ratio in faeces, hence underestimating the amount of *N. ossifragum* consumed daily by sheep and lambs. Correction factors can be applied to address this observation: (i) a correction for the *m/z* 315 ion $^{13}C_2$ isotope contribution (*ca.* 4.8 % of the *m/z* 315 ion intensity) and (ii) a correction for the $^{13}C_2$ isotope contribution from the *m/z* 319 ion (corresponding to deuterated material containing four deuterium atoms), i.e. including the *m/z* 321 ion intensity. However, applying these factors was not seen as critical due to the prime objective of the methodology being to determine daily intake levels to within 20-30%. It was also considered likely that variations in the distribution of the deuterium labelled sapogenins in faecal material would be subject to a greater uncertainty than that which might be addressed by the inclusion of $^{13}C_2$ isotope ion current corrections in the methodology.

It is apparent from the g DM/day results (Table 4.4 and Appendix I) that there is no obvious relationship between the amount of *N. ossifragum* consumed daily by lambs that developed alveld symptoms and the amount consumed by lambs that did not develop symptoms. For example, lamb-26 (Table 4.4) consumed an average of 321 g DM/day of *N. ossifragum* and did not develop alveld symptoms over the 21 day trial period, whereas lamb-128 (Appendix I) consumed an average of 198 g DM/day of *N. ossifragum* and developed signs of photosensitization on day 16 and was subsequently euthanised. No adult mother ewes developed photosensitization symptoms. On this basis, intakes based on live-weights were calculated.

4.3.8 Live-weight *N. ossifragum* Intake

Taking into account individual weights of the animals across the trial period, an estimate of the mean intakes of *N. ossifragum* dry matter (g) per day per kg live-weight can be obtained (g DM day/kg), see Table 4.5. Mother ewes showed a mean *N. ossifragum* intake of 9.8 g DM day/kg (STD = 1.8 g DM day/kg), healthy lambs (i.e. lambs that did not develop alveld symptoms) showed a mean *N. ossifragum* intake of 10.9 g DM day/kg (STD = 1.4 g DM day/kg) and lambs that developed alveld symptoms showed a mean *N. ossifragum* intake of

13.4 g DM day/kg (STD = 1.0 g DM day/kg). The mean *N. ossifragum* intake (g DM day/kg), presented in Table 4.5, for alveld lambs is calculated without inclusion of lamb-67 data. Lamb-67 developed alveld very early in the trial period (first signs of photosensitization seen on day 5) and had low *N. ossifragum* intake up until its death on day 11 (117 g DM/day, see Appendix I). Lamb-67 is therefore assumed to be an erroneous result.

Table 4.5. Intake of *N. ossifragum* per day per kilogram live-weight of sheep (g DM day/kg) for sheep involved in the trial.

Animal	Weight (kg) Day 1	Day 10	Day 20	Mean wt (kg)	Mean[a] *N. oss* g DM/day		Mean *N. oss* g DM day/kg
Ewes							
844	89	-	83	86	717		8.3
8056	65	-	69	67	700		10.4
952	106	-	98	102	847		8.3
8012	58	-	60	59	709		12.0
						Mean	9.8
						STD	1.8
						% CV	18
Healthy Lambs							
129	18	22	19	20	219		11.1
39	26	30	32	29	269		9.2
40	21	25	26	24	265		11.0
122	22	26	29	26	242		9.4
26	24	28	30	27	321		11.7
45	22	23	20	22	280		12.9
						Mean	10.9
						STD	1.4
						% CV	13
Alveld Lambs							
128	15	18	dead	17	198		12.0
121	21	24	dead	23	297		13.2
27	24	25	dead	25	344		14.0
54	18	17	dead	18	250		14.3
67	16	16	dead	16	117		7.3
						Mean[b]	13.4
						STD	1.0
						% CV	8

[a] means for day 5 data onwards, calculated using Equation (2); [b] mean, STD and % CV calculated omitting lamb-67 data. *N. oss* = *Narthecium ossifragum*.

The data in Table 4.5, at a 96% confidence level, show that the healthy lambs' *N. ossifragum* intake spans 9.5-12.3 g DM day/kg, compared to the alveld lambs' *N. ossifragum* intake of 12.4-14.4 g DM day/kg. Based on this, a definitive conclusion that lambs that developed alveld consumed, on average, more *N. ossifragum* (g DM day/kg) than lambs that did not develop alveld cannot be confidently drawn.

4.4 Conclusions and Future Work

(i) Until the present study no method has existed for measuring the daily uptake of saponins in animals grazing pastures containing steroidal saponin containing plants. The results obtained show that the isotope-dilution methodology of Loader *et al.* (2003) can be applied under field conditions to estimate the uptake of sapogenins from plant material where only a rectum sample collected once daily is available, and the total amount of daily faeces produced can not be ascertained.

(ii) Both lambs and ewes consumed higher than expected levels of *N. ossifragum* plant material (g DM/day), hence corresponding to higher intakes of saponins and genins than were originally anticipated.

(iii) Mother ewes, on average, consumed 2-3 times the daily weight of *N. ossifragum* (*ca.* 700-750 g DM/day) compared to that consumed by the lambs (*ca.* 200-250 g DM/day).

(iv) Percentage deuterium ratios, although lower than anticipated, were internally consistent across the animals involved in the trial and lay between *ca.* 2-3%.

(v) Mean live-weight *N. ossifragum* intakes were determined to be: 9.8 g DM day/kg for the mother ewes, 10.8 g DM day/kg for healthy lambs that did not develop alveld symptoms and 13.4 g DM day/kg for lambs that developed alveld symptoms (excluding lamb-67 data). The conclusion that lambs that developed alveld consumed more *N. ossifragum* per kg of live-weight (g DM day/kg) than lambs that did not develop alveld could not be definitively drawn.

(vi) Future field dosing trials utilizing the deuterium isotope-dilution methodology, should assume daily *N. ossifragum* consumption levels in the vicinity of *ca.* 700-750 g DM/day for adult sheep and *ca.* 200-250 g DM/day for lambs. The amount of deuterated genin substrate to be dosed daily should then be calculated prior to the commencement of the trial, taking into account average genin levels (mg/kg DM) of *N. ossifragum* in the pasture(s) to be grazed, to yield percentage deuterium ratios in faeces to the order of 10-15%.

Chapter Five

Synthesis

5.1 Introduction

In recent years, interest in new, safer and more effective antifungal, antibacterial and antiviral agents has grown and there has been a revival in traditional and natural products (as opposed to synthetic compounds) in medicine and in the preservation of food and cosmetics. In part, this is due to the increasing frequency of opportunistic human infections that affect immuno-compromised patients and the increasing incidence of drug-resistant strains to traditional antibiotics (De Lucca *et al.*, 2002; Escalante *et al.*, 2002; Kalemba and Kunicka, 2003). Despite the development, advances and significant benefits of antibiotics and the primary drugs used to combat such infections, much is left undesired, such as adverse side effects and toxicity. Also, plant pathogens, especially fungi, are responsible for yield reductions in crops around the world (Carpinella *et al.*, 2003). Although food and crop fungi can be controlled in one sense by the use of synthetic fungicides, they are partly responsible for the generation of toxic residues and are implicated in the development of pathogen resistance (Knight *et al.*, 1997; Wilson *et al.*, 1997).

Saponins are widely distributed throughout the natural world, especially the plant kingdom. They have been shown to possess a wide variety of biological activities (Section 1.5), including activity against human and plant pathogenic fungi (Flavel *et al.*, 1994; De Lucca *et al.*, 2002). For example, avenacins are a family of 4 structurally similar triterpenoid saponins that possess potent antifungal activity and are found in roots of a diploid oat species *Avena strigose* (Crombie *et al.*, 1986a; Osbourn, 2003). Avenacin A-1, the most abundant of the 4, fluoresces under UV light and can readily be visualised in the roots of young oat seedlings (Crombie *et al.*, 1986b). Papadopoulou *et al.* (1999) exploited this fact and isolated mutants from an avenacin-producing diploid oat species *A. strigose* that lacked the ability to synthesis avenacins. These saponin-deficient mutants showed compromised

resistance to a variety of fungal pathogens and evidence suggested this reduced disease resistance was a direct consequence of saponin deficiency (Papadopoulou *et al.*, 1999; Osbourn, 2003).

Although there is a large body of accumulated knowledge on the properties of saponins, much remains to be learnt about their structure-activity relationships (Bedir *et al.*, 2002; Favel *et al.*, 1994; Rao and Gurfinkel, 2000). Comparison and interpretation of accumulated data is difficult due to the variety of compounds, structures and diversity of biological activities tested.

In order to further explore and add to the knowledge of saponins, the synthesis of 3 steroidal saponins and 2 triterpenoidal saponins and their preliminary screening against some fungi and bacteria organisms was undertaken. It was envisaged that by comparing simple sapogenin glycosides an idea of their structure-activity relationships might be drawn if activity is shown. It was also of interest to investigate the activity of a monodesmosidic saponin and a bidesmosidic saponin, possessing the same genin backbone, for any difference in activity.

Sarsasapogenin saponins are present in *Yucca schidigera* (Mohave Yucca) and have been shown to have potent growth inhibitory activities against certain food deteriorating yeasts, film-forming yeasts and dermatophytic yeasts and fungi (Miyakoshi *et al.*, 2000). Mohave yucca extract is on sale in Japan as a food anti-deteriorating agent and is recognised as 'a safe food adjuvant' by the U.S. Food and Drug Administration (Miyakoshi *et al.*, 2000). Apart from other sarsasapogenin saponins present in Mohave Yucca and Norwegian *N. ossifragum*, a common monodesmosidic sarsasapogenin glycoside in both Mohave Yucca (Miyakoshi *et al.*, 2000) and Norwegian *N. ossifragum* (Deng, 1999) is 25*S*-5β-spirostan-3β-ol 3-*O*-β-D-xylopyranosyl-(1→3)-[β-D-glucopyranosyl-(1→2)]-β-D-galactopyranoside, which has been shown to possess antiyeast activity (Miyakoshi *et al.*, 2000).

The monodesmosidic steroidal saponin compounds synthesized in the present investigation were episarsasapogenin β-D-glucoside, episarsasapogenin β-D-galactoside and sarsasapogenin β-D-galactoside. It is of note that episarsasapogenin glycosides do not occur

naturally in Norwegian *N. ossifragum*, but arise after ruminal metabolism of sarsasapogenin saponins. Steroidal glycosides with an episarsasapogenin skeleton are not present in *Y. schidigera*.

Betulin (lup-20(29)-ene-3β,28-diol) is a well known triterpene and is used in the synthesis of many compounds that show varying degrees of bioactivity. It was envisaged that mono- and bidesmosidic glycosides could be readily prepared from this substrate.

Also of interest was the possibility that 1 or more of the synthesized saponins might be used as an internal standard or spike-recovery standard for the analysis of saponin containing materials. The majority of saponins that occur naturally in plants and in other natural media are monodesmosidic or bidesmosidic saponins containing 2 or more sugar residues. It can be envisaged that monodesmosidic saponins containing only a single sugar residue (e.g. a glucose or galactose residue) will elute from C-18 reverse phase LC columns after most naturally occurring, more polar, monodesmosidic or bidesmosidic saponins.

5.2 Synthesis of 2,3,4,6-Tetra-*O*-acetyl-α-D-glycopyranosyl bromides

5.2.1 Introduction

The conversion of a β-D-glucopyranose pentaacetate to the corresponding 2,3,4,6-tetra-*O*-acetyl-α-D-glycopyranosyl halide is a common and frequently encountered reaction and is the first step in the synthesis of β-D-glycosides. Axial (α-) orientation of the halide group is required for the formation of β-D-glycosides via the S_N2-like mechanism of the Koenig-Knorr reaction (Section 5.3).

Syntheses were undertaken using adaptations of the methodology reported by Deng (1999). 1,2,3,4,6-Penta-*O*-acetyl-β-D-glucopyranose or 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose, were reacted with HBr in glacial acetic acid at 6°C for 24-36 h (or until GC-MS analysis showed the reaction to be complete) to yield 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (Figure 5.1) or 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide respectively.

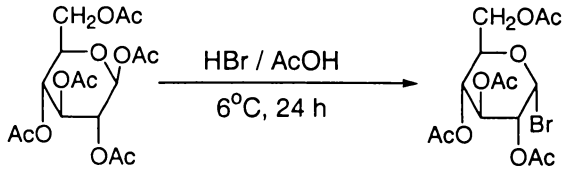


Figure 5.1. Reaction of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose with HBr.

The temperature at which the Koenig-Knorr type synthesis is performed is known to be a critical factor. Deng (1999) found that reaction of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose with 37% HBr in glacial acetic acid at room temperature (20°C) yielded 1,2,3,4,6-penta-*O*-acetyl-α-D-glucopyranose [Figure 5.2 (**A**)] and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide [Figure 5.2 (**B**)] as major products and only a minor amount of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (Figure 5.2).

Figure 5.2. Product structures (**A** and **B**) obtained from the reaction of 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose with HBr in glacial acetic acid at room temperature.

The ^{1}H NMR resonances of the H-1 (anomeric) protons of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide occurred at 6.58 (d, J = 4.0 Hz) and 6.70 (d, J = 4.0 Hz) ppm respectively. These coupling constants define the anomeric protons of these compounds to be equatorially oriented (Agrawal, 1992), hence their bromine atoms are axially orientated. Thus, replacement of the anomeric acetoxyl group by bromine proceeds with inversion at C-1. The α-bromo-acetyl-glucoside and galactoside were stable at 5°C and could be stored for several months in sealed vessels.

Complete ^{1}H and ^{13}C NMR assignments for 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide are given in Table 5.1. The data reported in Table 5.1 is consistent with data reported by Deng (1999) for 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide.

Table 5.1. ^{1}H and ^{13}C NMR signal assignments (ppm in $CDCl_3$), and COSY correlations determined for 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide.

Atom	2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide		2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide		COSY
	^{1}H	^{13}C	^{1}H	^{13}C	
1	6.58 (d, *J* = 4.0 Hz)	86.6	6.70 (d, *J* = 4.0 Hz)	88.2	H-2
2	4.80 (dd, *J* = 10.0, 4.0 Hz)	70.6	5.05 (dd, *J* = 4.0, 10.6 Hz)	67.9	H-1, H-3
3	5.52 (~t, *J* = 9.7 Hz)	70.2	5.41 (dd, *J* = 3.3, 10.6 Hz)	68.1	H-2, H-4
4	5.12 (~t, *J* = 9.9 Hz)	67.2	5.52 (dd, *J* = 1.3, 3.3 Hz)	67.1	H-3, H-5
5	4.27 (m)	72.2	4.48 (br t, *J* = 6.6 Hz)	71.1	H-4, H-6_A, H-6_B
6_A	4.30 (m)	60.9	4.11 (dd, *J* = 6.7, 11.4 Hz)	60.9	H-5, H-6_B
6_B	4.09 (m)		4.19 (dd, *J* = 6.4, 11.4 Hz)		H-5, H-6_A
$COCH_3$	1.99 (s)	20.5	2.01 (s)	20.6	
	2.01 (s)	20.5	2.05 (s)	20.6	
	2.06 (s)	20.6	2.11 (s)	20.7	
	2.06 (s)	20.6	2.15 (s)	20.8	
$COCH_3$		169.4		169.8	
		169.7		169.9	
		169.7		170.1	
		170.4		170.3	

5.3 Synthesis of the Steroidal β-D-glycosides

5.3.1 Introduction

A variety of methods for the preparation of β-D-glycosides have been reported. Formation of a glycoside bond is usually accomplished by reacting an α-D-acetoglycopyranosyl halide (normally the bromide or chloride) with a hydroxylic substance (in this case a sapogenin) in the presence of various catalysts, and is based on the Koenig-Knorr synthesis (Koenig-Knorr, 1901). Promotion of glycoside formation proceeds via a S_N2-like mechanism (Figure 5.3).

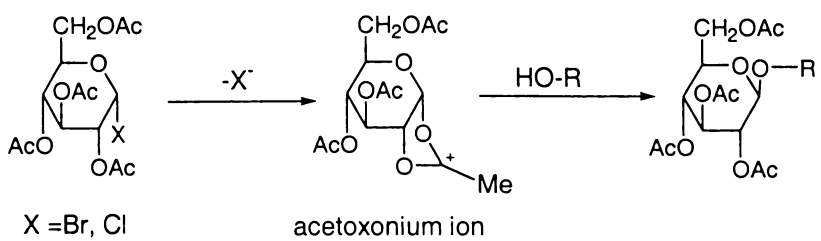


Figure 5.3. Reaction of α-glucosyl halides with an alcohol to form β-D-glucosides.

The oldest variant of the Koenig-Knorr synthesis utilizes insoluble silver catalysts such as Ag_2O and Ag_2CO_3 to promote glycoside formation and to neutralize the hydrohalogenic acid formed with the *O*-acetylglucosyl halide and the hydroxylic reagents.

Deng (1999) has reviewed some of the catalysts and solvents used in variants of this synthesis. Deng (1999) synthesized a series of sapogenin β-D-glycosides using cadmium carbonate ($CdCO_3$) as the glycosylation catalyst. $CdCO_3$ also facilitates the removal of any acid and water formed and/or present during the reaction. The synthetic procedure of Deng (1999) with minor modifications was utilized in this investigation.

The following steroidal saponins were synthesized:

(i) episarsasapogenin β-D-glucoside

(ii) episarsasapogenin β-D-galactoside

(iii) sarsasapogenin β-D-galactoside

In addition the triterpene saponins, betulin 3-(β-D-glucoside) and betulin 3,28-(β-D-diglucoside) were also synthesized. The structures of these saponins can be found on a foldout page in Appendix X.

Sarsasapogenin β-D-galactoside has been synthesized before (Kawasaki and Yamauchi, 1963), as with the 2 betulin β-D-glucoside compounds. However, it appears this could be the first time the synthesis of episarsasapogenin β-D-glucoside and episarsasapogenin β-D-galactoside is reported.

5.3.2 Synthesis of Episarsasapogenin β-D-glucoside

The sequence of reactions utilized in the synthesis of episarsasapogenin β-D-glucoside is shown in Figure 5.4.

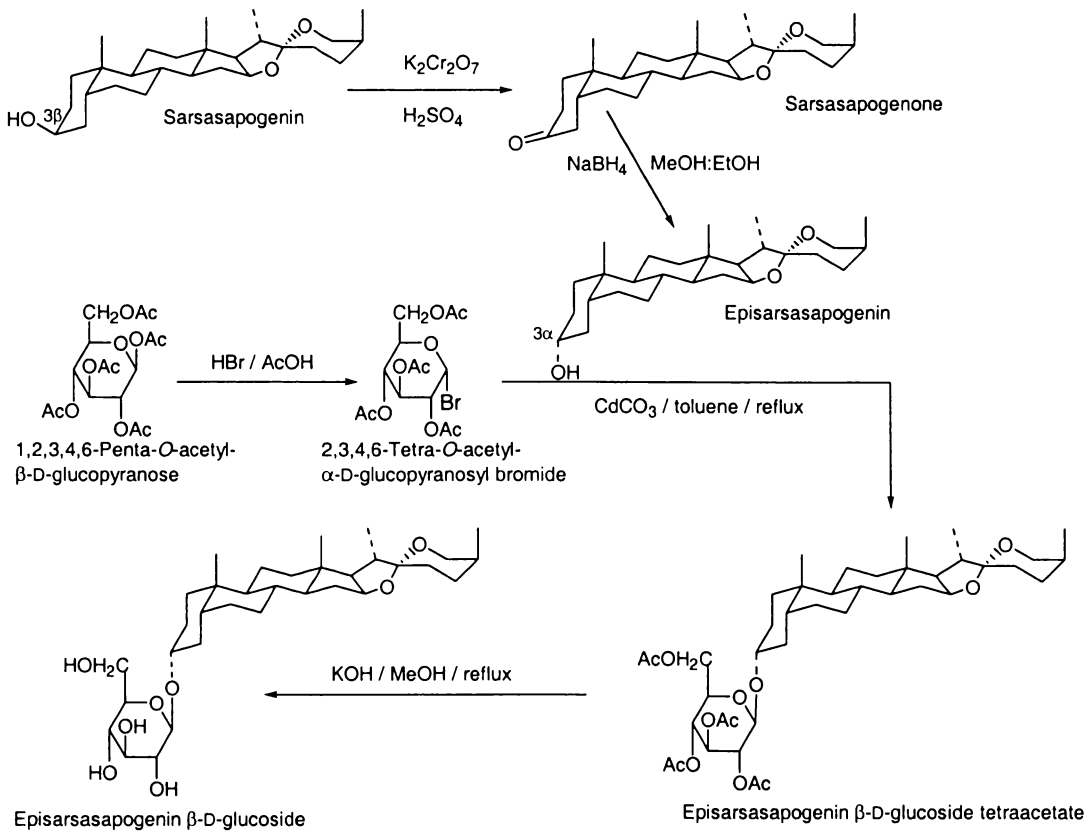


Figure 5.4. Reaction steps involved in the synthesis of episarsasapogenin β-D-glucoside.

Coupling of episarsasapogenin with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide was performed in toluene at reflux temperature, utilizing $CdCO_3$ as the catalyst (Deng, 1999). When TLC analyses showed the reaction to be complete (24-36 h), the reaction mixture was hot filtered to remove cadmium salts and solvent removed by rotary evaporation. Fractionation of the resulting residue by radial chromatography on silica gel using mixtures of petroleum spirits (40:60) and diethyl ether afforded episarsasapogenin β-D-glucoside tetraacetate.

5.3.2.1 Episarsasapogenin β-D-glucoside tetraacetate

The positive ion ES-MS of episarsasapogenin β-D-glucoside tetraacetate determined in MeOH:CH_2Cl_2 (1:1) as solvent, showed pseudo-molecular ions at *m/z* 769 $(M+Na)^+$ and 1515 $(M_2+Na)^+$. These ions are consistent with the molecular weight of the glucoside tetraacetate being 746 Daltons.

The ^1H NMR spectrum of episarsasapogenin β-D-glucoside tetraacetate, determined in $CDCl_3$, included 4 angular methyl signals, typical of sapogenins, at 0.74 ppm (s, H-18), 0.92 ppm (s, H-19), 0.99 ppm (d, J = 6.5 Hz, H-21) and 1.08 ppm (d, J = 6.8 Hz, H-27), 4 methyl signals corresponding to the sugar acetyl groups in the region 2.00-2.08 ppm and glucosyl proton signals and genin H-26 (CH_2-O) signals appeared in the region 3.20-5.30 ppm. The H-1' NMR signal of the anomeric glucosyl proton appeared as a sharp doublet at 4.59 ppm (J = 8.0 Hz). This coupling demonstrated that H-1' was 1,2-*trans*-diaxially orientated with respect to H-2' (Agrawal, 1992), and showed that the glycosidic linkage was therefore β-(equatorially) orientated.

The ^{13}C NMR spectrum of episarsasapogenin β-D-glucoside tetraacetate showed 41 carbon signals while the DEPT135 NMR spectrum showed the presence of 8 methyl (CH_3), 12 methylene (CH_2) and 14 methine (CH) signals. 27 of the ^{13}C NMR signals were attributable to genin carbons, 6 to glucosyl carbons and 8 to carbons to the 4 acetyl groups (which included 4 acetyl methyl signals in the region 20.6-20.8 ppm and 4 carbonyl signals in the region 169.4-170.7 ppm).

The H-2' to H-6' glucosyl resonances were identified via correlation pathways observed in the COSY spectrum (Figure 5.5), starting with the H-1' proton (4.59 ppm) and from signals observed in a series of 1D-SELTOCSY spectra (Figure 5.6) determined by selective excitation of the H-1' signal and the use of a range of correlation (mixing) times (30 to 150 msec). Glucosyl carbon resonances where subsequently identified via correlations observed in the HSQC ($^1J_{^{13}C\text{-}^1H\ \text{correlated}}$) spectrum between the glucosyl protons and the corresponding carbon atoms.

A significant advantage of the 1D-SELTOCSY experiment is that proton coupling pathways can be recognised much faster than in the corresponding 2D COSY and TOCSY experiment. By increasing the correlation times, progressive tracking of the proton spin system can be defined stepwise, for example (H-1') ↔ (H-2') ↔ (H-3') ↔ (H-4') ↔ (H-5') ↔ (H-6'$_A$) ↔ (H-6'$_B$) around a glucosyl ring, rather than the overall correlation result as afforded from a 2D experiment.

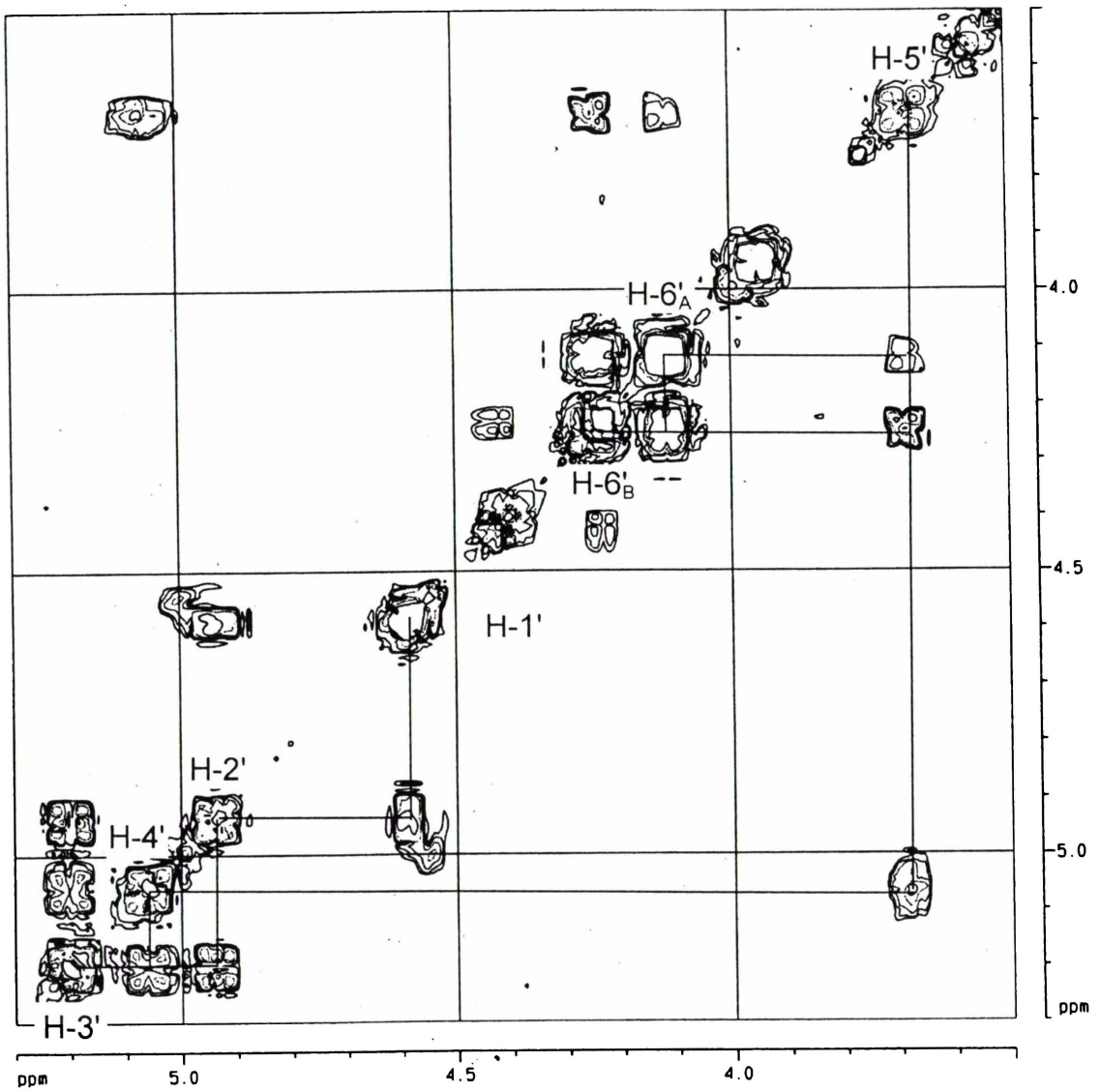


Figure 5.5. Glucosyl region COSY spectrum of episarsasapogenin β-D-glucoside tetraacetate illustrating the H-1' to H-6'$_A$/H-6'$_B$ correlations.

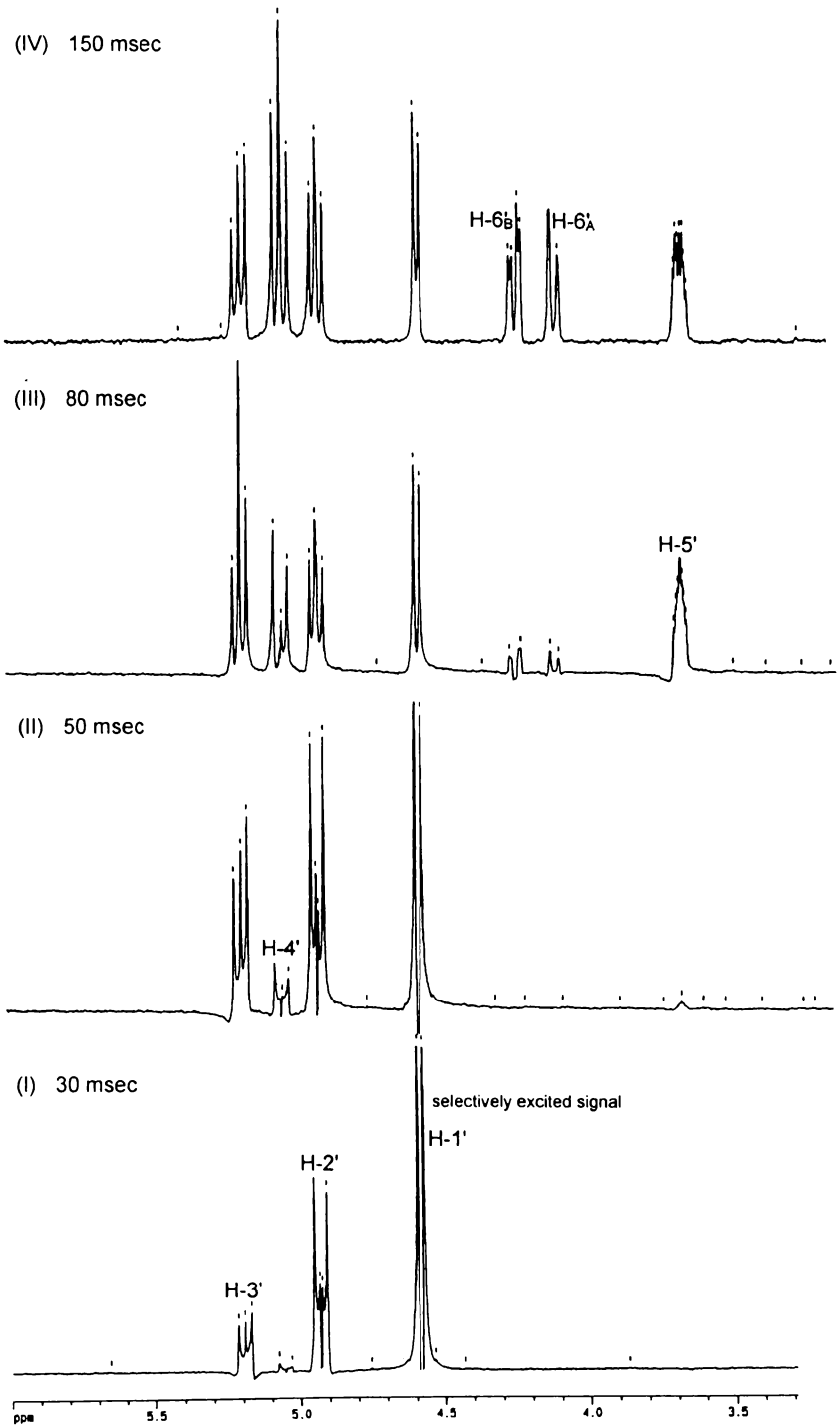


Figure 5.6. 1D-SELTOCSY spectra of episarsasapogenin β-D-glucoside tetraacetate. Selective excitation of the H-1' anomeric proton showing progressive tracking of glucosyl ring protons at a range of correlation (mixing times); (I) 30 msec, (II) 50 msec, (III) 80 msec and (IV) 150 msec.

HMBC correlations exhibited by the 4 genin methyl group resonances allowed their unambiguous assignment. 2 of the methyl group signals [0.74 ppm (s) and 0.99 ppm (d, J = 6.5 Hz)] showed correlations to C-17 (62.1 ppm). This identified the H-18 (0.74 ppm, s) and the H-21 (0.99 ppm, d, J = 6.5 Hz) resonances and showed that the remaining 2 methyl group resonances were attributable to H-19 (0.92 ppm, s) and H-27 (1.06 ppm, d, J = 6.8 Hz), respectively. The correlations observed in the HMBC spectrum between H-1' (4.59 ppm) and C-3 (80.9 ppm) and between H-3 (3.56 ppm) and C-1' (100.0 ppm) confirmed the presence of an *O*-glycosidic linkage at C-3 of episarsasapogenin. Other HMBC correlations observed for episarsasapogenin β-D-glucoside tetraacetate were consistent with the assignments presented in Figure 5.7 and Table 5.2.

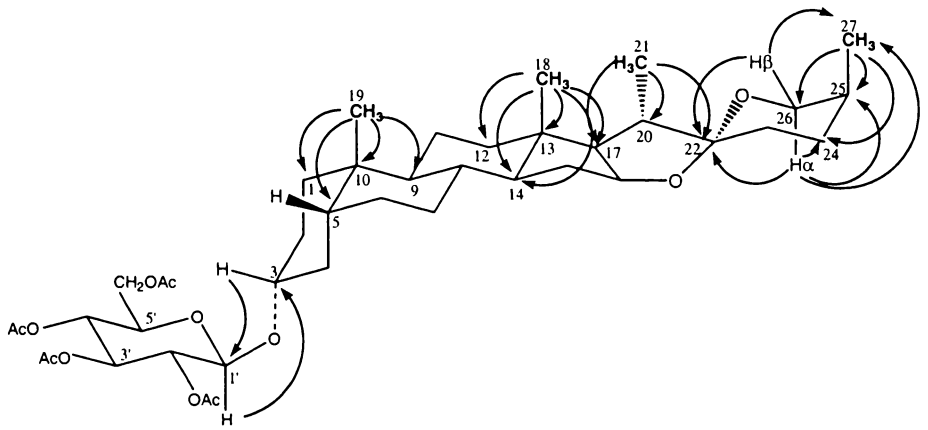


Figure 5.7. Selected HMBC correlations observed for episarsasapogenin β-D-glucoside tetraacetate (in $CDCl_3$).

Table 5.2. Selected HMBC correlations observed for episarsasapogenin β-D-glucoside tetraacetate (ppm in $CDCl_3$).

^{1}H signal	correlated ^{13}C signal(s)
0.74 (H-18)	40.2 (C-12), 40.7 (C-13), 56.2 (C-14), 62.1 (C-17)
0.92 (H-19)	35.3 (C-1), 34.9 (C-10), 42.2 (C-5), 40.5 (C-9)
0.99 (H-21)	62.1 (C-17), 42.2 (C-20), 109.7 (C-22)
1.08 (H-27)	25.9 (C-24), 27.2 (C-25), 65.2 (C-26)
3.30 (H-26α)	109.7 (C-22), 25.9 (C-24), 27.2 (C-25), 16.1 (C-27)
3.56 (H-3)	100.0 (C-1')
3.96 (H-26β)	109.7 (C-22), 16.1 (C-27)
4.59 (H-1')	80.9 (C-3)

Other genin ^{1}H and ^{13}C resonances were assigned using an array of NMR data, including HSQC, HMBC, DEPT135, COSY and NOE-difference spectra. For example, the COSY spectrum included correlations between H-3 (3.56 ppm) and the H-2 and H-4 methylene signals that resonated at 1.24, 1.58, 1.61 and 1.85 ppm. The HSQC spectrum showed that the ^{1}H signals that occurred at 1.24 and 1.61 ppm were associated with the ^{13}C signal which occurred at 27.3 ppm while the ^{1}H signals which occurred at 1.58 and 1.85 ppm were associated with the ^{13}C signal which occurred at 34.3 ppm.

A COSY correlation between one of the H-1 methylene protons (0.91 ppm) and the signal that occurred at 1.24 ppm unequivocally established the chemical shifts of the C-2 and C-4 methylene protons and their corresponding ^{13}C signals. The C-1 resonance was readily defined from HMBC spectral data that included a strong correlation between the H-19 methyl protons (0.92 ppm) and the C-1 methylene carbon signal, which resonated at 35.3 ppm (C-1) (Table 5.2 and Figure 5.7).

The ^{13}C resonances of C-6, C-7, C-23 and C-24, and their corresponding proton resonances were established from a combination of NOE-difference and HSQC spectral data. Irradiation of the methyl group protons of episarsasapogenin β-D-glucoside afforded the NOE enhancements presented in Table 5.3 and depicted in Figure 5.8.

Table 5.3. NOE-difference enhancements determined for methyl group protons of episarsasapogenin β-D-glucoside tetraacetate (ppm in $CDCl_3$).

Irradiated signal	Enhanced signals
0.74 (H-18)	1.57 (H-8β), 1.23 (H-11β), 1.20 (H-15β)
0.92 (H-19)	1.23 (H-11β), 1.57 (H-8β), 1.85 (H-6β) 1.35 (H-11α), 1.79 (H-1β)
0.99 (H-21)	1.80 (H-20), 1.88 (H-23β), 1.76 (H-17)
1.08 (H-27)	1.68 (H-25), 1.88 (H23β), 1.38 (H-24β)

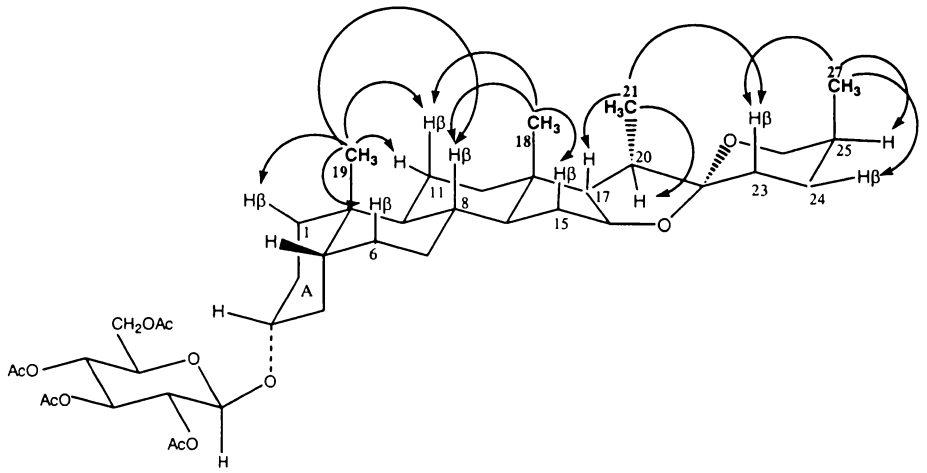


Figure 5.8. NOE-difference enhancements determined for the methyl group protons of episarsasapogenin β-D-glucoside tetraacetate (in $CDCl_3$).

The ^1H NMR resonance of H-16 (4.40 ppm) is characteristic of its proximity to an oxygen atom. H-16 (4.40 ppm) showed COSY correlations to the H-15 methylene protons (1.20 and 1.96 ppm) and to the H-17 methine proton (1.76 ppm). These protons exhibited HSQC correlations to the methylene carbon signal which occurred at 31.8 ppm (C-15) and the methine carbon signal that occurred at 62.1 ppm (C-17), respectively.

A complete assignment of ^1H and ^{13}C NMR signals for episarsasapogenin β-D-glucoside tetraacetate are presented in Table 5.4, Section 5.3.2.2.

Genin signal assignments were facilitated by comparisons with previously reported signal assignments for episarsasapogenin, sarsasapogenin, sarsasapogenin β-D-glucoside tetraacetate and sarsasapogenin β-D-glucoside (Agrawal *et al*, 1985; Deng, 1999; Loader, 2001), since only ring A carbon and proton atoms were sensitive to the replacement of the 3-OH group by a 3-*O*-glucosyl group. Assignments for sarsasapogenin and episarsasapogenin are presented in Appendix II.

5.3.2.2 Episarsasapogenin β-D-glucoside

Hydrolysis of episarsasapogenin β-D-glucoside tetraacetate was performed with *ca.* 0.70 $molL^{-1}$ methanolic potassium at reflux temperature (24 h). Separation of the crude reaction product by radial plate chromatography on silica gel, using mixtures of 5% aqueous MeOH and $CHCl_3$ as eluent yielded episarsasapogenin β-D-glucoside.

The negative ion ES-MS spectrum of episarsasapogenin β-D-glucoside in MeOH as solvent showed pseudo-molecular ions at *m/z* 623 $(M+COOH)^-$, 637 $(M+CH_3COO)^-$ and 1155 $(M_2-H)^-$. These ions are consistent with the molecular weight of the glucoside being 578 Daltons and demonstrate the hydrolysis procedure removed all of the acetoxyl groups.

The ^1H NMR spectrum of episarsasapogenin β-D-glucoside, determined in $CDCl_3$, included 4 methyl group signals at 0.74 ppm (s, H-18), 0.92 ppm (s, H-19), 0.98 ppm (d, J = 6.5 Hz, H-21) and 1.06 ppm (d, J = 6.9 Hz, H-27). Glucosyl proton signals and genin H-26 (CH_2-O) signals appeared in the region 3.20-4.40 ppm. The anomeric H-1' glucosyl proton appeared as a well defined doublet at 4.39 ppm (d, J = 7.7 Hz). This coupling constant showed that the glucosyl linkage was β-oriented (Agrawal, 1992).

The ^{13}C NMR spectrum of episarsasapogenin β-D-glucoside showed 33 signals, 6 of which were attributable to glucosyl carbons and 27 to genin carbons. The DEPT135 NMR spectrum showed the presence of 4 methyl (CH_3), 12 methylene (CH_2) and 14 methine (CH) signals. The ^{13}C NMR signals attributable to 8 carbon atoms from the 4 acetyl groups of episarsasapogenin β-D-glucoside tetraacetate were absent. This is consistent with complete hydrolysis of the acetoxyl groups.

A complete assignment of ^1H and ^{13}C NMR signals for episarsasapogenin β-D-glucoside are presented in Table 5.4. These assignments were established in a manner analogous to that described above for episarsasapogenin β-D-glucoside tetraacetate (Section 5.3.2.1).

In summary, COSY and 1D-SELTOCSY spectral data revealed the following correlation pathways; 4.39 ppm (H-1') ↔ 3.34 ppm (H-2') ↔ 3.51 ppm (H-3') ↔ 3.57 ppm (H-4') ↔ 3.27 ppm (H-5') ↔ 3.82 ppm ($H\text{-}6'_A/H\text{-}6'_B$). Only a single resonance was observed for the two H-6' methylene protons. The HSQC spectrum of episarsasapogenin β-D-glucoside verified this observation in that C-6' (61.6 ppm) showed only a single correlation to the signal that occurred at 3.82 ppm. Correlations observed in the HMBC spectrum between H-1' (4.39 ppm) and C-3 (79.5 ppm) and between H-3 (3.65 ppm) and C-1' (100.9 ppm) confirmed the presence of an *O*-glycosidic linkage at C-3 of episarsasapogenin.

Table 5.4. ^{1}H and ^{13}C NMR assignments determined for episarsasapogenin β-D-glucoside tetraacetate and episarsasapogenin β-D-glucoside (ppm in $CDCl_3$).

