Research Commons

http://researchcommons.waikato.ac.nz/

Research Commons at the University of Waikato

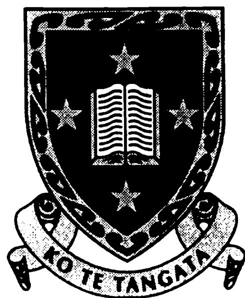
Copyright Statement:

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

The thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognise the author's right to be identified as the author of the thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from the thesis.

CHEMICAL ASPECTS OF SOME OVINE HEPATOGENOUS PHOTOSENSITIZATION DISEASES



A thesis submitted in fulfilment of the requirements for the Degree of

Doctor of Philosophy

in Chemistry

at the University of Waikato

by

Dawei Deng

1999
Hamilton, New Zealand

ABSTRACT

Current understandings of the chemistry, aetiology of saponin-associated hepatogenous photosensitization diseases of ruminants (sheep, cattle, goats and deer), the distribution of saponins in causative plants (*Agave lecheguilla, Brachiaria decumbens, Narthecium ossifragum, Nolina texana, Tribulus terrestris* and some *Panicum* species), and the ovine metabolism of saponins implicated in the development of hepatogenous photosensitization diseases, are reviewed.

Sarsasapogenin β-D-glucoside and 3β-D-epismilagenin β-D-glucoside were synthesised from sarsasapogenin and α-acetoglucosyl bromide via three and five step reaction sequences respectively. The structures of intermediates and the product glucosides were established using a combination of GC-MS, ES-MS and 400 MHz one- and two-dimensional NMR spectral data.

20,23,23-D_3-sarsasapogenin was prepared from sarsasapogenin using deuterioacetic acid, and the stability (exchangeability) of the deuterium atoms was determined in a series of pH 1-8 buffer solutions at 37°C, and in acetic acid at room temperature and 37°C.

Administration of an ethanol-water suspension of sarsasapogenin β-D-glucoside to a sheep afforded a maximum level of episarsasapogenin in bile samples 12 hours after dosing. Elevated levels of free sarsasapogenin and low levels of conjugated sarsasapogenin, free and conjugated episarsasapogenins and free and conjugated sarsasapogenones were detected in a rumen sample.

Administration of mixtures of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin to two sheep afforded a maximum level of episarsasapogenin in bile samples 7-16 hours after dosing. Unexpected loss of deuterium atoms occurred during ovine metabolism.

Analyses of mass spectral ion ratio data, determined for the mixed dosing experiment (using free and conjugated sarsasapogenins) and an experiment in which only 23,23,23-D_3-sarsasapogenin was dosed, showed that when administered as an ethanol-water suspension sarsasapogenin β-D-glucoside was ca 1.7 times more bioavailable than sarsasapogenin.

Dosing of 3β-D-epismilagenin β-D-glucoside to two sheep showed that oxidation and reduction at C-3 was a reversible process, and that epismilagenin (3α-OH), rather than

smilagenin (3β-OH), was the favoured reduction product of smilagen-3-one. Deuterium loss occurred at different rates for free and conjugated epismilagenins in different metabolic regions (e.g. the rumen and the jejunum). The ion ratio profile of jejunum epismilagenin conjugates was similar to that of bile epismilagenin conjugates.

Six saponins (faroecin, asiaticin, asiaticioside, narthecin, narthecioside and 22-methoxy-narthecioside) were isolated from a Norwegian collection of *N. ossifragum* and their structures were elucidated using ES-MS and one- and two-dimensional NMR spectral data. Three of these saponins, faroecin, asiaticioside and narthecioside, were identified as new compounds. Elevated levels of sarsasapogenin and smilagenin glycosides were found in some *N .ossifragum* samples (up to 2121 and 557 mg/kg respectively).

The furostenol tetrasaccharide, (25*R/S*)-furost-5-ene-3β,22α,26-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranoside, was isolated from a USA collection of *P. virgatum*. Variable levels of diosgenin and yamogenin glycosides were found in 13 USA *P. virgatum* collections (up to 288 and 42 mg/kg of diosgenin and yamogenin glycosides respectively).