	Episarsasapogenin β-D-glucoside tetraacetate		**Episarsasapogenin β-D-glucoside**	
Atom	^{13}C	^{1}H	^{13}C	^{1}H
1	35.3	0.91, 1.79	35.3	0.92, 1.80
2	27.3	1.24, 1.61	26.9	1.29, 1.75
3	80.9	3.56 (m)	79.5	3.65 (m)
4	34.3	1.58, 1.84	34.2	1.56, 1.84
5	42.2	1.35	42.2	1.37
6	27.2	1.24, 1.85	27.2	1.25, 1.85
7	26.6	1.09, 1.42	26.7	1.09, 1.42
8	35.5	1.57	35.5	1.56
9	40.5	1.38	40.5	1.39
10	34.9	-	34.9	-
11	20.7	1.23, 1.35	20.7	1.23, 1.37
12	40.2	1.14, 1.70	40.2	1.17, 1.70
13	40.7	-	40.7	-
14	56.2	1.16	56.3	1.18
15	31.8	1.20, 1.96	31.8	1.22, 1.96
16	81.0	4.40 (m)	81.0	4.39 (m)
17	62.1	1.76	62.2	1.75
18	16.5	0.74 (s)	16.4	0.74 (s)
19	23.4	0.92 (s)	23.5	0.92 (s)
20	42.2	1.80	42.2	1.79
21	14.4	0.99 (d, J = 6.5 Hz)	14.4	0.98 (d, J = 6.5 Hz)
22	109.7	-	109.7	-
23	26.0	1.38, 1.88	26.0	1.36, 1.87
24	25.9	1.38, 2.02	25.8	1.38, 2.01
25	27.2	1.68	27.1	1.67
26	65.2	3.30 (d, J = 11.2 Hz)	65.1	3.28 (d, J = 11.2 Hz)
		3.96 (dd, J =2.4, 10.6 Hz)		3.93 (br d, J = 10.6 Hz)
27	16.1	1.08 (d, J = 6.8 Hz)	16.1	1.06 (d, J = 6.9 Hz)
1'	100.0	4.59 (d, J = 8.0 Hz)	100.9	4.39 (d, J = 7.7 Hz)
2'	71.7	4.94 (~t, J = 8.0 Hz)	73.4	3.34 (m)
3'	73.0	5.21 (t, J = 9.5 Hz)	76.5	3.51 (m)
4'	68.8	5.06 (t, J = 9.6 Hz)	69.5	3.57 (m)
5'	71.7	3.68 (m)	75.6	3.27 (m)
6'	62.3	4.11 (dd, J = 2.3, 12.1 Hz)	61.6	3.82 (m)
		4.25 (dd, J = 5.2, 12.1 Hz)		
$COCH_3$	20.6	2.00 (s)		
	20.7	2.02 (s)		
	20.8	2.03 (s)		
	20.8	2.07 (s)		
$COCH_3$	169.4			
	169.4			
	170.4			
	170.7			

^{1}H NMR data readily distinguishes 25*R*- and 25*S*-genins. The H-26α and H-26β proton signals of sarsasapogenin and episarsasapogenin (25*S* genins) appear at *ca.* 3.30 and 3.96 ppm respectively (see Appendix II), whereas the H-26α and H-26β proton signals of smilagenin and epismilagenin (25*R* genins) occur at *ca.* 3.36 and 3.46 ppm respectively (Deng, 1999). The H-26α and H-26β proton signals determined here for episarsasapogenin β-D-glucoside, 3.28 and 3.93 ppm (Table 5.4), correspond to a 25*S*-orientation, thereby confirming that epimerisation of the episarsasapogenin skeleton to epismilagenin had not occurred under the synthetic conditions. On the other hand it is well known that acidic conditions can lead to the conversion of a 25*S*-genin to a 25*R*-genin (Deng, 1999; Marker and Rohrmann, 1939; Wall *et al.*, 1955). For example, sarsasapogenin (a 25*S*-genin) can be converted to smilagenin (a 25*R*-genin) by refluxing in ethanol and concentrated HCl for an extended period.

The orientation of the C-3 hydroxyl group of sarsasapogenin and episarsasapogenin (3α- and 3β-respectively), can be identified by the ^{1}H NMR chemical shifts of the H-1 protons. In sarsasapogenin (3β-OH) the H-1 protons occur at 1.41 and 1.51 ppm whereas in episarsasapogenin they occur at 0.96 and 1.78 ppm. Deng (1999) has previously prepared sarsasapogenin β-D-glucoside and observed that the H-1 protons of this glucoside resonated at 1.50 and 1.82 ppm. The occurrence of the H-1 proton signals of episarsasapogenin β-D-glucoside at 0.92 and 1.80 ppm is consistent with the conclusion that glucoside formation has proceeded with retention of configuration at C-3.

5.3.3 Synthesis of Episarsasapogenin β-D-galactoside

The synthetic procedure utilized to prepare episarsasapogenin β-D-galactoside was analogous to that used to prepare episarsasapogenin β-D-glucoside (Figure 5.4, Section 5.3.2), other than the use of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide, rather than 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide.

In summary, the coupling of episarsasapogenin with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide was performed in toluene at reflux temperature, utilizing $CdCO_3$ as the catalyst. When TLC analyses showed the reaction to be complete the reaction

mixture was hot filtered and solvent removed by rotary evaporation. Fractionation of the resulting residue by radial chromatography on silica gel using mixtures of petroleum spirits (40:60) and diethyl ether afforded episarsasapogenin β-D-galactoside tetraacetate.

5.3.3.1 Episarsasapogenin β-D-galactoside tetraacetate

The positive ion ES-MS of episarsasapogenin β-D-galactoside tetraacetate, determined in MeOH:CH_2Cl_2 (1:1) as solvent, showed pseudo-molecular ions at *m/z* 769 $(M+Na)^+$ and 1515 $(M_2+Na)^+$. These ions are consistent with the molecular weight of the galactoside tetraacetate being 746 Daltons.

The ^1H NMR spectrum of episarsasapogenin β-D-galactoside tetraacetate, determined in $CDCl_3$, included 4 angular methyl signals at 0.72 ppm (s, H-18), 0.90 ppm (s, H-19), 0.96 ppm (d, J = 6.5 Hz, H-21) and 1.05 ppm (d, J = 7.0 Hz, H-27) and 4 sugar acetyl group signals in the region 1.95-2.11 ppm. Galactosyl protons signal and genin H-26 (CH_2-O) signals appeared in the region 3.20-5.40 ppm. The anomeric H-1' galactosyl proton signal appeared as a sharp doublet at 4.52 ppm (J = 8.0 Hz). This coupling constant showed that the galactosidic linkage was β-orientated (Agrawal, 1992).

The ^{13}C NMR spectrum (ppm in $CDCl_3$) of episarsasapogenin β-D-galactoside tetraacetate showed 41 carbon signals while the DEPT135 NMR spectrum showed the presence of 8 methyl, 12 methylene and 14 methine signals. 27 of the ^{13}C NMR signals were attributable to genin carbons, 6 to galactosyl carbons and 8 to carbons to the 4 acetyl groups (which included 4 acetyl methyl signals in the region 20.5-20.8 ppm and 4 carbonyl signals in the region 169.1-170.3 ppm).

Complete assignments of ^1H and ^{13}C NMR signals of episarsasapogenin β-D-galactoside tetraacetate are presented in Table 5.5. These assignments were established in an analogous fashion to that described above for episarsasapogenin β-D-glucoside tetraacetate and episarsasapogenin β-D-glucoside (Section 5.3.2) using an array of NMR data including ^1H, ^{13}C, DEPT135, 1D-SELTOCSY, COSY, HSQC, HMBC and NOE-difference experiments.

Specifically, the H-2' to H-6' galactosyl resonances were identified using a combination of peak correlations observed in the COSY spectrum beginning with the H-1' proton (4.52 ppm). The following correlation pathways were identified; 4.52 ppm (H-1') ↔ 5.15 ppm (H-2') ↔ 4.99 ppm (H-3') ↔ 5.35 ppm (H-4') ←(weak)→ 3.87 ppm (H-5') ↔ 4.11 ppm ($H\text{-}6'_A/H\text{-}6'_B$). A series of 1D-SELTOCSY spectra determined using correlation times of 30-150 msec and selective excitation of H-1' verified these assignments. Subsequently HSQC data revealed the resonances of the corresponding galactosyl carbon atoms and showed that the two H-6' methylene proton resonances coincided. HMBC correlations observed between H-1' (4.52 ppm) and C-3 (81.1 ppm) and H-3 (3.53 ppm) and C-1' (100.7 ppm) confirmed the presence of an *O*-glycosidic linkage at C-3.

The H-26α (3.27 ppm) and H-26β (3.92) signals reported in Table 5.5 are in accord with those expected for a 25*S*-genin (Deng, 1999). The occurrence of the H-1 methylene proton signals at 0.90 and 1.77 ppm are consistent to those expected for an episarsasapogenin rather a sarsasapogenin adduct (Deng, 1999; Loader, 2001), thereby confirming that galactopyranosylation proceeded with retention of configuration at C-3.

Table 5.5. ^{1}H and ^{13}C NMR assignments determined for episarsasapogenin β-D-galactoside tetraacetate and episarsasapogenin β-D-galactoside (ppm in $CDCl_3$).

	Episarsasapogenin β-D-galactoside tetraacetate		**Episarsasapogenin β-D-galactoside**	
Atom	^{13}C	^{1}H	^{13}C	^{1}H
1	35.2	0.90, 1.77	34.2	0.90, 1.77
2	27.3	1.27, 1.59	26.8	1.27, 1.73
3	81.1	3.53 (m)	79.2	3.64 (m)
4	34.4	1.58, 1.84	34.2	1.54, 1.81
5	42.1	1.33	42.1	1.35
6	27.1	1.22, 1.84	27.2	1.22, 1.82
7	26.5	1.08, 1.40	26.7	1.06, 1.40
8	35.5	1.53	35.5	1.53
9	40.4	1.36	40.5	1.36
10	34.8	-	34.9	-
11	20.6	1.21, 1.33	20.6	1.19, 1.35
12	40.2	1.12, 1.67	40.2	1.14, 1.67
13	40.6	-	40.6	-
14	56.2	1.15	56.2	1.17
15	31.7	1.19, 1.94	31.8	1.18, 1.94
16	81.0	4.39 (m)	81.0	4.37 (m)
17	62.1	1.74	62.1	1.73
18	16.4	0.72 (s)	16.4	0.72 (s)
19	23.3	0.90 (s)	23.4	0.90 (s)
20	42.1	1.77	42.1	1.77
21	14.3	0.96 (d, J = 6.5 Hz)	14.3	0.95 (d, J = 6.7 Hz)
22	109.6	-	109.6	-
23	26.0	1.34, 1.85	25.9	1.33, 1.84
24	25.8	1.37, 1.99	25.8	1.36, 1.99
25	27.1	1.66	27.1	1.66
26	65.1	3.27 (d, J = 11.2 Hz) 3.92 (br d, J = 10.6 Hz)	65.0	3.25 (d, J = 11.2 Hz) 3.91 (br d J = 10.6 Hz)
27	16.0	1.05 (d, J = 7.0 Hz)	16.0	1.04 (d, J = 7.2 Hz)
1'	100.7	4.52 (d, J = 8.0 Hz)	101.3	4.29 (d, J = 7.8 Hz)
2'	69.2	5.15 (dd, J = 8.0, 10.5 Hz)	73.7	3.57 (m)
3'	71.0	4.99 (dd, J = 3.3, 10.6 Hz)	71.2	3.62 (m)
4'	67.1	5.35 (br d, J = 3.3 Hz)	68.6	4.02 (m)
5'	70.5	3.87 (~t, J = 6.7 Hz)	74.2	3.47 (m)
6'	61.4	4.11 (m)	60.9	3.78 (m)
$COCH_3$	20.5 20.6 20.6 20.8	1.95 (s) 2.01 (s) 2.02 (s) 2.11 (s)		
$COCH_3$	169.1 170.1 170.3 170.3			

5.3.3.2 Episarsasapogenin β-D-galactoside

Hydrolysis of episarsasapogenin β-D-galactoside tetraacetate with *ca.* 0.70 $molL^{-1}$ methanolic potassium at reflux temperature afforded episarsasapogenin β-D-galactoside. The crude reaction product was purified by radial plate chromatography on silica gel using mixtures of 5% aqueous MeOH and $CHCl_3$ as eluent.

The negative ion ES-MS spectrum of episarsasapogenin β-D-galactoside was obtained using MeOH as solvent. Pseudo-molecular ions observed at *m/z* 623 $(M+COOH)^-$, 1155 $(M_2-H)^-$ are consistent with the molecular weight of the galactoside being 578 Daltons and demonstrate that the hydrolysis procedure removed all of the acetoxyl groups.

Full ^1H and ^{13}C NMR assignments are presented in Table 5.5. These assignments were established in a manner analogous to that described above (Section 5.3.2). In summary, the ^1H NMR spectrum of episarsasapogenin β-D-galactoside, determined in $CDCl_3$, included 4 methyl group signals at 0.72 ppm (s, H-18), 0.90 ppm (s, H-19), 0.95 ppm (d, J = 6.7 Hz, H-21) and 1.04 ppm (d, J = 7.2 Hz, H-27). Galactosyl proton signals and genin H-26 (CH_2-O) signals appeared in the region 3.20-5.00 ppm. The anomeric H-1' galactosyl proton appeared as a well defined doublet at 4.29 ppm (d, J = 7.8 Hz) and corresponds to a β-orientation of the galactosidic linkage (Agrawal, 1992).

The ^{13}C NMR spectrum of episarsasapogenin β-D-galactoside showed 33 signals, 6 of which were attributable to galactosyl carbons and 27 to genin carbons. The DEPT135 NMR spectrum showed the presence of 4 methyl, 12 methylene and 14 methine signals. The ^{13}C NMR signals attributable to 8 carbon atoms of the 4 acetyl groups of the tetraacetate precursor were absent and is consistent with the complete hydrolysis of the acetoxyl groups.

Correlations observed in the COSY spectrum defined the resonances of the galactosyl methine and methylene protons. H-1' (4.29 ppm) showed a correlation to H-2' (3.57 ppm). This proton showed a correlation to H-3' (3.62 ppm), which in turn showed a correlation to H-4' (4.02 ppm). Only a very weak correlation was observed between H-4' and H-5' (3.47

ppm) which in turn showed correlations to H-6'$_A$/H-6'$_B$ (3.78 ppm). The resonances of the galactosyl protons were also revealed in a series of 1D-SELTOCSY spectra derived by irradiation of the H-1' signal (4.29 ppm). The HSQC spectral data subsequently identified the resonances of galactosyl carbons and confirmed that the resonances of the two H-6' protons coincided.

HMBC correlations observed between H-1' (4.29 ppm) and C-3 (79.2 ppm) and between H-3 (3.64 ppm) and C-1' (101.3 ppm) confirmed the presence of an *O*-glycosidic linkage at C-3. The H-26α (3.25 ppm) and H-26β (3.91 ppm) signals reported in Table 5.5 for episarsasapogenin β-D-galactoside are consistent with a 25*S*-genin configuration (Deng, 1999; Loader, 2001).

5.3.4 Synthesis of Sarsasapogenin β-D-galactoside

The procedure for the synthesis of sarsasapogenin β-D-galactoside was analogous to the procedure for episarsasapogenin β-D-glucoside and episarsasapogenin β-D-galactoside (Figure 5.4) and utilized sarsasapogenin and 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide to give the desired 3β-*O*-galactoside. Coupling of sarsasapogenin with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide was performed in toluene at reflux temperature, with $CdCO_3$ as the catalyst. Purification of the crude product material by radial chromatography on silica gel using petroleum spirits (40:60) and diethyl ether mixtures afforded sarsasapogenin β-D-galactoside tetraacetate.

5.3.4.1 Sarsasapogenin β-D-galactoside tetraacetate

The positive ion ES-MS of sarsasapogenin β-D-galactoside tetraacetate, determined in MeOH:CH_2Cl_2 (1:1) as solvent, showed ions consistent with the molecular weight of the galactoside tetraacetate being 746 Daltons at *m/z* 769 $(M+Na)^+$ and 1515 $(M_2+Na)^+$.

The ^{1}H NMR spectrum of sarsasapogenin β-D-galactoside tetraacetate, determined in $CDCl_3$, included 4 angular methyl signals typical of sapogenins at 0.70 ppm (s, H-18), 0.88 ppm (s, H-19), 0.93 ppm (d, J = 7.0 Hz, H-21) and 1.02 ppm (d, J = 7.2 Hz, H-27) and 4

methyl signals corresponding to the sugar acetyl groups in the region 1.93-2.09 ppm. Galactosyl proton signals and genin H-26 (CH_2-O) signals appeared in the region 3.20-5.40 ppm. The H-1' NMR signal of the anomeric galactosyl proton appeared as a sharp doublet at 4.48 ppm (J = 7.8 Hz). This coupling constant showed that the galactosidic linkage was β-orientated (Agrawal, 1992).

The ^{13}C NMR spectrum (in $CDCl_3$) of sarsasapogenin β-D-galactoside tetraacetate showed 41 carbon signals while the DEPT135 NMR spectrum showed the presence of 8 methyl, 12 methylene and 14 methine signals. 14 of the ^{13}C NMR signals were attributable to galactosyl carbon atoms and its 4 sugar acetyl groups, and 27 to genin carbon atoms. HMBC correlations observed between H-1' (4.48 ppm) and C-3 (74.2 ppm) and H-3 (3.95 ppm) and C-1' (99.0 ppm) confirmed the presence of an *O*-galactosidic linkage at C-3.

Complete assignments of ^{1}H and ^{13}C NMR signals of sarsasapogenin β-D-galactoside tetraacetate are presented in Table 5.6. These assignments were derived from detailed analyses of ^{1}H, ^{13}C, DEPT135, 1D-SELTOCSY, COSY, HSQC, HMBC and NOE-difference data in a manner analogous to those described above (Sections 5.3.2 and 5.3.3).

Table 5.6. ^{1}H and ^{13}C NMR assignments determined for sarsasapogenin β-D-galactoside tetraacetate and sarsasapogenin β-D-galactoside (ppm in $CDCl_3$).

	Sarsasapogenin β-D-galactoside tetraacetate		**Sarsasapogenin β-D-galactoside**	
Atom	^{13}C	^{1}H	^{13}C	^{1}H
1	30.2	1.29, 1.41	30.5	1.42, 1.50
2	26.3	1.42, 1.60	26.6	1.54, 1.68
3	74.2	3.95 (br s)	75.0	4.04 (m)
4	29.6	1.34, 1.76	30.3	1.47, 1.56
5	36.7	1.46	37.2	1.64
6	26.6	1.09, 1.84	26.7	1.17, 1.90
7	26.5	0.99, 1.39	26.6	1.05, 1.45
8	35.2	1.53	35.4	1.59
9	40.0	1.26	40.3	1.34
10	34.9	-	35.2	-
11	20.8	1.23, 1.34	21.0	1.25, 1.38
12	40.2	1.09, 1.66	40.4	1.15, 1.72
13	40.6	-	40.8	-
14	56.4	1.11	56.5	1.16
15	31.6	1.17, 1.91	31.8	1.24, 1.97
16	80.9	4.34 (m)	81.1	4.40 (~q, *J* = 7.6, 6.6, 7.8 Hz)
17	62.1	1.70	62.2	1.76
18	16.4	0.70 (s)	16.5	0.76 (s)
19	23.8	0.88 (s)	23.8	0.97 (s)
20	42.0	1.75	42.2	1.81
21	14.2	0.93 (d, *J* = 7.0 Hz)	14.4	0.99 (d, *J* = 6.8 Hz)
22	109.6	-	109.8	-
23	25.9	1.31, 1.83	26.0	1.37, 1.87
24	25.7	1.35, 1.96	25.9	1.42, 2.03
25	27.0	1.64	27.2	1.69
26	65.0	3.23 (d, *J* = 11.2 Hz) 3.89 (br d, *J* = 10.6 Hz)	65.2	3.30 (d, *J* = 11.2 Hz) 3.95 (m)
27	16.0	1.02 (d, *J* = 7.2 Hz)	16.1	1.08 (d, *J* = 7.2 Hz)
1'	99.0	4.48 (d, *J* = 7.8 Hz)	101.5	4.30 (d, *J* = 7.6 Hz)
2'	69.0	5.14 (~t, *J* = 7.8 Hz)	72.1	3.65 (m)
3'	71.0	4.98 (dd, *J* = 3.3, 10.4 Hz)	73.5	3.62 (m)
4'	67.1	5.32 (br d, *J* = 3.3 Hz)	69.5	4.00 (br d, *J* = 2.1 Hz)
5'	70.5	3.83 (~t, *J* = 6.8 Hz)	74.4	3.54 (~t, *J* = 5.4 Hz)
6'	61.2	4.09 (m)	62.7	3.85 (m) 3.99 (m)
$COCH_3$	20.5	1.93 (s)		
	20.5	1.96 (s)		
	20.6	1.99 (s)		
	20.7	2.09 (s)		
$COCH_3$	169.1			
	170.1			
	170.2			
	170.3			

5.3.4.2 Sarsasapogenin β-D-galactoside

Hydrolysis of sarsasapogenin β-D-galactoside tetraacetate with *ca.* 0.70 $molL^{-1}$ methanolic potassium at reflux temperature afforded sarsasapogenin β-D-galactoside. The crude reaction product was purified by radial plate chromatography on silica gel using mixtures of 5% aqueous MeOH and $CHCl_3$ as eluent.

The negative ion ES-MS spectrum of sarsasapogenin β-D-galactoside, in MeOH as solvent (Figure 5.9), showed pseudo-molecular ions at *m/z* 577 $(M-H)^-$, 623 $(M+COOH)^-$, 637 $(M+CH_3COO)^-$ and 1155 $(M_2-H)^-$. These ions are consistent with the molecular weight of the galactoside being 578 Daltons, and demonstrate entire removal of the acetoxyl groups by the hydrolysis procedure.

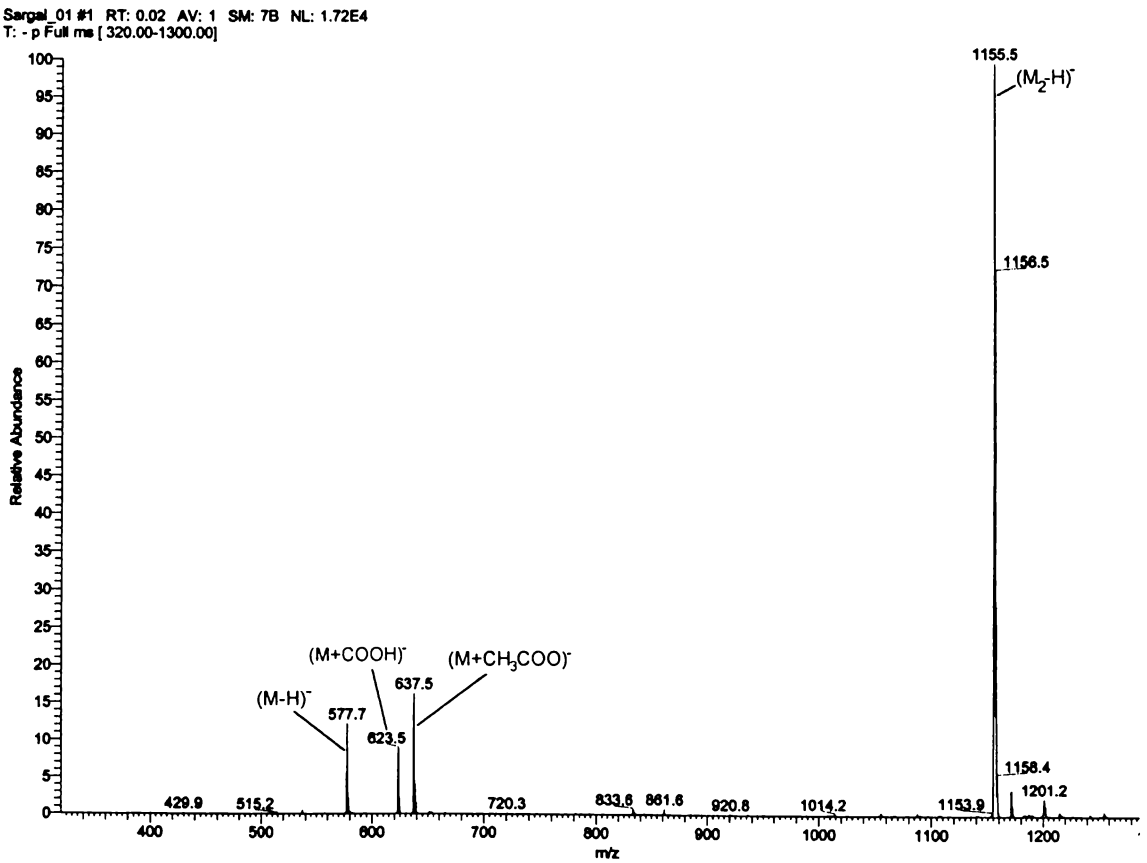


Figure 5.9. Negative ion ES-MS spectrum of sarsasapogenin β-D-galactoside, determined in MeOH.

Full ^{1}H and ^{13}C NMR assignments are presented in Table 5.6. These assignments were established in a manner analogous to that described above (Sections 5.3.2 and 5.3.3). In summary, the ^{1}H NMR spectrum of sarsasapogenin β-D-galactoside, determined in $CDCl_3$, included 4 methyl group signals at 0.76 ppm (s, H-18), 0.97 ppm (s, H-19), 0.99 ppm (d, J = 6.8 Hz, H-21) and 1.08 ppm (d, J = 7.2 Hz, H-27). Galactosyl proton signals and genin H-26 (CH_2-O) signals appeared in the region 3.20-4.50 ppm. The anomeric H-1' galactosyl proton appeared at 4.30 ppm as a well defined doublet (J = 7.6 Hz) and is consistent with a β-orientation of the galactosidic linkage (Agrawal, 1992).

The ^{13}C NMR spectrum of sarsasapogenin β-D-galactoside showed 33 signals, 6 of which were attributable to galactosyl carbons and 27 to genin carbons. The DEPT135 NMR spectrum showed the presence of 4 methyl, 12 methylene and 14 methine signals. The ^{13}C NMR signals attributable to 8 carbon atoms from the 4 acetyl groups which were present in the tetraacetate precursor before hydrolysis were absent.

Galactosyl proton signals; H-1' (4.30 ppm), H-2' (3.65 ppm), H-3' (3.62 ppm), H-4' (4.00 ppm), H-5' (3.54 ppm) and H-6'$_A$/H-6'$_B$ (3.85 and 3.95 ppm) were revealed by analyses of COSY and 1D-SELTOCSY spectral data. The HSQC spectrum confirmed the H-6'$_A$ and H-6'$_B$ methylene protons occurred at discrete chemical shifts of 3.85 and 3.95 ppm and that each of these protons were correlated to the ^{13}C signal which occurred at 62.7 ppm. HMBC correlations observed between H-1' (4.30 ppm) and C-3 (75.0 ppm) and H-3 (4.04 ppm) and C-1' (101.5 ppm) confirmed the presence of the *O*-galactosidic linkage at C-3.

The H-1 methylene protons (1.42 and 1.50 ppm) and the H-26α (3.30 ppm) and H-26β (3.90 ppm) proton signals determined for sarsasapogenin β-D-galactoside (Table 5.6), are respectively consistent with a 3β,25*S* genin configuration (Deng, 1999; Loader, 2001).

5.4 Synthesis of the Betulin β-D-glucosides

5.4.1 Introduction

Betulin (lup-20(29)-ene-3β,28-diol), a pentacyclic triterpene, is the main triterpenoid constituent of birch bark, from the birch family (Betulaceae). The white colour of birch bark is attributable to betulin and typically the betulin content varies between 10-30% (O'Connell *et al.*, 1988).

Betulin was one of the first natural products to be isolated from plants as a pure chemical substance. Lowitz achieved this in 1788 via sublimation from birch bark. This technology is still in use today (Patočka, 2003).

Betulin is used as an additive in cosmetics and shampoos. In Finland, where up to 2500 tonnes per year of betulin is obtained from birch bark waste, intensive research is being undertaken on the use of betulin in the manufacture of lacquers and 'first rate' protective coatings with high water, chemical and biological resistance (Patočka, 2003).

Betulin is a rather inert and stable organic compound that is not utilized by the majority of microorganisms and mycelial fungus, giving it a unique stability in the environment. Despite its relative inactivity, betulin has proved to be biologically active in a mammalian environment. Amongst other properties, betulin possesses anti-inflammatory, anticancer and antiviral properties (Recio *et al.*, 1995).

5.4.2 Synthesis of Betulin 3-(β-D-glucoside)

Coupling of betulin with 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide was performed in toluene at reflux temperature, utilizing $CdCO_3$ as the catalyst (Deng, 1999). When TLC analyses showed the reaction to be complete (12-36 h), the reaction mixture was hot filtered to remove cadmium salts and solvent removed by rotary evaporation. Fractionation of the resulting residue by radial chromatography on silica gel using mixtures of petroleum spirits (40:60) and diethyl ether afforded mainly betulin 3-(β-D-glucoside tetraacetate) and betulin 3,28-(β-D-diglucoside tetraacetate). Other products formed were betulin 28-(β-D-glucoside tetraacetate) and a 3-(β-D-glucoside tetraacetate) with a rearranged ring E structure. This 'rearranged' product was shown to be allobetulin β-D-glucoside tetraacetate, i.e. 19,28-oxido-18α-H-oleanane β-D-glucoside tetraacetate, see Section 5.5.2.

Figure 5.10 outlines the sequence of reactions utilized in the synthesis of the betulin 3-(β-D-glucoside) and betulin 3,28-(β-D-diglucoside) (the 2 major reaction products) and shows the structures of 2 minor by-products, betulin 28-(β-D-glucoside tetraacetate) and allobetulin β-D-glucoside tetraacetate. The synthesis of both betulin 3-(β-D-glucoside), betulin 3,28-(β-D-diglucoside) and their acetylated intermediates have previously been reported (Ivanov *et al.*, 1987; Dzizenko *et al.*, 1973; Ohara and Ohira, 2003).

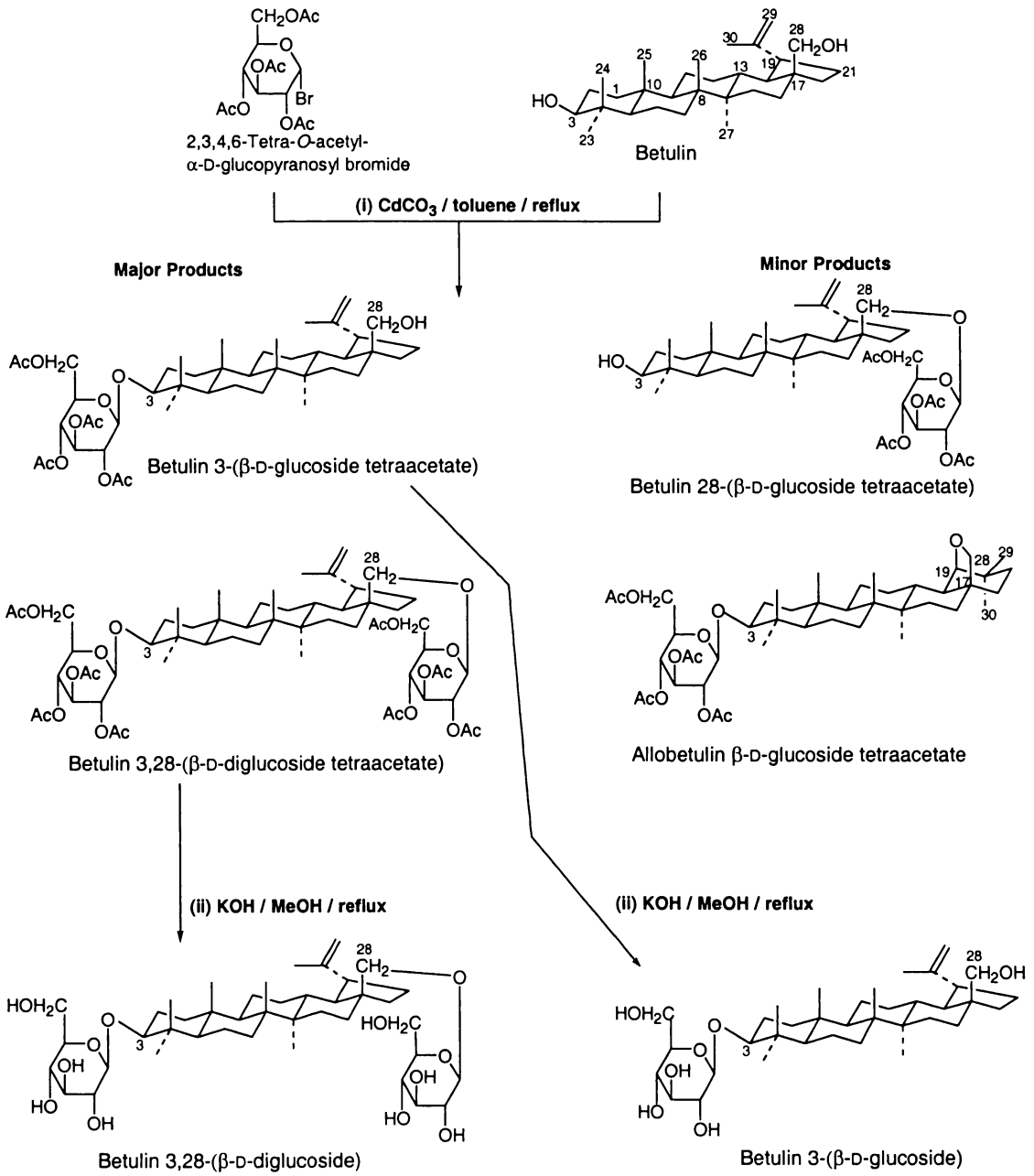


Figure 5.10. Reaction steps involved in the synthesis of betulin 3-(β-D-glucoside) and betulin 3,28-(β-D-diglucoside). The minor products betulin 28-(β-D-glucoside tetraacetate) and allobetulin β-D-glucoside tetraacetate are shown.

In each of several repetitions of the synthesis, betulin 3,28-(β-D-diglucoside tetraacetate) was the dominant product formed. It was however found that when using shorter reflux times (i.e. 12 h) combined with a smaller excess of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide with respect to betulin, a higher percentage of betulin 3-(β-D-glucoside tetraacetate) could be obtained. Longer reflux times (36 h) and a greater ratio of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide, yielded more betulin 3,28-(β-D-diglucoside tetraacetate).

Betulin 28-(β-D-glucoside tetraacetate) was also formed in the ratio of *ca.* 1:2 relative to betulin 3-(β-D-glucoside tetraacetate). This was unexpected since it was anticipated that an *O*-glycosidic linkage would be formed more readily at the C-28 primary hydroxyl group rather than the sterically hindered C-3 secondary hydroxyl group.

5.4.2.1 Betulin 3-(β-D-glucoside tetraacetate)

The positive ion ES-MS of betulin 3-(β-D-glucoside tetraacetate), determined in MeOH:CH_2Cl_2 (1:1) as solvent, showed pseudo-molecular ions at *m/z* 795 $(M+Na)^+$, 1567 $(M_2+Na)^+$ and 1583 $(M_2+K)^+$. These ions are consistent with the molecular weight of the glucoside tetraacetate being 772 Daltons.

The ^1H NMR spectrum of betulin 3-(β-D-glucoside tetraacetate), determined in $CDCl_3$, included 6 angular methyl signals, typical of betulin, at 0.71 ppm (s, H-24), 0.81 ppm (s, H-25), 0.89 ppm (s, H-23), 0.96 ppm (s, H-27), 1.02 ppm (s, H-26) and 1.67 ppm (s, H-30), 4 methyl signals attributable to the sugar acetyl groups at 2.00-2.11 ppm, and glucosyl proton signals and H-29 olefinic proton signals in the region 3.50-5.30 ppm. A single anomeric glucosyl proton signal appeared as a sharp doublet at 4.52 ppm (J = 8.0 Hz). This coupling constant demonstrated that the glucosidic linkage was β-orientated (Agrawal, 1992).

The ^{13}C NMR spectrum showed a total of 44 signals, 30 of which were attributable to the genin skeleton, 6 to glucosyl carbon atoms and 8 to carbon atoms of the 4 sugar acetyl groups (which included 4 acetyl methyl signals in the region 20.6-20.8 ppm and 4 carbonyl signals in the region 169.2-170.6 ppm). The DEPT135 spectrum revealed the presence of

10 methyl, 13 methylene, 11 methine carbon signals. 10 quaternary carbon signals (by difference with respect to the ^{13}C spectrum), were absent.

Glucosyl proton resonances were identified via correlated peak pathways in the COSY spectrum, starting with the H-1' proton (4.52 ppm), and from a series of 1D-SELTOCSY spectra determined with a range of mixing (correlation) times (30 to 150 msec) which enabled progressive tracking of proton resonances around the glucosyl residue starting with the H-1' signal. The following correlation pathways were observed; 4.52 ppm (H-1') ↔ 5.01 ppm (H-2') ↔ 5.24 ppm (H-3') ↔ 5.04 ppm (H-4') ↔ 3.67 ppm (H-5') ↔ 4.10 and 4.22 ppm (H-6'$_A$/H-6'$_B$). Glucosyl carbon resonances were subsequently identified via HSQC correlations between the glucosyl protons and the respective carbon atoms.

The ^1H NMR signals of the two H-29 olefinic protons occurred at 4.57 ppm (m) and 4.67 ppm (br s). In the HSQC spectrum these protons correlated to the carbon signal which occurred at 109.7 ppm. This is consistent with the retention of the 20(29)-olefinic bond. The C-28 (CH_2OH) resonance appeared at 60.6 ppm, slightly up-field of the glycosyl C-6' resonance (62.4 ppm). HSQC spectral data showed that the C-28 methylene proton signals occurred at 3.32 ppm (br d, J = 10.7 Hz) and 3.78 ppm (br d, J = 10.7 Hz). HMBC correlations between H-1' (4.52 ppm) and C-3 (90.7 ppm) and between H-3 (3.05 ppm) and C-1' (103.0 ppm) confirmed the presence of an *O*-glucosidic linkage at C-3 of betulin.

HMBC correlations exhibited by the 5 non-olefinic methyl group signals allowed their unambiguous assignment. 2 of the methyl group signals 0.71 ppm (s) and 0.89 ppm (s) showed correlations to C-3 (90.7 ppm), C-4 (39.0 ppm), C-5 (55.7 ppm) and to 27.7 ppm (C-23) or 16.2 ppm (C-24) respectively. This data, along with the observation of an NOE-enhancement between H-25 (s, 0.81 ppm) and H-24 (s, 0.71 ppm) unambiguously identified the H-24 (s, 0.71 ppm), H-25 (s, 0.81 ppm) and H-23 (s, 0.89 ppm) resonances. H-25 (s, 0.81 ppm) and H-26 (s, 1.02 ppm) showed correlations to C-9 (50.5 ppm) which allowed the identification of H-26 (s, 1.02 ppm). Both H-26 (s, 1.02 ppm) and H-27 (s, 0.96 ppm) showed correlations to C-8 (41.0 ppm) and C-14 (42.8 ppm) while the olefinic H-30 methyl group signal (s, 1.67 ppm) showed correlations to C-19 (47.9 ppm), C-20 (150.5 ppm) and C-29 (109.7 ppm). Other HMBC correlations, consistent with these assignments, are presented in Figure 5.11 and Table 5.7.

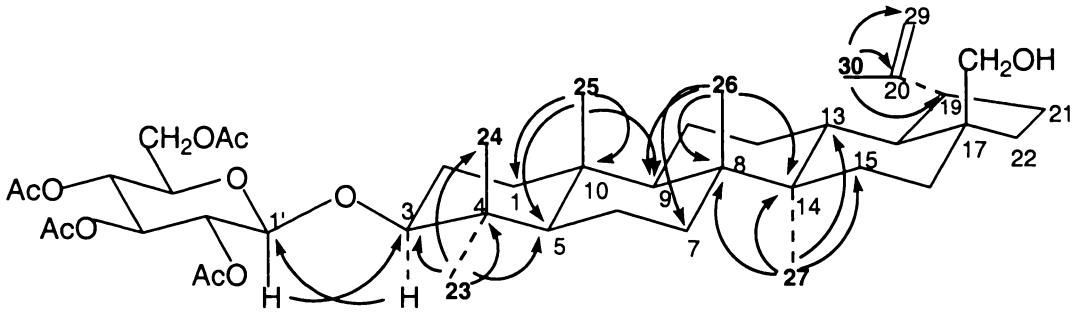


Figure 5.11. Selected HMBC correlations observed for betulin 3-(β-D-glucoside tetraacetate) (in $CDCl_3$).

Table 5.7. Selected HMBC correlations observed for betulin 3-(β-D-glucoside tetraacetate) (ppm in $CDCl_3$).

^{1}H signal	correlated ^{13}C signal(s)
0.71 (H-24)	90.7 (C-3), 39.0 (C-4), 55.7 (C-5), 27.7 (C-23)
0.81 (H-25)	38.7 (C-1), 55.7 (C-5), 50.5 (C-9), 36.9 (C-10)
0.89 (H-23)	90.7 (C-3), 39.0 (C-4), 55.7 (C-5), 16.2 (C-24)
0.96 (H-27)	41.0 (C-8), 37.4 (C-13), 42.8 (C-14), 27.1 (C-15)
1.02 (H-26)	34.3 (C-7), 41.0 (C-8), 50.5 (C-9), 47.9 (C-17)
1.57 (H-18)	37.3 (C-13), 47.9 (C-17/C-19), 150.5 (C-20), 60.6 (C-28)
1.67 (H-30)	47.9 (C-19), 150.5 (C-20), 109.7 (C-29)
3.05 (H-3)	103.0 (C-1'), 27.7 (C-23), 16.2 (C-24)
3.32 (H-28)	29.3 (C-16), 34.0 (C-22)
3.78 (H-28)	34.0 (C-22)
4.52 (H-1')	90.7 (C-3)

The remaining genin resonances were assigned using an array of NMR spectral data including HSQC, HMBC, DEPT135, COSY, TOCSY, and ROESY data. For example, H-5 (0.67 ppm) exhibited a COSY correlation to one of the two H-6 methylene protons (1.49 ppm). This proton in turn showed a COSY correlation to the 2 essentially co-incident H-7 methylene proton signals centred at 1.37 ppm. Subsequent inspection of the HSQC spectrum identified the resonance of the second H-6 (1.38 ppm) methylene proton and showed the C-6 and C-7 resonances occurred at 18.2 and 34.3 ppm respectively.

Similarly, H-19 (2.37 ppm) identified via an HSQC correlation to C-19 (47.9 ppm) [originally identified from an HMBC correlation from the H-30 methyl protons (1.67 ppm)] (see Table 5.7) showed COSY correlations to H-18 (1.57 ppm) and to one of the H-21 methylene protons (1.39 ppm). HSQC spectral data subsequently identified the resonance of the other H-21 methylene proton (1.95 ppm) and the C-21 resonance (29.8 ppm). The H-3 (3.05 ppm) proton showed COSY correlations to the H-2 methylene protons at 1.69 and 1.77 ppm. The HSQC spectrum subsequently showed that the C-2 signal occurred at 26.0 ppm.

The H-11 and H-12 resonances were distinguished via COSY correlations. H-9 (1.24 ppm) showed a correlation to one of the H-11 methylene protons (1.40 ppm) while H-13 (1.63 ppm) showed a correlation to one of the H-12 methylene protons (1.03 ppm). Subsequent inspection of the HSQC spectrum revealed the resonances of the other H-11 and H-12 methylene protons (1.21 and 1.63 ppm respectively) and showed the C-11 and C-12 resonances to occur at 20.9 and 25.3 ppm respectively.

A complete assignment of ^{1}H and ^{13}C NMR signals for betulin 3-(β-D-glucoside tetraacetate), in $CDCl_3$, is presented in Table 5.8. Signal assignments were facilitated by comparisons with assignments reported for lupeol (Burns *et al.*, 2000), and for assignments established locally for betulin, in both $CDCl_3$ and DMSO-D_6 (Appendix III). Replacement of the 3-OH group by a 3-*O*-glucosyl group had little effect on carbon and proton chemical shifts of ring B-E atoms.

Table 5.8. ^{1}H and ^{13}C NMR assignments determined for betulin 3-(β-D-glucoside tetraacetate) (ppm in $CDCl_3$) and betulin 3-(β-D-glucoside) (ppm in DMSO-D_6).

	Betulin 3-(β-D-glucoside tetraacetate)		**Betulin 3-(β-D-glucoside)**	
Atom	^{13}C	^{1}H	^{13}C	^{1}H
1	38.7	0.85, 1.65	38.3	0.93, 1.65
2	26.0	1.69, 1.77	25.7	1.60, 1.91
3	90.7	3.05 (m)	88.0	3.10 (m)
4	39.0	-	38.8	-
5	55.7	0.67	55.1	0.76
6	18.2	1.38, 1.49	17.8	1.44, 1.56
7	34.3	1.37 (2H)	33.8	1.43 (2H)
8	41.0	-	40.5	-
9	50.5	1.24	49.8	1.33
10	36.9	-	36.4	-
11	20.9	1.21, 1.40	20.4	1.26, 1.44
12	25.3	1.03, 1.63	24.8	1.07, 1.67
13	37.4	1.62	36.7	1.71
14	42.8	-	42.2	-
15	27.1	1.03, 1.69	26.7	1.01, 1.76
16	29.3	1.19, 1.92	29.1	1.12, 2.00
17	47.9	-	47.4	-
18	48.9	1.57	48.2	1.58
19	47.9	2.37	47.3	2.47
20	150.5	-	150.3	-
21	29.8	1.39, 1.95	29.4	1.36, 1.96
22	34.0	1.02, 1.86	33.8	0.98, 1.95
23	27.7	0.89 (s)	27.5	1.06 (s)
24	16.2	0.71 (s)	16.2	0.83 (s)
25	16.0	0.81 (s)	15.9	0.88 (s)
26	16.1	1.02 (s)	15.7	1.08 (s)
27	14.8	0.96 (s)	14.5	1.03 (s)
28	60.6	3.32 (br d, J = 10.7 Hz) 3.78 (br d, J = 10.7 Hz)	58.0	3.62 (br d, J = 10.4 Hz) 3.18 (br d, J = 10.4 Hz)
29	109.7	4.57 (m), 4.67 (br s)	109.5	4.63 (br s), 4.76 (br s)
30	19.2	1.67 (s)	18.8	1.73 (s)
1'	103.0	4.52 (d, J = 8.0 Hz)	105.3	4.23 (d, J = 7.8 Hz)
2'	71.7	5.01 (m)	74.9	3.05 (m)
3'	72.9	5.24 (~t, J = 9.4 Hz)	76.9	3.21 (m)
4'	68.9	5.04 (m)	70.1	3.14 (m)
5'	71.5	3.67 (m)	76.5	3.14 (m)
6'	62.4	4.10 (dd, J = 2.3, 12.5 Hz) 4.22 (dd, J = 4.6, 12.5 Hz)	62.2	3.52 (m) 3.73 (d, J = 11.2 Hz)
$COCH_3$	20.6 (s)	2.00 (s)		
	20.6 (s)	2.02 (s)		
	20.7 (s)	2.08 (s)		
	20.8 (s)	2.11 (s)		
$COCH_3$	169.2			
	169.4			
	170.1			
	170.6			

5.4.2.2 Betulin 3-(β-D-glucoside)

Hydrolysis of betulin 3-(β-D-glucoside tetraacetate) was performed in *ca.* 0.70 $molL^{-1}$ methanolic potassium at reflux temperature (24 h). Purification of the crude reaction product by radial plate chromatography on silica gel using mixtures of 5% aqueous MeOH and $CHCl_3$ yielded betulin 3-(β-D-glucoside).

The negative ion ES-MS spectrum of betulin 3-(β-D-glucoside) in MeOH as solvent showed pseudo-molecular ions at *m/z* 603 $(M-H)^-$, 649 $(M+COOH)^-$, 663 $(M+CH_3COO)^-$ and 1207 $(M_2-H)^-$, These ions are consistent with the molecular weight of the glucoside being 604 Daltons and demonstrate that hydrolysis proceeded with removal of the acetoxyl groups.

The ES-MS spectrum also showed ions at *m/z* 617, 1221 and 1825. These ions, showing mass gains of 14 Daltons to the $(M-H)^-$, $(M_2-H)^-$ and $(M_3-H)^-$ pseudo-molecular ions respectively, are believed to arise from matrix solvation effects under ES-MS conditions on the C-28 ($-CH_2OH$) functional group. The addition of a single methanol residue (CH_3OH, *m/z* 32), accompanied by the loss of water (*m/z* 18) on the $-CH_2OH$ functional group to yield a $-CH_2OCH_3$ group equates to mass gains of 14 Daltons, thus giving rise to the *m/z* 617 $(M+CH_3OH-H_2O-H)^-$, 1221 $(M_2+CH_3OH-H_2O-H)^-$ and 1825 $(M_3+CH_3OH-H_2O-H)^-$ ions. Furthermore, an ion observed at *m/z* 1235 can be rationalized as the addition of 2 methanol residues to the pseudo-molecular ion *m/z* 1207 $(M_2-H)^-$, hence *m/z* 1235 $(M_2+(CH_3OH-H_2O)_2-H)^-$.

ES-MS data for the synthesized steroidal saponins (Section 5.3), which do not possess a corresponding $-CH_2OH$ functionality, lack pseudo-molecular ions exhibiting the 14 Dalton mass gain characteristics. Similarly, betulin 3,28-(β-D-diglucoside) (Section 5.4.3), where an *O*-glucosyl residue is attached at C-28, also fails to exhibit ions attributable to addition of a methoxy group. Corroborating evidence that these ions are artefacts from ES-MS matrix effects comes from the absence of a methoxy signal in the ^1H, ^{13}C and HSQC NMR spectra of betulin 3-(β-D-glucoside).

The ^{1}H NMR spectrum of betulin 3-(β-D-glucoside), in DMSO-D_6, included 6 angular methyl signals at 0.83 ppm (s, H-24), 0.88 ppm (s, H-25), 1.03 ppm (s, H-27), 1.06 ppm (s, H-23), 1.08 ppm (s, H-26) and 1.73 ppm (s, H-30), glucosyl proton signals in the region 3.00-4.00 ppm and the broad singlet H-29 olefinic signals at 4.63 and 4.76 ppm. A single H-1' anomeric glucosyl proton signal appeared as a sharp doublet at 4.23 ppm (J = 7.8 Hz). This coupling constant showed that the glucosyl linkage was β-orientated (Agrawal, 1992). Methyl proton signals attributable to the 4 acetyl groups of the precursor tetraacetate were absent.

The ^{13}C NMR spectrum, in DMSO-D_6, showed a total of 36 signals, 30 of which were attributable to the genin skeleton and 6 to glucosyl carbon atoms. The 4 sugar acetyl groups, from the tetraacetate precursor were absent. The DEPT135 spectrum revealed the presence of 6 methyl, 13 methylene, 11 methine carbon signals. 6 signals corresponding to quaternary carbons, were absent with respect to the ^{13}C spectrum.

A complete assignment of ^{1}H and ^{13}C NMR signals for betulin 3-(β-D-glucoside), in DMSO-D_6, is presented in Table 5.8. These assignments were established in an analogous manner as described above (Section 5.4.2.1) and are consistent with the assignments of Ohara and Ohira (2003). In general only atoms in the immediate vicinity of the 3-*O*-glucoside group exhibited shifts which differed significantly from those reported for betulin determined in DMSO-D_6 (Appendix III).

In summary, COSY, 1D-SELTOCSY and ^{1}H spectral data identified the signals arising from H-1' (4.23 ppm, d, J = 7.8 Hz), H-2' (3.05 ppm, m), H-3' (3.21 ppm, m), H-4' (3.14 ppm, m), H-5' (3.14 ppm, m), H-6'$_A$ (3.52 ppm, m) and H-6'$_B$ (3.73 ppm, d, J = 11.2 Hz). In the HSQC spectrum the overlapping H-4' and H-5' signals (3.14 ppm) showed correlations to the ^{13}C signals which occurred at 70.1 and 76.5 ppm. H-1' (4.23 ppm) showed an HMBC correlation to C-5' (76.5 ppm), thereby distinguishing C-4' (70.1 ppm) from C-5' (76.5 ppm). Other glucosyl carbon assignments were established via HSQC correlations observed for the corresponding protons.

HMBC correlations between H-1' (4.23 ppm) and C-3 (88.0 ppm) and between H-3 (3.10 ppm) and C-1' (105.3 ppm) confirmed the presence of the *O*-glycosidic linkage at C-3 of betulin, while H-29 olefinic proton resonances at 4.63 ppm (br s) and 4.76 ppm (br s) confirmed the presence of the 20(29)-olefinic bond. The olefinic protons showed an HSQC correlation to the C-29 signal, which occurred at 109.5 ppm. H-28 showed two distinct proton resonances at 3.62 ppm (br d, J = 10.4 Hz) and 3.18 ppm (br d, J = 10.4 Hz). These protons showed an HSQC correlation to C-28 that resonated at 58.0 ppm.

5.4.3 Synthesis of Betulin 3,28-(β-D-diglucoside)

5.4.3.1 Betulin 3,28-(β-D-diglucoside tetraacetate)

The positive ion ES-MS of betulin 3,28-(β-D-diglucoside tetraacetate) determined in MeOH:CH_2Cl_2 (1:1) as solvent showed pseudo-molecular ions at *m/z* 1125 $(M+Na)^+$ and 1141 $(M+K)^+$, consistent with the molecular weight of the diglucoside tetraacetate being 1102 Daltons.

The ^1H NMR spectrum of betulin 3,28-(β-D-diglucoside tetraacetate), determined in $CDCl_3$, included 6 angular methyl signals, typical of betulin, at 0.73 ppm (s, H-24), 0.83 ppm (s, H-25), 0.90 ppm (s, H-23), 0.96 ppm (s, H-27), 1.02 ppm (s, H-26) and 1.67 ppm (s, H-30), 8 methyl signals attributable to the sugar acetyl groups at 2.00-2.09 ppm, and glucosyl proton signals and C-29 olefinic proton signals in the region 3.50-5.30 ppm. 2 anomeric glucosyl proton signals appeared as 2 well defined sharp doublets at 4.53 ppm (H-1', J = 8.0 Hz) and 4.46 ppm (H-1", J = 8.0 Hz). This coupling demonstrated that both *O*-glucosidic linkages, H-1' and H-1" (of the 2 glucosyl units attached to C-3 and C-28 of betulin respectively), were β-orientated (Agrawal, 1992). The occurrence of the H-29 proton resonances at 4.57 ppm (m) and 4.67 ppm (br d, J = 2.0 Hz) confirmed the presence of a 20(29) olefinic bond. Each of the H-28 (–CH_2-O) protons appeared as well defined doublets, centred at 3.53 ppm (J = 9.7 Hz) and 3.62 ppm (J = 9.7 Hz).

The ^{13}C NMR spectrum, in $CDCl_3$, showed a total of 58 signals, 30 of which were attributable to the genin skeleton, 12 to glucosyl carbon atoms and 16 to carbon atoms of the 8 sugar acetyl groups (which included 8 acetyl methyl signals in the region 20.7-20.8 ppm and 8 carbonyl signals in the region 169.2-170.7 ppm). The DEPT135 spectrum

revealed the presence of 14 methyl, 14 methylene, 16 methine carbon signals. 14 signals corresponding to quaternary carbons were absent with respect to the ^{13}C spectrum.

The 2 anomeric proton signals, H-1' (4.53ppm) and H-1" (4.46 ppm), were assigned to glucosyl residues attached at C-3 and C-28 of betulin respectively. Correlations observed in the HMBC spectrum between H-1' (4.53 ppm) and C-3 (90.7 ppm) and between H-3 (3.06 ppm) and C-1' (103.0 ppm) showed the presence of an *O*-glycosidic linkage at C-3. These chemical shifts are consistent with values determined for betulin 3-(β-D-glucoside tetraacetate) (Table 5.8). Similarly, correlations between H-1" (4.46 ppm) and C-28 (68.6 ppm) and between each of the H-28 protons (3.53 and 3.62 ppm) and C-1" (101.7 ppm) revealed the presence of an *O*-glycosidic linkage at C-28 of betulin. HSQC data showed that C-28 occurred at 68.6 ppm, compared to 60.7 ppm in betulin (in $CDCl_3$). This downfield shift is consistent with C-28 glucosylation (Agrawal, 1992).

Glucosyl proton resonances were identified via correlated peak pathways in the COSY spectrum, starting with the H-1' and H-1" protons, and from a series of 1D-SELTOCSY spectra determined with a range of mixing (correlation) times (30 to 150 msec). These spectra enabled the progressive tracking of proton resonances around the respective glucosyl residues. Beginning with H-1' (4.53 ppm) the following correlation pathways were observed for the 3-*O*-glucosyl residue; 4.53 ppm (H-1') ↔ 5.03 ppm (H-2') ↔ 5.21 ppm (H-3') ↔ 5.03 ppm (H-4') ↔ 3.68 ppm (H-5') ↔ 4.11 and 4.23 ppm ($H\text{-}6'_A/H\text{-}6'_B$). Similarly, starting with H-1" (4.46 ppm), the following correlation pathways were observed for the 28-*O*-glucosyl residue; 4.46 ppm (H-1") ↔ 5.00 ppm (H-2") ↔ 5.21 ppm (H-3") ↔ 5.08 ppm (H-4") ↔ 3.68 ppm (H-5") ↔ 4.15 and 4.27 ppm ($H\text{-}6''_A/H\text{-}6''_B$). All glucosyl carbon resonances were subsequently identified via HSQC correlations between the foregoing glucosyl protons and the corresponding carbon atoms.

Overlapping COSY signals for H-3'/H-3", H-4'/H-4" and H-5'/H-5" and overlapping HSQC correlations for the carbon resonances corresponding to these proton shifts did not enable the unambiguous assignment of resonances to the respective glucosyl residues (Table 5.9).

Complete ^1H and ^{13}C NMR assignments of remaining genin signals of betulin 3,28-(β-D-diglucoside tetraacetate) were established using analogous methods to those described above (Section 5.4.2), and are presented in Table 5.9.

Table 5.9. ^{1}H and ^{13}C NMR assignments determined for betulin 3,28-(β-D-diglucoside tetraacetate) (ppm in $CDCl_3$) and betulin 3,28-(β-D-diglucoside) (ppm in DMSO-D_6).

Atom	Betulin 3,28-(β-D-diglucoside tetraacetate)		Betulin 3,28-(β-D-diglucoside)	
	^{13}C	^{1}H	^{13}C	^{1}H
1	38.7	0.85, 1.65	38.3	0.94, 1.66
2	26.0	1.70, 1.78	25.7	1.61, 1.92
3	90.7	3.06 (dd, *J* = 4.7, 11.4 Hz)	87.9	3.11 (m)
4	39.1	-	38.8	-
5	55.7	0.68	55.0	0.77
6	18.2	1.39, 1.50	17.7	1.44, 1.56
7	34.3	1.37 (2H)	33.7	1.44 (2H)
8	41.0	-	40.4	-
9	50.4	1.25	49.7	1.35
10	37.0	-	36.3	-
11	20.9	1.21, 1.41	20.3	1.28, 1.44
12	25.2	1.03, 1.63	24.8	1.09, 1.69
13	37.7	1.59	36.9	1.70
14	42.8	-	42.2	-
15	27.1	1.01, 1.58	26.8	1.04, 1.77
16	29.5	1.16, 1.84	29.1	1.20, 2.15
17	47.2	-	46.7	-
18	48.8	1.52	48.3	1.59
19	48.0	2.35	47.2	2.50
20	150.5	-	150.2	-
21	29.8	1.36, 1.95	29.2	1.38, 2.01
22	34.7	1.00, 1.87	34.3	1.06, 1.99
23	27.7	0.90 (s)	27.5	1.06 (s)
24	16.2	0.73 (s)	16.2	0.84 (s)
25	16.1	0.83 (s)	15.9	0.88 (s)
26	16.1	1.02 (s)	15.7	1.09 (s)
27	14.9	0.96 (s)	14.4	1.03 (s)
28	68.6	3.53 (d, *J* = 9.7 Hz) 3.62 (d, *J* = 9.7 Hz)	66.7	3.57 (d, *J* = 9.6 Hz) 3.63 (d, *J* = 9.6 Hz)
29	109.7	4.57 (m), 4.67 (br d, *J* = 2.0 Hz)	109.7	4.64 (br s), 4.78 (br s)
30	19.2	1.67 (s)	18.7	1.74 (s)
1'	103.0	4.53 (d, *J* = 8.0 Hz)	105.3	4.23 (d, *J* = 7.7 Hz)
2'	71.8	5.03 (m)	74.0	3.05 (m)
3'	72.9[a]	5.21 (m)[a]	76.8[a]	3.21 (m)[a]
4'	68.9[a]	5.03 (m)[a]	70.2[a]	3.14 (m)[a]
5'	71.6[a]	3.68 (m)[a]	76.5[a]	3.15 (m)[a]
6'	62.4	4.11 (m) 4.23 (m)	61.2[a]	3.53 (m)[a] 3.75 (m)[a]
1"	101.7	4.46 (d, *J* = 8.0 Hz)	103.7	4.18 (d, *J* = 7.7 Hz)
2"	71.4	5.00 (m)	73.5	3.06 (m)
3"	73.0[a]	5.21 (m)[a]	76.8[a]	3.21 (m)[a]
4"	68.7[a]	5.08 (m)[a]	70.2[a]	3.14 (m)[a]
5"	71.8[a]	3.68 (m)[a]	76.5[a]	3.15 (m)[a]
6"	62.2	4.15 (m) 4.27 (m)	61.2[a]	3.53 (m)[a] 3.75 (m)[a]

cont...

Table 5.9 cont...

Atom	Betulin 3,28-(β-D-diglucoside tetraacetate) ^{13}C	Betulin 3,28-(β-D-diglucoside tetraacetate) ^{1}H	Betulin 3,28-(β-D-diglucoside) ^{13}C	Betulin 3,28-(β-D-diglucoside) ^{1}H
$COCH_3$	20.7 (s)[b] 20.8 (s)[b]	2.00 (s)[d] 2.02 (s)[d]		
	20.7 (s)[b] 20.8 (s)[b]	2.00 (s)[d] 2.03 (s)[d]		
	20.7 (s)[b] 20.8 (s)[b]	2.01 (s)[d] 2.06 (s)[d]		
	20.7 (s)[b] 20.8 (s)[b]	2.02 (s)[d] 2.09 (s)[d]		
$COCH_3$	169.2[c] 170.4[c]			
	169.2[c] 170.4[c]			
	169.5[c] 170.4[c]			
	169.5[c] 170.7[c]			

[a, b, c, d] denote interchangeable assignments between respective atoms in the C-3- and C-28-*O*-glucosyl residues. Unambiguous assignments not established.

5.4.3.2 Betulin 3,28-β-D-diglucoside

Hydrolysis of betulin 3,28-(β-D-diglucoside tetraacetate) was performed in *ca.* 0.70 $molL^{-1}$ methanolic potassium at reflux temperature. The reaction mixture, separated by radial plate chromatography on silica gel, using mixtures of 5% aqueous MeOH and $CHCl_3$, yielded betulin 3,28-(β-D-glucoside).

The negative ion ES-MS of betulin 3,28-(β-D-diglucoside), in MeOH as solvent, showed pseudo-molecular ions at *m/z* 765 $(M-H)^-$, 811 $(M+COOH)^-$, 825 $(M+CH_3COO)^-$, 1531 $(M_2-H)^-$ and 1577 $(M_2+COO)^-$. These ions are consistent with the molecular weight of the diglucoside being 766 Daltons and demonstrate that hydrolysis proceeded with removal of all the acetoxyl groups.

The ^{1}H NMR spectrum of betulin 3,28-(β-D-diglucoside), determined in DMSO-D_6, included 6 angular methyl signals, typical of betulin, at 0.84 ppm (s, H-24), 0.88 ppm (s, H-25), 1.03 ppm (s, H-27), 1.06 ppm (s, H-23), 1.09 ppm (s, H-26) and 1.74 ppm (s, H-30). Glucosyl proton signals and H-29 olefinic proton signals were present in the region 3.00-5.00 ppm. The 2 anomeric glucosyl proton signals appeared as 2 well defined sharp doublets at 4.23 ppm (H-1', J = 7.7 Hz) and 4.18 ppm (H-1", J = 7.7 Hz). This coupling demonstrated that both glycosidic linkages, at C-3 and C-28 respectively, were β-orientated (Agrawal, 1992).

The ^{13}C NMR spectrum in DMSO-D_6 showed a total of 42 signals, 30 of which attributable to the genin skeleton and 12 to glucosyl carbon atoms. The DEPT135 spectrum revealed the presence of 6 methyl, 14 methylene, and 16 methine signals. Signals, corresponding to the 6 quaternary carbons of the genin were not seen in the DEPT135 spectrum.

A complete assignment of ^{1}H and ^{13}C NMR signals of betulin 3,28-(β-D-diglucoside), in DMSO-D_6, are presented in Table 5.9. The glucosyl proton resonances of the 2 sugar residues were assigned via COSY and 1D-SELTOCSY spectra peak correlation pathways. Beginning with the 2 anomeric protons H-1' (4.23 ppm) and H-1" (4.18 ppm) the respective COSY correlations identified the H-2' (3.05 ppm) and H-2" (3.06 ppm) resonances. HSQC correlations subsequently identified C-2' (74.0 ppm) and C-2" (73.5 ppm).

Overlapping COSY signals for the H-3'/H-3" (3.21 ppm), H-4'/H-4" (3.14 ppm), H-5'/H-5" (3.15 ppm) methine protons and the H-6'/H-6" (3.53 and 3.75 ppm) methylene protons prevented their unambiguous assignment and limited the interpretability of the corresponding HSQC correlations.

In the HSQC spectrum H-4'/H-4" (3.14 ppm) and H-5'/H-5" (3.15 ppm) showed correlations to signals centred at 70.2 and 76.5-76.8 ppm. The C-4'/C-4" and C-5'/C-5" resonances were distinguished via HMBC correlations from H-1' (4.23 ppm) and H-1" (4.18 ppm) to carbon signals centred at 76.5-76.8 ppm (C-5'/C-5"). C-3'/C-3" (76.8 ppm) and C-5'/C-5" (76.5 ppm) and their respective proton signals (3.21 and 3.15 ppm) were identified by comparison with assignments determined for betulin 3-(β-D-glucoside) (Table 5.8).

HMBC correlations between H-1' (4.23 ppm) and C-3 (87.9 ppm) and between H-3 (3.11 ppm) and C-1' (105.3 ppm) confirmed the presence of a 3-*O*-glucosidic linkage while correlations between H-1" (4.18 ppm) and C-28 (66.7 ppm) and between the H-28 methylene protons (3.57 and 3.63 ppm) to C-1" (103.7 ppm) confirmed the presence of a 28-*O*-glucosidic linkage. The occurrence of H-29 olefinic proton resonances at 4.64 ppm (br s) and 4.78 ppm (br) and the C-29 resonance at 109.7 ppm showed the 20(29)-olefinic bond was intact.

2 methylene carbons exhibited almost identical chemical shifts, 29.1 ppm and 29.2 ppm. Their corresponding methylene protons (identified from the HSQC spectrum) occurred at 1.20 and 2.15 ppm and at 1.38 and 2.01 ppm, respectively. These signals were distinguished on the basis of COSY correlations. H-19 (2.50 ppm) showed COSY correlations to the protons which resonated at 1.38 and 2.01 ppm. These proton resonances were therefore assigned to H-21, hence C-21 occurred at 29.2 ppm. On the other hand, one of the H-15 methylene protons (1.77 ppm) showed correlations to the protons at 1.20 and 2.15 ppm. These proton resonances were therefore assigned to H-16; hence C-16 occurred at 29.1 ppm.

5.5 Minor Tetraacetate Products

During the coupling of betulin and 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (Figure 5.10), 2 minor products, in addition to betulin 3-(β-D-glucoside tetraacetate) and betulin 3,28-(β-D-diglucoside tetraacetate), were also formed (Section 5.4.2). One of the minor products, betulin 28-(β-D-glucoside tetraacetate) was an expected reaction product; however the other, allobetulin β-D-glucoside tetraacetate was at the time an unexpected reaction product (Section 5.5.2).

As these tetraacetates were minor synthetic components, they were not hydrolyzed to yield the corresponding glucosides.

5.5.1 Betulin 28-(β-D-glucoside tetraacetate)

Separation of the coupling reaction residue by radial chromatography using mixtures of petroleum spirits (40:60) and $CHCl_3$ afforded betulin 28-(β-D-glucoside tetraacetate). This tetraacetate eluted slightly after betulin 3-(β-D-glucoside tetraacetate). The synthesis of betulin 28-(β-D-glucoside tetraacetate) has been previously reported (Uvarova *et al.*, 1980).

The positive ion ES-MS of betulin 28-(β-D-glucoside tetraacetate), in MeOH:CH_2Cl_2 (1:1), showed pseudo-molecular ions at *m/z* 795 $(M+Na)^+$, 1567 $(M_2+Na)^+$ and 1583 $(M_2+K)^+$.

These ions are consistent with the molecular weight of the glucoside tetraacetate being 772 Daltons.

Complete ^{1}H and ^{13}C NMR assignments for betulin 28-(β-D-glucoside tetraacetate) (in $CDCl_3$), were determined in an analogous fashion to that described for betulin 3-(β-D-glucoside) and betulin 3,28-(β-D-glucoside) (Sections 5.4.2 and 5.4.3), and are presented in Table 5.10. In summary, the ^{1}H NMR spectrum showed included 6 angular methyl singlet signals (typical of betulin), 4 methyl signals attributable to the sugar acetyl groups, H-29 olefinic proton signals and a single β-orientated anomeric proton H-1" (4.46 ppm, d, J = 8.0 Hz) (Agrawal, 1992).

The ^{13}C NMR spectrum showed a total of 44 signals, 30 belonging to the genin skeleton, 6 to the glucosyl carbon atoms and 8 to the 4 sugar acetyl groups. The DEPT135 spectrum revealed the presence of 10 methyl, 13 methylene, 11 methine carbon signals. 10 signals corresponding to quaternary carbons were absent with respect to the ^{13}C NMR spectrum. Glucosyl proton resonances were identified via correlated peak pathways in the COSY spectrum, starting with the H-1" proton (4.46 ppm), and from a series of 1D-SELTOCSY spectra. The following correlation pathways were observed; 4.46 ppm (H-1") ↔ 5.01 ppm (H-2") ↔ 5.22 ppm (H-3") ↔ 5.09 ppm (H-4") ↔ 3.69 ppm (H-5") ↔ 4.15 and 4.29 ppm ($H\text{-}6''_A/H\text{-}6''_B$). The location of the glycosidic linkage was determined via HMBC correlations between H-1" (4.46 ppm) and C-28 (68.7) and H-28 (3.55 and 3.63 ppm) and C-1" (101.7 ppm).

Table 5.10. ^{1}H and ^{13}C NMR assignments determined for betulin 28-(β-D-glucoside tetraacetate) (ppm in $CDCl_3$).

Atom	^{13}C	^{1}H
1	38.8	090, 1.66
2	27.5	1.59 (2H)
3	79.0	3.18 (m)
4	38.9	-
5	55.4	0.68
6	18.4	1.40, 1.53
7	34.3	1.39 (2H)
8	41.0	-
9	50.5	1.27
10	37.2	-
11	20.9	1.20, 1.42
12	25.2	1.04, 1.63
13	37.8	1.60
14	42.8	-
15	27.2	1.02, 1.59
16	29.5	1.19, 1.83
17	47.1	-
18	48.7	1.54
19	48.0	2.35
20	150.5	-
21	29.7	1.37, 1.95
22	34.7	1.00, 1.87
23	28.0	0.97 (s)
24	15.4	0.77 (s)
25	16.2	0.83 (s)
26	16.1	1.03 (s)
27	14.9	0.97 (s)
28	68.7	3.54 (d, *J* = 9.7 Hz)
		3.63 (d, *J*= 9.7 Hz)
29	109.7	4.57 (m), 4.67 (br d, *J* = 2.1 Hz)
30	19.1	1.66 (s)
1"	101.7	4.46 (d, *J* = 8.0 Hz)
2"	71.4	5.01 (dd, *J* = 8.0, 9.6 Hz)
3"	72.9	5.22 (~t, *J* = 9.6 Hz)
4"	68.7	5.09 (~t, *J* = 9.7 Hz)
5"	71.9	3.69 (m)
6"	62.2	4.15 (dd, *J* = 4.7, 12.3 Hz)
		4.29 (dd, *J* = 2.6, 12.3 Hz)
$COCH_3$	20.6 (s)	2.01 (s)
	20.6 (s)	2.01 (s)
	20.7 (s)	2.02 (s)
	20.8 (s)	2.09 (s)
$COCH_3$	169.2	
	169.4	
	170.4	
	170.7	

5.5.2 Allobetulin β-D-glucoside tetraacetate

The positive ion ES-MS, in MeOH:CH_2Cl_2 (1:1), of a fraction that eluted from the silica gel radial plate before betulin 28-(β-D-glucoside tetraacetate) and betulin 3-(β-D-glucoside tetraacetate), showed pseudo-molecular ions at *m/z* 795 $(M+Na)^+$, 1567 $(M_2+Na)^+$ and 1583 $(M_2+K)^+$. These ions are consistent with the molecular weight expected for a betulin-like monoglucoside tetraacetate (772 Daltons), a molecular weight equivalent to those for betulin 3-(β-D-glucoside tetraacetate) and betulin 28-(β-D-glucoside tetraacetate) (Sections 5.4.2.1 and 5.5.1).

The ^1H NMR spectrum of the glucoside tetraacetate included 7 singlet methyl signals at 0.72, 0.79, 0.84, 0.90, 0.90, 0.92 and 0.96 ppm, 4 methyl signals attributable to sugar acetyl groups at 2.00-2.07 ppm, a series of glucosyl proton signals in the region 4.10-5.20 ppm and a broad singlet signal at 3.52 ppm. A single anomeric glucosyl proton signal appeared at 4.53 ppm as a well defined doublet (H-1', J = 7.9 Hz). This coupling showed that the glucosidic linkage was β-orientated (Agrawal, 1992). Notable features of the ^1H NMR spectrum of this compound were the absence of the characteristic H-29 olefinic signals, which in betulin occur in the vicinity of *ca.* 4.50-4.70 ppm and the olefinic methyl signal at *ca.* 1.60-1.70 ppm.

Careful analyses of ^1H and ^{13}C NMR spectral data showed that rearrangement in ring E had occurred to afford 19,28-oxido-18α-H-oleanane β-D-glucoside tetraacetate, i.e. allobetulin β-D-glucoside tetraacetate. This rearrangement can be envisaged to have occurred via acid catalyzed initiation at the olefinic carbon C-29 (Figure 5.12). It is thought that residual acid in the reaction mixture (HBr eliminated during the reaction and not instantly neutralised by $CdCO_3$) initiated the rearrangement process.

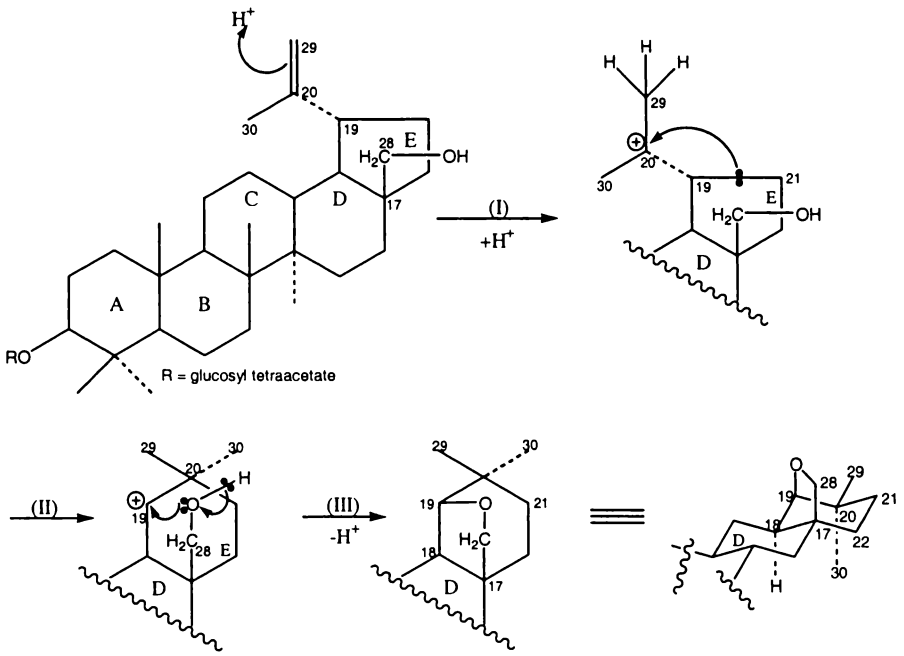


Figure 5.12. Proposed mechanism of ring E rearrangement via acid catalyzed initiation to afford the allobetulin analogue of betulin 3-(β-D-glucoside tetraacetate).

Initiation, via H^+ addition to C-29 (Step I, Figure 5.12) can be envisaged to afford a tertiary carbocation centre at C-20. Subsequent ring E expansion may occur via the migration of the 19(21) bond electrons to the carbocation centre, with the formation of the C-20 to C-21 bond and a secondary carbocation centre at C-19 (Step II). Although the proposed pathway requires the rearrangement of a tertiary carbocation to a less stable secondary carbocation, it is possible that this transformation allows ring E expansion from a 5-membered to a lesser strained 6-membered ring system. Steric relief may also be a contributing factor. Thereafter, attack of C-19 carbocation centre by a lone pair of electrons on the C-28 oxygen (Step III) and subsequent loss of H^+, affords the 19,28-oxido bridge. The above mechanism (Figure 5.12) is consistent with the mechanism reported by Li *et al.* (1998) for the formation of A-*neo*-lup-3(5)-en-28→19β-olide and A-*neo*-18α-taraxast-3(5)-en-28→19β-olide from betulinic acid.

Signals attributable to the presence of two H-28, C**H**$_2$-O- (oxido) protons appeared in the ^{1}H NMR spectrum at 3.43 ppm (d, J = 7.7 Hz) and 3.76 ppm (d, J = 7.7 Hz). The broad singlet-like nature of the H-19 signal which occurred at 3.52 ppm can be attributed to a dihedral angle between H-18 and H-19 of *ca.* 81.1°, as revealed by a molecular modelling analysis (Figure 5.13) performed using CS CHEM3D ULTRA® software and MM2 parameters (Allinger, 1977).

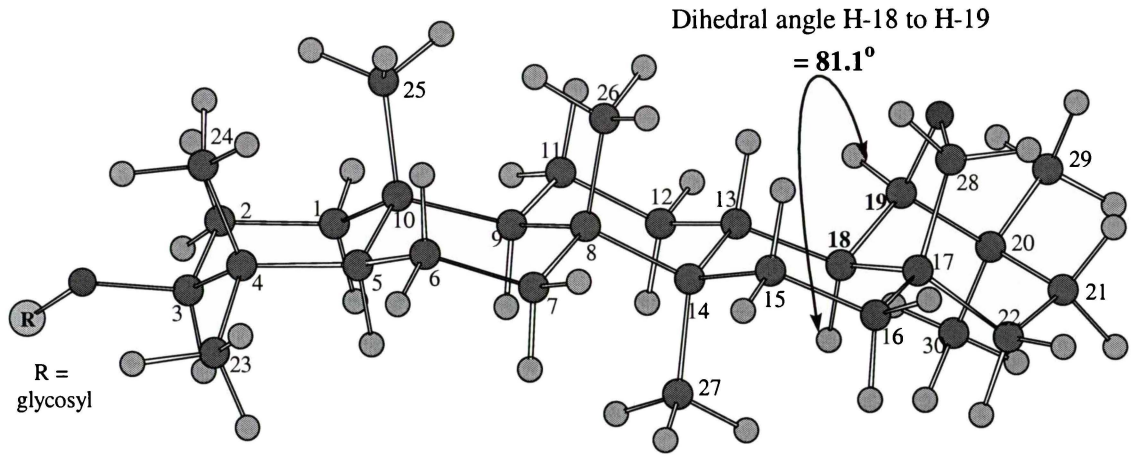


Figure 5.13. 3D model representation of allobetulin β-D-glucoside tetraacetate illustrating the dihedral angle between H-18 and H-19.

The Karplus equation (Karplus, 1962, 1963) describes the relationship between the NMR coupling constant and the dihedral angle between vicinal hydrogens. Protons having a dihedral angle of 90° possess no vicinal interaction (hence no coupling), whereas protons with dihedral angles between 0° and 90° interact variably. For dihedral angles $0^\circ \leq \Phi \leq 90^\circ$ the Karplus equation takes the form:

$$^3J_{HH'} = k_1 + k_2 \cos^2 \Phi$$

where: k_1 = *ca.* 0 Hz and k_2 = *ca.* 10 Hz

and Φ = dihedral angle between vicinal protons H and H'.

For a dihedral angle of 81.1°, the Karplus equation above predicts ^3J = *ca.* 0.25 Hz, indicating that little coupling will occur between these two protons. Typically, the line width of ^{1}H NMR signals is *ca.* 0.5 Hz or greater; therefore no resolvable coupling is

expected for H-19 (br s, 3.52 ppm). This H-19 signal assignment is consistent with the H-19 signal assignment reported by Li *et al.* (1998) for allobetulin acetate in $CDCl_3$, i.e. 3.53 ppm (1H, s, H-19).

The ^{13}C NMR spectrum contained a total of 44 signals 30 of which were attributable to the genin skeleton, 6 to glycosyl carbon atoms and 8 to carbon atoms of the 4 sugar acetyl groups. The DEPT135 spectrum revealed the presence of 11 methyl, 12 methylene, 11 methine and, by difference, 10 quaternary carbon signals. C-3, the location of the *O*-glucosidic linkage was confirmed from HMBC correlations between H-1' (4.53 ppm) and C-3 (90.7 ppm) and between H-3 (3.07 ppm) and C-1' (103.0 ppm).

COSY glucosyl proton correlations, and subsequent confirmation by a series of 1D-SELTOCSY spectra derived by irradiation of the H-1' signal (4.53 ppm), revealed the following pathways; 4.53 ppm (H-1') ↔ 5.02 ppm (H-2') ↔ 5.20 ppm (H-3') ↔ 5.04 ppm (H-4') ↔ 3.67 ppm (H-5') ↔ 4.12 and 4.24 ppm ($H\text{-}6'_A/H\text{-}6'_B$). HSQC spectral correlations assigned glucosyl proton resonances with the respective carbon chemical shifts.

HMBC correlations exhibited by the 7 genin methyl group protons allowed their unambiguous assignments. 5 methyl groups, H-23 (s, 0.90 ppm), H-24 (s, 0.72 ppm), H-25 (s, 0.84 ppm), H-26 (s, 0.96 ppm) and H-27 (s, 0.90 ppm) were assigned in a manner analogous to that described earlier for betulin 3-(β-D-glucoside) (Section 5.4.2). The remaining 2 methyl groups, H-29 (s, 0.92 ppm) and H-30 (s, 0.79 ppm), showed correlations to C-19 (88.0 ppm), C-20 (36.3 ppm), C-21 (32.8 ppm) and respectively to each other, i.e. C-30 (24.6 ppm) and C-29 (28.9 ppm). An NOE-enhancement from H-30 (s, 0.79 ppm) to H-18 (1.46 ppm) led to the unambiguous assignment of H-30 as the axially orientated methyl group. C-8 (40.7 ppm) and C-14 (40.8 ppm) were barely resolvable in the HMBC spectrum hence their respective assignments are interchangeable (Table 5.12).

3 methylene carbons, C-12, C-15 and C-16, exhibited very similar carbon resonances (Table 5.12) and were assigned (with their respective protons) as follows. The H-15 protons (1.09 and 1.56 ppm) were distinguished from an NOE-enhancement from the H-26 methyl protons (s, 0.96 ppm) to H-15β (1.56 ppm). The H-15 protons showed HSQC correlations

to a ^{13}C signal, which occurred at 26.5 ppm (C-15). The H-16 protons (1.31 and 1.43 ppm) were subsequently identified via a COSY correlation from an H-15 proton (1.56 ppm) to a proton signal, which occurred at 1.31 ppm. The HSQC spectrum linked this methylene proton, and its companion proton (1.43 ppm) to the ^{13}C signal which occurred at 26.3 ppm (C-16). The H-12 proton (0.90 and 1.64 ppm) assignments were confirmed via a COSY correlation from H-13 (1.45 ppm) to 0.90 ppm (H-12). The H-12 protons showed an HSQC correlation to a ^{13}C signal that occurred at 26.5 ppm (C-12).

NOE-enhancements observed for allobetulin β-D-glucoside tetraacetate (Table 5.11) were consistent with the NMR assignments presented in Table 5.12.

Table 5.11. Selected NOE-difference enhancements determined for allobetulin β-glucoside tetraacetate (ppm in $CDCl_3$).

Irradiated signal	Enhanced signals
0.79 (H-30)	0.92 (H-29), 1.21 (H-21α), 1.46 (H-18), 3.52 (H-19)
0.84 (H-25)	0.72 (H-24), 0.96 (H-26), 1.35 (H-6β), 1.48 (H-11β)
0.92 (H-29)	0.79 (H-30), 1.50 (H-21β), 3.52 (H-19)
0.96 (H-26)	0.84 (H-25), 1.45 (H-13), 1.56 (H-15β)
3.43 (H-28α)	1.29 (H-22β), 1.39 (H-22α), 1.50 (H-21β), 3.76 (H-28β)
3.52 (H-19)	0.79 (H-30), 0.92 (H-29), 1.45 (H-13), 1.64 (H-12β)
3.76 (H-28β)	0.96 (H-26), 1.31 (H-16β), 1.45 (H-13), 1.56 (H-15β), 3.43 (H-28α)

Table 5.12. ^{1}H and ^{13}C NMR assignments determined for allobetulin β-D-glucoside tetraacetate (ppm in $CDCl_3$).

Atom	^{13}C	^{1}H
1	38.9	0.88, 1.71
2	26.0	1.71, 1.79
3	90.7	3.07 (dd, *J* = 5.0, 11.3 Hz)
4	39.1	-
5	55.9	0.68
6	18.2	1.35, 1.47
7	34.0	1.38 (2H)
8	40.7[a]	-
9	51.2	1.29
10	37.1	-
11	21.1	1.25, 1.48
12	26.5	0.90, 1.64
13	34.2	1.45
14	40.8[a]	-
15	26.5	1.09, 1.56
16	26.3	1.31, 1.43
17	41.5	-
18	46.9	1.46
19	88.0	3.52 (br s)
20	36.3	-
21	32.8	1.21, 1.50
22	36.8	1.29, 1.39
23	27.7	0.90 (s)
24	16.2	0.72 (s)
25	16.5	0.84 (s)
26	15.8	0.96 (s)
27	13.5	0.90 (s)
28	71.3	3.43 (d, *J* = 7.7 Hz) 3.76 (br d, *J* = 7.7 Hz)
29	28.9	0.92 (s)
30	24.6	0.79 (s)
1'	103.0	4.53 (d, *J* = 7.9 Hz)
2'	71.8	5.02 (m)
3'	73.0	5.20 (~t, *J* = 9.5 Hz)
4'	68.9	5.04 (m)
5'	71.6	3.67 (ddd, *J* = 2.7, 5.3, 9.9 Hz)
6'	62.3	4.12 (dd, *J* = 2.6, 12.2 Hz) 4.24 (m)
$COCH_3$	20.6 (s) 20.7 (s) 20.7 (s) 20.8 (s)	2.00 (s) 2.01 (s) 2.06 (s) 2.07 (s)
$COCH_3$	169.2 169.4 170.4 170.7	

[a] denotes interchangeable assignments.

At the time of synthesis, rearrangement of the betulin skeleton to the allobetulin skeleton was not expected. However, once the compound was identified, a literature search revealed that the transformation of betulin to allobetulin is a well known reaction. Reported as early as 1922 by Schulze and Pieroh, the transformation was achieved using formic acid, to give a moderate yield of allobetulin (Li *et al.*, 1998). A number of acidic reagents have been employed for the betulin-allobetulin transformation including hydrobromic acid in chloroform (Dischendorfer, 1923), sulphuric acid in acetic acid (Barton and Holness, 1952), concentrated hydrochloric acid in ethanol (Lawrie *et al.*, 1960) and on solid acid supports (Li *et al.*, 1998; Lavoie *et al.*, 2001). The presence of allobetulin β-D-glucoside tetraacetate has been reported previously under Koenig-Knorr glycosylation conditions (Odinokova *et al.*, 1984).

5.6 Isolation of 4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside)

A sample of freeze-dried Scottish *N. ossifragum* leaves, collected in July 2001 from Auchtertyre Farm, Strathfillian, West Perthshire, Scotland, during the ISOPP6 field trip (see Chapter 3), was extracted with $CHCl_3$ for 4 h using a SoxTech system HT 1043 Extraction Unit (Tecator, Sweden) apparatus and yielded a free sapogenin extract which was discarded. Subsequent SoxTech extraction with MeOH for 4 h afforded a conjugated sapogenin extract. Initial separation of the crude conjugated extract by silica gel column chromatography was performed using mixtures of 5% aqueous MeOH and $CHCl_3$. Relevant fractions were then combined and separated by radial chromatography on silica gel using mixtures of 5% aqueous MeOH and $CHCl_3$ which yielded 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside).

The structure of 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) was confirmed by NMR. Complete ^{1}H and ^{13}C NMR assignments, determined in deutero-pyridine, are presented in Table 5.13. Assignments are consistent with those of Inoue *et al.* (1995) and Langseth *et al.* (1999).

Table 5.13. ^{1}H and ^{13}C NMR assignments determined for 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (ppm in C_5D_5N).