The furostenol tetrasaccharide, (25*R/S*)-furost-5-ene-3β,22α,26-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranoside and the corresponding spiro-stenol tetrasaccharide, (25*R/S*)-spirost-5-en-3β-ol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside}, were isolated from a New Zealand collection of *P. miliaceum*.

Hydrolysis of the ethanol-water extracts of a USA collection of *N. texana* afforded three dihydroxy sapogenins, ruscogenin, neoruscogenin and spirosta-5,25(27)-diene-1β,3β-diol, in a ratio of ca 1:1.2:2.5. Three monoglycosylated saponins, spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-fucopyranoside, spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-α-L-arabinopyranoside and spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-galactopyranoside, were identified as constituents of the *N. texana* ethanol-water extract.

The levels of saponins in 82 Papua New Guinea collections of *B. decumbens* and 4 Iranian collections of *Tribulus terrestris* were determined. Variable levels of diosgenin and yamogenin glycosides (*B. decumbens* samples), and diosgenin, smilagenin, sarsasapogenin, tigogenin, neotigogenin, hecogenin and neohecogenin glycosides (*T. terrestris* samples) were identified.

ACKNOWLEDGEMENTS

I wish to thank the large number of people who contributed and helped me to complete this thesis. In particularly, I would like to thank:

–Professor Alistair Wilkins, my chief supervisor, for his invaluable experimental knowledge, patience, guidance, encouragement and help throughout my study.

–Dr Chris Miles, my second supervisor, in the Toxicology and Food Safety Research Group, AgResearch, Ruakura, Hamilton, for his very valuable, critical advice and comments during this project.

–Dr Barry Smith, Toxicology and Food Safety Research Group, AgResearch Ruakura, Hamilton, for veterinary guidance and help handling the lambs and collecting samples during the dosing experiments.

–Associate Professor Lyndsay Main and Professor Brain Nicholson for welcoming me when I first visited the Department, and for their help during my time in the Department.

–Mrs Jannine Sims for training and assistance with GC-MS and relevant programs, Dr Ralph Thomson for training and assistance with NMR, Mrs Wendy Jackson for assistance with ES-MS, Dr Merilyn Manley-Harris and Ted Christian for assistance with HPLC.

–Mrs Caroline Huang (now of the Department of Earth Sciences), Mrs Pat Gread, Mrs Annie Barker and Mrs Amu Upreti for their help in carrying our laboratory work.

–Mr Peter Embling, Toxicology and Food Safety Research Group, AgResearch Ruakura, Hamilton, for assistance in handling the lambs and collecting samples during the dosing experiments.

–Dr Arne Flåøyen, National Veterinary Institute, Olso, Norway, for collecting and supplying *N. ossifragum* samples.

–Mrs Sue Low, University of Technology, Lae, Papua New Guinea, for collecting and supplying the Papua New Guinea *B. decumbens* samples.

–Dr R. L. Reid, Division of Animal and Veterinary Sciences, West Virginia University, Morgantown, USA, for collecting and supplying the USA *P. virgatum* samples.

– Professor Murl Bailey, College of Veterinary Medicine, Texas A&M University, USA, for collecting and supplying the USA *N. texana* sample.

–Dr Lance McLeay, Department of Biological Sciences, for useful discussions.

–Dr Fengming (Frank) Tian, Department of Earth Sciences, for his helpful suggestions and editing of this thesis.

–Dr Ken Richards and Mrs Eileen Richards, Elim Church, Hamilton, for their love and excellent proof-reading of this thesis.

–Peter Charlton and Dorothee Götz, PhD students in Department of Biological Sciences, for their excellent proof-reading of this thesis.

–Simon Berndt, Lucy Meagher, Mindy Wang, Ian Graves, Julie Zhu, Lucy Feng, Nick Goodwin and all my C block colleagues for their help and companionship in the research laboratory.

–The Scholarship Committee, University of Waikato, for the award of a scholarship which made it possible for me to complete this thesis.