Atom	^{13}C	^{1}H
2	178.3	-
3	90.3	6.45 (s)
4	170.6	-
5	98.2	5.40 (s)
4*	59.6	3.69 (s)
1'	103.5	5.27 (d, J = 7.8 Hz)
2'	74.9	4.07 (t, J = 8.3 Hz)
3'	78.3	4.20 (t, J = 8.7 Hz)
4'	71	4.25 (t, J = 9.0 Hz)
5'	79.2	3.90 (ddd, J = 2.4, 5.0, 9.2 Hz)
$6'_A$	62.2	4.32 (dd, J = 4.9, 11.9 Hz)
$6'_B$		4.46 (dd, J = 2.3, 11.9 Hz)

4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (Figure 5.14) has previously been isolated from *N. ossifragum* (Stabursvik, 1959; Tschesche and Hoppe, 1971) and from *N. asiaticum* (Inoue *et al.*, 1995) although its structure was not determined until 1995 (Inoue *et al.*, 1995). Of the 3 known furanones reported in *N. ossifragum* and *N. asiaticum*; 3-methoxyfuran-2(5*H*)-one, 4-methoxyfuran-2(5*H*)-one and 5-hydroxy-4-methoxyfuran-2(5*H*)-one, the latter is the only one that is present naturally in a glycosidic form (Langseth *et al.*, 1999).

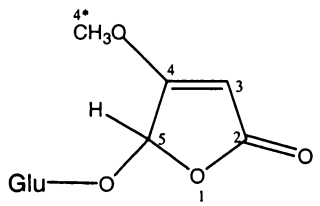


Figure 5.14. Structure of 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside).

Toxicology studies performed on goats have shown 3-methoxyfuran-2(5*H*)-one is the substance principally responsible for the nephrotoxic effect of *N. ossifragum* plants on cattle, moose, goats and other ruminants (Langseth *et al.*, 1999). Interestingly, 4-methoxyfuran-2(5*H*)-one, when dosed at twice the level of 3-methoxyfuran-2(5*H*)-one (i.e. 60 mg/kg live weight), was not found to be toxic to goats (Langseth *et al.*, 1999). Following this, it was of interest to ascertain if 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) would demonstrate desirable bioactive properties and was therefore included in the bioactivity testing reported in Chapter 6.

Chapter Six

Bioactivity Assessment

6.1 Introduction

Saponins are widely distributed in the plant kingdom and are well known for their wide spectrum of biological activities. Many have potent antimicrobial and antifungal activities (Miyakoshi *et al.*, 2000; Renault *et al.*, 2003). Members of the saponin family are exploited commercially for a range of purposes including medicines and drugs, synthetic hormone precursors, food and beverage additives and cosmetics (Osbourn, 2003). Although scores of saponins exhibit a collection of active properties, the ability to accurately predict the properties or bioactivities of individual saponins is presently wanting due to the lack of knowledge concerning saponin structure-activity relationships. A few structure-activity relationship studies have been performed (Bedir *et al.*, 2002; Ohara and Ohira, 2003; Escalante *et al.*, 2002; Favel *et al.*, 1994; Sindambiwe *et al.*, 1998). However, due to the extremely wide range of structures encountered, the assortment of testing regimes utilized and the diversity of biological activities tested, inter-study comparisons are often not possible. For example, Favel *et al.* (1994) reported that 3 monodesmosidic triterpenoid saponins (possessing oleanolic acid as the genin) were devoid of antifungal activities. However, the monodesmosidic oleanolic saponin phytolaccoside B has been reported to possess a broad spectrum of activity against a range of fungal organisms (Kobayashi *et al.*, 1995; Escalante *et al.*, 2002).

As a contribution to structure-activity knowledge, the saponin compounds synthesized in Chapter 5 where subjected to a preliminary assessment of their bioactivities against a variety of fungi and bacteria organisms. It was envisaged that this might lead to the identification of some structure-activity relationships amongst the synthesized compounds.

The agar gel well diffusion technique (AGWD) utilized in these investigations was developed and carried out, in person, during a 3-month visit to the National Veterinary Institute (NVI), Oslo, Norway, during 2003.

6.2 Materials and Methods

6.2.1 Test Compounds

The following compounds (the syntheses of which are described in Chapter 5) were subjected to the bioactivity assessments reported in this chapter.

sarsasapogenin β-D-glucoside	episarsasapogenin β-D-galactoside
episarsasapogenin β-D-glucoside	betulin 3-(β-D-glucoside)
sarsasapogenin β-D-galactoside	betulin 3,28-(β-D-diglucoside)

Sarsasapogenin β-D-glucoside was an existing sample previously synthesized by Deng (1999). This glucoside has been isolated from asparagus (*Asparagus officinalis*) along with 8 other steroidal glycosides (Goryanu *et al.*, 1976).

4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside), isolated from a Scottish collection of *Narthecium ossifragum* (see Chapter 5, Section 5.6), was also included in the bioactivity assessments, as were the following extracts:

(i) *Yucca schidigera* 70° Brix viscous liquid extract.
Batch N°: #w22/2001; produced by NOR-VET, ApS, Kanalholmen 2, 2650 Hvidovre, Denmark.

(ii) A methanol conjugate extract from 12 g of freeze-dried Norwegian *N. ossifragum* leaves (SoxTech extracted as in Chapter 3, Section 3.2.2).

(iii) Manuka oil active concentrate.
Batch No. TE03.2; March 2003; obtained from Tairawhiti Pharmaceuticals Ltd, Te Araroa, East Cape, New Zealand. Relative density (20°C): 1.072; refractive index (20°C): 1.501. Triketone content by GC-MS analysis: flaversone 18.98%, leptospermone 17.68%, isoleptospermone 61.51%.

Stock test solutions of each compound and extract were prepared in neat DMSO and stored in sealed test tubes at 4°C until required.

6.2.2 Test Organisms and Media

Bacterial organisms, fungal organisms and agar mediums were prepared and supplied, respectively, by the Microbiology Department and the Media Department, NVI, Oslo. Organisms were stored on agar gel Petri dishes at 4°C until needed.

Bacterial and fungal bioactivity assessments were performed using combined methods utilized in the Microbiology Department, NVI, Oslo, modified versions of Etest procedures (Etest Technical Manual, 2000), and adaptations from various literature sources. Details of testing procedures for each test organism can be found in Section 6.2.

Tetracycline in DMSO was used as the positive control drug for bacteria testing while amphotericin B in DMSO was employed as the positive control drug for all the fungal assessments (Figure 6.1). Amphotericin B solutions were protected from sunlight using aluminium foil. Fresh positive control solutions were made no more than 48 h prior to each bioactivity assessment. Neat DMSO was used as the negative control across the battery of tests. Positive and negative control wells were run in duplicate.

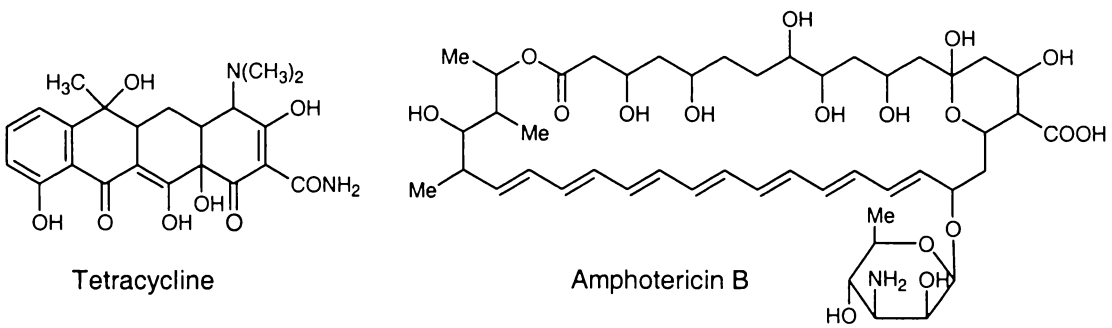


Figure 6.1. Structures of the positive control drugs tetracycline and amphotericin B.

6.2.3 Bacterial Strains

6.2.3.1 *Staphylococcus aureus* and *Escherichia coli*

Two bacterial strains were supplied, *S. aureus* CCUG 9129 and *E. coli* CCUG 17620 and were maintained on blood agar. Fresh cultures were streaked onto blood agar plates and incubated at 30°C for 30 h. Inoculums were prepared from picking isolated colonies from each isolate and suspending in 9 mL sterile saline water (0.9% NaCl). The turbidity of the cell suspension was measured on a spectrophotometer at 530 nm and was adjusted to match a 0.5 McFarland inoculum density standard, i.e. 1×10^6 to 5×10^6 colony forming units/mL (CFU/mL) (Matar *et al.*, 2003; Kirkpatrick *et al.*, 1998; Rubio *et al.*, 2003). Mueller Hinton agar plates (90 mm in diameter) were inoculated by flooding the surface with the standardized inoculum suspension and drawing off the excess. Plates were allowed to dry (*ca.* 15 min). 4 equally spaced wells (7 mm diameter) were punched into the agar gel and 75 μL of each test compound was introduced into each well. Each test concentration was run in duplicate. Test plates were incubated at 37°C for 24 h and then inspected visually. Tetracycline (8 ppm) was used as the positive control agent.

6.2.4 Fungal Strains

6.2.4.1 *Candida albicans* and *Candida glabrata*

C. albicans VI 02828 and *C. glabrata* VI 02817 isolates were supplied and maintained on Sabourauds agar (SAB). Fresh cultures were prepared by selecting a single colony and streaking onto fresh SAB agar plates and incubating at 25°C for 48 h. Standard test inoculums were made by suspending the 2 day old cell cultures in 9 mL sterile saline water (0.9% NaCl) and the optical densities were adjusted to correspond to a 0.5 McFarland standard. SAB plates (90 mm diameter) were inoculated with 400 μL of inocula, spread evenly in 3 directions over the agar surface with a sterile swab. Plates were allowed to dry (*ca.* 15 min) before wells were punched into the agar gel (7 mm diameter) and 60 μL of each test compound was introduced into the wells in duplicate. Test plates were incubated at 30°C for 48 h and inspected visually. Amphotericin B (50 ppm) was used as the positive control agent.

6.2.4.2 *Aspergillus fumigatus*

3-point inoculations onto malt extract agar (MEA) plates were performed from the *A. fumigatus* 1309.2 strain which was supplied and maintained on MEA. Plates were incubated at 25°C for 5-7 days. Both spore and hyphae from the 5-7 day old cultures were suspended in sterile saline water (0.9% NaCl) with Tween 80 and adjusted to a 0.5 McFarland standard. MEA test plates were swabbed in 3 directions using a sterile swab with 400 μL of inocula and allowed to dry (*ca.* 15 min). Holes were punched in the agar and 75 μL of each test compound was introduced into each well in duplicate. Plates were incubated at 30°C for 24 h and inspected visually. Amphotericin B (500 ppm) was used as the positive control agent.

6.2.4.3 *Penicillium rogueforti* and *Penicillium expansum*

P. rogueforti VI 01614 and *P. expansum* 1235-2 were supplied and maintained on MEA. Both organisms were freshly cultured via 3-point inoculation on MEA plates and incubated at 25°C for 7 days. Suspensions of the test fungi were made in sterile saline water (0.9% NaCl) with Tween 80 and adjusted to a 0.5 McFarland standard. Each MEA test plate was then inoculated with 400 μL of inocula, spread in 3 directions with a sterile swab and left to dry (*ca.* 15 mins) before the wells were punched in the agar. 75 μL of each test compound was introduced into each well in duplicate. Plates were incubated at 30°C for 48 h before being visually inspected. Amphotericin B (500 ppm) was used as the positive control agent for both organisms.

6.2.4.4 *Microsporum canis*

M. canis VI 02823 was supplied and maintained on mycobiotic agar (MY). *M. canis* was freshly cultured via 3-point inoculation onto MY plates and incubated at 30°C for 14 days. Fungi suspensions in sterile water (0.9% NaCl) with Tween 80, adjusted to a 0.5 McFarland standard were used to inoculate fresh MY agar plates. 400 μL of inocula was spread in 3 directions on the agar surface with a sterile swab and left to dry (*ca.* 15 mins) before the wells were punched in the agar. 75 μL of each test compound was introduced into each well in duplicate and plates were incubated at 30°C for 72 h before being visually inspected. Amphotericin B (500 ppm) was used as the positive control agent.

6.2.4.5 *Trichophyton mentagrophytes*

T. mentagrophytes VI 02136, maintained on SAB agar plates was freshly cultured onto SAB agar plates via 3-point inoculation and incubated at 30°C for 11 days. Suspension of the fungi in sterile water (0.9% NaCl) with Tween 80, adjusted to a 0.5 McFarland standard provided the inoculum that was used to inoculate fresh SAB agar test plates. 400 μL of inocula was applied in 3 directions with a sterile swab and left to dry (*ca.* 15 mins) before the wells were punched in the agar. 75 μL of each test compound was introduced into the wells. Plates were incubated at 30°C for 48 h and then visually inspected. Amphotericin B (500 ppm) was used as the positive control agent.

6.3 Results and Discussion

6.3.1 Inhibitory Zones and Concentrations

A positive result was interpreted as being a compound test well that showed, from visual inspection, an inhibitory effect on organism lawn growth over and above that of the DMSO negative control wells. Inhibition zones are reported as diameters (mm) which encase the well diameter (7 mm) and have two sub-designations;

(i) Fully clear - where the agar surface in the inhibitory zone around the well is fully clear of organism lawn growth, i.e. 12.

(ii) Reduced growth – where organism lawn growth is reduced but the agar surface is not completely void of growth. Values are bracketed, i.e. (12).

In cases where positive results showed an inner fully clear inhibition zone followed by an outer reduced growth zone, both diameters are reported, with the reduced growth diameter including the fully clear diameter. For example, a positive result that shows an overall inhibition diameter of 15 mm with an inner fully clear inhibition zone of 12 mm is presented as: 12 (15).

The synthesized saponin compounds; sarsasapogenin β-D-glucoside, sarsasapogenin β-D-galactoside, episarsasapogenin β-D-glucoside, episarsasapogenin β-D-galactoside, betulin 3,28-(β-D-diglucoside) (betulin-diglu) and betulin 3-(β-D-glucoside) (betulin-glu) were tested at concentrations of 100, 200 and 400 ppm. 4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (furanone-glu) was tested at 500, 1000 and 2000 ppm and the *Y. schidigera* 70° Brix extract (*Y. schidigera* Brix), *N. ossifragum* conjugate extract (*N. oss* extract) and the Manuka oil active concentrate (triketone oil) were tested at 1000, 2000, 5000 and 10000 ppm. Where test compounds showed activity at the lowest initial test concentration, concentrations were lowered to find the concentration where inhibition activity ceased.

It was noticed for solutions of the 6 synthesized saponins, that the use of concentrations above 400 ppm in DMSO as solvent resulted in residues in the agar wells once diffusion of the test solutions had occurred into the agar. Since only 1 hydrophilic group (the glycoside residue) is present in the synthesized saponins, the solubility of these saponins seems to be dominated by the hydrophobic genin constituent, which is consistent with the observation that they lack advantageous aqueous solubility properties. The agar gels employed in the bioactivity testing were all aqueous based mediums.

At concentrations of up to and including 400 ppm, the synthesized saponins diffused into the agar medium. However at concentrations greater than 400 ppm diffusion of the total amount of saponin did not seem to be possible and deposition of saponin residues in the agar wells was observed. Betulin 3,28-(β-D-diglucoside) seemed, at least to some extent, more able to diffuse into the agar mediums than the other 5 saponins. This may be a consequence of it being more hydrophilic compared to the other saponins, due to the presence of 2, rather than 1, glycosidic residues.

The upper concentration limit for the synthesized saponins was therefore set at 400 ppm. It was also found that where the synthesized saponins showed effect at 400 ppm (i.e. against *C. glabrata*, Section 6.3.3), raising the concentration, for example to 2000 ppm, showed no increase in inhibition zones and lead to the deposition of residues on the well bottoms.

Where the synthesized saponins showed no inhibition at 400 ppm it would have been desirable to test at higher concentrations in order to gauge the threshold inhibition concentration limits, however due to the apparent diffusion limits this was not possible. 4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside), the triketone oil, the *Y. schidigera* Brix and *N. ossifragum* extracts all diffused without difficulty into the agar mediums at their respective test concentrations.

6.3.2 Bacteria

6.3.2.1 *S. aureus* and *E. coli*

The two bacterial strains tested (*S. aureus* and *E. coli*) were only susceptible to the triketone oil. All of the other tested compounds and extracts showed no discernible effects up to the maximum tested concentrations (Table 6.1). All experiments were performed in duplicate.

Table 6.1. Bacterial inhibition zones (mm) after 24 h incubation. Reduced growth zones (mm) are bracketed.

Compound	Conc (ppm)	Organism and Inhibition Zone (mm) *S. aureus*	*E. coli*
Tetracycline	8	19 (25)	16 (18)
DMSO	neat	-	-
Triketone oil	500	(11)	-
	1000	10 (14)	-
	2000	12 (13)	-
	5000	14 (15)	-
	10000	15 (18)	(9)

S. aureus = *Staphylococcus aureus*; *E. coli* = *Escherichia coli*; Triketone oil = Manuka oil active concentrate. '-' = no inhibition of organism growth.

The triketone oil reduced the *S. aureus* lawn growth when applied at concentrations down to 500 ppm, and displayed slightly reduced inhibition of *E. coli* lawn growth at 10000 ppm. These findings are consistent with previously reported results of East Cape Manuka oil and its triketone fraction, both of which have been shown to exhibit antimicrobial activity against *S. aureus* and *E. coli* (Perry *et al.*, 1997; Porter and Wilkins, 1999). The lack of inhibition by the synthesized saponin compounds is consistent with previous knowledge,

that the majority of steroidal and triterpenoidal saponins have little, or no, antibacterial activity (Chapter 1, Section 1.5.2.3).

An inhibition dose response curve for the triketone oil against *S. aureus* is presented in Figure 6.2 and illustrates an increasing inhibition of *S. aureus* as the concentration of triketone oil increases. The area of organism inhibition was calculated as the area (mm^2) of the fully clear inhibition zones (see Table 6.1) minus the area of the test well.

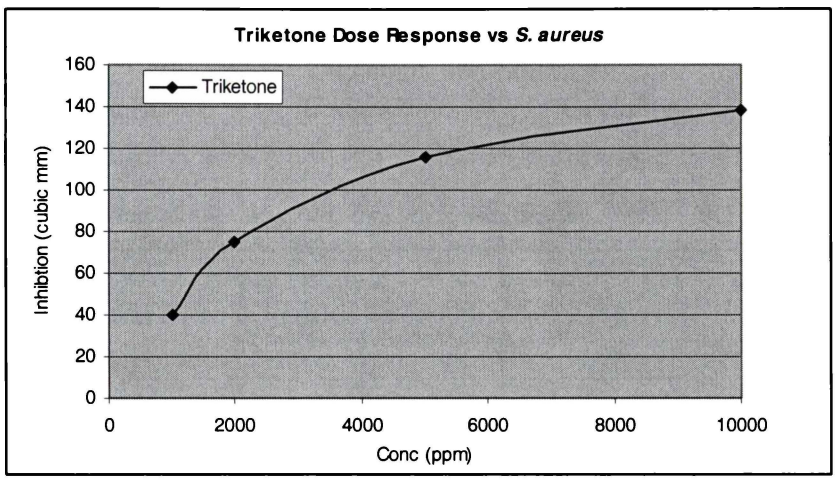


Figure 6.2. Triketone oil dose response curve observed against *S. aureus*.

6.3.3 Yeasts

6.3.3.1 *C. albicans* and *C. glabrata*

Antiyeast activities obtained for the tested compounds against *C. albicans* and *C. glabrata* are presented in Table 6.2.

Table 6.2. Antiyeast activity inhibition zones (mm) after 24 h incubation. Reduced growth zones (mm) are bracketed.

Compound	Conc (ppm)	Organism and Inhibition Zone (mm) *C. albicans*	*C. glabrata*
Amphotericin B	50	21	18
DMSO	neat	-	-
Sar-glu	400	-	(10-11)
Sar-gal	400	-	(10-11)
Episar-glu	400	-	(10-11)
Episar-gal	400	-	(10-11)
Betulin-glu	400	-	(10-11)
Betulin-diglu	400	-	(10-11)
Furanone-glu	2000	-	(10-11)
N. oss extract	5000	-	(9)
	10000	(11)	10 (11)
Y. schidigera	2000	(11)	(9)
	5000	16	14
	10000	18	19
Triketone oil	2000	(9)	(9)
	5000	10	10
	10000	11	12

C. albicans = *Candida albicans*; *C. glabrata* = *Candida glabrata*; Sar-glu = sarsasapogenin β-D-glucoside; Sar-gal = sarsasapogenin β-D-galactoside; Episar-glu = episarsasapogenin β-D-glucoside; Episar-gal = episarsasapogenin β-D-galactoside; Betulin-glu = betulin 3-(β-D-glucoside); Betulin-diglu = betulin 3,28-(β-D-diglucoside); Furanone-glu = 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside); *N. oss* extract = *Narthecium ossifragum* conjugate extract; *Y. schidigera* = *Yucca schidigera* 70° Brix extract; Triketone oil = Manuka oil active concentrate. '-' = no inhibition of organism growth.

Y. schidigera Brix showed the best activity of the tested compounds against the 2 yeast strains. *Y. schidigera* saponins have been previously reported to possess antiyeast activities against *C. albicans*, *C. famata* and a number of other yeasts (*Miyakoshi et al.*, 2000). The *N. ossifragum* conjugate extract showed slight activity against both yeast strains. Deng (1999) identified a steroidal saponin from Norwegian *N. ossifragum* that has also been identified in *Y. schidigera* and has been shown to have antiyeast activity (*Miyakoshi et al.*, 2000).

None of the synthesized saponins, or 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside), showed antiyeast activity against *C. albicans*. However these compounds all showed similar visual reduction growth when tested against *C. glabrata*. The effects, although not potent, showed reduced growth areas that were visually different to the DMSO controls, which themselves had full *C. glabrata* lawn growth to the well edges. As mentioned in Section 6.3.1, raising the concentrations of the synthesized saponins from 400 ppm to 2000 ppm resulted in residues on the well bottoms and no effective visual increase in the inhibition zones of *C. glabrata*. This can be interpreted as showing the effective concentration diffusion limit of the synthesized saponins into the agar medium was 400 ppm.

Photographic representations of a selection of *C. glabrata* test wells are presented in Figure 6.3. An episarsasapogenin β-D-galactoside 2000 ppm test well illustrates the residue left behind post diffusion. The inhibition zone for the episarsasapogenin β-D-galactoside 400 ppm well was equivalent to the 2000 ppm result.

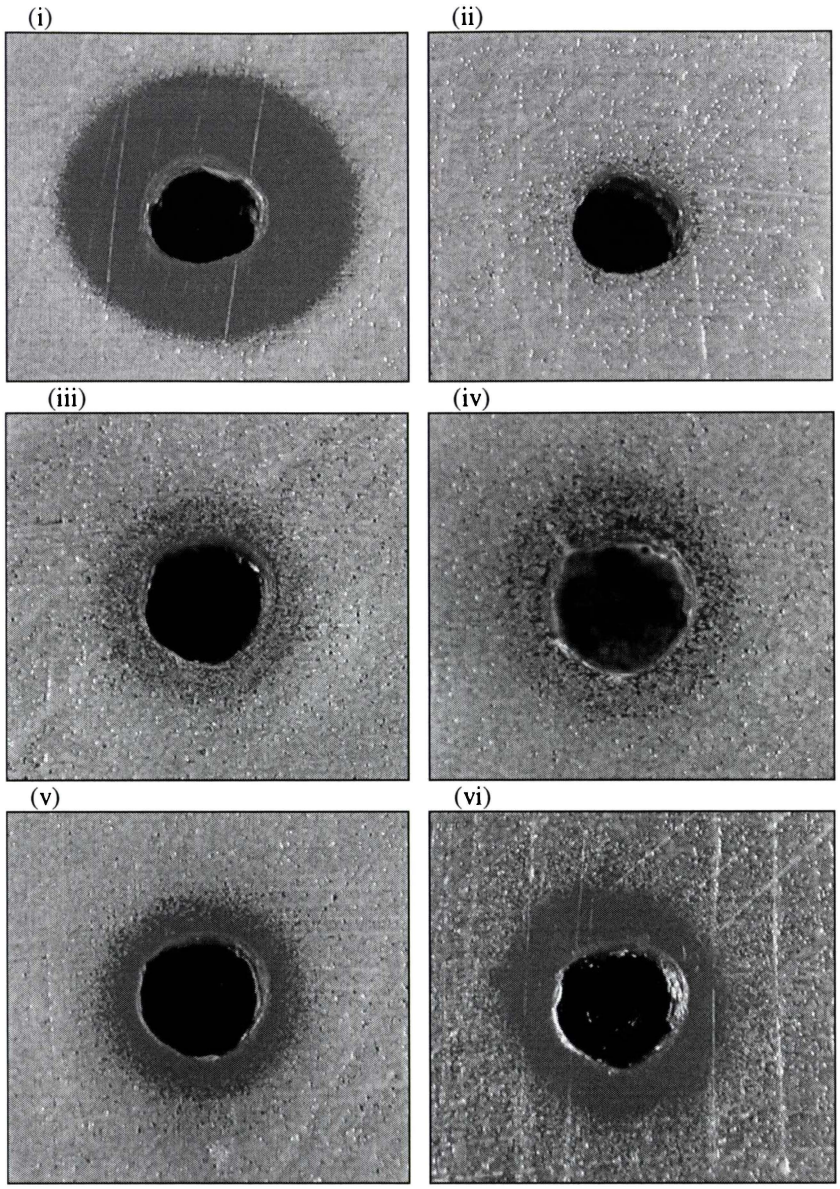


Figure 6.3. *C. glabrata* test wells illustrating inhibition zones after 24 h for; (i): amphotericin B (50 ppm) positive control, (ii): DMSO negative control, (iii): 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) at 2000 ppm, (iv): episarsasapogenin β-D-galactoside at 2000 ppm, showing residue on the well bottom, (v): *N. ossifragum* conjugate extract at 10000 ppm and (vi): triketone oil at 10000 ppm.

The ability of the 6 synthesized saponins to inhibit *C. glabrata* growth was only very slight. Possibly the activity threshold concentration of the 6 synthesized saponins against *C. glabrata* is in the vicinity of 400 ppm. When tested at 200 ppm, none of the 6 saponins

inhibited *C. glabrata* growth. Due to diffusion limitations, concentrations above 400 ppm could not be reliably assessed. If the saponins could be made more diffusible into the agar, it may be possible to obtain dose response curves and therefore comparison data for the different saponins.

The antiyeast dose response curves determined for the *Y. schidigera* 70° Brix extract are presented in Figure 6.4.

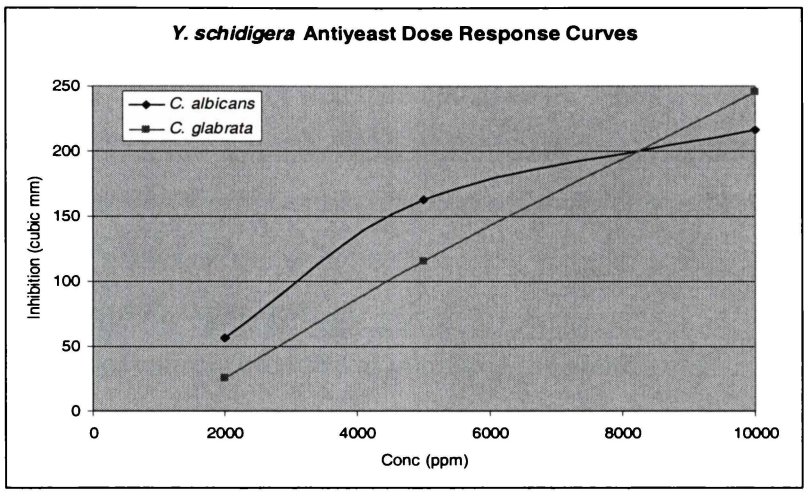


Figure 6.4. *Y. schidigera* 70° Brix extract antiyeast dose response curves observed against *C. albicans* and *C. glabrata.*

6.3.4 Fungi

6.3.4.1 *A. fumigatus*

Presented in Table 6.3 are the *A. fumigatus* assessment results. The only test substance to show an inhibitory effect against *A. fumigatus* after 24 h incubation was the triketone oil. It was observed that after 48 h of incubation, *A. fumigatus* lawn growth was more pronounced than at 24 h and that inhibition zones were reduced in size, indicating that *A. fumigatus* growth was proceeding slowly back into the inhibition zone after initial suppression. This occurred for both the triketone oil and amphotericin B. Inhibition zones of the triketone 10000 ppm wells and amphotericin B wells were not fully clear of *A. fumigatus* growth. A diameter of *ca.* 10 mm was fully clear and the remainder of the inhibition zones had reduced growth of *A. fumigatus.* Amphotericin B did not appear to have potent activity against the *A. fumigatus* strain.

Table 6.3. *A. fumigatus* inhibition zones (mm) after 24 h incubation. Reduced growth zones (mm) are bracketed.

Compound	Conc (ppm)	Organism and Inhibition Zone (mm) *A. fumigatus*
Amphotericin B	500	10 (15)
DMSO	neat	-
Triketone oil	5000	(13)
	10000	10 (16)

A. fumigatus = *Aspergillus fumigatus*; Triketone oil = Manuka oil active concentrate. '-' = no inhibition of organism growth.

New Zealand Manuka oil possessing triketone components, mainly sourced from the East Cape (Perry *et al.*, 1997), has been reported to show antifungal activity against 2 other *Aspergillus* species, namely *A. niger* and *A. ochraceus* (Lis-Balchin *et al.*, 2000).

6.3.4.2 *P. rogueforti* and *P. expansum*

Amphotericin B was not entirely effective at inhibiting the growth of *P. rogueforti*. At 500 and 1000 ppm inhibition zones were very similar. Accordingly, a concentration of 500 ppm amphotericin B was used as the positive control agent. Amphotericin B at 500 ppm was more effective in inhibiting the growth of *P. expansum* than *P. rogueforti* (Table 6.4).

Table 6.4. *Penicillium spp.* inhibition zones (mm) after 48 h incubation. Reduced growth zones (mm) are bracketed.

Compound	Conc (ppm)	Organism and Inhibition Zone (mm) *P. rogueforti*	*P. expansum*
Amphotericin B	500	(15)	18
DMSO	neat	-	-
Furanone-glu	2000	(10-11)	-
N. oss extract	5000	(10)	-
	10000	12 (14)	-
Y. schidigera	5000	16 (18)	-
	10000	21	-
Triketone oil	10000	-	12 (14)

P. rogueforti = *Penicillium rogueforti*; *P. expansum* = *Penicillium expansum*; Furanone-glu = 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside); *N. oss* extract = *Narthecium ossifragum* conjugate extract; *Y. shidigera* = *Yucca schidigera* 70° Brix extract; Triketone oil = Manuka oil active concentrate. '-' = no inhibition of organism growth.

4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) (2000 ppm) and the *N. ossifragum* conjugate extract (5000 ppm) showed limited inhibition effects against *P. rogueforti*, with the *N. ossifragum* conjugate extract showing slightly improved inhibition at 10000 ppm. *Y. schidigera* 70° Brix extract showed remarkable inhibition of *P. rogueforti* at 5000 and 10000 ppm, but no effect was seen at 2000 ppm. The 6 synthetic saponins showed no discernable inhibition of the two *Penicillium* species at concentrations up to their apparent diffusion limit of 400 ppm.

6.3.4.3 *M. canis*

Inhibition zones were recorded 72 h after incubation. After 96 h, lawn growth of *M. canis* was thicker and inhibition zones were observed to have decreased in diameter by *ca.* 2-3 mm with respect to 72 h inhibition zones, which indicates growth advancement of *M. canis*. Maximum inhibition zones observed for *M. canis* (72 h) are presented in Table 6.5.

Table 6.5. *M. canis* inhibition zones (mm) after 72 h incubation. Reduced growth zones (mm) are bracketed.

Compound	Conc (ppm)	Organism and Inhibition Zone (mm) *M. canis*
Amphotericin B	500	18
DMSO	neat	-
Sar-glu	200	(10)
	400	(12)
Sar-gal	200	(10)
	400	(14)
Episar-glu	400	(11-12)
Furanone-glu	1000	(10)
	2000	19
N. oss extract	1000	(12-13)
	2000	18
	5000	23
	10000	25
Y. schidigera	1000	12-13
	2000	19
	5000	23
	10000	33
Triketone oil	1000	(10-11)
	2000	(12-13)
	5000	13 (14)
	10000	14 (15)

M. canis = *Microsporum canis*; Sar-glu = sarsasapogenin β-D-glucoside; Sar-gal = sarsasapogenin β-D-galactoside; Episar-glu = episarsasapogenin β-D-glucoside; Furanone-glu = 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside); *N. oss* extract = *Narthecium ossifragum* conjugate extract; *Y. schidigera* = *Yucca schidigera* 70° Brix extract; Triketone oil = Manuka oil active concentrate. '-' = no inhibition of organism growth.

Of the 6 synthesized saponins compounds, sarsasapogenin β-D-glucoside, sarsasapogenin β-D-galactoside and episarsasapogenin β-D-glucoside showed a small but promising effect against *M. canis* at 400 ppm, resulting in reduced growth inhibition zones (Table 6.5). Slight inhibition effects were seen for sarsasapogenin β-D-glucoside and sarsasapogenin β-D-galactoside at 200 ppm, and no inhibition effects on the growth of *M. canis* were observed at 100 ppm. No inhibition was seen at 200 ppm for episarsasapogenin β-D-glucoside. Sarsasapogenin β-D-galactoside showed marginally better inhibition results against *M. canis* at 400 ppm than sarsasapogenin β-D-glucoside and episarsasapogenin β-D-

glucoside. From the results obtained it appears that the episarsasapogenin skeleton does not result in an increase in activity with respect to the corresponding glycosides containing the sarsasapogenin skeleton tested herein. The 2 betulin glucosides showed no effect against *M. canis* up to 400 ppm.

The 400 ppm synthesized saponin *M. canis* test plates, after 96 h incubation, are shown in Figure 6.5. Inhibition zones were visually smaller than the 72 h inhibition zones reported in Table 6.5.

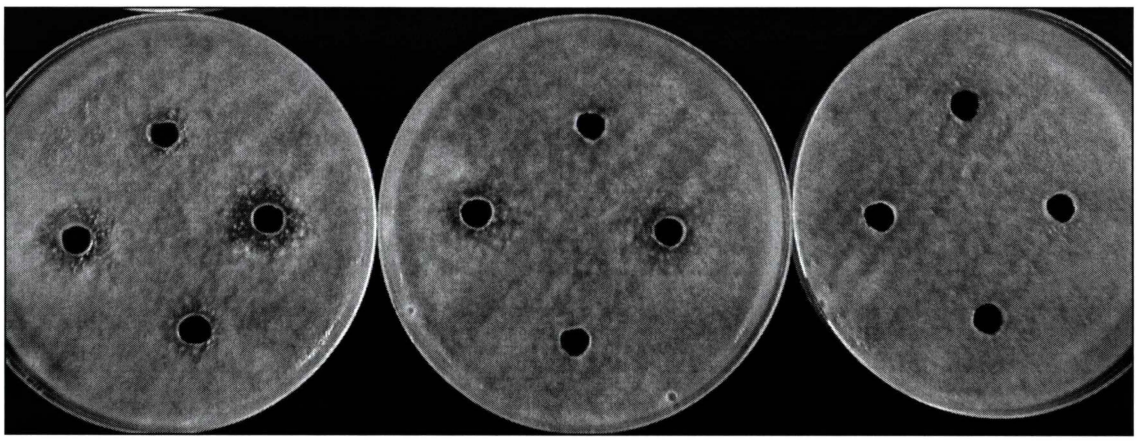


Figure 6.5. *M. canis* inhibition zones for the 6 synthesized saponin compounds at 400 ppm after 96 h incubation. Plates from left to right; sarsasapogenin β-D-glucoside (top/bottom wells) and sarsasapogenin β-D-galactoside; episarsasapogenin β-D-glucoside (top/bottom wells) and episarsasapogenin β-D-galactoside; betulin 3,28-(β-D-diglucoside) (top/bottom wells) and betulin 3-(β-D-glucoside).

4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) showed a limited inhibition effect at 1000 ppm and exhibited good inhibition zones at 2000 ppm (19 mm), just larger than the 500 ppm amphotericin B positive control (18 mm). The triketone oil showed inhibition effects which slowly increased from 1000 to 10000 ppm.

The *N. ossifragum* conjugate extract and the *Y. schidigera* Brix extract showed remarkable inhibition of *M. canis* from 2000 to 10000 ppm. No report of the effects of *Y. schidigera* extract(s) on *M. canis* was found in the literature. However, a *Y. schidigera* saponin fraction has been reported to show activity towards a range of dermatophytic fungi and to exhibit a range of antifungal activities (Miyakoshi *et al.*, 2000).

The steroidal spirostanol saponins (based on sarsasapogenin and smilagenin skeletons) identified in *Y. schidigera* (Miyakoshi *et al.*, 2000), Norwegian *N. ossifragum* (Deng, 1999) and Japanese *N. asiaticum* (Kobayashi *et al.*, 1993; Inoue *et al.*, 1995) are monodesmosides consisting of 2 or 3 sugar residues, comprised from 4 primary residues (galactose, glucose, arabinose and xylose), with the sugar chain attached at C-3 of the genin. For *N. ossifragum* and *N. asiaticum* saponins, the first sugar residue (glycosidically bonded to the steroidal skeleton) is galactose (Deng, 1999; Kobayashi *et al.*, 1993; Inoue *et al.*, 1995), whereas in *Y. schidigera*, the first sugar residue is galactose or glucose (Miyakoshi *et al.*, 2000).

Based on the activities reported here for the *N. ossifragum* and *Y. schidigera* extracts against *M. canis* growth, it appears that in order for monodesmosidic sarsasapogenin and smilagenin saponins to exhibit antifungal activity of some potency, the presence of a sugar moiety containing 2 or more glycosidic residues is necessary. The antifungal activities displayed by the 4 synthesized steroidal saponins containing only 1 glycosidic residue lacked inhibitory potency against *M. canis*.

The inactivity of the 2 betulin glycosides against *M. ca*nis can be rationalised in the same manner. Ohara and Ohira (2003) reported the biological activity of a series of betulin 3-(β-D-glycosides) when submitted to germination and growth regulation tests on alfalfa seeds. It was found that betulin glycosides with a sugar moiety comprised of 2 to 4 glucose residues exhibited the greatest inhibitory effects.

Growth inhibition results for selected compounds and extracts, against *M. canis* are depicted in Figure 6.6. Large inhibitory effects were observed for the *N. ossifragum* conjugate and *Y. schidigera* Brix extracts.

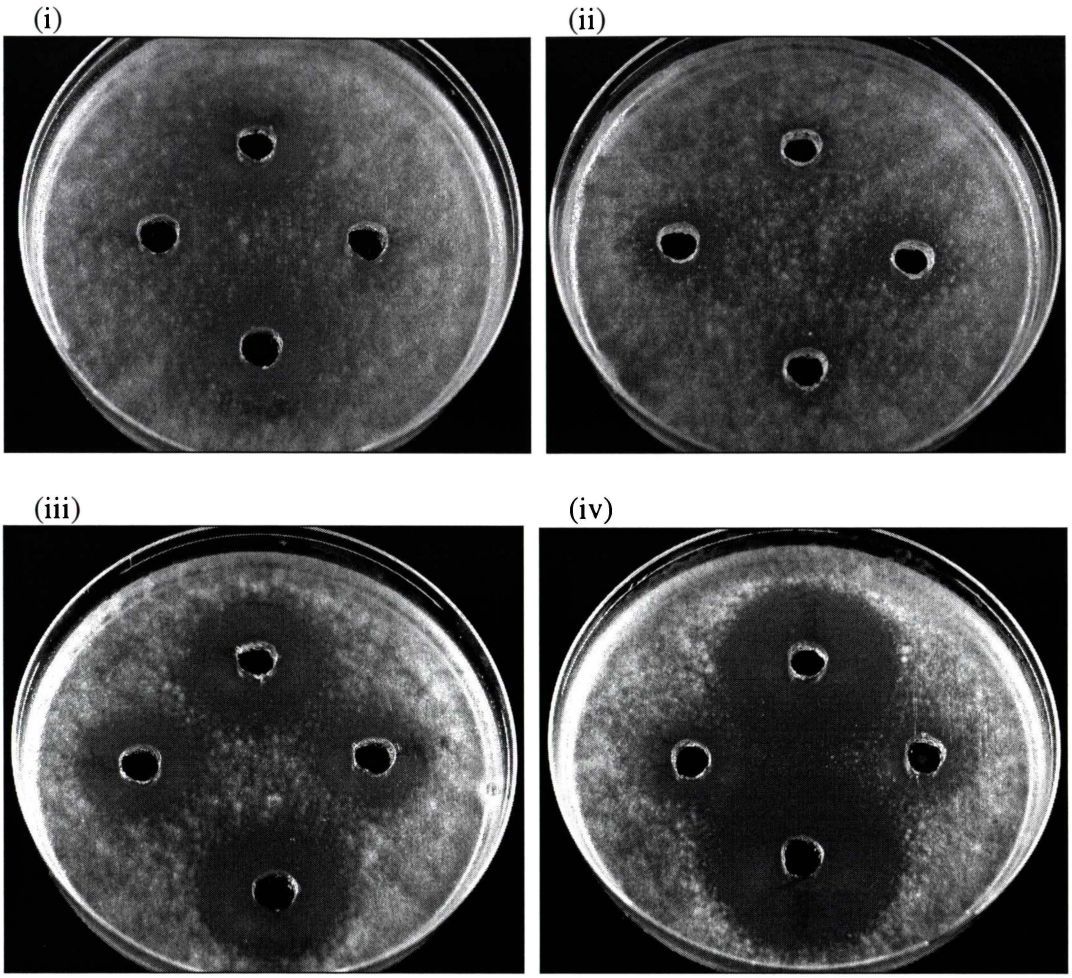


Figure 6.6. *M. canis* 72 h inhibition zones for; (i): amphotericin B at 500 ppm (top/bottom wells) and DMSO control, (ii): sarsasapogenin β-D-glucoside (top/bottom wells) and sarsasapogenin β-D-galactoside both at 400 ppm, (iii): *N. ossifragum* conjugate extract at 10000 ppm (top/bottom wells) and 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) at 2000 ppm and (iv): *Y. schidigera* 70° Brix extract (top/bottom wells) and the triketone oil at 5000 ppm.

6.3.4.4 *T. mentagrophytes*

T. mentagrophytes appeared to be the fungi most susceptible to the compounds and extracts tested. Inhibition results are presented in Table 6.6.

Table 6.6. *T. mentagrophytes* inhibition zones (mm) after 48 h incubation. Reduced growth zones (mm) are bracketed.

Compound	Conc (ppm)	Organism and Inhibition Zone (mm) *T. mentagrophytes*
Amphotericin B	500	15
DMSO	neat	-
Sar-glu	200	(10-11)
	400	(13)
Sar-gal	200	(10-11)
	400	(13)
Furanone-glu	1000	(10-11)
	2000	17
N. oss extract	1000	(12-13)
	2000	13 (15)
	5000	17 (21)
	10000	22
Y. schidigera	1000	(12-13)
	2000	15 (17)
	5000	24
	10000	29
Triketone oil	1000	(10-11)
	2000	(13)
	5000	14
	10000	17

T. mentagrophytes = *Trichophyton mentagrophytes*; Sar-glu = sarsasapogenin β-D-glucoside; Sar-gal = sarsasapogenin β-D-galactoside; Furanone-glu = 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside); *N. oss* extract = *Narthecium ossifragum* conjugate extract; *Y. schidigera* = *Yucca schidigera* 70° Brix extract; Triketone oil = Manuka oil active concentrate. '-' = no inhibition of organism growth.

Y. schidigera saponins and East Cape Manuka oil have been reported to exhibit inhibition of *T. mentagrophytes* growth (Miyakoshi *et al.*, 2000; Perry *et al.*, 1997) which is consistent with the results obtained above. The *Y. schidigera* Brix extract showed excellent growth inhibition qualities against *T. mentagrophytes* (Table 6.6), as did the *N. ossifragum* conjugate extract. The saponins found in *Y. schidigera* and *N. ossifragum* are predominantly monodesmosidic steroidal saponins, with 2 or 3 glycoside residues in the sugar moiety (Miyakoshi *et al.*, 2000; Deng, 1999; Kobayashi *et al.*, 1993; Inoue *et al.*, 1995).

Of the synthesized saponins, only sarsasapogenin β-D-glucoside and sarsasapogenin β-D-galactoside exhibited inhibitory growth effects against *T. mentagrophytes* with similar sized reduced growth inhibition zones of 13 mm at a concentration of 400 ppm. At 200 ppm growth inhibition zones were smaller (10-11 mm).

The episarsasapogenin saponins showed no discernible inhibitory effects at 400 ppm and suggest that the episarsasapogenin skeleton does not result in increased activity against *T. mentagrophytes*, at least with respect to the monoglycosidic saponins tested in this investigation. These results support the proposition that monodesmosidic sarsasapogenin and smilagenin saponins require sugar moieties containing 2 or more sugar residues in order to display antifungal activity.

4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) showed a limited inhibition effect at 1000 ppm but exhibited fully clear inhibition zones at 2000 ppm (17 mm). This zone was larger than the inhibition zones of the 500 ppm amphotericin B positive control drug. The 2 betulin glucosides exhibited no antifungal effect against *T. mentagrophytes*.

Visual results for selected test compounds against *T. mentagrophytes* are presented in Figure 6.7.

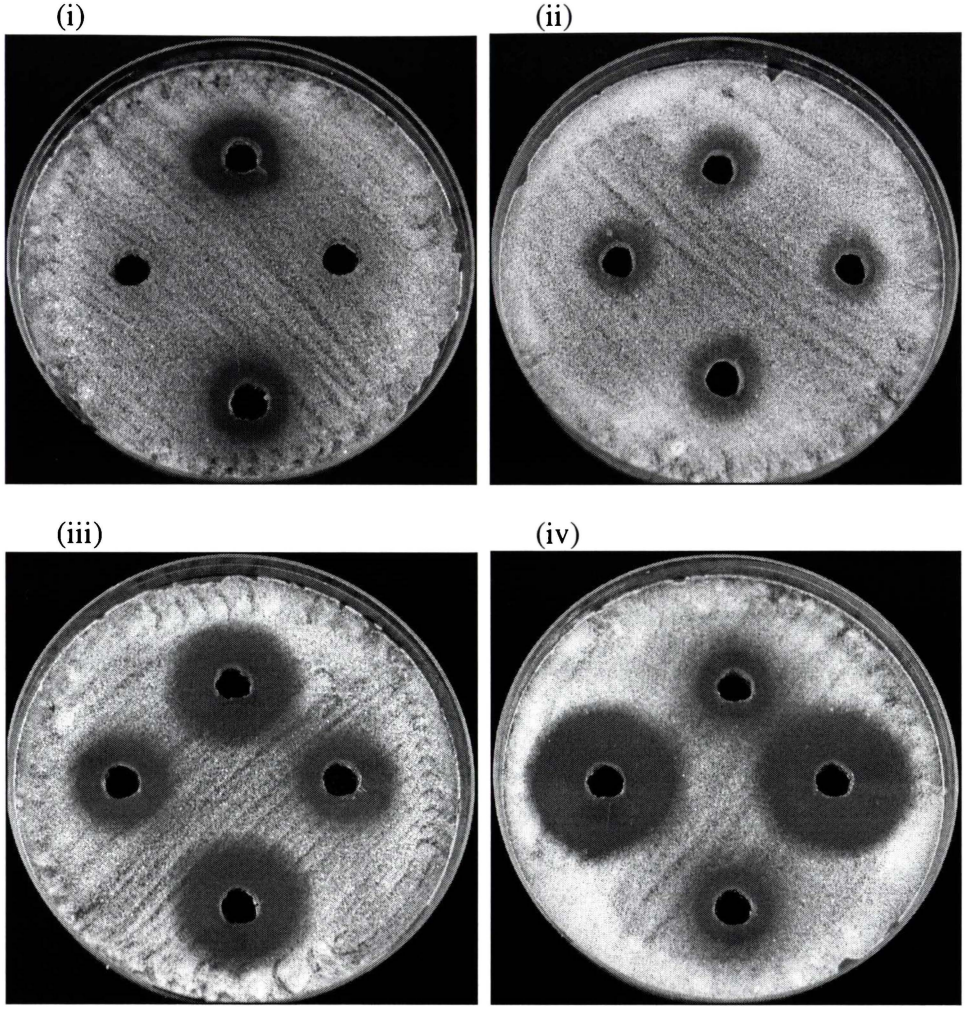


Figure 6.7. *T. mentagrophytes* 48 h inhibition zones for; (i): amphotericin B at 500 ppm (top/bottom wells) and DMSO control, (ii): sarsasapogenin β-D-glucoside (left/right wells) and sarsasapogenin β-D-galactoside both at 400 ppm, (iii): *N. ossifragum* conjugate extract at 10000 ppm (top/bottom wells) and 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) at 2000 ppm and (iv): *Y. schidigera* 70° Brix extract at 10000 ppm (left/right wells) and the triketone oil at 5000 ppm.

6.4 University of Canterbury Assays

6.4.1 Introduction

The 6 synthesized saponins utilized in the foregoing investigations were also forwarded to the University of Canterbury, Christchurch, New Zealand for a second round of antimicrobial testing. Testing was performed using the disk diffusion technique (DD) as described below.

6.4.2 University of Canterbury Methodology

Bacteria or fungi, at a known concentration, are mixed with Mueller Hinton or Potato dextrose agar and poured in Petri dishes and incubated to grow a lawn of bacteria or fungi over the dish. Samples are pipetted (40 μL) onto 6 mm diameter paper disks and the solvent evaporated. The disks are then placed on the inoculated agar Petri dishes, with appropriate positive and negative controls, and incubated. Activities are observed as zones of inhibition outside the disk and are measured in millimetres as the radius of inhibition.

2 organisms were used; *Candida albicans* ATCC 14053 and *Trichophyton mentagrophytes* ATCC 28185.

6.4.3 Results and Discussion

The assay results provided by the University of Canterbury showed that when tested at 10 mg/mL (10000 ppm) the only saponin to show antimicrobial activity, with slight inhibition observed against *T. mentagrophytes,* was sarsasapogenin β-D-galactoside. Its radius of inhibition was 1 mm. This result is in accord with results obtained using the agar gel well diffusion technique (AGWD) performed at the NVI. AGWD results showed that sarsasapogenin β-D-galactoside was slightly more effective against *M. canis* than sarsasapogenin β-D-glucoside, but no difference in activity could be seen between the 2 when tested against *T. mentagrophytes*. The DD results indicate that sarsasapogenin β-D-galactoside is more effective against *T. mentagrophytes* than sarsasapogenin β-D-glucoside.

The DD technique employed by the University of Canterbury affords only low activity levels for the synthesized saponin compounds when compared to the AGWD technique employed at the NVI. Activity was only seen at 10000 ppm against *T. mentagrophytes* for sarsasapogenin β-D-galactoside using the DD technique whereas activity was observed at 200 and 400 ppm using the AWGD technique. It is likely the DD technique suffered from greater diffusion difficulties concerning the synthesized saponin compounds into and across the agar than was the case for the AWGD technique.

Use of the DD technique was investigated at the NVI against the bacteria *S. aureus* and *E. coli* and the yeast organism *C. albicans*, using the positive control drugs tetracycline and amphotericin B. Results obtained indicated that the DD technique gave lower quality results than the AGWD method. The DD trial methodology is outlined below for *C. albicans*.

2 square paper disks (10 mm × 10 mm) were soaked with 20 μL of amphotericin B (50 ppm) and placed (wet) on inoculated agar gel dishes in duplicate. A single well (7 mm) containing 60 μL of amphotericin B (50 ppm) and a 'puddle' cut into the agar surface with 20 μL injected directly onto the surface were run in unison with the paper disks. Presented in Figure 6.8 is a visual representation of the Petri dish after 24 h incubation at 30°C.

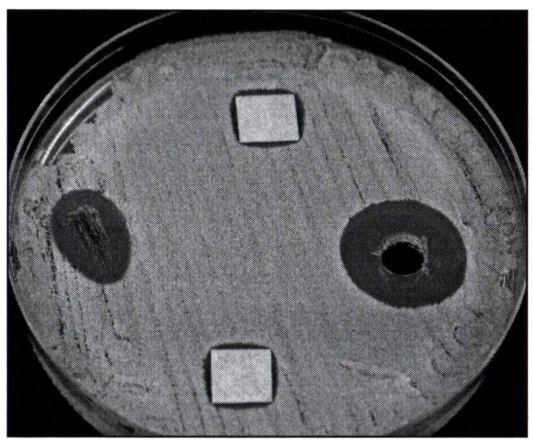


Figure 6.8. *C. albicans* 24 h disk diffusion trial results. Paper disks show minimal inhibition zones (top and bottom) compared to the well (right) and the 'puddle' (left).

Paper disk inhibition zones were more pronounced against *S. aureus* (with respect to well inhibition zones) but were poor for *E. coli*. The results obtained aided the decision to adopt the agar gel well diffusion technique for testing, and also enforces the hypothesis that the disk diffusion technique may be troubled by compound diffusion difficulties.

6.5 Conclusions

Although antiyeast and antifungal responses of the *N. ossifragum* conjugate and the *Y. schidigera* 70° Brix extracts often paralleled each other, direct comparisons of activities and concentrations cannot be made as detailed information of individual components contained in each respective extract was not determined. For example overall saponin concentrations, ratio of spirostanol/furostanol forms, monodesmosidic/bidesmosidic ratios and the presence of other non-saponin compounds.

Y. schidigera 70° Brix extract exhibited antiyeast and antifungal activities that paralleled activities determined for *Y. schidigera* (Mohave Yucca) saponins reported by Miyakoshi *et al.* (2000). However direct comparisons between activity concentration levels observed here and levels reported by Miyakoshi *et al.* (2000) cannot be made. In addition to applying a different bioassay technique, assessments by Miyakoshi *et al.* (2000) employed a concentrated saponin fraction and individual saponin compounds extracted from Mohave Yucca.

Miyakoshi *et al.* (2000) concluded that the Mohave Yucca steroidal saponins that exhibited the most potent antiyeast activities were those that possessed a branched-chain trisaccharide moiety. Sarsasapogenin and smilagenin saponins with a disaccharide moiety possessed moderate activities and saponins with additional 2β-hydroxyl or 12-keto functionalities showed little or no antiyeast activities.

The 6 synthesized saponins did not possess a broad spectrum of antibacterial, antiyeast and antifungal activities against the range of organisms tested in this investigation. All the saponins did however show limited antiyeast activity at 400 ppm against *C. glabrata*.

The principal conclusions of the activity assessments presented in this chapter, and possible structure-activity relationships are summarised below:

(i) The finding that the 2 episarsasapogenin glycosides were less effective against *M. canis* and *T. mentagrophytes* than the 2 sarsasapogenin glycosides can be interpreted as indicating that the presence of a 3α-*O*-(β-D-glycoside) linkage leads to reduced activity relative to that of the corresponding 3β-*O*-(β-D-glycoside).

(ii) The agar gel well diffusion technique found sarsasapogenin β-D-galactoside to be slightly more effective against *M. canis* than sarsasapogenin β-D-glucoside and episarsasapogenin β-D-glucoside. No distinction was apparent between sarsasapogenin β-D-galactoside and sarsasapogenin β-D-glucoside when tested against *T. mentagrophytes*.

(iii) The disk diffusion technique found sarsasapogenin β-D-galactoside to be more effective than sarsasapogenin β-D-glucoside when tested against *T. mentagrophytes*.

(iv) Comparison of results obtained for the 4 synthesized sapogenin glycosides and the *Y. schidigera* 70° Brix and *N. ossifragum* conjugate extracts indicates that for sarsasapogenin and smilagenin saponins to exhibit antifungal activities the sugar moiety should contain two or more glycoside residues.

(v) Betulin 3-(β-D-glucoside) and betulin 3,28-(β-D-diglucoside) lacked activity against the fungal organisms tested.

(vi) 4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside) exhibited good antifungal activity against *M. canis* and *T. mentagrophytes* at 2000 ppm and limited antifungal and antiyeast activity against *P. rogueforti* and *C. glabrata* at 2000 ppm.

(vii) The disk diffusion technique was seen as inferior to the agar well gel diffusion technique for the testing of these synthesized saponin compounds, believed to be due to diffusion difficulties and limitations of the compounds into and across the agar medium.

Chapter Seven

Saprolegnia parasitica

7.1 Introduction

Saprolegnia (pronounced: "Sap-ro-leg-ni-ah") is the main genus of water moulds responsible for significant fungal infections of freshwater fish (Beakes *et al.*, 1994). Infection of fish with *Saprolegnia*, termed 'saprolegniasis', is an increasing problem in the fresh water stage of salmonoid aquaculture (Beakes *et al.*, 1994; Meyer, 1991). This fungal infection is visible as white or grey patches on the skin of the fish and has a cotton-like appearance, see Figure 7.1 (Bruno and Poppe, 1996; Beakes *et al.*, 1994). *Saprolegnia* can spread over the entire body surface and generally invades epidermal tissues beginning around the head and fins. Infections typically occur when fish are stressed or otherwise have weakened immune systems and after disruption of skin membrane integrity by UV irradiation (sunburn) or mechanical damage (Bruno and Wood, 1999; Pickering, 1994). Since fungus is almost always present in freshwater ecosystems, it is thought that some change in the fish must occur for infection to take hold (Bruno and Wood, 1999).

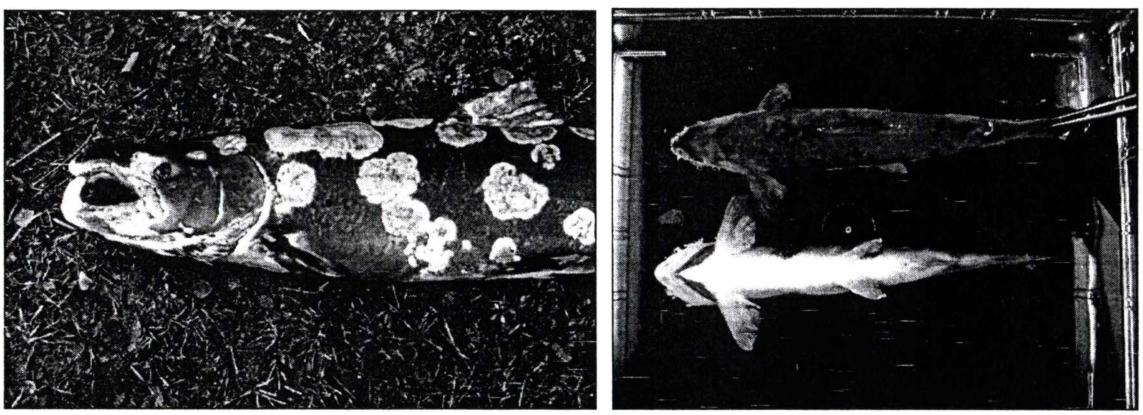


Figure 7.1. Left: A fish infected with *Saprolegnia*. Right: Atlantic salmon (*Salmon salar*) parr infected with *S. parasitica* strain N12 used in testing.

Saprolegniasis, if untreated, leads to death via haemodilution, i.e. osmoregulatory failure (Hatai and Hoshiai, 1994). Saprolegniasis causes tissue destruction and epithelial integrity loss as a result of cellular necrosis or dermal and epidermal damage. Mortality timelines are dependent on the sites of infection, fungal growth rate, tissue type damaged and individual fish resistance to the invasion (Pickering and Willoughby, 1982). Infected fish appear lethargic, lose equilibrium and generally do not recover (Bruno and Poppe, 1996). *S. parasitica* is the most pathogenic species of *Saprolegnia*, and can grow in dilute nutrient mediums such as fish mucous (Murphy, 1991; Pickering and Willoughby, 1982).

Disease is the single greatest source of economic losses in aquaculture (Meyer, 1991). It has been indicated (Hatai and Hoshiai, 1994) that in Miyagi Prefecture, Japan, *S. parasitica* is responsible for annual mortality rates of 50% in coho salmon (*Oncohynchus kisutch* Walbaum). Southeastern US channel catfish farmers have reported losses of up to 50%, due to a *Saprolegnia* condition called 'winter kill', an economic loss of 40 million dollars US (Bruno and Wood, 1999). One Norwegian fish farming company has alone estimated losses in the order of 2-3 million dollars US annually in Norway and Chile due to *Saprolegnia* infections (Stueland, *pers commun*).

Saprolegnia infections are difficult to prevent and treat. Malachite green is an organic dye considered to be the most effective chemical for controlling *Saprolegnia*, and has been used extensively throughout much of the world as a fungicide and ectoparasiticide (Bruno and Wood, 1999; Alderman, 1985). It has however been banned for use in Norway, the US and other parts of the world due to concerns of its potential toxicological, teratogenic and mutagenic properties (Alderman, 1985; Bruno and Wood, 1999; Fitzpatrick *et al.*, 1995; Meyer and Jorgenson, 1983). The increasing problem of *Saprolegnia* infection in freshwater aquaculture is partly due to this banning of malachite green.

Despite extensive research, no alternative parallelling the effectiveness of malachite green has yet been found. Alternative treatments for *Saprolegnia*, although less effective, are in use such as: formalin, a 37% formaldehyde solution, registered for use in the US (Bruno and Wood, 1999; Bly *et al.*, 1997); Pyceze®, the active ingredient of which is bronopol (2-bromo-2-nitropropan-1,3-diol), developed in the UK and permitted for use by the EU; and

hydrogen peroxide. However some concerns exist with the use of these treatments. For example, formalin carries concerns about its effects on the environment and persons who handle it (Fitzpatrick *et al.*, 1995). Currently the most effective strategy for controlling and preventing *Saprolegnia* outbreaks is good fish management and husbandry techniques combined with chemical treatment, most importantly 2-4 days after handling (Bruno and Wood, 1999; Hatai and Hoshiai, 1994; Meyer *et al.*, 1991). Stress reduction seems to be the single largest factor in aiding fish resistance to saprolegniasis.

A desirable property (especially from a farming viewpoint) of a *Saprolegnia* treatment substance/chemical is one that exhibits a large gap between the concentration required to inhibit *Saprolegnia* and the concentration at which it is toxic towards fish. Therefore, if fish are accidentally exposed to an increased dose during treatment, the risk of loss (accidental overdose) of entire stocks is minimised.

The triketone fraction of East Cape Manuka oil is known to exhibit antimicrobial activity, including activity against a range of bacteria and fungi (Porter and Wilkins, 1998; van Klink *et al.*, 1999); Perry *et al.*, 1997). The structures of the three major triketones found in East Cape Manuka oil, including their tautomeric enol forms are illustrated in Figure 7.2. The triketone enolic forms are believed to be the active forms and are present as the corresponding enolate anions at basic pH.

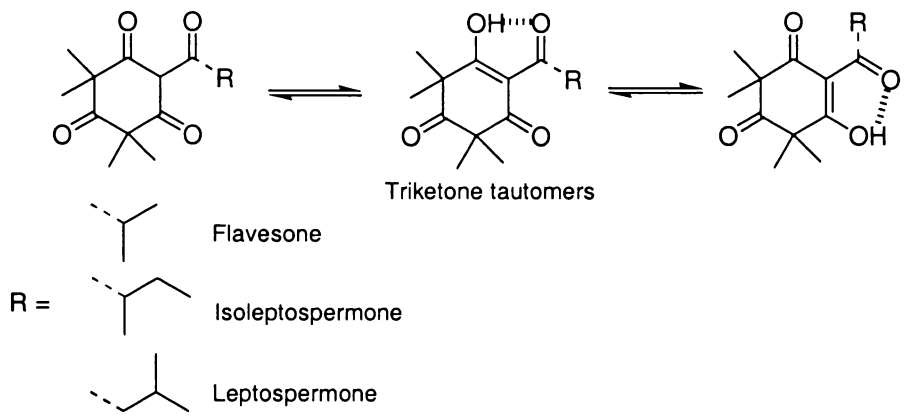


Figure 7.2. Triketones present in East Cape Manuka oil active fraction. Triketone enol tautomers are illustrated.

It was of interest to ascertain if the triketone oil possessed activity against *S. parasitica*. In part, interest in the Manuka triketones arose from the availability in the market place of both the oil and the triketone fraction and their use in a wide variety of products including antibacterial soaps, creams, oils etc. If the triketone oil showed potential as a candidate for *Saprolegnia* treatment, the time and cost associated with gaining approval for the use of a 'natural extract' would be minimal compared to the time and cost of gaining approval for the use of a synthetic substance.

A simple *in vitro* screening method has been jointly developed at the National Veterinary Institute (NVI), Oslo, Norway, by Svein Stueland (PhD student) and Alpharma AS, Animal Health Division, Oslo, Norway, for the preliminary screening of compounds and substances for fungistatic and fungicidal effects on *Saprolegnia* species. The method has been demonstrated to be simple, effective and reproducible. Results from the *in vitro* testing of the East Cape triketone oil against *S. parasitica* are presented in this Chapter. Testing was undertaken in person, during a 3-month visit to the NVI during 2003.

7.2 Methodology

7.2.1 *In vitro* Testing Regime

The basic methodology of the *in vitro* screening procedure is as follows. Sterilized hemp seeds (*Cannabis sativa*) colonised with strains of *S. parasitica* are used to model infected fish. Petri dishes containing Sabourauds medium (SAB) are inoculated with a pure culture of a fish-pathogenic strain of *S. parasitica*. Hemp seeds are applied to the Petri dishes and incubated at 20°C for 2-3 days (Figure 7.3). The colonised hemp seeds are transferred to 48-well tissue culture plates (1 seed per well) and exposed to different concentrations of the test substance dissolved in water.

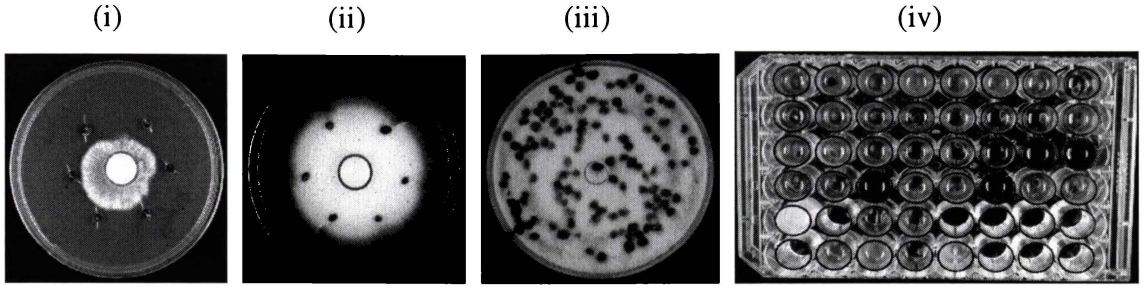


Figure 7.3. *S. parasitica* cultured growth onto sterilized hemp seeds; (i) and (ii): the *Saprolegnia* strain in the centre of the agar spreads out, inoculating the hemp seeds, (iii): typical dish with inoculated hemp seeds and (iv): a 48-well tissue culture plate.

Exposure is a continuous static exposure for 72 h. To ensure nutrition for *S. parasitica* growth, all wells contain 1% Sabourauds medium. Seeds are inspected for growth under microscope after 24 and 72 h exposure. *S. parasitica* hyphal growth is measured and quantified according to 4 gradations, ranging from zero to abundant hyphal growth (Figure 7.4).

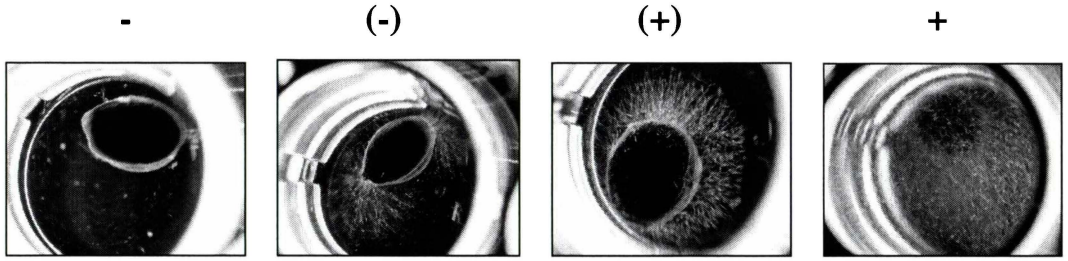


Figure 7.4. Visual representations of hyphal growth grading; **-**: zero or much reduced hyphal growth, **(-)**: strongly reduced growth, small hypha or not covering the hemp seed, **(+)**: reduced growth, but hypha completely covering the seed and **+**: full hypha growth.

Water and malachite green (MG) are used as negative and positive controls respectively. Unhindered growth of *Saprolegnia* is observed in the negative controls, compared to fully inhibited growth in the positive (MG) controls. Visual differences between negative and positive control wells (water and MG respectively) across a 72 h time period are presented in Figure 7.5.

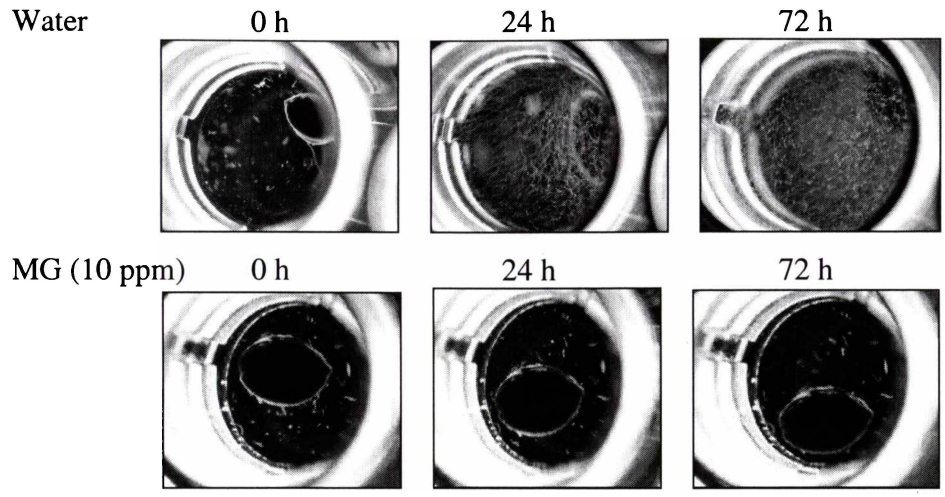


Figure 7.5. Effects of negative (water) and positive (malachite green (MG)) controls on *S. parasitica* hyphal growth over a 72 h time period.

The triketone oil was tested at 50, 100, 150 and 200 ppm, with all testing performed in duplicate.

7.2.2 *S. parasitica* Cultures and Media

Two pathogenic strains of *S. parasitica* were used in the *in vitro* assessments; N6 – a Norwegian isolate, and N12 – a Scottish isolate. The pure strains were sampled from diseased salmon parr, isolated, recultured and supplied by Svein Stueland, NVI. SAB liquid and media plates were supplied by the Media Department, NVI.

The Manuka oil active concentrate (triketone oil) was obtained from Tairawhiti Pharmaceuticals Ltd, Te Araroa, East Cape, New Zealand;

> Batch No. TE03.2; March 2003. Relative density (20°C): 1.072; refractive index (20°C): 1.501. Triketone content by GC-MS analysis: flaversone 18.98%, leptospermone 17.68%, isoleptospermone 61.51%.

All test solutions were sterilized (autoclaved) and stored in sealed tubes until utilized. Water for test solutions was sterilized water from salmon fishponds at the NVI.

7.3 *In vitro* Results and Discussion

The 6 synthesized saponins, prepared as described in Chapter 5 and assessed for bioactivity in Chapter 6, were also scheduled for preliminary screening against *S. parasitica*. However due to aqueous solubility difficulties, results proved inconclusive and are not reported.

It was immediately apparent that at neutral (natural) pH the triketone oil was not completely soluble in aqueous solution. Various strategies, including the use of a buffer solution and 2 water miscible organic solvents, were employed in order to afford a homogenous aqueous solution medium. An essential requirement was that the aqueous test solutions containing organic solvents or buffers (used to solubilize the triketone oil) exhibited little or no *in vitro* inhibition on *S. parasitica* hyphal growth. The lethality limits against *S. parasitica* for the 2 organic solvents investigated, DMSO and 1-methyl-2-pyrrolidinone (pyrrolidinone), expressed as % by volume aqueous solution, were determined to be 2% and 1% respectively.

Solvent levels of DMSO (1%), or of pyrrolidinone (0.5% or 1%), required the use of 0.5% Tween 80 by volume to fully solubilize the triketone oil in aqueous solutions. At levels less than 1% DMSO and 0.5% pyrrolidinone the triketone oil was not completely soluble, even with 0.5% Tween 80. The addition of Tween 80 had no inhibitory effect on the growth of *S. parasitica*. Aqueous controls containing 1.0% pyrrolidinone however had a slight effect on *S. parasitica* growth, i.e. a grading of (-) to (+), see Figure 7.4. At 0.5% pyrrolidinone, and also at 1% DMSO, full *S. parasitica* growth was observed (Table 7.1).

Duplicate *in vitro* growth results for the triketone oil against *S. parasitica* in aqueous solutions containing;

(i) 1% DMSO / 0.5% Tween 80

(ii) 1% pyrrolidinone / 0.5% Tween 80

(iii) 0.5% pyrrolidinone / 0.5% Tween 80

are presented in Table 7.1. Pyceze® (bronopol) at 50 ppm, the currently registered treatment for *Saprolegnia*, was also included in the assessments as a second positive control.

Table 7.1. *In vitro* grading results of triketone oil versus *S. parasitica*.

Solution	Conc (ppm)	Growth Results N6 Strain 24h	N6 Strain 72h	N12 Strain 24h	N12 Strain 72h
Water		+	+	+	+
MG	10	-	-	-	-
Pyceze®	50	-	-	-	-
DMSO + T80 control	1% + 0.5%	+	+	+	+
Triketone oil	50	(+)	+	+	+
Triketone oil	100	(+)	+	(+)	+
Triketone oil	150	(+)	+	(+)	+
Triketone oil	200	(-)	+	(+)	+
Pyrrol + T80 control	0.5% + 0.5%	+	+	(+)	+
Triketone oil	50	(+)	(+)	(+)	+
Triketone oil	100	(+)	(+)	(+)	(+)
Triketone oil	150	(-)	(-)	(-)	(+)
Triketone oil	200	(-)	(-)	(-)	(-)
Pyrrol + T80 control	1% + 0.5%	(-)	(-)	(-)	(+)
Triketone oil	50	(-)	(-)	(-)	(-)
Triketone oil	100	(-)	(-)	(-)	(-)
Triketone oil	150	-	-	-	-
Triketone oil	200	-	-	-	-

MG = malachite green; T80 = Tween 80; Pyrrol = pyrrolidinone; For growth grading descriptions see Section 7.2.1, Figure 7.4.

It was apparent from the results presented in Table 7.1 that, at all of the tested concentrations, the triketone oil in aqueous DMSO (99:1) resulted in zero or minimal inhibition on both strains of *S. parasitica* hyphal growth after 24 and 72 h, i.e. growth gradings of (+) and +.

The 0.5% pyrrolidinone:aqueous control solution, after 72 h, showed no inhibitory effect on hyphal growth. Promising inhibition was observed for 150 and 200 ppm triketone solutions, with (-) growths (Figure 7.4), except against strain N12 at 150 ppm, which showed (+) growth at 72 h.

It can be hypothesized that the presence of pyrrolidinone (C_5H_9NO) enhances the activity of the triketone oil (via a synergic effect) when compared to the DMSO results. Possibly this may be a consequence of the ability of pyrrolidinone to abstract a proton from the

triketones, thus promoting the formation of the enolate anions of the enol tautomers of the triketones (Figure 7.6). The enol tautomers are believed to be the most bioactive forms of the Manuka triketones.

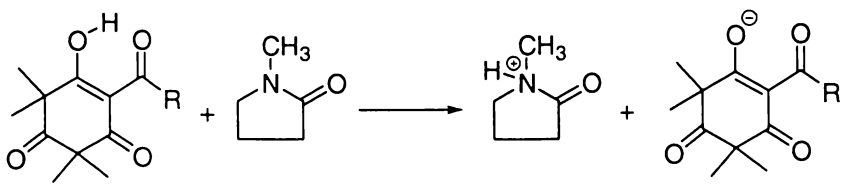


Figure 7.6. Pyrrolidinone assisted triketone enolate anion formation.

When the pyrrolidinone level was increased from 0.5% to 1%, an increased inhibitory effect was noted for the triketone oil at concentrations of 150 and 200 ppm, indicating a stronger synergic effect, possibly due to an increased triketone enolate anion level (Figure 7.6).

The 1% pyrrolidinone:aqueous control solution (with no added triketone oil) had inhibitory effects on *S. parasitica* growth. However, at 150 and 200 ppm triketone oil, in the presence of 1% pyrrolidinone, an inhibitory effect against the N6 and N12 *S. parasitica* strains over and above that of the control solution was observed (see Table 7.1). At concentrations of 50 and 100 ppm the inhibitory effect against strain N6 was comparable to that of the control solution, while a slight increase in inhibition was noted against strain N12.

In order to further investigate the potential of the triketone oil as a commercial treatment of *Saprolegnia*, a fish toxicity test was required. Toxicity protocols and detailed results are presented later in Section 7.4. In summary, the toxicity tests showed that 0.5% or 1% pyrrolidinone:aqueous solutions containing 0.5% Tween 80 by volume were toxic to Atlantic salmon in their own right, without the addition of triketone oil. Further testing showed that the acceptable pyrrolidinone level in aqueous solution, in which fish displayed normal behaviour, was 0.2% by volume. The presence of Tween 80 in the solutions was found to be unsuitable, due to excessive foaming from the fish test baths during the toxicity assessments.

Since at levels less than 0.5% pyrrolidinone, the triketone oil was not completely soluble, the use of basic buffer solutions were investigated in order to overcome the problem of the aqueous insolubility of the triketone oil. At basic pH's the enolate anion forms of the enol tautomers depicted in Figure 7.2 are completely soluble. Sodium phosphate dibasic ($Na_2HPO_4.7H_2O$) and potassium phosphate monobasic (KH_2PO_4) solutions were utilized to make a series of phosphate buffers at concentrations of 0.01, 0.10 and 0.25 $molL^{-1}$, with each concentration buffered to pH 7.0, 7.5, 8.0 and 8.5.

At the test concentrations required (50, 100, 150 and 200 ppm), the triketone oil was found to be soluble at each buffer concentration and at each pH. Since the presence of pyrrolidinone was thought to have a synergic effect on the inhibitory performance of the triketone oil, 0.1% by volume (a non-toxic concentration towards Atlantic salmon) was added to each respective buffer test solution containing triketone oil. The presence of pyrrolidinone also aided in solubilizing the triketone oil.

Presented in Table 7.2 are the *in vitro* average duplicate growth results of the triketone oil tested against *S. parasitica* in 3 phosphate buffers of concentrations 0.01, 0.10 and 0.25 $molL^{-1}$, and at pH 7.0, 7.5, 8.0 and 8.5.

Table 7.2. *In vitro* grading results of triketone oil versus *S. parasitica* using phosphate buffer solutions.

Solution	Conc (ppm)	Growth Results N6 Strain 24h	N6 Strain 72h	N12 Strain 24h	N12 Strain 72h
Water		+	+	+	+
MG	10	-	-	-	-
Pyceze®	50	-	-	-	-
0.01 molL^{-1} buffer					
pH 7.0 control	0.1% pyrrol	+	+	+	+
Triketone oil	50	+	+	+	+
Triketone oil	100	+	+	+	+
Triketone oil	150	(+)	+	+	+
Triketone oil	200	(+)	+	(+)	+
pH 7.5 control	0.1% pyrrol	+	+	+	+
Triketone oil	50	+	+	+	+
Triketone oil	100	+	+	+	+
Triketone oil	150	+	+	+	+
Triketone oil	200	+	+	(+)	+
pH 8.0 control	0.1% pyrrol	+	+	+	+
Triketone oil	50	+	+	+	+
Triketone oil	100	+	+	+	+
Triketone oil	150	+	+	+	+
Triketone oil	200	+	+	+	+
pH 8.5 control	0.1% pyrrol	+	+	+	+
Triketone oil	50	+	+	+	+
Triketone oil	100	+	+	+	+
Triketone oil	150	+	+	+	+
Triketone oil	200	+	+	+	+
0.10 molL^{-1} buffer					
pH 7.0 control	0.1% pyrrol	+	+	+	+
Triketone oil	50	+	+	+	+
Triketone oil	100	+	+	+	+
Triketone oil	150	(+)	+	(-)	+
Triketone oil	200	(-)	+	(-)	+
pH 7.5 control	0.1% pyrrol	+	+	+	+
Triketone oil	50	+	+	+	+
Triketone oil	100	+	+	+	+
Triketone oil	150	(+)	+	+	+
Triketone oil	200	(+)	+	+	+
pH 8.0 control	0.1% pyrrol	+	+	+	+
Triketone oil	50	+	+	+	+
Triketone oil	100	+	+	(+)	+
Triketone oil	150	(+)	+	(+)	+
Triketone oil	200	(-)	(+)	(+)	+
pH 8.5 control	0.1% pyrrol	(+)	+	(+)	+
Triketone oil	50	(+)	+	(+)	+
Triketone oil	100	(+)	+	(+)	(+)
Triketone oil	150	(-)	+	(+)	(+)
Triketone oil	200	(-)	+	(+)	(+)

cont...

Table 7.2 cont...

Solution	Conc (ppm)	Growth Results N6 Strain 24h	N6 Strain 72h	N12 Strain 24h	N12 Strain 72h
0.25 molL^{-1} buffer					
pH 7.0 control	0.1% pyrrol	(+)	+	+	+
Triketone oil	50	-	(-)	(-)	(+)
Triketone oil	100	-	-	(-)	(+)
Triketone oil	150	-	-	-	-
Triketone oil	200	-	-	-	-
pH 7.5 control	0.1% pyrrol	-	(-)	(+)	+
Triketone oil	50	-	(-)	(-)	(+)
Triketone oil	100	-	(-)	-	(-)
Triketone oil	150	-	-	-	-
Triketone oil	200	-	-	-	-
pH 8.0 control	0.1% pyrrol	(-)	(+)	(+)	(+)
Triketone oil	50	-	(-)	(-)	(+)
Triketone oil	100	-	(-)	(-)	(+)
Triketone oil	150	-	-	(-)	(-)
Triketone oil	200	-	-	-	-
pH 8.5 control	0.1% pyrrol	(-)	(+)	(-)	(+)
Triketone oil	50	-	(+)	(-)	(-)
Triketone oil	100	-	(-)	(-)	(-)
Triketone oil	150	(-)	(+)	(-)	(+)
Triketone oil	200	-	-	(-)	(-)

MG = malachite green; Pyrrol = pyrrolidinone; For growth grading descriptions see Section 7.2.1, Figure 7.4.

The 0.01 and 0.10 molL^{-1} phosphate buffer solutions at pH 7.0, 7.5, 8.0 and 8.5 showed little or no inhibition of *S. parasitica* hyphal growth across the range of triketone oil concentrations tested (see Table 7.2).

The 0.25 molL^{-1} phosphate buffer control solutions displayed minimal inhibition of *S. parasitica*, the exception being the pH 7.5 control which showed much reduced (-) growth against strain N6. The most promising results were seen at pH 7.5, in which 100, 150 and 200 ppm triketone oil concentrations gave full or much reduced hyphal growth. At pH 7.0, (150 and 200 ppm) and at pH 8.0 (200 ppm) full hyphal inhibitions were also observed. While these results were promising, from a commercial viewpoint, a buffer concentration of 0.25 molL^{-1} was not seen as an economically viable option, nor were the concentrations of 150 to 200 ppm triketone oil.

Although not potently active against *S. parasitica* under the conditions tested herein, the toxicity of the triketone oil against Atlantic salmon was investigated (Section 7.4.4) in a phosphate buffer solution of 0.01 molL^{-1} buffered to pH 7.5.

7.4 Toxicological Testing

7.4.1 Test Design

The toxicity assessment was performed as an *in vivo* single dose acute toxicity study on Atlantic salmon fry (*Salmon salar*) by static bath exposure. This protocol exposes Atlantic salmon to test substances at 2 concentrations (50 and 200 ppm) with exposure times of 30 min and 2 h. Control solution baths are also run. If the test substances are acutely toxic at 50 ppm for 30 min, they are not tested at 50 ppm for 2 h, or at 200 ppm. If substances are acutely toxic at 50 ppm after 2 h exposure, but not toxic after 30 min exposure, they are only tested at 200 ppm with an exposure time of 30 min. If control solutions are found to be acutely toxic or distressing to fish then test substances are not added and subjected for assessment. Ethical consent for the toxicology assessment was obtained by Svein Stueland, NVI, Oslo, Norway.

7.4.2 Methodology

25 L buckets, containing the test or control solutions and an oxygen supply, had 20 fish randomly selected from a large holding tank added. 10 fish were removed after 30 min exposure, anaesthetised with a benzocaine (ethyl aminobenzoate) solution (0.3 g/10 L), tagged by tail clipping, and transferred to a small fresh water observation tank. After 2 h exposure, the remaining 10 fish were placed, untagged, in the fresh water observation tank. Fish were monitored 24 h post exposure.

Fish behaviour patterns (i.e. loss of equilibrium, distress, lethargy etc), and mortality rates at each concentration were recorded for each exposure time, and 24 h post exposure. Surviving fish were euthanised 24 h post exposure by an overdose of benzocaine solution (1.2 g/10 L) and disposed of accordingly. All fish used in the trial were not considered fit for human consumption.

6 mth old Atlantic salmon fry were used in the toxicity assessments and had an average post-mortem weight of 25 g. Fish were acclimatised in a large holding tank for 14 days and

had a 3 day starvation period before exposure. Water temperature was *ca.* 6°C in the 25 L test buckets.

Figure 7.7. Left: Holding tank of 6 mth old Atlantic salmon fry. Right: 25 L test buckets (yellow) and larger fresh water recovery/observation tanks.

7.4.3 Pyrrolidinone Toxicity

As noted previously (Section 7.2) the triketone oil showed inhibition of *S. parasitica* growth in 0.5% and 1% pyrrolidinone:aqueous solutions, with each solution containing 0.5% Tween 80. These control solutions were subjected to the toxicity assessment outlined above and were found to be unsuitable mediums for triketone oil testing.

The 1% pyrrolidinone:aqueous solution was acutely toxic to 20 Atlantic salmon fry (100% fish mortality) after 30 min of exposure. Although the 0.5% pyrrolidinone:aqueous solution was not acutely toxic after 30 min and 2 h exposure all fish were very distressed and lethargic with many displaying loss of equilibrium at both exposure periods. 24 h post exposure all fish exhibited normal behaviour in the observation tank, i.e. full recovery. Tween 80 was determined to be an unsuitable aqueous medium component since excessive foaming of the test baths occurred on both occasions.

Upon further assessment, it was found that the safe limit, percentage by volume, of pyrrolidinone was 0.2%. At this concentration, and without the presence of Tween 80, fish exhibited normal behaviour at both exposure times (30 min and 2 h).

7.4.4 Triketone Oil Toxicity

A 0.01 $molL^{-1}$ phosphate buffer control solution (made from 0.01 $molL^{-1}$ solutions of $Na_2HPO_4.7H_2O$ and KH_2PO_4) containing 0.1% pyrrolidinone and buffered to pH 7.5 showed no detrimental effects on fish after 30 min and 2 h exposure, i.e. fish exhibited normal behaviour. Using identical phosphate buffer solutions, the triketone oil was tested at 50 and 200 ppm.

At 50 ppm and 2 h exposure, the fish showed normal behaviour and no signs of distress. At 200 ppm and 30 min exposure, all fish showed normal behaviour. However at 2 h exposure, 5 of the remaining 10 fish died (50% mortality), with the remaining 5 live fish exhibiting signs of distress and lethargy. 24 h post exposure, fish in the observation tank had recovered and exhibited normal behaviour.

From the toxicity tests it was determined that a triketone oil concentration of 200 ppm was acutely toxic and had a 50% mortality rate on Atlantic salmon fry after 2 h of exposure. At 50 ppm, the triketone oil was not acutely toxic after a 2 h exposure period.

7.5 Conclusions

The principal conclusions from the *in vitro* assessment of East Cape triketone oil versus *S. parasitica*, and the *in vivo* toxicity trials reported in this Chapter are:

(i) The aqueous insolubility of triketone oil at neutral (natural) pH required the use of small percentages of organic solvents and phosphate buffer solutions to obtain homogenous aqueous solutions.

(ii) The triketone oil showed no inhibition of *S. parasitica* when tested at concentrations up to 200 ppm, in an aqueous solution containing 1% DMSO and 0.5% Tween 80.

(iii) Promising inhibition of *S. parasitica* growth was seen at concentrations of 150 and 200 ppm using two aqueous solutions containing 0.5% and 1.0% pyrrolidinone, each containing 0.5% Tween 80.

(iv) The pyrrolidinone/Tween 80:aqueous solutions proved toxic and distressful to Atlantic salmon fry (*Salmon salar*) and were unsuitable as solvent mediums.

(v) At phosphate buffer concentrations of 0.01 and 0.10 $molL^{-1}$, buffered to pH's of 7.0-8.5, the triketone oil showed no inhibition of *S. parasitica* growth. Although inhibition was seen at 150 and 200 ppm, at pH 7.0 and 7.5 of a 0.25 $molL^{-1}$ buffer solution, these concentrations are not seen as commercially viable.

(vi) East Cape triketone oil at 200 ppm was found to be acutely toxic to Atlantic salmon, with a 50% mortality rate after a 2 h single dose static bath exposure.