Finally, special thanks to my wife Jing Yuan, my son Yuefeng Deng and my daughter Holly Deng for their love, encouragement, patience and help during my time at Waikato University.

TABLE OF CONTENTS

Abstract ii
Acknowledgments iv
Table of Contents vi
List of Figures xi
List of Tables xvii
Abbreviations xxiii

Chapter One INTRODUCTION 1
1.1 Photosensitization Diseases of Livestock 1
1.1.1 Introduction 1
1.1.2 Types of Photosensitizations 2
1.2 Biliary Crystals 3
1.3 Saponins 6
1.3.1 Chemical Structures 6
1.3.2 Biological Characteristics 9
1.3.2.1 General 9
1.3.2.2 Role of Saponins in Photosensitizations 10
1.4 Plants 11
1.5 Ovine Saponin Metabolism 21
1.6 Aims and Objectives of the Present Investigation. 25

Chapter Two SYNTHESES 27
2.1 Introduction 27
2.2 Synthesis of Sarsasapogenin β-D-glucoside 28
2.2.1 Introduction 28
2.2.2 Results and Discussion 30
2.3 Synthesis of 3β-D-Epismilagenin β-D-glucoside 37
2.3.1 Introduction 37
2.3.2 Results and Discussion 38
2.4 Preparation of 20,23,23-2D_3-Sarsasapogenin 48
2.4.1 Introduction 48
2.4.2 Results and Discussion 49

2.5 Stability Tests 55
2.5.1 Introduction 55
2.5.2 Results and Discussion 55
2.6 Summary 60

Chapter Three ANIMAL TRIALS 60
3.1 Introduction 60
3.2 Methodology 62
3.2.1 Sheep Trials 62
3.2.2 Sample Preparation 64
3.2.3 GC-MS Analysis 64
3.3. Trial 1 - Dosing of Sarsasapogenin β-D-glucoside 66
3.3.1 Introduction 66
3.3.2 Results and Discussion 66
3.3.3 Conclusions 74
3.4 Trial 4 - Dosing with 20,23,23-D_3-Sarsasapogenin 75
3.4.1 Introduction 75
3.4.2 Results and Discussion 75
3.4.2.1 Levels of Sapogenins in Rumen, Jejunum, Faeces and Bile Samples 75
3.4.2.2 Contributions to Bile Samples 80
3.4.2.3 Contributions to Rumen, Jejunum and Faeces Samples 82
3.4.2.4. Metabolism 87
3.4.3 Summary and Conclusions 89
3.5 Trial 2 - Dosing of a Mixture of Sarsasapogenin β-D-glucoside and 20,23,23-D_3-Sarsasapogenin 90
3.5.1 Introduction 90
3.5.2 Results and Discussion 91
3.5.2.1 Levels of Sapogenins in Rumen, Jejunum and Faeces Samples 98
3.5.2.2 Levels of Sapogenins in Bile Samples 100
3.5.2.3 Contributions of Deuterated and non-Deuterated Sapogenins to Bile Samples 102
3.5.2.4 Contributions in Rumen, Jejunum and Faeces Samples 121
3.5.3 Summary and Conclusions 128
3.6 Trial 3 - Dosing with 3β-D-Epismilagenin β-D-glucoside 129
3.6.1 Introduction 129
3.6.2 Results and Discussion 129