(vii) No further work is presently planned for the development of triketone oil as a commercially viable treatment for *Saprolegnia*.

Chapter Eight

Experimental

8.1 General

8.1.1 Nuclear Magnetic Resonance (NMR) Experiments

One- and two-dimensional ^{1}H and ^{13}C NMR spectra were obtained from $CDCl_3$, DMSO-D_6 or C_5D_5N solutions using a Bruker DRX-400 instrument with a 5 mm inverse probehead operating at 400.13 (^{1}H) and 100.62 (^{13}C) MHz. Chemical shifts are reported relative to internal tetramethylsilane (TMS), where δ (^{1}H) TMS + δ (^{1}H) $CDCl_3$ = 7.26 ppm and δ (^{13}C) TMS + δ (^{13}C) $CDCl_3$ = 77.06 ppm, or δ (^{1}H) TMS + δ (^{1}H) DMSO-D_6 = 2.60 ppm and δ (^{13}C) TMS + δ (^{13}C) DMSO-D_6 = 39.50 ppm, or δ (^{1}H) TMS + δ (^{1}H) C_5D_5N = 7.20, 7.57 and 8.72 ppm and δ (^{13}C) TMS + δ (^{13}C) C_5D_5N = 123.5, 135.6 and 149.8 ppm.

8.1.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

Sample Preparation

Sheep faecal material and plant samples were extracted and hydrolyzed at The National Veterinary Institute (NVI), Oslo, Norway as described in the relevant chapters and forwarded (air freight) to the University of Waikato, Hamilton, New Zealand for GC-MS analysis, except for the Scottish *N. ossifragum* samples which were analyzed at The NVI, Oslo.

Primary Standard Solutions

Standard 1: 249.0 mg of sarsasapogenin propionate was weighted into a 10 mL glass vial, dissolved in *ca.* 5 mL $CHCl_3$, and transferred with repeated washing into a 250 mL volumetric flask and made up to the mark with $CHCl_3$ to give a standard solution of 0.996 mg/mL of sarsasapogenin propionate.

Standard 2: 29.7 mg of sarsasapogenin propionate was weighted into a 10 mL glass vial, dissolved in *ca.* 5 mL $CHCl_3$, and transferred with repeated washing into a 100 mL volumetric flask and made up to the mark with $CHCl_3$ to give a standard solution of 0.297 mg/mL of sarsasapogenin propionate.

Standard 3: 7.5 mg of sarsasapogenin acetate was weighted into a 10 mL glass vial, dissolved in *ca.* 5 mL $CHCl_3$, and transferred with repeated washing into a 25 mL volumetric flask and made up to the mark with $CHCl_3$ to give a standard solution of 0.30 mg/mL of sarsasapogenin acetate.

Sapogenin Analyses

The National Veterinary Institute, Oslo

GC-MS analysis of sapogenin acetates was performed using a 0.22 mm id (internal diametre) × 25 m HP-5 (Hewlett Packard) methylsilicone capillary column installed in a Karlo-Erba 8000 GC and interfaced to a VG Trio 1000 mass spectrometer. GC-MS analyses were oven programmed from 200°C (1 min hold) to 265°C at 40°C/min, and then to 290°C at 5°C/min with a 16 min hold. Column head pressure was typically set at 15 kPa.

The University of Waikato

GC-MS analysis of sapogenin acetates was performed using a 0.22 mm id (internal diametre) × 25 m HP-1 (Hewlett Packard) methylsilicone capillary column installed in a HP-5980 GC instrument fitted with a HP-7673A auto-injector and interfaced to a HP-5970B mass selective detector (MSD) operating with electron impact ionisation (EI) at 70 eV. The GC oven temperature was routinely programmed from 200°C to 250°C at 35°C/min and then to 295°C at 3°C/min with a 15 min hold. Column head pressure was typically set at 15 kPa.

Identification of sapogenin acetates was carried out by comparison of the retention times of a series of standard samples, and by examination of the total ion chromatograms (TIC) and the selected ion mode (SIM) profiles of a series of characteristic ions: *m/z* 139, 255/315, 269/329, 284/344 and 458 for saturated sapogenin acetates, *m/z* 282 and 396 for unsaturated sapogenin acetates, and *m/z* 271 and 300 for saturated 3-keto-sapogenins.

Quantitation of the acetylated sapogenins was performed by integrating the *m/z* 139 ion profile extracted for selected ion mode (SIM) chromatograms. The level of deuterium retention in sapogenin substrates was determined by assessment of the *m/z* 315 to 320 ion cluster of the corresponding acetates. Concentrations of sapogenins were determined relative to an internal standard (sarsasapogenin propionate) using appropriate response factors (R_F).

Calculations

Calculations were performed using purpose written Microsoft Excel™ spreadsheets.

Response factor (R_F)

Response factors (R_F) were determined from slopes of routinely run 3-point calibration curves using standard solutions of sarsasapogenin acetate (0.30 mg/mL) and sarsasapogenin propionate (0.297 mg/mL) in the ratios 1:1, 1:2.5 and 2.5:1 respectively.

Levels (mg/kg)

The level (mg/kg) of compound (x) in each sample (y) is calculated by the formula:

$$\text{Level (x)} = \frac{A\,(x)}{A\,(std)} \times \frac{Wt\,(std)}{Wt\,(y)} \times \frac{1}{R_F\,(x)} \times R_M\,(x) \times 1000$$

where: A (x) is the integrated peak area of compound x

A (std) is the integrated peak area of internal standard added to the sample

Wt (std) is the mass of internal standard added to the sample (mg)

Wt (y) is the mass of plant or animal sample

R_M (x) is the ratio of the molecular weights of the target alcohol and the corresponding acetate

1000 is a multiplier included in the equation to convert a level of mg/g to a level of mg/kg (ppm)

8.1.3 Chromatography

Thin Layer Chromatography (TLC)

TLC was performed on silica gel plates (E. Merck 5554) using the following solvent systems; (i) hexane:ethyl acetate (2:1) for synthesized glycoside tetraacetates, (ii) $CHCl_3$:5% aqueous MeOH (85:15) for synthesized glycosides and (iii) $CHCl_3$:MeOH:H_2O (40:19:1) for *N. ossifragum* saponins. Plates were developed with 10% H_2SO_4 solution and heating on a hot plate.

Radial Chromatography (RC)

Radial chromatography was performed using a Chromatotron 7924T and 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plates.

Column Chromatography (CC)

Column chromatography was performed using a 2.5 × 23 cm silica gel 60 (0.063-0.200 mm) column, and wet packed using petroleum spirits (40:60).

8.1.4 Electrospray-Mass Spectrometry (ES-MS)

ES-MS was performed on a Thermo Finnigan LCQ Advantage instrument equipped with a quadrupole ion trap mass analyzer, a Pfeiffer TMH 261-130 split-flow turbomolecular pump, and interfaced to a Gateway 2000® PC with an Intel® Pentium® III processor. Negative ion mode was performed with a cone voltage of -30 V to -40 V and a capillary temperature of 280°C. Positive ion mode was performed with a cone voltage of +30 V to +40 V and a capillary temperature of 280°C. Samples were dissolved in MeOH (-ve ion mode) or MeOH:CH_2Cl_2 (1:1) (+ve ion mode) and introduced via a direct infusion syringe pump.

8.1.5 High Resolution-Mass Spectrometry (HR-MS)

HR-MS analysis of the five synthesized saponins (see Chapter 5) was performed at the University of Auckland, New Zealand, utilizing Fast Atom Bombardment (FAB) and *m*-nitrobenzyl alcohol as the liquid matrix.

8.2 Chapter Five – Synthesis

8.2.1 2,3,4,6-Tetra-*O*-acetyl-α-D-glucopyranosyl bromide

Typically, 1,2,3,4,6-penta-*O*-acetyl-β-D-glucopyranose (5.0 g, 0.013 moles), glacial acetic acid (10 mL) and 45% hydrobromic acid in acetic acid (2.5 mL, 0.02 moles) were mixed and held at 6°C for 24-36 h until TLC and/or GC-MS showed complete reaction. The solution was poured into a mixture of $CHCl_3$ (40 mL) and ice water (125 mL), shaken and separated. The aqueous layer was further extracted with $CHCl_3$ (2 × 20 mL). The combined $CHCl_3$ extracts were combined, washed with a saturated solution of $NaHCO_3$ (3 × 50 mL) and dried over anhydrous Na_2SO_4 for 16 h. Solvent removal by rotary evaporator gave an oil. Recrystallization from diethyl ether afforded 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide as white needles (4.59 g, 87.2%), m.p. 87-88°C [lit. 88-89°C (Merck Index 13th edn.)]; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.1.

8.2.2 2,3,4,6-Tetra-*O*-acetyl-α-D-galactopyranosyl bromide

Typically, 1,2,3,4,6-penta-*O*-acetyl-β-D-galactopyranose (5.0 g, 0.013 moles), glacial acetic acid (10 mL) and 45% hydrobromic acid in acetic acid (2.5 mL, 0.02 moles) were mixed and held at 6°C for 24-36 h until TLC and/or GC-MS showed complete reaction. The solution was poured into a mixture of $CHCl_3$ (40 mL) and ice water (125 mL), shaken and separated. The aqueous layer further extracted with $CHCl_3$ (2 × 20 mL). The combined $CHCl_3$ extracts were combined, washed with a saturated solution of $NaHCO_3$ (3 × 50 mL) and dried over anhydrous Na_2SO_4 for 16 h. Solvent removal by rotary evaporator gave an oil. Recrystallization from diethyl ether afforded 2,3,4,6-tetra-O-acetyl-α-D-galactopyranosyl bromide as white needles (4.34 g, 82.4%), m.p. 77-79°C [lit. 77-80°C (Mary *et al.*, 1990)]; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.1.

8.2.3 Synthesis of Episarsasapogenin β-D-glucoside

Episarsasapogenin β-D-glucoside tetraacetate

For a typical synthesis, a mixture of episarsasapogenin (1.0 g, 2.40×10^{-3} moles), $CdCO_3$ (1.0 g, 5.8×10^{-3} moles) and toluene (50 mL) was refluxed in a 100 mL round bottom flask fitted with a Dean and Stark trap and an anhydrous $CaCl_2$ water trap fitted to the condenser for 30 min. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.0 g, 4.87×10^{-3} moles) in toluene (15 mL) was added dropwise to the reaction mixture over 30 min and

the mixture was refluxed for 24-36 h until TLC showed complete reaction. The reaction mixture was hot filtered and solvent removal via rotary evaporation gave an oil. A sub-sample of the oil was then separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of petroleum spirits (40:60):diethyl ether (1:0, 3:1, 3:2, 1:1, 1:4, 0:1). Episarsasapogenin β-D-glucoside tetraacetate eluted in the late 40% to 50% diethyl ether portions, m.p. 125-130°C; ES-MS (+30 V, MeOH:CH_2Cl_2): *m/z* 769 $(M+Na)^+$ and *m/z* 1515 $(M_2+Na)^+$; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.4.

Episarsasapogenin β-D-glucoside

The episarsasapogenin β-D-glucoside tetraacetate oil was dissolved in MeOH (100 mL) in a 250 mL round bottom flask, KOH pellets (4 g, 0.71 $molL^{-1}$) were added and the mixture refluxed for 24 h. After cooling the mixture was poured into water (600 mL) and $CHCl_3$ (200 mL) shaken and separated. The aqueous layer was further extracted with $CHCl_3$ (150 mL and 100 mL). The combined extracts were dried over anhydrous Na_2SO_4 for 16 h. Solvent removal by rotary evaporator gave an oily residue, which was separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of $CHCl_3$:5% aqueous MeOH (1:0, 9:1, 17:3, 4:1, 3:1, 1:1, 0:1). Episarsasapogenin β-D-glucoside eluted in the late 10% to early 15% aqueous MeOH fractions, (276 mg, 19.9%), m.p. 213-217°C; ES-MS (-30 V, MeOH): *m/z* 623 $(M+COOH)^-$, *m/z* 637 $(M+CH_3COO)^-$ and *m/z* 1155 $(M_2-H)^-$; HR-MS: *m/z* 579.3886 $(M+H)^+$ ($C_{33}H_{55}O_8$ requires 579.3897); ^1H and ^{13}C NMR (δ ppm, DMSO-D_6): see Table 5.4.

8.2.4 Synthesis of Episarsasapogenin β-D-galactoside

Episarsasapogenin β-D-galactoside tetraacetate

A mixture of episarsasapogenin (2.1 g, 5.05×10^{-3} moles), $CdCO_3$ (2.5 g, 0.0145 moles) and toluene (60 mL) was refluxed in a 100 mL round bottom flask fitted with a Dean and Stark trap and an anhydrous $CaCl_2$ water trap fitted to the condenser for 30 min. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (4.0 g, 9.73×10^{-3} moles) in toluene (15 mL) was added dropwise to the reaction mixture over 30 min and the mixture was refluxed for 24-36 h until TLC showed complete reaction. The reaction mixture was hot filtered and solvent removal via rotary evaporation gave an oil. A sub-sample of the oil was then separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of petroleum spirits (40:60):diethyl ether (1:0, 3:1, 3:2, 1:1, 1:4, 0:1). Episarsasapogenin β-D-galactoside tetraacetate eluted in the late 40% to

50% diethyl ether portions, m.p. 102-105°C; ES-MS (+30 V, MeOH:CH_2Cl_2): *m/z* 769 $(M+Na)^+$ and *m/z* 1515 $(M_2+Na)^+$; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.5.

Episarsasapogenin β-D-galactoside

The episarsasapogenin β-D-galactoside tetraacetate oil was dissolved in MeOH (100 mL) in a 250 mL round bottom flask, KOH pellets (4 g, 0.71 $molL^{-1}$) were added and the mixture refluxed for 24 h. After cooling the mixture was poured into water (600 mL) and $CHCl_3$ (200 mL) shaken and separated. The aqueous layer was further extracted with $CHCl_3$ (150 mL and 100 mL). The combined extracts were dried over anhydrous Na_2SO_4 for 16 h. Solvent removal by rotary evaporator gave an oily residue, which was separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of $CHCl_3$:5% aqueous MeOH (1:0, 9:1, 17:3, 4:1, 3:1, 1:1, 0:1). Episarsasapogenin β-D-galactoside eluted in the late 10% to early 15% aqueous MeOH fractions, (890 mg, 30.5%), m.p. 223-227°C; ES-MS (-30 V, MeOH): *m/z* 623 $(M+COOH)^-$ and *m/z* 1155 $(M_2-H)^-$; HR-MS: *m/z* 579.3902 $(M+H)^+$ ($C_{33}H_{55}O_8$ requires 579.3897); ^1H and ^{13}C NMR (δ ppm, DMSO-D_6): see Table 5.5.

8.2.5 Synthesis of Sarsasapogenin β-D-galactoside

Sarsasapogenin β-D-galactoside tetraacetate

A mixture of sarsasapogenin (1.5 g, 3.61×10^{-3} moles), $CdCO_3$ (1.6 g, 9.3×10^{-3} moles) and toluene (55 mL) was refluxed in a 100 mL round bottom flask fitted with a Dean and Stark trap and an anhydrous $CaCl_2$ water trap fitted to the condenser for 30 min. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (3.0 g, 7.3×10^{-3} moles) in toluene (15 mL) was added dropwise to the reaction mixture over 30 min and the mixture was refluxed for 24-36 h until TLC showed complete reaction. The reaction mixture was hot filtered and solvent removal via rotary evaporation gave an oil. A sub-sample of the oil was then separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of petroleum spirits (40:60):diethyl ether (1:0, 3:1, 3:2, 1:1, 1:4, 0:1). Sarsasapogenin β-D-galactoside tetraacetate eluted in the late 40% to 50% diethyl ether portions, m.p. 180-184°C; ES-MS (+30 V, MeOH:CH_2Cl_2): *m/z* 769 $(M+Na)^+$ and *m/z* 1515 $(M_2+Na)^+$; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.6.

Sarsasapogenin β-D-galactoside

The sarsasapogenin β-D-galactoside tetraacetate oil was dissolved in MeOH (100 mL) in a 250 mL round bottom flask, KOH pellets (4.1 g, 0.71 $molL^{-1}$) were added and the mixture refluxed for 24 h. After cooling the mixture was poured into water (600 mL) and $CHCl_3$ (200 mL) shaken and separated. The aqueous layer was further extracted with $CHCl_3$ (150 mL and 100 mL). The combined extracts were dried over anhydrous Na_2SO_4 for 16 h. Solvent removal by rotary evaporator gave an oily residue, which was separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of $CHCl_3$:5% aqueous MeOH (1:0, 9:1, 17:3, 4:1, 3:1, 1:1, 0:1). Sarsasapogenin β-D-galactoside eluted in the 10% to early 15% aqueous MeOH fractions, (660 mg, 31.7%), m.p. 218-224°C; ES-MS (-30 V, MeOH): *m/z* 577 $(M-H)^-$, *m/z* 623 $(M+COOH)^-$, *m/z* 637 $(M+CH_3COO)^-$ and *m/z* 1155 $(M_2-H)^-$; HR-MS: *m/z* 579.3895 $(M+H)^+$ ($C_{33}H_{55}O_8$ requires 579.3897); ^1H and ^{13}C NMR (δ ppm, DMSO-D_6): see Table 5.6.

8.2.6 Synthesis of the Betulin β-D-glucosides

In a typical synthesis, a mixture of betulin (0.95 g, 2.15 × 10^{-3} moles), $CdCO_3$ (2.2 g, 0.0128 moles) and toluene (70 mL) was refluxed in a 100 mL round bottom flask fitted with a Dean and Stark trap and an anhydrous $CaCl_2$ water trap fitted to the condenser for 30 min. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (2.6 g, 6.32 × 10^{-3} moles) in toluene (15 mL) was added dropwise to the reaction mixture over 30 min and the mixture was then refluxed for 24-36 h until TLC showed complete reaction. The reaction mixture was hot filtered and solvent removal via rotary evaporation gave an oil. Careful separation of the oil by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of petroleum spirits (40:60):diethyl ether (1:0, 13:7, 3:2, 11:9, 1:1, 9:11, 2:3. 7:13. 1:3, 0:1) yielded four glycosidic products (see below);

(i) Allobetulin β-D-glucoside tetraacetate eluted in the 45% diethyl ether fraction, (48 mg, 2.9%), m.p. 88-92°C; ES-MS (+30 V, MeOH:CH_2Cl_2): *m/z* 795 $(M+Na)^+$, *m/z* 1567 $(M_2+Na)^+$ and *m/z* 1583 $(M_2+K)^+$; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.12.

(ii) Betulin 3-(β-D-glucoside tetraacetate) eluted in the early 50% diethyl ether fraction, (240 mg, 14.0%), m.p. 118-122°C; ES-MS (+30 V, MeOH:CH_2Cl_2): *m/z* 795 $(M+Na)^+$, *m/z* 1567 $(M_2+Na)^+$ and *m/z* 1583 $(M_2+K)^+$; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.8.

(iii) Betulin 28-(β-D-glucoside tetraacetate) eluted in the late 50% diethyl ether fraction, (109 mg, 6.6%), m.p. 116-120°C; ES-MS (+30 V, MeOH:CH_2Cl_2): *m/z* 795 $(M+Na)^+$, *m/z* 1567 $(M_2+Na)^+$ and *m/z* 1583 $(M_2+K)^+$; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.10.

(iv) Betulin 3,28-(β-D-diglucoside tetraacetate) eluted in the 65% to 70% diethyl ether fraction, (659 mg, 27.9%), m.p. 226-228°C; ES-MS (+40 V, MeOH:CH_2Cl_2): *m/z* 1125 $(M+Na)^+$ and *m/z* 1141 $(M_2+K)^+$; ^1H and ^{13}C NMR (δ ppm, $CDCl_3$): see Table 5.9.

Betulin 3-(β-D-glucoside)

Betulin 3-(β-D-glucoside tetraacetate) (220 mg) was dissolved in MeOH (25 mL) in a 250 mL round bottom flask, KOH pellets (1.0 g, 0.71 $molL^{-1}$) were added and the mixture refluxed for 24 h. After cooling the mixture was poured into water (300 mL) and $CHCl_3$ (100 mL) shaken and separated. The aqueous layer was further extracted with $CHCl_3$ (2 × 50 mL). The combined extracts were dried over anhydrous Na_2SO_4 for 16 h. Solvent removal by rotary evaporator gave an oily residue which was separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of $CHCl_3$:5% aqueous MeOH (1:0, 19:1, 9:1, 17:3, 3:1, 0:1). Betulin 3-(β-D-glucoside) eluted in the late 10% to early 15% MeOH fractions, (141 mg, 81.9%), m.p.158-164°C. ES-MS (-30 V, MeOH): *m/z* 603 $(M-H)^-$, *m/z* 617 $(M+CH_3OH-H_2O-H)^-$, *m/z* 649 $(M+COOH)^-$, *m/z* 663 $(M+CH_3COO)^-$, *m/z* 1207 $(M_2-H)^-$, *m/z* 1221 $(M_2+CH_3OH-H_2O-H)^-$, *m/z* 1235 $(M_2+(CH_3OH-H_2O)_2-H)^-$ and *m/z* 1825 $(M_3+CH_3OH-H_2O-H)^-$; HR-MS: *m/z* 605.4407 $(M+H)^+$ ($C_{36}H_{61}O_7$ requires 605.4417); ^1H and ^{13}C NMR (δ ppm, DMSO-D_6): see Table 5.8.

Betulin 3,28-(β-D-diglucoside)

Betulin 3,28-(β-D-diglucoside tetraacetate) (640 mg) was dissolved in MeOH (50 mL) in a 250 mL round bottom flask, KOH pellets (2.0 g, 0.71 $molL^{-1}$) were added and the mixture refluxed for 24 h. After cooling the mixture was poured into water (400 mL) and $CHCl_3$ (100 mL) shaken and separated. The aqueous layer was further extracted with $CHCl_3$ (2 × 50 mL). The combined extracts were dried over anhydrous Na_2SO_4 for 16 h. Solvent removal by rotary evaporator gave an oily residue, which was separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate and mixtures of $CHCl_3$:5% aqueous MeOH (1:0, 9:1, 4:1, 7:3, 3:2, 1:1, 0:1). Betulin 3,28-(β-D-diglucoside) eluted in the 30% to 40% MeOH fractions, (398 mg, 89.4%), m.p. 203-206°C; ES-MS (-40 V, MeOH): *m/z* 765 $(M-H)^-$, *m/z* 811 $(M+COOH)^-$, *m/z* 825 $(M+CH_3COO)^-$, *m/z* 1531 $(M_2-H)^-$ and 1577 $(M_2+COOH)^-$; HR-MS: *m/z* 767.4944 $(M+H)^+$ ($C_{42}H_{71}O_{12}$ requires 767.4946); ^1H and ^{13}C NMR (δ ppm, DMSO-D_6): see Table 5.9.

8.2.7 Isolation of 4-Methoxyfuran-2(5*H*)-one 5-(β-D-glucoside)

A freeze-dried leaf sample of Scottish *N. ossifragum* (10.9 g) was sequentially extracted with $CHCl_3$ (50 mL) for 4 h using a SoxTech apparatus, then for 4 h with MeOH (50 mL). The crude MeOH extract was absorbed onto silica gel and separated by column chromatography (2.5 × 23 cm silica gel 60 (0.063-0.200mm) column) using mixtures of $CHCl_3$:5% aqueous MeOH (1:0, 17:3, 3:1, 1:1, 1:3, 0:1). Fractions 9, 10 and 11 were combined and further separated by radial chromatography using a 22.5 cm diametre × 2 mm thick silica gel (E. Merck 938) plate with mixtures of $CHCl_3$:5% aqueous MeOH (1:0, 17:3, 3:1, 13:7, 11:9, 3:7, 3:17, 0:1). Fractions 5 and 6 (25 to 35% MeOH portions) contained 4-methoxyfuran-2(5*H*)-one 5-(β-D-glucoside), (*ca.* 50 mg), m.p. 88-94°C; GC-MS (acetate): *m/z* 113 (100%), 347 (8); ^1H and ^{13}C NMR (δ ppm, C_5D_5N): see Table 5.13.

8.2.8 East Cape Manuka Active Concentrate – Triketone Oil

25 mL of the active fraction of East Cape Manuka oil was purchased from Tairawhiti Pharmaceuticals Ltd and was supplied with the following physical and chemical data; relative density: 1.072; refractive index: 1.501; GC-MS; flavasone (18.98%), isoleptospermone (17.68%), leptospermone (61.51%).

8.3 Chapter Six – Bioactivity Assessment

Bacterial organisms, fungal organisms and agar mediums were prepared and supplied by the Microbiology Department and the Media Department, National Veterinary Institute (NVI), Oslo, Norway. Testing protocols can be found in Section 6.2.

All chemicals used in the bioactivity assessments were available from the Chemistry and Media Departments, NVI.

8.4 Chapter Seven – *Saprolegnia parasitica*

Two fish-pathogenic strains of *S. parasitica* were isolated, cultured and supplied by Svein Stueland, Microbiology Department, The NVI, Oslo. Sabouraud's media was supplied by the Media Department, The NVI, Oslo. All chemicals used in the bioactivity assessments were available from the Chemistry and Media Departments, The NVI, Oslo.

8.4.1 *In vitro* Phosphate Buffers at 0.01, 0.10 and 0.25 molL^{-1}

A Mettler Delta 320 pH meter was calibrated prior to use, using Merck calibration buffer solutions; (i): citric acid/sodium hydroxide/hydrogen chloride, pH 4 and (ii): disodium hydrogen phosphate/potassium dihydrogen phosphate, pH 7.

Buffers of pH 7.0-8.5, used for *in vitro* assessments were typically made as described below for the 0.25 molL^{-1} phosphate buffer solutions. 0.25 molL^{-1} solutions of potassium phosphate monobasic and sodium phosphate dibasic were used, i.e. 8.5 g KH_2PO_4 in 250 mL salmon pond (dechlorinated) water and 16.75g $Na_2HPO_4.7H_2O$ in 250 mL salmon pond (dechlorinated) water. All buffer solutions were autoclaved prior to *in vitro* testing.

Phosphate Buffer pH 7.0
100 mL potassium phosphate monobasic solution (pH 4.37) was adjusted to pH 7.00 by slow addition of sodium phosphate dibasic solution, with stirring. End volume *ca.* 300 mL.

Phosphate Buffer pH 7.5
100 mL sodium phosphate dibasic solution (pH 8.90) was adjusted to pH 7.50 by slow addition of potassium phosphate monobasic solution with stirring. End volume *ca.* 115 mL.

Phosphate Buffer pH 8.0
100 mL sodium phosphate dibasic solution (pH 8.91) was adjusted to pH 8.01 by slow addition of potassium phosphate monobasic solution with stirring. End volume *ca.* 107 mL.

Phosphate Buffer pH 8.5
100 mL sodium phosphate dibasic solution (pH 8.91) was adjusted to pH 8.50 by slow addition of potassium phosphate monobasic solution with stirring. End volume *ca.* 102 mL.

8.4.2 *In vivo* Toxicity Testing Phosphate Buffers

Standard 0.01 $molL^{-1}$ sodium phosphate dibasic ($Na_2HPO_4.7H_2O$) and potassium phosphate monobasic (KH_2PO_4) solutions were used to create the toxicity testing bath aqueous solutions, as described below:

Triketone Oil 50 ppm Assessment

17 L sodium phosphate dibasic solution (pH 8.85) was lowered to pH 7.51 via the addition of *ca.* 6 L of potassium phosphate monobasic solution. Triketone oil (1.25 mL) was dissolved in 1-methyl-2-pyrrolidinone (25 mL) and added with vigorous stirring to the test bath. The test bath was topped up to 25 L with water.

Triketone Oil 200 ppm Assessment

17 L sodium phosphate dibasic solution (pH 8.85) was lowered to pH 7.48 via the addition of *ca.* 6 L of potassium phosphate monobasic solution. Triketone oil (25 mL) was dissolved in 1-methyl-2-pyrrolidinone (25 mL) and added with vigorous stirring to the test bath. The test bath was topped up to 25 L with water.

Appendix

Appendix I. Total genin levels (mg/kg DM), daily % deuterated 25*S*-genins, faecal 25*S*-genins from *rome* [Equation (1)] and calculated *rome* plant material consumed [Equation (2)] found in sheep faeces during the dosing trial period, see Chapter 4.

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Sub T	Total (mg/kg DM)	% deuterated 25*S*-genins	Feacal 25*S*-genins from *rome* (mg/day)[a]	Calculated consumed *rome* (g DM/day)[b]
Ewe-844											
1	-	21	22	-	548	147		738			
2	-	49	15	-	272	368		704			
3	20	158	44	245	620	2210		3297	5.2	1472	221
4	47	620	246	-	6007	3677		10597	2.2	3524	529
5	32	346	269	-	6348	5101		12095	2.2	3567	536
6free	50	449	423	-	10472	6106	17501		2.2	3567	536
6conj	-	27	16	-	263	332	639	18140	-	-	-
7	41	349	360	-	7930	4925		13605	2.0	3891	585
8	52	578	543	-	11505	5089		17768	1.8	4406	662
9	63	589	451	-	9287	5355		15745	1.8	4299	646
10	48	472	652	-	13131	6933		21236	1.7	4563	686
11	52	424	532	-	10208	5790		17006	1.6	4974	747
12	82	740	1160	-	21889	7481		31351	1.3	6073	912
13free	75	871	984	-	20770	8888	31588		1.7	4684	704
13conj	-	24	15	-	234	218	490	32078	-	-	-
14	60	660	753	-	14882	7764		24119	1.7	4647	698
15	72	920	801	-	18129	8483		28406	1.9	4118	619
16	61	675	836	-	16149	9403		27124	1.7	4706	707
17	52	642	550	-	12650	9539		23433	2.1	3764	566
18	77	990	851	-	19871	11498		33287	1.4	5558	835
19	35	621	625	-	15041	6464		22785	1.3	5940	892
20free	38	921	747	-	17680	7641	27027		1.4	5610	843
20conj	-	26	14	-	265	291	596	27623	-	-	-
21	41	759	677	-	14453	6163		22094	1.4	5575	838
22	41	721	785	-	15609	6530		23687	1.3	5954	894
									Mean[c] 1.7	4772	717
Lamb-128 (developed alveld)											
1	-	-	-	-	-	-		0			
2	-	52	-	-	53	248		354			
3	14	95	12	126	77	810		1135	49.4	41	6
4	28	368	40	-	906	2480		3822	8.0	461	69
5	43	633	330	-	7289	7431		15727	3.6	1085	163
6free	38	468	326	-	6861	7531	15223		3.3	1187	178
6conj	3	31	7	-	167	564	772	15995	-	-	-
7	42	456	269	-	5708	7598		14072	3.4	1146	172
8	43	590	343	-	7721	10557		19254	3.4	1135	171
9	53	697	412	-	9231	11198		21591	2.5	1576	237
10	52	458	192	-	4342	9812		14856	2.7	1421	214
11	33	376	159	-	3545	6338		10451	2.7	1443	217
12	51	704	202	-	4804	7896		13656	1.8	2233	336
13free	46	406	121	-	2835	7568	10976		2.5	1556	234
13conj	-	28	12	-	216	573	829	11805	-	-	-
14	27	360	179	-	3256	4860		8682	3.5	1091	164
15	21	172	115	-	1901	2350		4559	6.0	631	95
									Mean[c] 3.2	1319	198

cont...

Appendix I. cont...

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Sub T	Total (mg/kg DM)	% deuterated 25*S*-genins	Feacal 25*S*-genins from *rome* (mg/day)[a]	Calculated consumed *rome* (g DM/day)[b]
Lamb-129											
1	-	-	-	-	-	-		-			
2	-	42	-	-	79	280		401	57.8	29	4
3	-	88	-	107	71	512		779	52.2	37	5
4	36	270	21	267	188	1455		2237	14.7	233	35
5	39	410	22	124	2719	4252		7566	4.9	773	116
6free	35	471	239	-	5811	8286	14843		3.4	1147	172
6conj	-	-	-	-	-	-	-	14843	-	-	-
7	21	283	190	-	4496	5127		10117	2.9	1353	203
8	42	454	290	-	6852	6792		14431	2.8	1376	207
9	62	693	236	-	5282	8195		14469	3.4	1145	172
10	50	487	34	-	7062	8334		15968	3.6	1069	161
11	19	174	207	-	4020	7655		12075	4.7	806	121
12	42	550	548	-	11836	8280		21255	2.1	1833	275
13free	37	474	458	-	10378	10285	21631		2.2	1767	266
13conj	-	15	-	-	88	181	283	21914	-	-	-
14	53	623	486	-	10347	8815		20323	2.0	1917	288
15	87	1049	593	-	13791	10172		25692	1.8	2205	331
16	72	706	495	-	10889	8887		21050	1.9	2014	303
17	56	595	332	-	7835	10407		19225	2.0	1967	296
18	-	-	-	-	-	-		-	-	-	-
19	48	608	370	-	8377	11930		21333	2.6	1509	227
20free	-	59	445	-	11594	10345	22443		2.4	1595	240
20conj	-	188	9	-	164	410	770	23213	-	-	-
21	34	493	324	-	7367	10875		19094	3.3	1187	178
22	-	454	248	-	6244	10175		17121	3.4	1125	169
									Mean[c] 2.9	1458	219
Ewe-952											
1	-	-	-	-	-	-		-			
2	-	60	-	79	168	501		809			
3	-	-	-	-	-	-		-			
4	36	496	313	-	6819	4813		12476	1.6	5077	763
5	53	493	366	-	8180	5145		14238	1.7	4656	700
6free	30	609	423	-	10165	6935	18162		1.5	5361	805
6conj	-	25	10	-	214	361	610	18772	-	-	-
7	56	592	636	-	13090	7048		21422	1.3	6236	937
8	86	846	472	-	10147	5994		17544	1.3	6001	902
9	92	1023	527	-	10764	5650		18056	1.5	5342	803
10	94	919	771	-	14582	7332		23697	1.2	6744	1013
11	48	469	423	-	7892	5486		14318	1.8	4253	639
12	76	1036	711	-	14367	7404		23593	1.3	5948	894
13free	85	1162	730	-	16623	9690	28290		1.5	5113	768
13conj	1	22	5	1	131	257	415	28705	-	-	-
14	75	803	416	-	8031	7038		16364	1.5	5317	799
15	104	1169	438	-	8561	6364		16637	1.5	5314	798
16	91	939	682	-	13532	8228		23472	1.3	6286	944
17	57	698	341	-	7081	6231		14409	1.5	5242	788

cont...

Appendix I. cont...

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Sub T	Total (mg/kg DM)	% deuterated 25*S*-genins	Feacal 25*S*-genins from *rome* (mg/day)[a]	Calculated consumed *rome* (g DM/day)[b]
Ewe-952											
18	108	1413	955	-	19111	9048		30635	1.3	5970	897
19	46	1058	970	-	19582	9402		31058	1.4	5525	830
20free	76	1154	1146	-	23164	11869	37410		1.4	5686	854
20conj	-	28	12	-	196	400	636	38046	-	-	-
21	84	1069	949	-	17965	8363		28429	1.2	6513	978
22	103	1374	1116	-	19816	11017		33425	1.3	5969	897
								Mean[c]	1.4	5637	847
Lamb-121 (developed alveld)											
1	-	-	-	-	-	-		-			
2	10	130	10	-	181	395		726			
3	49	495	88	-	1700	2642		4972	7.5	493	74
4	-	-	-	-	-	-		-	-	-	-
5	47	619	185	-	4084	6167		11102	2.8	1392	209
6free	58	701	409	-	8551	10344	20063		2.9	1350	203
6conj	3	29	10	-	189	412	642	20705	-	-	-
7	42	506	475	-	9739	7778		18541	2.2	1784	268
8	99	1081	930	-	21195	8777		32082	1.9	2101	316
9	129	1407	870	-	20546	7961		30914	1.6	2394	360
10	84	808	917	-	18940	10652		31400	2.0	1974	297
11	58	630	730	-	13444	7661		22523	2.1	1862	280
12	57	898	809	-	17787	6935		26487	1.3	2964	445
								Mean[c]	2.1	1977	297
Lamb-122											
1	-	-	-	-	-	-		-			
2	-	47	-	-	100	309		456			
3	17	140	16	173	180	1227		1753	16.5	202	30
4	28	354	35	-	1004	2627		4048	8.4	435	65
5	36	322	70	-	1597	5084		7110	3.6	1080	162
6free	48	533	187	-	4001	9464	14234		3.3	1154	173
6conj	-	29	8	-	165	505	706	14940	-	-	-
7	63	693	459	-	8583	10163		19962	2.7	1464	220
8	70	1208	684	-	16180	11425		29567	1.7	2375	357
9	119	1727	426	-	10248	11362		23882	2.1	1893	284
10	57	822	295	-	7826	11124		20123	2.4	1617	243
11	37	376	128	-	3101	8630		12272	3.8	1008	152
12	74	880	401	-	8840	10699		20894	1.8	2181	328
13free	44	755	389	-	10670	14393	26251		1.7	2334	351
13conj	-	17	5	-	118	255	394	26645	-	-	-
14	61	786	314	-	7940	13619		22720	1.8	2192	329
15	71	827	177	-	4452	10336		15863	1.8	2215	333
16	50	575	221	-	5312	9609		15767	2.1	1853	278
17	40	427	161	-	3727	10111		14467	2.3	1681	253
18	-	-	-	-	-	-		-	-	-	
19	40	575	354	-	8599	11001		20569	2.8	1410	212
20free	16	219	93	-	2496	9127	11951		4.2	917	138
20conj	-	10	4	-	68	137	219	12170	-	-	-

cont...

Appendix I. cont...

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Sub T	Total (mg/kg DM)	% deuterated 25*S*-genins	Feacal 25*S*-genins from *rome* (mg/day)[a]	Calculated consumed *rome* (g DM/day)[b]
Lamb-122											
21	16	239	104		2694	7632		10684	3.8	1011	152
22	15	198	89		2290	7802		10394	3.7	1051	158
								Mean[c]	2.7	1614	242
Ewe-8056											
1	-	-	-	-	-	-		-			
2	18	43	15	98	129	246		550			
3	30	176	179	-	3663	3190		7238	3.3	2345	352
4	-	-	-	-	-	-		-	-	-	-
5	48	356	336	-	6863	4460		12063	1.8	4484	674
6free	48	509	532	-	11732	5469	18290		1.9	4061	610
6conj	-	22	16	-	261	321	619	18909	-	-	-
7	62	605	768	-	14918	6472		22826	1.4	5762	866
8	83	757	520	-	10219	5118		16696	1.4	5537	832
9	89	650	735	-	13039	6152		20665	1.5	5322	800
10	75	439	429	-	7501	5108		13552	1.9	4075	612
11	62	397	288	-	5905	5387		12039	2.6	3001	451
12	101	736	352	-	6431	4544		12164	2.0	3874	582
13free	83	1084	941	-	19575	7649	29332		1.7	4626	695
13conj	-	20	12	-	159	221	411	29743	-	-	-
14	107	1196	1202	-	20837	8963		32306	1.4	5732	861
15	141	1268	986	-	17813	8282		28490	1.7	4497	676
16	73	622	752	-	12967	6341		20755	1.2	6370	957
17	60	470	401	-	7901	5894		14727	2.3	3406	512
18	89	642	460	-	8073	6177		15440	1.7	4737	712
19	71	581	519	-	9791	6241		17203	1.7	4655	699
20free	52	422	404	-	7632	5620	14129		1.9	4170	627
20conj	-	-	-	-	-	-	-	14129	-	-	-
21	65	445	444	-	7974	7237		16165	1.6	4852	729
22	99	628	610	-	9145	7095		17576	1.7	4664	701
								Mean[c]	1.7	4657	700
Lamb-39											
1	-	-	-	-	33	82		115			
2	-	15	-	-	39	102		156			
3	12	73	13	118	97	464		777	36.3	70	11
4	64	772	79	-	1765	4190		6870	4.9	784	118
5	59	589	120	-	2733	5863		9363	3.6	1085	163
6free	65	775	298	-	7378	9052	17567		3.2	1197	180
6conj	-	30	6	-	174	519	729	18296	-	-	-
7	55	590	364	-	8836	7615		17460	2.9	1327	199
8	77	723	377	-	9388	8182		18747	2.4	1601	240
9	121	976	258	-	6212	6881		14448	2.8	1393	209
10	50	434	137	-	3151	6543		10315	3.0	1283	193
11	69	603	252	-	5651	6973		13547	2.5	1579	237
12	94	727	438	-	9366	7404		18028	2.2	1783	268
13free	84	852	420	-	10002	7546	18905		1.7	2342	352
13conj	-	29	6	68	114	643	860	19765	-	-	-

cont...

Appendix I. cont...

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Sub T	Total (mg/kg DM)	% deuterated 25*S*-genins	Feacal 25*S*-genins from *rome* (mg/day)[a]	Calculated consumed *rome* (g DM/day)[b]
Lamb-39											
14	61	716	488	-	11638	8820		21723	1.7	2275	342
15	102	905	478	-	11767	9311		22562	1.7	2337	351
16	88	687	521	-	10709	9838		21844	1.9	2047	308
17	65	595	271	-	6399	8984		16314	2.0	2004	301
18	122	860	371	-	7971	7706		17030	1.8	2143	322
19	80	832	709	-	15819	8950		26388	1.8	2244	337
20free	70	894	518	-	13290	10384	25156		2.0	1941	292
20conj	-	22	5	52	112	526	717	25873	-	-	-
21	61	723	422	-	9777	9764		20747	2.1	1897	285
22	67	587	277	-	6473	8061		15465	2.3	1729	260
								Mean[c]	2.3	1789	269
Lamb-40											
1	11	21	-	31	41	83		187			
2	-	21	-	-	64	94		179			
3	16	95	18	202	164	1045		1539	23.0	134	20
4	53	361	38	300	430	1780		2962	11.2	319	48
5	13	97	25	-	604	857		1595	15.1	225	34
6free	19	161	36	315	450	1917	2898		12.1	291	44
6conj	-	13	3	-	66	131	213	3111	-	-	-
7	40	504	274	-	7208	6690		14717	3.1	1235	186
8	49	592	357	-	9812	7324		18133	2.0	1959	294
9	111	1443	255	-	6601	6157		14568	2.4	1620	243
10	46	307	140	-	3157	6941		10591	3.7	1040	156
11	48	395	184	-	3965	5867		10460	3.4	1143	172
12	89	704	454	-	9004	6994		17245	2.1	1901	286
13free	90	1053	816	-	18289	9265	29513		1.8	2174	327
13conj	-	36	17	-	379	479	911	30424	-	-	-
14	57	887	728	-	19063	9667		30402	1.7	2353	354
15	97	1189	594	-	16035	10144		28058	1.5	2591	389
16	80	79	477	-	11395	9958		21988	1.9	2053	308
17	99	1125	686	-	16149	11846		29905	1.7	2343	352
18	131	1825	917	-	20349	11373		34595	1.6	2399	360
19	89	1903	921	-	21838	11383		36134	1.5	2620	394
20free	63	1384	823	-	21510	11622	35402		1.7	2262	340
20conj	-	-	-	-		-	-	35402	-	-	-
21	76	1057	828	-	18169	10421		30550	1.9	2071	311
22	55	637	469	-	11354	9108		21624	2.6	1514	227
								Mean[c]	3.4	1766	265
Lamb-45											
1	-	-	-	-	-	-		-			
2	-	41	13	-	208	454		716	24.5	123	19
3	35	471	146	-	2918	3066		6636	3.1	1254	188
4	80	1211	262	-	6127	3903		11583	2.1	1827	274
5	-	-	-	-	-	-		-	-	-	-
6free	57	760	542	-	11683	5902	18944		1.7	2341	352
6conj	25	146	60	-	873	1150	2254	21198	-	-	-

cont...

Appendix I. cont...