3.6.2.1 Levels of Sapogenins in Rumen, Jejunum and Faeces Samples	131
3.6.2.2 Stereoselectivity of Reduction at C-3	135
3.6.2.3 Levels of Conjugated Epismilagenins in Bile Samples	138
3.6.2.4 Bile Cholesterol Levels	142
3.6.2.5 Ion Ratio Analyses	144
3.6.3 Summary and Conclusions	153
Chapter Four SAPONIN CONSTITUENTS OF SELECTED PLANTS	154
4.1 Introduction	154
4.2 *Narthecium ossifragum*	155
4.2.1 Introduction	155
4.2.2 Results and Discussion	157
4.3 *Panicum virgatum*	172
4.3.1 Introduction	172
4.3.2 Results and Discussion	173
4.4 *Panicum miliaceum*	181
4.4.1 Introduction	181
4.4.2 Results and Discussion	181
4.5 *Nolina texana*	187
4.5.1 Introduction	187
4.5.2 Results and Discussion	190
4.5.2.1 Characterisation of Sapogenin Constituents	190
4.5.2.2 Quantitative Analyses	195
4.5.2.3 Characterisation of Saponin Constituents	196
4.6 *Tribulus terrestris*	205
4.6.1 Introduction	205
4.6.2 Results and Discussion	205
4.7 *Brachiaria decumbens*	209
4.7.1 Introduction	209
4.7.2 Results and Discussion	209
4.8 Summary and Conclusions	219
Chapter Five EXPERIMENTAL	220
5.1 General	220
5.1.1 Nuclear Magnetic Resonance (NMR) Experiments	220
5.1.2 Gas Chromatography-Mass Spectrometry (GC-MS) Analysis	221
5.1.2.1 Sample Preparation	221

5.1.2.2 Primary Standard Solutions 222
5.1.2.3 GC-MS 223
5.1.2.4 Calculations 225
5.1.3 Electrospray Mass Spectroscopy (ES-MS) 225
5.1.4 Chromatography 226
5.1.5 Chemicals 226
5.2 Experimental, Chapter Two 227
5.2.1 Synthesis of Sarsasapogenin β-D-glucoside 227
5.2.2 Synthesis of 3β-D-Epismilagenin β-D-glucoside 229
5.2.3 Preparation of 20,23,23-D_3-Sarsasapogenin 232
5.2.4 20,23,23-D_3-Sarsasapogenin Stability Tests 233
5.3 Experimental, Chapter Three 233
5.3.1 Trial 1 - Dosing Trial with Sarsasapogenin β-D-glucoside 233
5.3.2 Trial 2 - Dosing Trial with a Mixture of Sarsasapogenin β-D-glucoside and 20,23,23-D_3-Sarsasapogenin 235
5.3.3 Trial 3 - Dosing Trial with 3β-D-Epismilagenin β-D-glucoside 235
5.3.4 Trial 4 - Dosing Trial with 20,23,23-D_3-Sarsasapogenin 236
5.4 Experimental, Chapter Four 236
5.4.1 *Narthecium ossifragum* 236
5.4.1.1 Isolation of *N. ossifragum* Saponins 236
5.4.1.2 GC-MS Analyses of *N. ossifragum* 238
5.4.2 *Panicum virgatum* 239
5.4.2.1 Isolation of *P. Virgatum* Saponins 239
5.4.2.2 GC-MS Analyses of *P. Virgatum* 239
5.4.3 *Panicum miliaceum* 240
5.4.3.1 Isolation of *P. miliaceum* Saponins 240
5.4.3.2 GC-MS Analyses of *P. miliaceum* 241
5.4.4 *Nolina texana* 241
5.4.4.1 Characterisation of *N. texana* Sapogenins 241
5.4.4.2 Characterisation of *N. texana* Saponins 242
5.4.4.3 GC-MS Analyses of *N. texana* 244
5.4.5 *Tribulus terrestris* 244
5.4.6 *Brachiaria decumbens* 244

APPENDIX 245
Figure I. ^{1}H NMR spectra of sarsasapogenin β-D-glucoside tetraacetate (A) and 3β-D-epismilagenin β-D-glucoside tetraacetate (B) 246

Figure II. ES-MS spectra of saponin **4-24** (A) and saponin **4-23** (B) isolated from the ethanol-water extract of *N. ossifragum* 247

Figure III. ^{1}H NMR spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside **4-24** isolated from the ethanol-water extract of *N. ossifragum* 248

Figure IV. COSY spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum* 249

Figure V. ^{13}C NMR spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum* 250

Figure VI. HSQC spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum* 251

Figure VII. HMBC spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum* 252

Figure VIII. Partial HOHAHA spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum* 253

Figure IX. ROESY spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum* 254

Figure X. Sample Excel spreadsheet used to calculate the levels of free and conjugated sapogenins and sterols in animal and plant samples 255