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Sub T	Total (mg/kg DM)	% deuterated 25*S*-genins	Feacal 25*S*-genins from *rome* (mg/day)[a]	Calculated consumed *rome* (g DM/day)[b]
Lamb-45											
7	65	935	1146	-	22140	7664		31950	1.4	2781	418
8	97	1284	1043	-	20687	7990		31099	1.6	2466	371
9	134	1648	843	-	16401	6440		25466	2.0	1947	293
10	50	698	721	-	14728	6852		23050	2.3	1724	259
11	48	673	936	-	16532	6604		24793	2.2	1758	264
12	76	958	1055	-	18945	6946		27980	1.6	2406	362
13free	-	-	-	-	-	-			-	-	-
13conj	-	-	-	-	-	-		-	-	-	-
14	44	462	548	-	9771	3423		14248	2.1	1866	280
15	103	933	652	-	11678	5165		18531	2.6	1501	226
16	42	431	433	-	7650	4583		13138	2.4	1642	247
17	40	447	400	-	6902	4594		12382	2.5	1556	234
18	60	709	281	-	5169	5295		11515	3.5	1105	166
19	61	1015	558	-	11262	7291		20187	3.0	1286	193
20free	73	1673	887	-	18875	9308	30816		2.0	1986	298
20conj	-	21	6	-	110	163	299	31115	-	-	-
21	102	1930	785	-	16095	9919		28831	2.2	1806	271
22	102	1831	577	-	11672	8506		22688	2.3	1681	253
								Mean[c]	2.2	1866	280
Lamb-54 (developed alveld)											
1	-	24	9	-	173	110		316			
2	-	3	5	-	121	245		374	34.4	76	11
3	21	193	63	-	1472	3125		4875	6.4	582	87
4	37	394	229	-	4789	6341		11790	3.3	1162	175
5	46	669	258	-	6082	8018		15073	2.9	1332	200
6free	44	654	308	-	7903	11087	19996		2.8	1375	207
6conj	4	38	15	-	300	420	776	20772	-	-	-
7	56	701	384	-	8048	9302		18491	2.3	1695	255
8	133	1190	343	-	6410	10085		18160	3.7	1036	156
9	116	1197	685	-	10603	8431		21034	2.5	1548	233
10	115	1386	571	-	9558	10039		21670	2.7	1438	216
11	80	833	548	-	9330	6590		17381	2.2	1740	261
12	155	1844	1217	-	19561	9028		31804	1.5	2563	385
13free	219	2937	1283	-	21810	10723	36971		1.7	2246	337
13conj	-	20	8	-	114	166	307	37278	-	-	-
								Mean[c]	2.5	1664	250
Lamb-67 (developed alveld)											
1	-	5	-	-	82	52		139			
2	-	-	-	-	34	91		125			
3	12	24	7	-	137	342		521	60.5	26	4
4	26	89	31	-	483	1332		1962	22.6	137	21
5	31	224	50	-	724	3153		4182	6.8	546	82
6free	52	695	296	-	6563	6315	13921		3.7	1037	156
6conj	5	34	10	66	155	720	991	14912	-	-	-
7	65	651	255	-	5919	8362		15252	4.0	955	144
8	58	587	160	-	3509	6670		10985	4.7	809	122

cont...

Appendix I. cont...

Day	Smil-CO	Sar-CO	Smil	Epismil	Sar	Episar	Sub T	Total (mg/kg DM)	% deuterated 25*S*-genins	Feacal 25*S*-genins from *rome* (mg/day)[a]	Calculated consumed *rome* (g DM/day)[b]
Lamb-67 (developed alveld)											
9	123	1164	154		3116	4972		9529	5.0	758	114
10	33	329	107		2064	3676		6209	6.5	576	86
								Mean[c]	5.1	780	117

Smil-CO = smilagenone (mg/kg DM);); Sar-CO = sarsasapogenone (mg/kg DM); Smil = smilagenin (mg/kg DM); Epismil = epismilagenin (mg/kg DM); Sar = sarsasapogenin (mg/kg DM); Episar = episarsasapogenin (mg/kg DM); free = free genins (mg/kg DM); conj = conjugated genins (mg/kg DM); Sub T = genin sub total; Total = total free and conjugated genins (mg/kg DM).

% deuterated 25*S*-genins = percentage ratio of deuterated 25*S*-genins to non-deutrated 25*S*-genins, = Σ(*m/z* 317-320) / Σ(*m/z* 315-320).

Note: [a] calculated using Equation (1); [b] calculated using Equation (2) – see Chapter 4, Section 4.3.6; [c] mean calculated for day 5 data onwards.

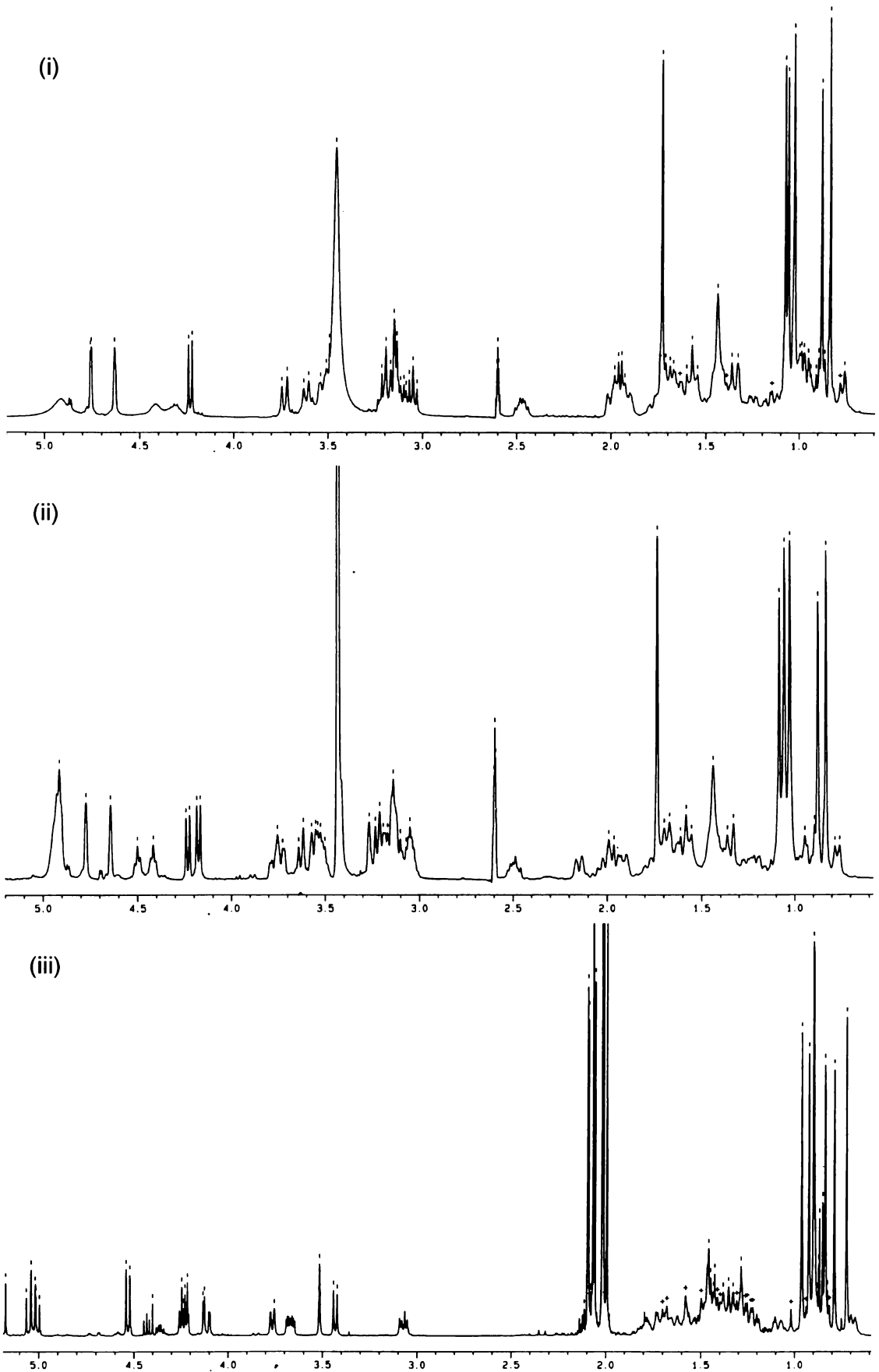
Appendix II. ^{1}H and ^{13}C NMR assignments determined for sarsasapogenin and episarsasapogenin (ppm in $CDCl_3$) at the University of Waikato.

	Sarsasapogenin		Episarsasapogenin	
Atom	^{13}C	^{1}H	^{13}C	^{1}H
1	30.0	1.41, 1.51	35.3	0.96, 1.78
2	27.8	1.50, 1.59	30.6	1.31, 1.67
3	67.2	4.11	71.5	3.61
4	33.6	1.34, 1.99	36.6	1.51, 1.74
5	36.6	1.73	42.0	1.39
6	26.6	1.17, 1.92	27.1	1.22, 1.87
7	26.6	1.06, 1.45	26.8	1.10, 1.45
8	35.4	1.60	35.4	1.59
9	40.0	1.34	40.7	1.42
10	35.4	-	34.7	-
11	21.0	1.29, 1.39	20.7	1.25, 1.41
12	40.4	1.16, 1.73	40.3	1.16, 1.72
13	40.8	-	40.8	-
14	56.6	1.17	56.4	1.18
15	31.8	1.24, 1.98	31.8	1.23, 1.99
16	81.1	4.42	81.1	4.42
17	62.2	1.77	62.2	1.78
18	16.5	0.77 (3H)	16.5	0.76 (3H)
19	24.0	0.99 (3H)	23.4	0.95 (3H)
20	42.2	1.81	42.2	1.81
21	14.4	1.00 (3H)	14.4	1.00 (3H)
22	109.8	-	109.8	-
23	26.0	1.38, 1.88	26.0	1.38, 1.89
24	25.8	1.41, 2.03	25.9	1.42, 2.04
25	27.2	1.70	27.2	1.70
26	65.2	3.30, 3.96	65.2	3.31, 3.96
27	16.1	1.08 (3H)	16.1	1.08 (3H)

Appendix III. ^{1}H and ^{13}C NMR assignments determined for lupeol (ppm in $CDCl_3$) (Burns *et al.*, 2000) and betulin (ppm in $CDCl_3$ and DMSO-D_6) (the University of Waikato).

	Lupeol ($CDCl_3$)		Betulin ($CDCl_3$)		Betulin (DMSO-D_6)	
Atom	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	38.7	0.90, 1.67	38.8	0.90, 1.66	38.3	0.92, 1.63
2	27.4	1.56, 1.60	27.5	1.56, 1.60	27.2	1.54 (2H)
3	79.0	3.19	79.1	3.19 (dd, J = 5.0, 11.2 Hz)	76.9	3.07 (m)
4	38.9	-	39.0	-	38.5	-
5	55.3	0.68	55.4	0.67	54.9	0.72
6	18.3	1.39, 1.51	18.4	1.40, 1.52	18.0	1.44, 1.57
7	34.3	1.39 (2H)	34.1	1.40 (2H)	33.9	1.44 (2H)
8	40.8	-	41.0	-	40.5	-
9	50.4	1.27	50.5	1.26	49.9	1.32
10	37.2	-	37.3	-	36.7	-
11	20.9	1.23, 1.41	20.9	1.21, 1.43	20.4	1.25, 1.43
12	25.2	1.07, 1.67	25.3	1.04, 1.63	24.9	1.08, 1.67
13	38.1	1.66	37.4	1.64	36.8	1.73
14	42.9	-	42.8	-	42.2	-
15	27.5	1.00, 1.68	27.2	1.06, 1.70	26.7	1.01, 1.75
16	35.6	1.37, 1.47	29.3	1.21, 1.93	29.1	1.15, 2.01
17	43.0	-	47.9	-	47.3	-
18	48.3	1.36	48.9	1.59	48.2	1.57
19	48.0	2.38	47.9	2.38	47.4	2.48
20	151.0	-	150.6	-	150.4	-
21	29.9	1.32, 1.92	29.8	1.41, 1.95	29.4	1.36, 1.95
22	40.0	1.19, 1.38	34.4	1.04, 1.86	33.9	1.00, 1.94
23	28.0	0.97	28.1	0.97 (s)	28.1	0.97 (s)
24	15.4	0.76	15.4	0.76 (s)	15.8	0.75 (s)
25	16.1	0.83	16.2	0.83 (s)	15.9	0.86 (s)
26	16.0	1.03	16.1	1.03 (s)	15.7	1.07 (s)
27	14.6	0.95	14.9	0.98 (s)	14.5	1.02 (s)
28	18.0	0.79	60.7	3.32 (d, J = 10.8 Hz)	58.0	3.61 (d, J = 10.6 Hz)
				3.79 (dd, J = 1.7, 10.8 Hz)		3.18 (d, J = 10.6 Hz)
29	109.3	4.56	109.8	4.58 (m)	109.6	4.63 (br s),
		4.69		4.68 (d, J = 2.2 Hz)		4.76 (br s)
30	19.3	1.68	19.2	1.68 (s)	18.8	1.73 (s)

Appendix IV. ^{1}H NMR spectra of: (i) betulin 3-(β-D-glucoside), (ii) betulin 3,28-(β-D-diglucoside) and (iii) allobetulin β-D-glucoside tetraacetate.



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Ovine Metabolism of Lithogenic Sapogenins. Synthesis of [2,2,4,4-$^2H_4$]Sarsasapogenone, [2,2,4,4-$^2H_4$]Sarsasapogenin, and [2,2,4,4-$^2H_4$]Episarsasapogenin and Evaluation of Deuterium Retention in a Sheep-Dosing Trial

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The suitability of [2,2,4,4-$^2H_4$]sarsasapogenone (1b), [2,2,4,4-$^2H_4$]sarsasapogenin (2b), and [2,2,4,4-$^2H_4$]episarsasapogenin (3b) as isotopically labeled dosing substrates to determine the levels of free and conjugated sapogenins present in feces from sheep grazing saponin-containing plants implicated in the development of ovine hepatogenous photosensitization diseases was investigated. A 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin (2b) and [2,2,4,4-$^2H_4$]episarsasapogenin (3b), obtained by reduction of [2,2,4,4-$^2H_4$]sarsasapogenone (1b), was found to retain 94% of incorporated deuterium, when dosed to one sheep. The recovery of the dosed mixture of genins 2b and 3b was calculated to be 85%. Considerable loss of deuterium and a lower recovery of genin material were observed when [2,2,4,4-$^2H_4$]sarsasapogenone (1b) was dosed.

KEYWORDS: Deuterated sapogenins; dosing trial; sheep; fecal material; *Narthecium ossifragum*; episarsasapogenin; sarsasapogenin; sarsasapogenone

INTRODUCTION

Hepatogenous photosensitization of sheep associated with grazing of plants containing steroidal saponins is both economically important and an animal welfare problem in many parts of the world (*1*). At least nine plants containing steroidal saponins have been reported to cause hepatogenous photosensitizations of sheep (*2*). Common features of all of the diseases associated with the grazing of saponin-containing plants are their sporadic occurrence, the difficulty in reproducing symptoms during dosing experiments (*1*, *3*), and the appearance of calcium salts of episapogenin glucuronides in the liver and in the bile ducts (*4*−*10*). The sporadic occurrence of hepatogenous photosensitization diseases, and the difficulties of reproducing them, may be related to variations in the saponin content of the plants and the proportion of saponin-containing plants in the diet.

Bog asphodel (*Narthecium ossifragum*), a member of the lily family, is one of the saponin-containing plants known to cause severe problems to the sheep industry in Norway and on the British Isles (*1*, *2*). Photosensitization has been reproduced in sheep by dosing them with crude saponins from *N. ossifragum* (*11*, *12*), but the doses necessary for causing the typical symptoms have been unrealistically large and far beyond those that probably could have been ingested by grazing. It has been shown that dosing with relatively large (but not unrealistically so) doses of *N. ossifragum* daily for 21 days did not cause liver damage in lambs (*13*). An attempt to reproduce the typical lesions in the liver by dosing with pure sarsasapogenin and diosgenin has also failed (*14*).

Improved knowledge of the normal uptake of saponins by sheep grazing pastures possessing saponin-containing plants and of saponin doses that induce photosensitization under field conditions is required if the etiology of saponin-associated photosensitization diseases is to be better understood. We have proposed that by dosing a known amount of deuterium-labeled sapogenin once per day to sheep that were additionally dosed with *N. ossifragum* and determining the ratio of labeled sapogenin to unlabeled sapogenins in feces, it would be possible to estimate the amount of sapogenins originating from the ingested plant material (*15*). This proposal presupposed that no deuterium, or a very small amount of deuterium, was lost during ovine metabolism of the labeled sapogenin and that the concentrations of sapogenins in feces were reasonably constant, regardless of time of feces collection or the time of dosing and ingestion of saponins. Flåøyen et al. (*15*) showed that although consistent fecal sapogenin levels were present during days 4−9 of a dosing trial in which [20,23,23-$^2H_3$]sarsasapogenin and *N. ossifragum* plants were administered once and three times daily, respectively, to two sheep, [20,23,23-$^2H_3$]sarsasapogenin was not a suitable dosing substrate because the 20- and 23-deuterium atoms were lost during the dosing experiment and/or subsequent chemical analysis.

We now report the synthesis of [2,2,4,4-$^2H_4$]sarsasapogenone (1b), [2,2,4,4-$^2H_4$]sarsasapogenin (2b), and [2,2,4,4-$^2H_4$]episarsasapogenin (3b) (Figure 1) and the use of 1b and a 1:4 mixture of 2b and 3b in dosing experiments performed using one sheep.

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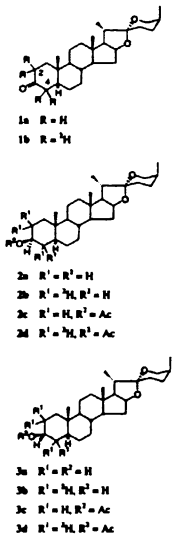

Figure 1. Structures of sapogenins referred to in this investigation.

MATERIALS AND METHODS

General Experimental Procedures. One- and two-dimensional ^1H and ^{13}C NMR spectra were obtained from $CDCl_3$ solutions using a Bruker DRX-400 instrument fitted with a 5 mm inverse probe head operating at 400.13 (^1H) and 100.62 (^{13}C) MHz. Chemical shifts are reported relative to internal tetramethylsilane (TMS), where δ (^1H) TMS + δ (^1H) $CHCl_3$ = 7.26 ppm and δ (^{13}C) TMS + δ (^{13}C) $CDCl_3$ = 77.06 ppm or δ (^1H) TMS + δ (^1H) C_5D_4HN = 8.72 ppm and δ (^{13}C) TMS + δ (^{13}C) C_5D_5N = 123.52 ppm. Radial chromatography was performed using a Chromatotron 7924T and a 22.5 cm diameter × 2 mm thick silica gel (60 G, Merck 7731) plate. GC-MS analyses were performed using a 25 m × 0.22 mm i.d. HP-5 column installed in an HP5890 GC instrument interfaced to an HP5970B mass selective detector, operated in selected ion mode.

Sarsasapogenone (1a). In a typical preparation, sodium dichromate (8 g) in water (50 mL) and concentrated sulfuric acid (10 mL) were added to a stirred solution of 95% sarsasapogenin (2a) (Sigma Chemical Co.) (10 g) in acetone (700 mL) and stirred for 24 h. Water (1 L) was added, and the mixture was extracted with $CHCl_3$ (1 × 400, 2 × 200, and 1 × 100 mL). The extracts were combined and dried over anhydrous Na_2SO_4 (24 h); solvent was removed using a rotary evaporator, and the product material was redissolved in $CHCl_3$ (150 mL) and filtered through an alumina column (Al_2O_3, Brockmann grade II). The column was washed with a further 50 mL of $CHCl_3$, and the solvent was evaporated using a rotary evaporator to afford crystalline material, which was dried for 3 h under high vacuum. Sarsasapogenone (1a) (8.13 g, 81.8%): mp 194−198 °C [lit. mp 212−214 °C (*16*)]; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.79 (3H, s, H-18), 1.00 (3H, d, J = 6.7 Hz, H-21), 1.04 (3H, s, H-19), 1.08 (3H, d, J = 7.1 Hz, H-27), 2.29 (1H, ddd, J = 5.3, 14.5, 14.5 Hz, H-2α), 2.68 (1H, dd, J = 13.6, 14.9 Hz, H-4α), 3.29 (1H, br d, J = 11.0 Hz, H-26α), 3.94 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.39 (1H, m, H-16); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.4 (C-21), 16.1 (C-27), 16.5 (C-18), 21.1 (C-11), 22.7 (C-19), 25.8 (C-24), 26.0 (C-23), 26.1 (C-7), 26.6 (C-6), 27.1 (C-25), 31.8 (C-15), 35.1 (C-10), 35.2 (C-8), 37.0 (C-1), 37.2 (C-2), 40.2 (C-12), 40.7 (C-13), 41.0 (C-9), 42.2 (C-20), 42.4 (C-4), 44.3 (C-5), 56.3 (C-14), 62.2 (C-17), 65.2 (C-26), 81.0 (C-16), 109.7 (C-22), 213.0 (C-3). MS (70 eV), *m/z* 414 (M$^+$, 5%), 300 (8), 285 (5), 271 (24), 139 (100).

[2,2,4,4-$^2H_4$]Sarsasapogenone (1b). In a typical preparation, sarsasapogenone (1a) (10 g), dioxane (300 mL), D_2O (25 mL), and sodium metal (0.35 g) were refluxed for 24 h with an anhydrous $CaCl_2$ water trap fitted to the condenser. Distilled water (500 mL) was added to the cooled solution, and the precipitate was recovered by filtration using a Büchner funnel. Product material was washed with distilled water and dried under high vacuum for 3 h. GC-MS data showed that the product material (9.42 g, 93.7%, mp 208−214 °C) consisted mainly of [^2H]-, [$^2H_2$]-, and [$^2H_3$]sarsasapogenone: MS (70 eV), *m/z* 418 (2%), 417 (5), 416 (7), 415 (5), 414 (0.6), 302 (5), 287 (3), 273 (12), 139 (100). Product material from several deuteration reactions (21.2 g) in dioxane (400 mL) was subjected to a second deuteration cycle using D_2O (20 mL) and sodium metal (0.8 g). Workup as described above afforded [2,2,4,4-$^2H_4$]sarsasapogenone (1b) (deuterated ketone) (19.88 g, 93.3%): mp 208−214 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.80 (3H, s, H-18), 1.01 (3H, d, J = 6.7 Hz, H-21), 1.05 (3H, s, H-19), 1.09 (3H, d, J = 7.1 Hz, H-27), 3.31 (1H, br d, J = 11.0 Hz, H-26α), 3.96 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.43 (1H, m, H-16); ^{13}C ($CDCl_3$, 400 MHz) δ 14.4 (C-21), 16.1 (C-27), 16.5 (C-18), 21.1 (C-11), 22.7 (C-19), 25.9 (C-24), 26.0 (C-23), 26.1 (C-7), 26.6 (C-6), 27.2 (C-25), 31.8 (C-15), 35.1 (C-10), 35.3 (C-8), 37.0 (C-1), 40.2 (C-12), 40.8 (C-13), 41.0 (C-9), 42.2 (C-20), 44.2 (C-5), 56.3 (C-14), 62.2 (C-17), 65.2 (C-26), 81.0 (C-16), 109.8 (C-22), 213.3 (C-3); MS (70 eV), *m/z* 418 (M$^+$, 3%), 304 (6), 289 (5), 275 (20), 139 (100).

[2,2,4,4-$^2H_4$]Sarsasapogenin (2b) and [2,2,4,4-$^2H_4$]Episarsasapogenin (3b). In a typical preparation [2,2,4,4-$^2H_4$]sarsasapogenone (1b) (10 g) and $NaBH_4$ (2 g) in EtOH/MeOH (1:1) (400 mL) were stirred at room temperature for 24 h, after which time the solvent was removed using a rotary evaporator to afford a 1:4 mixture of [2,2,4,4-$^2H_4$]-sarsasapogenin (2b) and [2,2,4,4-$^2H_4$]episarsasapogenin (3b) (deuterated alcohol mixture): mp 194−200 °C. Separation of a portion of the mixture using radial chromatography, with 20 mL portion mixtures of petroleum spirits and diethyl ether (9:1, 4:1, 1:1, 3:7, and 1:9) as eluents afforded specimens of [2,2,4,4-$^2H_4$]sarsasapogenin (2b) and [2,2,4,4-$^2H_4$]episarsasapogenin (3b).

[2,2,4,4-$^2H_4$]Sarsasapogenin (2b): mp 180−184 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.76 (3H, s, H-18), 0.98 (3H, s, H-19), 1.00 (3H, d, J = 6.7 Hz, H-21), 1.08 (3H, d, J = 7.1 Hz, H-27), 3.30 (1H, br d, J = 11.0 Hz, H-26α), 3.96 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.09 (1H, br s, H-3), 4.41 (1H, m, H-16); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.4 (C-21), 16.1 (C-27), 16.5 (C-18), 21.0 (C-11), 24.0 (C-19), 25.9 (C-24), 26.0 (C-23), 26.6 (C-6, C-7), 27.2 (C-25), 29.9 (C-1), 31.8 (C-15), 35.3 (C-10), 35.4 (C-8), 36.5 (C-5), 40.0 (C-9), 40.4 (C-12), 40.8 (C-13), 42.2 (C-20), 56.6 (C-14), 62.2 (C-17), 65.2 (C-26), 67.0 (C-3), 81.1 (C16), 109.8 (C-22). Acetylation of a subsample of 2b in a GC vial using pyridine/acetic anhydride (1:1) (0.5 mL) afforded [2,2,4,4-$^2H_4$]sarsasapogenin acetate (2d): MS (70 eV), *m/z* 462 (M$^+$, 8%), 348 (7), 333 (14), 319 (18), 288 (15), 273 (9), 259 (11), 139 (100).

[2,2,4,4-$^2H_4$]episarsasapogenin (3b): mp 193−197 °C; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.76 (3H, s, H-18), 0.95 (3H, s, H-19), 1.00 (3H, d, J = 6.7 Hz, H-21), 1.08 (3H, d, J = 7.1 Hz, H-27), 3.31 (1H, br d, J = 11.0 Hz, H-26α), 3.61 (1H, br s, H-3), 3.96 (1H, dd, J = 2.6, 11.0 Hz, H-26β), 4.42 (1H, m, H-16); ^{13}C NMR ($CDCl_3$, 400 MHz) δ 14.4

Table 1. Dry Matter (DM) and Free and Conjugated (conj) Sapogenins Determined for Fecal Material Recovered from a Sheep Administered Single Doses of 1b on Day 0 and a 1:4 Mixture of 2b and 3b on Day 9

		feces DM		sapogenins (mg/kg of DM)				
day	extract[a]	%	kg	1a + 1b	2a + 2b	3a + 3b	subtotal[b]	total[b]
				Ketone Dose (1000 mg)				
1	free	39.9	0.239	136	25	67	228	
	conj			5.7	7.1	20	33	261
2	free	39.4	0.217	458	110	254	823	
	conj			21	33	106	160	983
3	free	37.0	0.238	152	95	246	593	
	conj			8.4	19	75	102	695
4	free	35.8	0.220	40	37	125	202	
	conj			6.4	9.1	33	49	251
5	free	38.2	0.298	9.1	13	34	56	
	conj			1.5	2.5	5.4	9.4	65
6	free	39.3	0.286		1	26	37	
	conj				2	3.6	5.6	42
				Alcohol Dose (284 mg)				
10	free	43.6	0.184	3.5	10	54	68	
	conj				1	2.3	3.4	71
11	free	47.8	0.189	27	70	427	524	
	conj			6.6	12	55	74	598
12	free	41.8	0.221	19	52	213	284	
	conj			4.4	9.4	34	48	332
13	free	42.8	0.197	10	28	88	126	
	conj			3	6.4	20	29	155
14	free	41.2	0.224		11	32	43	
	conj				0.7	1.9	2.5	46

[a] Free = genin material obtained by CH_2Cl_2 extraction and acetylation; conj = genin material obtained by MeOH extraction, hydrolysis, and acetylation. [b] Subtotal = total free or conjugated genins; total = free + conjugated genins.

(C-21), 16.1 (C-27), 16.5 (C-18), 20.7 (C-11), 23.4 (C-19), 25.9 (C-24), 26.0 (C-23), 26.8 (C-7), 27.1 (C-6), 27.2 (C-25), 31.8 (C-15), 34.7 (C-10), 35.3 (C-1), 35.4 (C-8), 40.3 (C-12), 40.7 (C-9), 40.8 (C-13), 42.0 (C-5), 42.2 (C-20), 56.4 (C-14), 62.2 (C-17), 65.2 (C-26), 71.5 (C-3), 81.1 (C16), 109.8 (C-22). Acetylation of a subsample of 3b in a GC vial using pyridine/acetic anhydride (1:1) (0.5 mL) afforded [2,2,4,4-$^2H_4$]episarsasapogenin acetate (3d): MS (70 eV), *m/z* 462 (M^+, 11%), 348 (4), 333 (13), 319 (19), 288 (19), 273 (8), 259 (12), 139 (100).

Dosing Trial. One 7-month-old male sheep, Dala breed, was dosed intraruminally (day 0) with 1000 mg of deuterated ketone (1b), suspended in 100 mL of water/EtOH (80:20) and washed down with 100 mL of water. Nine days later (day 9) the same sheep was dosed in the same fashion with 284 mg of the 1:4 mixture of 2b and 3b. Hay was fed ad-libitum for 1 week before and throughout the dosing experiment. The sheep was kept in a cage allowing collection of all fecal output. Fecal material was collected once per day, weighed (wet weight), and frozen at −20 °C (Table 1). Liquid nitrogen was added to a representative subsample from each day's collection. The subsamples were then finely chopped using a Robot Coupe R301 Ultra chopper (Robot Coupe, S.A., Montceau en Bourgogne, France) and freeze-dried overnight. Percent dry matter (DM) was calculated as 100 × freeze-dried weight/wet weight (Table 1). A sample of the hay that was fed was also freeze-dried and prepared for chemical analysis.

Sapogenin Analyses. Soxhlet extraction of freeze-dried fecal material using dichloromethane (free sapogenin extract), followed by methanol (conjugated sapogenin extract), hydrolysis of the conjugated sapogenin extract for 90 min at 95 °C using 0.5 mol/L hydrochloric acid, and GC-MS analysis of acetylated sapogenin extracts were performed as previously reported (*17*, *18*) except that SIM GC-MS analyses were modified to include *m/z* 285−288, 315−320, 345−348, and 459−462 ions (deuterated sapogenin acetates) and *m/z* 271−275 ions (deuterated sarsasapogenone). Quantitation of the acetylated sapogenins was performed by integrating *m/z* 139 ion profiles extracted for SIM GC-MS chromatograms. The level of deuterium retention in sapogenin substrates was determined by assessment of the *m/z* 315−320 ion contributions determined for sapogenin acetates. Allowance was made for ^{13}C contributions, but not ^{18}O contributions, to these ions. Sapogenin concentrations (Table 1) were calculated relative to sarsasapogenin propionate as the internal standard using response factors (R_F) determined by comparison of the integrated peak areas recorded for a reference solution containing known weights of sarsasapogenin propionate and sarsasapogenin acetate. All calculations were performed using purpose-written Excel spreadsheets.

RESULTS AND DISCUSSION

Synthesis of Deuterated Sapogenins. Oxidation of sarsasapogenin (2a) using sodium dichromate and concentrated sulfuric acid afforded sarsasapogenone (1a). Thereafter, two cycles of deuteration afforded material (1b) that exhibited an M^+ ion at *m/z* 418 compared to *m/z* 414 in 1a. Similarly, the fragment ions that occurred at *m/z* 271, 285, and 300 in the mass spectrum of 1a occurred at *m/z* 275, 289, and 304, respectively, in the mass spectrum of 1b. Reduction of 1b using sodium borohydride afforded a 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin (2b) and [2,2,4,4-$^2H_4$]episarsasapogenin (3b). The relative contributions of the deuterated genins 2b and 3b in the synthetic mixture are comparable to those of the natural genins 2a and 3a detected in fecal samples following the dosing, in previous trials, of *N. ossifragum* plant material (*15*, *17*). Complete ^1H and ^{13}C NMR assignments (see Supporting Information) of 1b, 2b, and 3b were derived from detailed analyses of one- and two-dimensional NMR spectral data, including COSY, TOCSY, ROESY, g-HSQC, and g-HMBC spectra. The presence of deuterium atoms at C-2 and C-4 suppressed the ^{13}C signals attributable to these atoms. Comparison of the g-HSQC spectra of 1a and 1b revealed the absence of signals attributable to H-2α, H-2β, H-4α, and H-4β in the g-HSQC spectrum of 1b. In 1a these signals occurred at 2.29, 2.13, 2.65, and 1.99 ppm, respectively. Similarly, the H-2α, H-2β, H-4α, and H-4β signals of sarsasapogenin (2a) and episarsasapogenin (3a) were not seen in the HSQC spectra of 2b and 3b, respectively.

Deuterium Retention Calculations prior to Dosing. Because, in dosing trials, the levels of 2a and 3a are determined by GC-MS analyses of the corresponding acetylated genins, that is, sarsasapogenin acetate (2e) and episarsasapogenin acetate (3e), respectively (*18*), deuterium incorporation was assessed using specimens of the acetylated deuterated genins 2d and 3d, respectively. The incorporation of one, two, three, or four deuterium atoms afforded M^+ ions at *m/z* 459, 460, 461, and 462, respectively, whereas the presence of an *m/z* 463 ion can be attributed to a natural abundance ^{13}C contribution, rather than a pentadeuterated contribution. Nondeuterated and deuterated M^+ ion contributions were therefore calculated as the sum of the *m/z* 458 and 459 ions and the *m/z* 460−463 ion clusters, respectively. This approach, which includes the natural ^{13}C isotope contribution to the *m/z* 458 (M^+) and 462 ([2,2,4,4-$^2H_4$]-M^+) ions, respectively, also assumes a negligible level of monodeuteration and ignores minor ^{18}O contributions. The percent deuterium enrichment, expressed as the sum of the $^2H_2$, $^2H_3$, $^2H_4$ and corresponding ^{13}C isotope contributions were calculated as 100 × $\sum(m/z\ 460-463)/\sum(m/z\ 458-463)$. Two deuteration cycles afforded deuterated sapogenin material, which when acetylated and analyzed by SIM GC-MS showed 99.9% deuterium incorporation.

Because of the low relative intensity of the molecular ion cluster of deuterated sapogenin acetates, deuterium retention during the two dosing periods was determined by monitoring *m/z* 315−320 ion cluster contributions. This ion cluster was the most intense of six diagnostic ion clusters that occur at *m/z* 344−349, 329−334, 315−320, 284−289, 269−274, and 255−259 in the mass spectra of the deuterated acetates 2d and 3d. Because

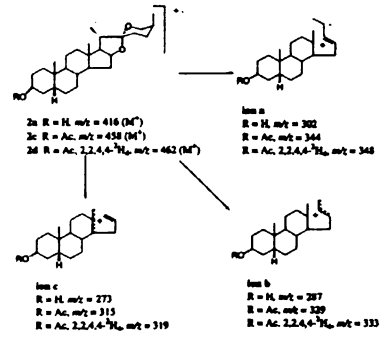

Figure 2. Postulated mass spectral fragmentation pathways for sapogenins 2a, 2c, 2d, 3a, 3c, and 3d leading to the ions referred to in the text.

the *m/z* 284−289, 269−274, and 255−259 ion clusters arise by loss of HOAc or [^{2}H]OAc from the *m/z* 344−349, 329−334, and 315−320 ion clusters, respectively, it was not appropriate to use the *m/z* 284−289, 269−274, and 255−259 ion clusters for quantitative deuterium retention calculations. The percent deuteration levels of sapogenins prior to dosing were calculated as 100 × $\sum(m/z\ 317-320)/\sum(m/z\ 315-320)$, on the basis of the ion intensities determined for subsamples of the acetylated variants of the parent genins. The average deuterium content of the *m/z* 315−320 ion cluster of an acetylated subsample of episarsasapogenin was determined to be 94.6%, compared to 99.9% for the *m/z* 458−463 (M^+) ion cluster. This difference can be attributed to a small part of the *m/z* 315−320 ion current originating from fragmentation ions other than those arising from the isotopically labeled variants of ion c (Figure 2). Experimentally determined deuterium retention levels were corrected (see below) for this discrepancy.

Dosing Trial Results. Previous investigations have shown that in the rumen of a dosed sheep sapogenins are partly oxidized and reduced to afford episapogenins, which, along with sapogenins and low levels of oxidized sapogenins, are ultimately secreted in fecal material but not in urine (*15*, *18*). Fecal samples were collected daily before and during the trial period. Representative subsamples were freeze-dried, extracted, and analyzed using previously reported SIM GC-MS methodology (*19*), modified to included *m/z* 271−275 (deuterated ketogenins) and *m/z* 315−320 ion clusters (deuterated acetates). Total genin levels were determined using *m/z* 139 ion responses (*19*), whereas the *m/z* 271−275 and 315−320 ion data were used to determine the deuterium retention levels of ketogenins and genins, respectively. The hay-fed ad libitum throughout the dosing trial was shown to contain no sapogenins. Similarly, no sapogenins were detected in feces collected prior to the commencement of the two dosing trials, that is, on day 0, prior to the dosing of the deuterated ketone, and on day 9, prior to the dosing of the deuterated alcohol mixture.

In each of the dosing trials, maximum genin levels were found in fecal material 2−3 days after the respective deuterated genins were dosed (Table 1). Thereafter, genin levels declined to negligible levels 5−6 days after dosing. Appreciable levels of ketogenins and genins were detected in day 2−3 fecal samples (Table 1). SIM GC-MS analyses showed that although episarsasapogenin and sarsasapogenin retained predominantly three or four deuterium atoms, as evidenced by strong *m/z* 318 and 319 ion contributions, recovered sarsasapogenone predominantly exhibited *m/z* 271, 272, and 273 ion responses corresponding to the presence of none, one, or two deuterium atoms, respectively. Deuterium retention analyses of stored batches of the deuterated ketone (1b), performed several months after the dosing trial, unexpectedly showed the presence of only one or two deuterium atoms; however, analyses of the genin alcohols derived by sodium borohydride reduction of the stored ketone material showed that the reduction products predominantly retained three or four deuterium atoms. These observations can be interpreted as indicating that although the majority of deuterium atoms incorporated into deuterated ketone (1b) were retained during ovine metabolism of 1b to 2b and 3b, deuterium loss from unmetabolized ketone may have occurred during the extraction, hydrolysis (conjugate extracts only), and/or derivatization steps during the GC-MS analysis protocol.

Table 2. Uncorrected and Corrected Percent $^2H_2$−$^2H_4$[a] Retention Levels Determined for the *m/z* 315−320 Ion Cluster of Acetylated Episarsasapogenin Recovered from Fecal Samples Collected during the Dosing Experiments

	uncorrected % $^2H_2$−$^2H_4$		corrected % $^2H_2$−$^2H_4$	
day	free	conjugated	free[b]	conjugated[b]
		Ketone[c] Experiment		
2	90.1	90.9	95.2	96.1
3	88.4	87.8	93.4	92.8
		Alcohol[d] Experiment		
11	91.5	90.5	96.7	95.6
12	87.4	85.6	92.4	90.4

[a] Uncorrected % $^2H_2$−$^2H_4$ = 100 × $\sum(m/z\ 317-320)/\sum(m/z\ 315-320)$; corrected % $^2H_2$−$^2H_4$ = 100 × $\sum(m/z\ 317-320)/\sum(m/z\ 315-320)$ − 0.946, where the average % $^2H_4$ incorporation in the dosed substrates was 94.6%. [b] Free = genin material obtained by CH_2Cl_2 extraction and acetylation; conjugated = genin material obtained by MeOH extraction, hydrolysis, and acetylation. [c] Ketone = [2,2,4,4-$^2H_4$]sarsasapogenone. [d] Alcohol = 4:1 mixture of [2,2,4,4-$^2H_4$]episarsasapogenin and [2,2,4,4-$^2H_4$]sarsasapogenin.

Our results showed that some loss of deuterium from the deuterated genin alcohols 2b and 3b occurs during ovine metabolism and/or extraction and chemical analyses, as evidenced by a moderate reduction in the relative $^2H_4$ contribution and an increased $^2H_3$ contribution (see Supporting Information), but the total $^2H_2$−$^2H_4$ contribution remains essentially unchanged because the reduced $^2H_4$ contribution is offset by an increased $^2H_3$ contribution. When corrected for an average $^2H_2$−$^2H_4$ content of 94.6%, calculated using the *m/z* 315−320 ion cluster of [2,2,4,4-$^2H_4$]episarsasapogenin, the recovered $^2H_2$−$^2H_4$ contribution was ~90−96% (average = 94%) of that dosed (Table 2). Although the principle objective of this trial was to assess the extent to which deuterium was retained in the deuterated genin substrates during ovine metabolism, and subsequent workup and analysis procedures, the results showed there to be a satisfactory overall recovery (241 mg, ~85%) of dosed sapogenin material, calculated as the sum of total sapogenins (mg/kg of DM) multiplied by the daily weight of fecal material (kg of DM) (Table 1). Because only a single sheep was dosed in the trials reported in this paper, care must be exercised when the results presented in Tables 1 and 2 are interpreted. The results are, however, consistent with levels of free and conjugated sapogenins and episapogenins detected in previous dosing trials (*15*, *17*). Prior to the use of the methodology in a field trial, there is a need to validate the

methodology in a multiple-sheep trial (e.g., $n = 3$) in which the deuterated substrate is dosed over an extended time period.

During the present and previous dosing trials (*15*, *17*) we have observed (but not reported or discussed) that ruminal metabolism of β-sitosterol (a constituent of many plants grazed by animals) affords three isomeric dihydroanalogues of β-sitosterol, which, when acetylated, exhibit significant m/z 458 (M^+) and m/z 315 ions. The GC-MS retention times of two of the three dihydro-β-sitosterol acetate isomers detected in the extracts are similar to those of sarsasapogenin and episarsasapogenin acetates; however, provided care was taken to adjust GC-MS acquisition conditions to minimize peak overlap, interference was not significant.

Our results show that the deuterated alcohol mixture consisting of 2b and 3b is a suitable dosing material for use in determining the percentage deuterium content of sapogenins recovered from sheep fecal material during a dosing trial. On the other hand, the lower recovery of [2,2,4,4-$^2H_4$]sarsasapogenone (1b) (~55%), possibly attributable to more extensive rumen metabolism, including complete degradation, and considerable loss of deuterium from 1b during the dosing trial show that this substrate is not a suitable dosing material.

ABBREVIATIONS USED

COSY, correlated spectroscopy; GC-MS, gas chromatography−mass spectrometry; g-HMBC, pulsed field gradient heteronuclear multiple bond correlation; g-HSQC, pulsed field gradient heteronuclear single quantum coherence; m/z, mass to charge ratio; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; ROESY, rotating frame Overhauser effect spectroscopy; SIM, selected ion mode; TOCSY, total correlation spectroscopy.

Supporting Information Available: Table of ^1H and ^{13}C NMR assigments determined for sarsasapogenin, [2,2,4,4-$^2H_4$]-sarsasapogenin, and [2,2,4,4-$^2H_4$]episarsasapogenin (δ in $CDCl_3$) and figures showing the relative percent contributions of ions contributing to ion a, ion a-HOAc (or [^{2}H]OAc), ion b, ion b-HOAc (or [^{2}H]OAc), ion c, and ion c-HOAc (or [^{2}H]OAc) clusters determined for sarsasapogenin acetate (2c) and [2,2,4,4-$^2H_4$]episarsasapogenin acetate (3d).

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Validation of Isotope-Dilution Methodology for Estimating the Ovine Uptake of Steroidal Saponins from *Narthecium ossifragum*

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ABSTRACT

The results of dosing experiments in which three sheep were dosed 72 g/dry matter of *Narthecium ossifragum* plants and a 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin and [2,2,4,4-$^2H_4$]episarsasapogenin once daily for 14 days are reported. The isotope-dilution methodology, based on integrated selected ion mode GC-MS profiles determined for deuterated and non-deuterated genins recovered from fecal samples and a knowledge of the average sapogenin content of the dosed plant material, was found to give more reliable and consistent results than previously reported mass balance methodology. Four days after one of the sheep refused part of the offered plant material, the isotope-dilution methodology identified a corresponding decrease in fecal sapogenin levels.

Keywords: deuterated sapogenins, *Narthecium ossifragum*, sheep, dosing experiments

Abbreviations: GC-MS, gas chromatography-mass spectroscopy; DM, dry matter

INTRODUCTION

Hepatogenous photosensitization of sheep is both economically important and an animal welfare problem in various parts of the world. The condition results when a toxin causes liver damage resulting in retention of the photosensitizing agent phylloerythrin (Clare, 1952; Flåøyen, 1999). Many hepatogenous photosensitization disorders are associated with ingestion of plants containing steroidal saponins. Typical of these diseases is the accumulation of insoluble calcium salts of episarsasapogenin or epismilagenin glucuronides in liver cells and bile ducts (Holland et al., 1991; Miles et al., 1991, 1992a, 1992b, 1993, 1994a, 1994b). All these diseases occur sporadically and are difficult to reproduce by dosing experiments (Kellerman et al., 1991; Flåøyen et al., 1991, 1993). Furthermore the saponin containing plants associated with them are not always toxic to grazing animals (Ender, 1955; Abdelkader et al., 1984; Flåøyen et al., 1996). The role of steroidal saponins in the aetiology of hepatogenous photosensitizations of sheep is unclear and needs to be clarified. One step in this process will be to measure the intake of steroidal saponins in sheep that become photosensitized under field conditions.

We have previously proposed that the daily intake of plant saponins from natural sources, can be estimated by dosing sheep once daily with a deuterated sapogenin (Flåøyen et al., 2001). For this approach to be successful the deuterated sapogenin must retain a high percentage of the incorporated deuterium atoms during passage through the animal and subsequent chemical analysis. This approach also presupposes the ratio of deuterated to non-deuterated sapogenins in feces is reasonably constant and not sensitive to changes in the time collection, or the time of dosing and ingestion of saponins and sapogenins.

Flåøyen et al. (2001) have reported that consistent fecal sapogenin levels were present during days 4–9 of a dosing trial in which [20,23,23-$^2H_3$]sarsasapogenin and *N. ossifragum* plants were administered. [20,23,23-$^2H_3$]sarsasapogenin was however not a suitable dosing substrate since the 20- and 23-deuterium atoms were exchanged with ^1H atoms during the dosing experiment or the subsequent chemical analysis. Similarly we have reported (Loader et al., 2003) that when [2,2,4,4-$^2H_4$]sarsasapogenone was dosed to a single sheep the deuterated ketone material recovered from collected fecal matter showed only the retention of one or two or the four, initially present, deuterium atoms. On the other hand, when a 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin and [2,2,4,4-$^2H_4$]episarsasapogenin was administered, >94% of the introduced deuterium atoms were present in sapogenins recovered from fecal samples from one sheep (Loader et al., 2003).

The aim of this study was to validate the isotope-dilution methodology of Loader et al. (2003) and to demonstrate that the method can be used under field conditions to estimate the uptake of plant sapogenins.

MATERIALS AND METHODS

Plant material

N. ossifragum plant material (bog asphodel) was hand picked from a pasture in Vest-Agder County, Southern Norway, in August 1-10, 1997 and frozen prior to the dosing trials. Three sub-samples (ca. 9 g) of the *N. ossifragum* material were freeze-dried overnight and the dry matter (DM) content calculated. Duplicate DM samples (ca. 0.2 g) were extracted with MeOH, hydrolyzed, acetylated and analyzed for total genin levels by GC-MS (Wilkins et al., 1994; Flåøyen and Wilkins, 1997; Loader et al., 2003).

Dosing Trial

The trial lasted 14 days from 2/5/00 to15/5/00. Three caged, castrated male sheep, Dala breed, each ca. two years of age were daily, on days 3 to14, given a 70 mg dose of a 1:4 mixture of [2,2,4,4-$^2H_4$]sarsasapogenin and [2,2,4,4-$^2H_4$]episarsasapogenin (Figure 1) into the rumen by a stomach tube. The deuterated sapogenin mixture was suspended in 100 mL of water:EtOH (80:20) and washed down with 50 mL water. The sheep were fed 300 g (72 g DM) of *N. ossifragum* on days 3 to 14. Hay, fed in controlled amounts during the experimental period, was offered daily after the ration of *N. ossifragum* had been consumed. Fecal material from each of the sheep was collected once daily on days 3 to 11, and 14, and at 6 h intervals (0800, 1400, 2000 and 0200 h) on days 12 and 13. Fecal production was recorded daily and frozen at -20°C on the day of collection. Dry matter content of fecal and *N. ossifragum* material was determined by freeze-drying. Urine samples were collected during the trial period.

GC-MS analyses

GC-MS analysis of acetylated sapogenin extracts was performed as previously reported (Wilkins et al., 1994; Flåøyen and Wilkins 1997; Loader et al., 2003).

Total genin analyses

Freeze-dried fecal sub-samples (ca. 0.5 g) collected on days 4 to 11, and 14 were extracted for 12 h with MeOH (150 mL) using a Soxhlet apparatus. The extracts were concentrated to dryness on a rotary evaporator and the resulting residues were taken up in 0.5 mol/L HCl (10 mL) and transferred, with repetitive washing (3 x 3 mL of 0.5 mol/L HCl), to a boiling tube that was heated in a water bath for 90 min at 85-90°C. After cooling, the hydrolysate solution was extracted with dichloromethane (4 x 4 mL) and filtered through a short silica column (ca. 3 cm) packed in a Pasteur pipette. The combined filtrates were evaporated to dryness under a stream of warm nitrogen and acetylated at room temperature for ca. 16 h using pyridine-acetic anhydride (1:1) (0.5 mL).

Free and conjugate genin analyses

Fecal material collected at 6 h intervals on days 12 and 13 were sequentially extracted with CH_2Cl_2 (free genins) and MeOH (conjugated genins) as previously reported (Wilkins et al., 1994; Flåøyen and Wilkins 1997; Loader et al., 2003). The % deuterium content of sapogenins found in fecal samples was determined according to Loader et al. (2003).

Calculation of plant genin content of fecal materials

The plant genin content of the fecal material collected daily (Table 1) was calculated using two equations.

Equation 1:

Plant genins (mg/day) = [(70 * (100 / %D)] – 70

Where 70 mg/day of deuterated genins were dosed once daily, and %D is the % deuterium content of the recovered fecal sample corrected for the 94.6% isotopic purity of the mixture of deuterated genins utilized in the dosing experiment.

Equation 2:

Plant genins (mg/day) = [fecal genins (mg/kg DM) * fecal weight (kg DM)] - 70

Equation 2 assumes that the genin level present in a fecal sub-sample, and the daily weight of fecal material is known.

RESULTS

The *N. ossifragum* leaves dosed contained 3655 mg/kg DM plant sapogenins (standard deviation 705 mg/kg DM). This afforded an estimate of the daily *N. ossifragum* genin intake by the sheep based on Equations 1 and 2 respectively (Tables 1 and 2). The calculated daily consumption of *N. ossifragum* plant material was determined by dividing plant genin intake levels by the average genin content of the dosed plant material. Mean daily consumption levels were calculated using day 4-14 data when Equation 1 was used and day 5-14 data when Equation 2 was used, since it was apparent that steady state sapogenin levels had not always been achieved after 4 days.

DISCUSSION

The results calculated using Equation 1 validate the isotope dilution methodology and demonstrate that it can be applied under field conditions to estimate the uptake of plant sapogenins where only a rectum sample collected once daily would be available, and the total daily amount of fecal matter produced can not be ascertained.

The fecal sampling procedure applied during the validation dosing trials reported in this paper was designed to model the situation that would be encountered under field trial conditions. A particularly important objective of the trial was the validation of our hypothesis (Flåøyen and Wilkins, 1997; Flåøyen et al., 2001) that ca. 96 h after dosing commenced, the ratio of deuterated to non-deuterated sapogenins in fecal material would be relatively constant, irrespective of the time of fecal material collection, and likely variations in sapogenin levels in individual fecal pellets collected during a 24 h period. Thus, a randomly selected sub-sample of fecal material, intended to model a once daily fecal sample that could be recovered from the rectum of a sheep under natural grazing conditions, rather than a sub-sample of homogenized daily fecal material as utilized in previous dosing trials (Flåøyen and Wilkins, 1997; Flåøyen et al., 2001), was extracted and its genin content determined.

Since only the total daily genin content was required, freeze-dried fecal samples were, with the exception of day 11 and 12 samples, extracted with MeOH to yield a combined free and conjugate genin extract which, after hydrolysis, afforded the total genin content. Day 11 and day 12 fecal samples, collected at 6 h intervals, were sequentially extracted with CH_2Cl_2 and MeOH, to afford free and conjugated genin fractions, respectively. Mean day 11 and 12 results (average of results determined for sub-samples of the 4 six hour samples) are included in Tables 1 and 2. Typically, four or five days were required for the fecal genin content to reach a steady state level. This finding is consistent with earlier results (Flåøyen and Wilkins, 1997; Flåøyen et al., 2001). No saponins or sapogenins were found in fecal samples collected prior to the commencement of the dosing experiment, in the urine samples collected before and throughout the experimental period, or in the hay fed during the experimental period. Sheep 2 and 3 ingested all *N. ossifragum* material offered. However on days 7-11, 13 and 14, sheep 1 consumed only part of the offered *N. ossifragum* plant material (Tables 1 and 2).

In accord with the results of earlier dosing experiments, episarsasapogenin was the dominant genin found in the fecal samples (Flåøyen and Wilkins, 1997; Flåøyen et al., 2001; Loader et al., 2003). Deuterated smilagenin and epismilagenin acetates were not detected showing that the ovine metabolism of sapogenins does not result in the epimerisation of 25*S*-genins (sarsasapogenin and episarsasapogenin) to 25*R*-genins (smilagenin and epismilagenin). As in previous dosing trials, levels low levels of sarsasapogenone and smilagenone (the intermediates whereby sapogenins are metabolised to the corresponding episapogenins) were detected in all of the extracts (Flåøyen and Wilkins, 1997; Flåøyen et al., 2001). Variations in the ratio of 25*S*- to 25*R*-genins (ca. 5 to 35%) observed for fecal sapogenins are considered to be indicative of variations in the ratio of sarsasapogenin (25*S*-) and smilagen (25*R*-) saponins in the dosed plant material.

The % deuterated genin contributions determined for day 4-14 fecal samples (Table 1) were remarkably consistent (average levels 19.8 to 23.1%) during the trial period, other than for sheep 1 on days 12 and 13. The elevated deuterium levels on these days are however consistent with sheep 1 ingesting a reduced quantity of *N. ossifragum*, ca. 3-4 days previously. For sheep 2 and 3, the calculated average daily dose values of 64 g DM and 79 g DM respectively (Table 1) correspond closely with that dosed (72 g DM). A pleasing aspect of the sheep 1 result is that, ca. 4 days after this sheep refused some of the offered plant material (Table 1) there was a corresponding decrease in the calculated daily *N. ossifragum* intake for this sheep (Figure 2). Since this sheep also refused part of the offered material on days 13 and 14, a similar decrease in calculated dosed material would have been expected on days 17 and 18.

The calculated daily plant intake values presented in Table 2 should, on the other hand, be interpreted with considerable caution since the mass balance approach, implicit in Equation 2, assumes that the sapogenin content of a randomly selected fecal sub-sample is constant over a 24 h period and that it should not vary significantly during consecutive 24 h periods. The substantially greater variations in calculated daily intake of plant material presented in Table 2 compared to those presented in Table 1 may be largely attributable to the inhomogenous distribution of sapogenins in individual fecal pallets. It is apparent from the data presented in Table 2 that typically 5 days (rather 4 days) were required to achieve steady state levels. Day 4 results (bracketed in Table 2) were therefore excluded from the calculation of mean levels presented in Table 2.

A significant advantage of the isotope-dilution method is that, provided isotope-labelled and unlabelled sapogenins are uniformly distributed in fecal material, a reliable estimate of the daily consumption of sapogenins can be made irrespective of whether or not of all of the sapogenins dosed 4-5 days previously have completely passed through the digestive tract. It is also apparent that the isotope-dilution method is intrinsically more accurate than the mass balance approach used in previous dosing experiments and that wherever possible (assuming the availability of isotopically labelled sapogenin material) isotope-dilution methodology should be used in future dosing experiments.

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Table 1. Daily weights of dosed plant material, % deuterated genins, plant genins, and calculated dosed plant material determined for sheep 1, 2 and 3 using %D data (Equation 1). Day 12 and 13 results were derived from values determined for four samples collected at 6 h intervals.

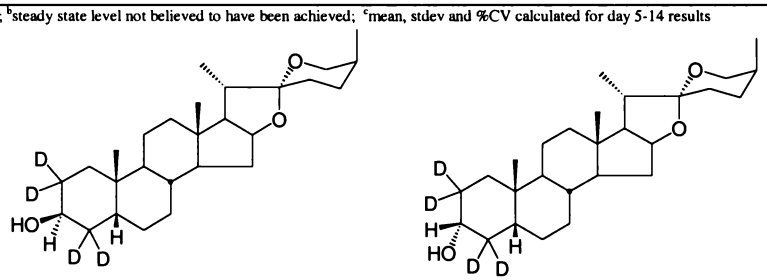
day	dosed plant (g DM/day)	% deuterated genins	plant genins[a] (mg/day)	calculated dosed plant (g DM/day)
sheep 1				
4	72	19.9	282	77
5	72	19.9	282	77
6	72	20.8	266	73
7	66	19.3	292	80
8	46	22.7	238	65
9	31	19.5	290	79
10	47	19.0	298	82
11	61	21.4	258	71
12	72	27.6	183	50
13	47	33.2	141	39
14	42	22.4	242	66
mean[b]	22.3	252	69	
stdev	4.2	47	13	
% CV		19	19	19
sheep 2				
4	72	22.1	247	68
5	72	28.4	176	48
6	72	24.2	219	60
7	72	23.3	231	63
8	72	21.0	263	72
9	72	21.6	255	70
10	72	22.4	242	66
11	72	23.8	224	61
12	72	21.9	250	68
13	72	23.5	228	62
14	72	22.7	238	65
mean[b]	23.1	235	64	
stdev	1.9	23	6	
%CV	8	10	10	
sheep 3				
4	72	18.9	300	82
5	72	18.6	306	84
6	72	19.5	290	79
7	72	18.3	313	86
8	72	20.4	273	75
9	72	16.9	344	94
10	72	21.6	255	70
11	72	22.4	242	66
12	72	22.6	239	66
13	72	19.3	292	80
14	72	20.6	270	74
mean[b]	19.8	287	79	
stdev	1.8	32	9	
%CV	9	11	11	

[a] calculated using Equation 1; [b] mean, stdev and %CV calculated for day 4-14 results

Table 2. Daily weights of dosed plant material, fecal material, fecal and plant genins, and calculated dosed plant material determined for sheep 1, 2 and 3 using fecal data (Equation 2). Days 12 and 13 results were derived from values determined for four samples collected at 6 h intervals.

day	dosed plant (g DM/day)	fecal material (g DM)	fecal material (% DM)	fecal genins (mg/kg DM)	plant genins[a] (mg/day)	calculated dosed plant (g DM/day)
sheep 1						
4	72	321	29.2	286[b]	-	-
5	72	712	27.4	427	234	64
6	72	359	33.9	815	222	61
7	66	544	25.1	772	349	95
8	46	360	28.6	846	235	64
9	31	418	26.9	501	140	38
10	47	456	27.8	443	132	36
11	61	369	33.2	429	88	24
12	72	870	28.1	451	322	88
13	47	525	31.0	599	245	67
14	42	451	27.2	393	107	29
mean[c]	506	28.9	568	207	57	
stdev		167	2.9	178	89	24
%CV	33	10	31	43	43	
sheep 2						
4	72	555	38.1	52[b]	-	-
5	72	528	38.7	361	120	33
6	72	448	40.9	404	111	30
7	72	389	37.8	734	215	59
8	72	450	37.5	678	235	64
9	72	648	31.7	507	258	71
10	72	402	30.7	362	76	21
11	72	529	35.1	354	117	32
12	72	868	34.0	754	585	160
13	72	651	31.9	662	361	99
14	72	681	31.3	491	265	73
mean[c]		559	35.0	531	234	64
stdev		151	3.6	162	152	42
%CV	27	10	31	50	65	
sheep 3						
4	72	699	35.4	232[b]	-	-
5	72	565	32.2	369	138	38
6	72	472	36.5	877	344	94
7	72	390	37.7	849	261	71
8	72	518	36.7	697	291	80
9	72	486	36.7	364	107	29
10	72	505	38.6	789	329	90
11	72	474	38.2	649	238	65
12	72	930	30.2	531	424	116
13	72	559	35.4	574	217	59
14	72	623	36.6	569	285	78
mean[c]	552	35.9	627	263	72	
stdev		147	2.7	181	95	26
%CV	27	7	29	36	36	

[a]calculated using Equation 2; [b]steady state level not believed to have been achieved; [c]mean, stdev and %CV calculated for day 5-14 results



[2,2,4,4-$^2H_4$]sarsasapogenin

[2,2,4,4-$^2H_4$]episarsasapogenin

Figure 1. Chemical structures of [2,2,4,4-$^2H_4$]sarsasapogenin and [2,2,4,4-$^2H_4$]episarsasapogenin

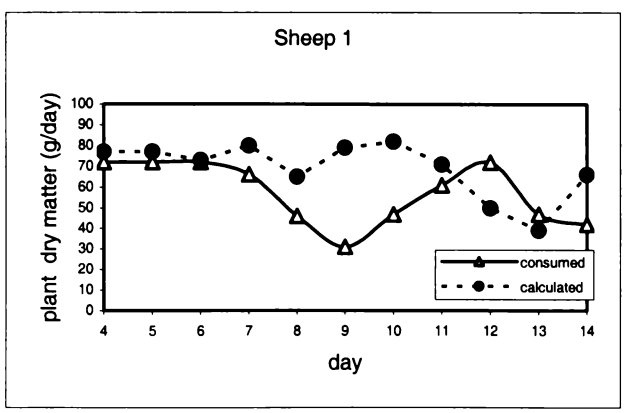


Figure 2. Consumed and calculated *N. ossifragum* plant material (g DM/day) determined for sheep 1.

Chapter 43

A Procedure for the Estimation of the Daily Intake of Saponins from Pasture by Sheep

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Introduction

Narthecium ossifragum (Eng.: Bog asphodel), a member of the lily family, is known to cause alveld (literally: elf-fire), a hepatogenous photosensitization of lambs in Norway, the British Isles and the Faroe Islands (Flåøyen, 1999). Steroidal saponins of the plant have been suggested to cause the liver lesions resulting in retention of the photosensitizing agent phylloerythrin (Flåøyen, 1999). Attempts to reproduce alveld and related diseases experimentally have been hampered by a lack of knowledge concerning the level of steroidal saponins ingested by animals during natural toxicity outbreaks (Flåøyen *et al.*, 1991). Hitherto we have proposed that it might be possible to determine the level of steroidal saponins ingested by dosing sheep once daily with a known amount of isotopically labelled sapogenins, and determine the ratio of deuterated to natural sapogenins in faecal material (Flåøyen *et al.*, 2001).

An attempt to exploit this approach using 20,23,23-D_3-sarsasapogenin failed due to loss of deuterium during ruminal metabolism of the dosed sapogenin, and/or the extraction, derivation and analytical procedures (Flåøyen *et al.*, 2001). An important observation during this trial was that about 96 h after the commencement of the dosing experiment in which a sheep was dosed once daily with 20,23,23-D_3-sarsasapogenin, and three times daily with *N. ossifragum* plant material, essentially steady state levels of sapogenins were present in faeces collected at varying times during the remaining trial period (Flåøyen *et al.*, 2001). This finding supported our hypothesis that, provided a suitable dosing substrate could be identified (i.e. one not prone to deuterium exchange under ruminal and analytical conditions), it would be possible to dose a sheep once daily with a

could be identified (i.e. one not prone to deuterium exchange under ruminal and analytical conditions), it would be possible to dose a sheep once daily with a

known amount of an isotopically labelled sapogenin and determine natural sapogenin intake by comparing the ratio of deuterated to natural genins present in faecal material.

In this chapter we report evaluations of the suitability of 2,2,4,4-D_4-sarsasapogenone and a *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin as isotopically labelled dosing substrates.

Materials and Methods

Synthesis of deuterated sapogenins

2,2,4,4-D_4-sarsasapogenone was prepared by refluxing sarsasapogenone (10 g) for 24 h in a mixture of dioxane (300 ml) and D_2O (25 ml) to which Na (0.35 g) had been cautiously added. Two cycles of deuterium exchange afforded material that consisted predominantly (> 96%) of 2,2,4,4-D_4-sarsasapogenone. Reduction of 2,2,4,4-D_4-sarsasapogenone (10 g) in ethanol:methanol (1:1, 400 ml) with sodium borohydride (2 g) afforded a *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin. A more detailed account of the synthesis of these substrates and their spectroscopic characterization will be reported elsewhere.

Preliminary trial (deuterium retention assessment)

A 2-year-old male Dala sheep was caged and administered a single intraruminal dose of 2,2,4,4-D_4-sarsasapogenone (1 g) suspended in 100 ml of water:ethanol (80:20) and washed down with 50 ml water. Eight days later, the same sheep was administered a single dose of a *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin (600 mg) suspended in 100 ml of water:ethanol (80:20) and washed down with 50 ml water. Hay was offered *ad libitum* during each of the experimental periods and 24 h faeces and urine samples were collected during the two dosing periods. Sub-samples of the faeces samples were freeze dried and analysed for free and conjugated sapogenins.

Validation trial (plant intake calibration)

Three 2-year-old male Dala sheep were caged and on each of days 0 to 10 were offered 300 g wet weight (= 72 g dry matter (DM)) of *N. ossifragum* plant material, and also administered a single 70 mg intraruminal dose of a *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin suspended in 100 ml of water:ethanol (80:20) and washed down with 50 ml water. Hay was offered *ad libitum* during the experimental period. Faeces and urine samples were collected once daily during the trial period. Sub-samples of the faeces samples were freeze dried and analysed for total sapogenins.

Sapogenin extraction and GC/MS analyses

Narthecium ossifragum and faecal samples were freeze dried, and either sequentially extracted with dichloromethane (free sapogenin extracts) and methanol (conjugated sapogenin extracts) (plant samples and preliminary trial samples), or with methanol alone (total sapogenin extracts) (calibration trial samples). Conjugated sapogenin and total sapogenin extracts were hydrolysed with 0.5 M HCl. All extracts were spiked with sarsasapogenin propionate as internal standard, acetylated with pyridine/acetic anhydride and analysed by selected ion mode (SIM) GC/MS as described previously (Wilkins *et al.*, 1994), other than that a 25 m x 0.25 mm id HP-5 (Hewlett Packard, USA) column was used and *m/z* 139, *m/z* 315-320 (genin acetates) and *m/z* 271-275 (sarsasapogenone) ions were monitored. The ratio of natural (non-deuterated) and deuterated genin acetates were determined using the sums of the *m/z* 315 + 316 (^{13}C isotope of the *m/z* 315 ion) and *m/z* 317-320 (D_2, D_3, D_4 + ^{13}C isotope of the *m/z* 320 ion) ion currents respectively. Plausible structures for the *m/z* 139 and 315 (319) ions of genin acetates are given in Fig. 43.1. Urine samples were extracted and analysed as reported previously (Flåøyen and Wilkins, 1997).

Results

The dosing experiments in which a sheep was administered a single dose of 2,2,4,4-D_4-sarsasapogenone on day 0, and 8 days later a *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin showed that maximum levels of free and conjugated sapogenins were, in each case, present in faecal material collected on days 2 and 3 (Tables 43.1 and 43.2). Sapogenin concentrations were determined as previously reported using integrated *m/z* 139 ion contributions (Wilkins *et al.*, 1994). The percentage deuterated sapogenins were determined by comparing the peak areas of the *m/z* 317+320 (deuterated sapogenin acetates) and *m/z* 315-320 ion currents (total sapogenin acetates).

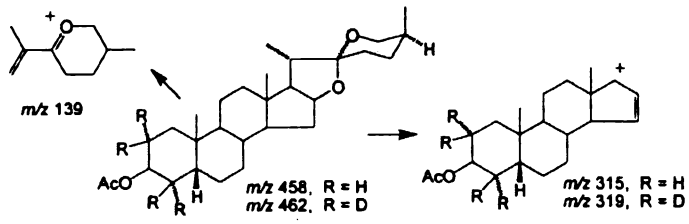

Fig. 43.1. Plausible structures for the *m/z* 139 and 315 (319) ions of genin acetates.

Table 43.1. Free and conjugated sapogenins levels (mg kg^{-1} DM) found in faecal samples after administration of a single dose of 2,2,4,4-D_4-sarsasapogenone.

Day	Extract	Sar-CO[a]	Sarsa[b]	Episar[c]	Total[d]	% D[e]
0	Free	–	–	–	–	
	Conjugated	–	–	–	–	
1	Free	136	25	67	228	
	Conjugated	5.7	7.1	20	33	
2	Free	459	110	353	923	95.2
	Conjugated	21	33	106	159	96.1
3	Free	152	95	346	592	93.4
	Conjugated	8.4	19	74	102	92.8
4	Free	40	37	125	202	
	Conjugated	6.4	9.1	32	48	
5	Free	9	13	34	56	
	Conjugated	1.5	2.5	5.4	9.4	
6	Free	–	11	26	37	
	Conjugated	–	2.0	3.6	5.6	
7	Free	–	–	–	–	
	Conjugated	–	–	–	–	

[a]sar-CO = sarsasapogenone; [b]sarsa = sarsasapogenin; [c]episar = episarsasapogenin; [d]total = total genins sar-CO, sarsa and episar levels; [e]% D = % deuterium retained in episarsasapogenin.

Table 43.2. Free and conjugated sapogenins levels (mg kg^{-1} DM) found in faecal samples after administration of a single dose of a *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin.

Day	Extract	Sar-CO[a]	Sarsa[b]	Episar[c]	Total[d]	% D[e]
0	Free	–	–	–	–	
	Conjugated	–	–	–	–	
1	Free	3.5	10	53	66	
	Conjugated	–	1.0	2.3	3.3	
2	Free	27	69	421	516	96.7
	Conjugated	6.6	12	54	73	95.6
3	Free	20	54	220	294	92.4
	Conjugated	4.6	10	35	49	90.4
4	Free	9.2	27	85	120	
	Conjugated	2.9	6.1	19	28	
5	Free	–	12	35	47	
	Conjugated	–	0.7	2.0	2.7	
6	Free	–	–	–	–	
	Conjugated	–	–	–	–	

[a]sar-CO = sarsasapogenone; [b]sarsa = sarsasapogenin; [c]episar = episarsasapogenin; [d]total = total genins sar-CO, sarsa and episar levels; [e]% D = % deuterium retained in episarsasapogenin.

Validation Trial

The total levels of deuterated and natural sapogenins detected in faeces recovered from one of the three sheep that consumed 72 g day^{-1} DM of *N. ossifragum*, and which were also dosed once daily for 11 days with a *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin are presented in Table 43.3. The percentage deuterated sapogenin content of the samples is presented in Table 43.3. A mean level of 3,655 (standard deviation 705) mg kg^{-1} DM was found in randomly selected *N. ossifragum* samples. Duplicate analyses of plant subsamples agreed to within 0.5–2.8%. In accord with other dosing trial results, episarsasapogenin was the dominant genin constituent detected in faecal material (Flåøyen and Wilkins, 1997; Flåøyen *et al.*, 2001). No sapogenins were detected in urine samples.

Since 70 mg of deuterated sapogenins were dosed daily and mean percentage D level faecal material was of 18.5% (Table 43.3) the calculated average daily intake of sapogenins was 378 mg day^{-1}, of which 308 mg day^{-1} could be attributed to the ingestion of *rome* sapogenins. Furthermore, since the average sapogenin level of the dosed plant material was found to be 3655 (standard deviation 705) mg kg^{-1} DM, the calculated mean daily intake of *N. ossifragum* was 84 g DM, compared to the 72 g DM (= 300 g wet weight) that was dosed. Generally similar results were obtained for sheep 2 and 3 (calculated mean plant intakes of 64 and 69 mg kg^{-1} DM respectively). Given variations in the saponin level of the dosed plant material, and some lack of homogeneity in the distribution of natural and

Table 43.3. Percentage deuterated sapogenins (% D), calculated total sapogenin levels in faecal samples collected from a sheep consuming 72 g DM *N. ossifragum* daily and dosed once daily with a c. 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin, and calculated daily consumption of *N. ossifragum* (g DM day^{-1}).

Day	% D	Calc. total sapogenins (mg kg^{-1} DM day^{-1})	Calc. plant sapogenins (mg kg^{-1} DM day^{-1})	Calc. plant (g DM day^{-1})
3	19.1	366	296	81
4	17.9	391	321	88
5	18.4	380	310	85
6	17.3	405	335	92
7	17.3	405	335	92
8	19.3	363	293	80
9	16.0	438	368	101
10	20.4	273	343	75
11	21.2	330	260	71
Mean	18.5	378	308	84
Std dev	1.6	48	32	9

deuterated sapogenins in faecal material, agreement to within 1 to 1.5 standard deviations was considered to be acceptable.

Conclusions

It is apparent from the results presented in Table 43.2 that the *c.* 1:4 mixture of 2,2,4,4-D_4-sarsasapogenin and 2,2,4,4-D_4-episarsasapogenin afforded by sodium borohydride reduction of 2,2,4,4-D_4-sarsasapogenone (*c.* 1:4 is the ratio at which these sapogenins are typically present in faeces samples from animals consuming *N. ossifragum*), can be utilized as an isotopically labelled dosing substrate for determining the intake of *N. ossifragum* sapogenins under normal grazing conditions.

Calculated intakes of 64–84 g DM day^{-1} of *N. ossifragum* (89–117% of actual intakes) were considered satisfactory for future use of this isotope dilution technique in measuring intake of *N. ossifragum* under natural conditions.

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Chapter 82

Steroidal Sapogenins and Saponins in *Narthecium ossifragum* from Scotland

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Introduction

Narthecium ossifragum (Eng.: Bog asphodel) is a saponin containing plant implicated in the hepatogenous photosensitization of sheep (Flåøyen, 1999) in Northern Europe. Most reported outbreaks of *N. ossifragum*-associated photosensitization of sheep originate from Norway (Ender, 1955; Ceh and Hauge, 1981; Abdelkader *et al.*, 1984; Flåøyen, 1999), however there are also reports of *N. ossifragum*-associated photosensitization of sheep grazing *N. ossifragum* containing pastures in the Faroe Islands, Scotland (Flåøyen *et al.*, 1995) and the North of England (Ford, 1964).

While there is some knowledge of the levels of saponins (steroidal sapogenin glycosides) in Norwegian collections of *N. ossifragum* (Flåøyen *et al.*, Chapter 11 this volume), there is no information concerning the levels of steroidal sapogenins in Scottish collections of *N. ossifragum*.

In this chapter we report an evaluation of the levels of free and conjugated sapogenins (saponins) found in *N. ossifragum* plants collected from Auchtertyre Farm, Strathfillian, West Perthshire, Scotland. At the time of sampling two photosensitized lambs had recently been removed from a pasture that included an appreciable quantity of *N. ossifragum*.

Experimental

Plant material (leaf, stems, flower heads and roots) were collected on 8 August 2001 from two sites on Auchtertyre Farm, Strathfillian, West Perthshire, Scotland (56° 26' North, 4° 39' West), adjacent to the access road leading to the West Highland Railway Line bridge, *c.* 100 and 150 m above the farm workshop area (sites 1 and 2, young, recently grazed plants) and from a hillside slope *c.* 500 m above the railway bridge (site 3, mature plants that had not been grazed). Plant

samples were frozen within 6 h of collection, and maintained at freezer temperature, other than for the period (10 h) when they were transported to the National Veterinary Institute, Oslo, where they were refrozen and freeze dried.

Accurately weighed portions of the freeze dried plant materials (*c.* 0.2–0.3 g) were placed in cellulose extraction thimbles and sequentially extracted for 3 h with dichloromethane, and (after overnight drying of the extraction thimble) with methanol, using a SoxTech extractor. After the evaporation of solvent under a stream of warm nitrogen, conjugated extracts were hydrolysed for 90 min at *c.* 90–95°C using 0.5 M HCl (7 ml), and extracted with dichloromethane (3 x 4 ml). All extracts were acetylated with pyridine/acetic anhydride, spiked with sarsasapogenin propionate as internal standard, and analysed by selected ion mode (SIM) GC/MS as described previously (Wilkins *et al.*, 1994), other than that a 25 m x 0.22 mm id HP-5 capillary column (Hewlett Packard, USA), a Karlo-Erba 8000 GC and a VG Trio 1000 mass spectrometer were used. SIM-GC/MS analyses were temperature programmed from 200°C (1 min hold) to 265°C at 40°C min^{-1} and then to 290°C at 5°C min^{-1} (16 min hold). Quantification was performed relative to sarsasapogenin propionate. The *m/z* 139 ion response factor of sarsasapogenin acetate relative to that of sarsasapogenin propionate was 1.15.

Results and Discussion

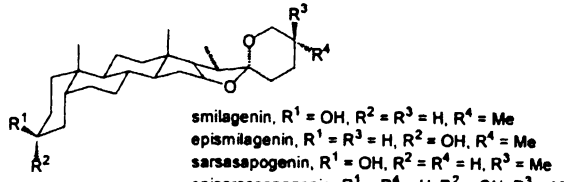
The dry matter (DM) content and levels of free and conjugated sapogenins determined for the freeze dried plant materials are presented in Table 82.1. Confirmation of the presence of smilagenin and sarsasapogenin acetates (25*R* and 25*S*-epimers respectively of the same sapogenin; see Fig. 82.1) in the acetylated extracts was established by comparison of total ion chromatogram mode mass spectra, *m/z* 255/*m/z* 315, *m/z* 269/*m/z* 329 and *m/z* 284/*m/z* 344 ion ratio (Wilkins *et al.*, 1994) and retention time data for the acetylated sapogenins and for authentic specimens of smilagenin and sarsasapogenin acetates. The total levels of conjugated (glycosylated) sapogenins found in leaf samples, with the exception of the basal region of the mature plants from site 3, (2040–4618 mg kg^{-1} DM) are comparable to those present in summer collections of Norwegian *N. ossifragum* (Flåøyen *et al.*, Chapter 11 this volume).

Since plant material was collected from only two sites on a single Scottish farm, caution must therefore be exercised when comparing the characteristics of Norwegian and Scottish collections of *N. ossifragum*. The *c.* 45–65% contribution of smilagenin to total sapogenins determined for the Scottish collections of *N. ossifragum* (Table 82.1) can be compared to the *c.* 10–15% contribution of smilagenin to total sapogenins in Norwegian collections. Miles *et al.* (1991) have suggested that following the ovine metabolism of sapogenins to episapogenins, epismilagenin (25*R*-epimer) may be more lithogenic (crystal-forming) than episarsasapogenin (25*S*-epimer) (Fig. 82.1).

Table 82.1. Percentage dry matter (DM) and free and conjugated sapogenin levels (mg kg^{-1} DM) determined for *N. ossifragum* plant samples.

Site/sample	Extract	% DM	Smilagenin	Sarsasapogenin	Total[c]
Site 1[a]					
Leaf (0–15 cm)	Free	34.3	tr	tr	tr
	Conjugated		1,047	993	2,040
Flower heads	Free	75.4	3	55	58
	Conjugated		8,139	10,245	18,384
Stems	Free	52.7	tr	tr	0
	Conjugated		26	28	54
Roots	Free	54.1	145	687	832
	Conjugated		44	167	211
Site 2[a]					
Leaf (0–15 cm)	Free	44.6	tr	tr	tr
	Conjugated		2,788	1,830	4,618
Site 3[b]					
Leaf (0–10.cm)	Free	30.4	tr	tr	tr
	Conjugated		75	36	111
Leaf (10–20 cm)	Free	30.8	tr	tr	tr
	Conjugated		2,119	1,454	3,573
Leaf (20–30 cm)	Free	40.2	tr	tr	tr
	Conjugated		1,870	1,408	3,278
Leaf (bulk)	Free	32.3	6	12	18
	Conjugated		1,262	1,029	2,291
Stems	Free	36.0	12	20	32
	Conjugated		23	24	47
Flower heads and	Free	57.5	24	32	56
stems	Conjugated		42	30	72
Roots	Free	62.0	341	841	1,182
	Conjugated		14	54	68

[a] Young plants; [b] mature plants; [c] total sapogenins; tr = trace.



smilagenin, R^1 = OH, R^2 = R^3 = H, R^4 = Me
epismilagenin, R^1 = R^3 = H, R^2 = OH, R^4 = Me
sarsasapogenin, R^1 = OH, R^2 = R^4 = H, R^3 = Me
episarsasapogenin, R^1 = R^4 = H, R^2 = OH, R^3 = Me

Fig. 82.1. Chemical structures of smilagenin, epismilagenin, sarsasapogenin and episarsasapogenin.

Intriguing aspects of the results were the detection of a substantial level of conjugated sapogenins (saponins) in the flower heads of young plants from site 1 (*c.* 18 g kg^{-1} DM) and the presence of significant levels of free sapogenins in the roots of plants from sites 1 and 3 (832 and 1184 mg kg^{-1} DM respectively). It is tempting to speculate that sapogenins are initially synthesized in the roots, and following glycosylation, they are transported to leaf tips and flower heads, where they may possibly act as antifungal agents (Flåøyen *et al.*, Chapter 11 this volume). More detailed studies are however required to validate these hypotheses.

Conclusions

The presence in Scottish *N. ossifragum* samples, from a pasture on which sheep were photosensitized, of elevated levels of conjugated steroidal sapogenins has been established. The percentage contribution of smilagenin (a 25*R*-genin) to the Scottish samples was typically two to four times greater than was the case for Norwegian collections of *N. ossifragum*.

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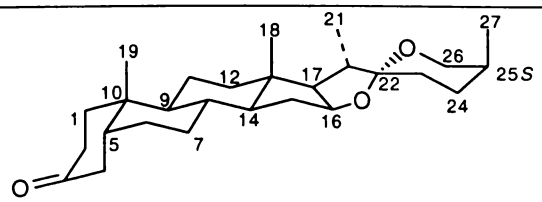
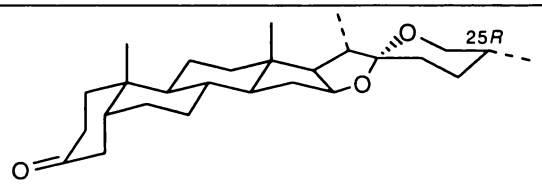
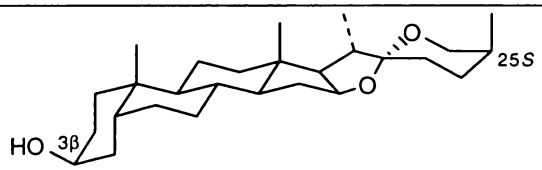
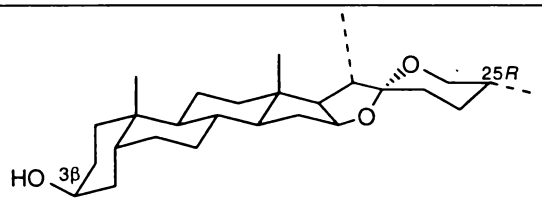
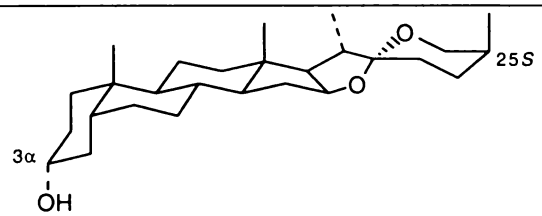
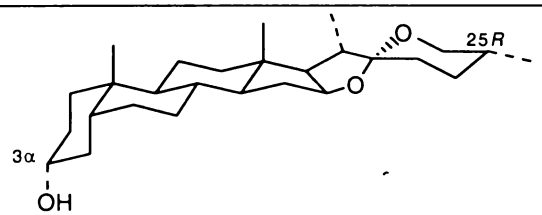
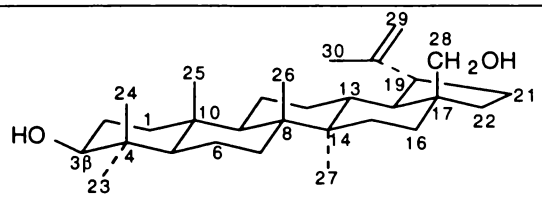
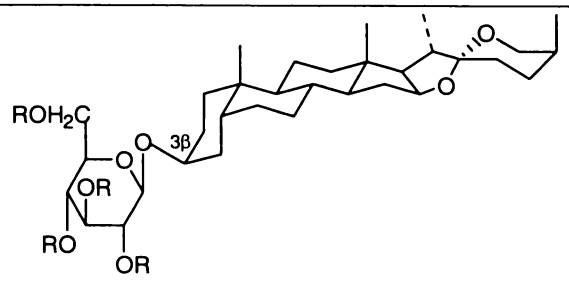
Appendix IX. Genin structures discussed in Chapters 2, 3, 4 and 5.

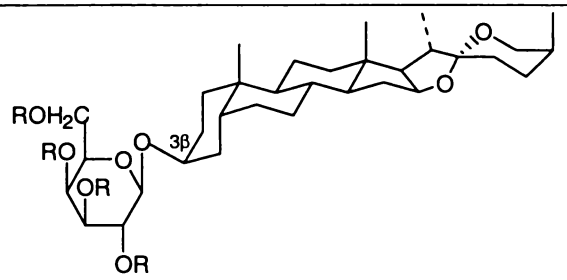
Sarsasapogenone (Sar-CO)	
Smilagenone (Smil-CO)	
Sarsasapogenin (Sar)	
Smilagenin (Smil)	
Episarsasapogenin (Episar)	
Epismilagenin (Epismil)	
Betulin	

Appendix X. Synthesized saponin structures discussed in Chapters 5 and 6.



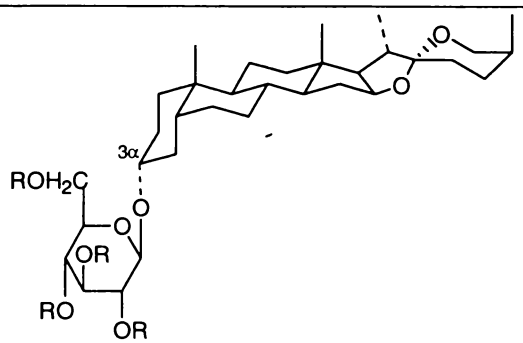
R = OAc Sarsasapogenin β-D-glucoside tetraacetate

R = H Sarsasapogenin β-D-glucoside



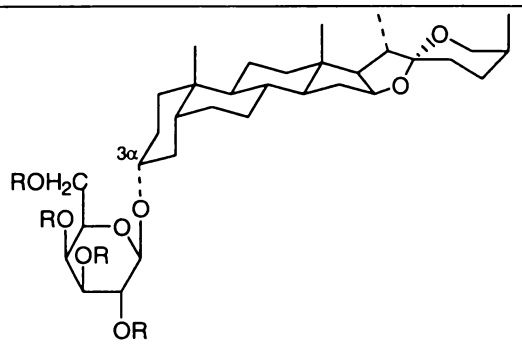
R = OAc Sarsasapogenin β-D-galactoside tetraacetate

R = H Sarsasapogenin β-D-galactoside



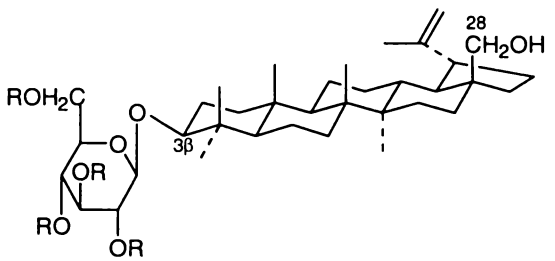
R = OAc Episarsasapogenin β-D-glucoside tetraacetate

R = H Episarsasapogenin β-D-glucoside



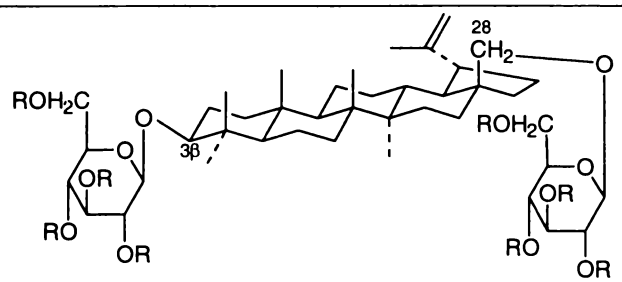
R = OAc Episarsasapogenin β-D-galactoside tetraacetate

R = H Episarsasapogenin β-D-galactoside



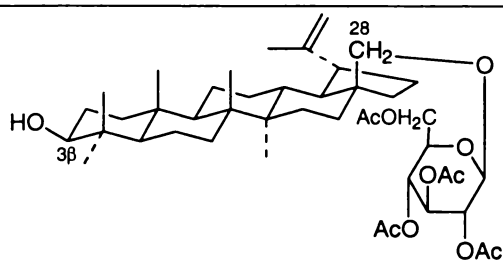
R = OAc Betulin 3-(β-D-glucoside tetraacetate)

R = H Betulin 3-(β-D-glucoside)



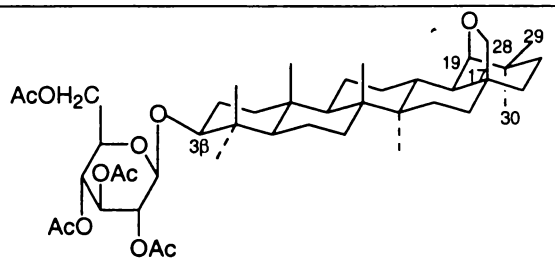
R = OAc Betulin 3,28-(β-D-diglucoside tetraacetate)

R = H Betulin 3,28-(β-D-diglucoside)



R = OAc Betulin 28-(β-D-glucoside tetraacetate)

R = H Betulin 28-(β-D-glucoside)



R = OAc Allobetulin β-D-glucoside tetraacetate

R = H Allobetulin β-D-glucoside

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