REFERENCES 256

LIST OF FIGURES

Chapter One

Figure 1-1 Structures of smilagenin (**1-1**) and epismilagenin (**1-2**) 4

Figure 1-2 Structures of calcium salts of the β-D-glucuronides of epismilagenin (**1-3**) and episarsasapogenin (**1-4**) 5

Figure 1-3 Structures of diosgenin (**1-5**) (a steroidal sapogenin) and hederagenin (**1-6**) (a triterpenoid sapogenin) 6

Figure 1-4 Structures of tigogenin (**1-7**) and sarsasapogenin (**1-8**) 7

Figure 1-5 Partial structures of spirostanol and furostanol saponins 8

Figure 1-6 Structures of some sugars often found in saponins 9

Figure 1-7 Structures of protodioscin (**1-10**) and protoyamoscin (**1-11**) (Berndt, 1997) 13

Figure 1-8 Structures of saponins isolated from *N. asiaticum* by Inoue et al. (1995) (*) and Kobayashi et al. (1993) (**) 15

Figure 1-9 Structures of saponins isolated from *N. recurvata* by Takaashi et al. (1995) (*) and Mimaki et al. (1996) (**) 16

Figure 1-10 Structures of dichotomin and its 22-methoxy analogue isolated from *P. dichotomiflorum* by Munday et al. (1993) 17

Figure 1-11 Structure of coloratin, as proposed by Wilkins (pers commun). 18

Figure 1-12 Structures of sapogenins and saponins isolated from *T. terrestris* 20

Figure 1-13 Proposed pathway for the ruminal metabolism of diosgenin-containing saponins to calcium salt of the β-D-glucuronide of epismilagenin (biliary crystals) (after Miles et al., 1994b) 22

Figure 1-14 Structures of the mono- and di-hydroxylated sapogenins mentioned in the text 23

Figure 1-15 Levels (mg/kg dry matter) of free and conjugated sapogenins identified in three metabolic regions and in the *N. ossifragum* sample (Flåøyen and Wilkins, 1997) 24

Chapter Two

Figure 2-1 Reaction of α-glucosyl halides with an alcohol to form β-glucosides 28

Figure 2-2 Reactions utilised in the synthesis of sarsasapogenin β-D-glucoside (**2-2**) 30

Figure 2-3 Structures of by-products **2-6** and **2-7** 31

Figure 2-4 Selected HMBC correlations observed for sarsasapogenin β-D-glucoside 35

Figure 2-5 Proposed pathway for the loss of the 3-deuterium atom of episapogenin during ovine metabolism 37

Figure 2-6 Reactions involved in the synthesis of 3β-D-epismilagenin β-D-glucoside (**2-8**) 38

Figure 2-7 Stereochemical outcomes of lithium aluminium hydride reduction of 3-keto-5α- and 3-keto-5β-steroids (from Shoppee and Summers, 1950) 40

Figure 2-8 Deuterium labelling at ring A or ring E/F of sapogenins 47

Figure 2-9 Structure of the *m/z* 139 fragment ion of sapogenins 48

Figure 2-10 Deuterium-labelling reaction of sarsasapogenin at C-20 and C-23 48

Figure 2-11 Ratio (%) of non-deuterated (*m/z* 139), mono-deuterated (*m/z* 140), di-deuterated (*m/z* 141) and tri-deuterated (*m/z* 142) sarsasapogenins in the reaction mixtures after refluxing. A: 0-168 hours. B: expansion of the 0-8 hours region of A 51

Figure 2-12 Percentage *m/z* 139-142 ion contributions (%) of deuterated sarsasapogenin samples in buffer solutions and acetic acids at 37°C, or at room temperature 58

Chapter Three

Figure 3-1 Sheep used in the dosing experiments (trials) in July 1997 62

Figure 3-2 Levels of conjugated and free episarsasapogenin in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside 69

Figure 3-3 Levels of free and conjugated cholesterol and cholic acid identified in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside 71

Figure 3-4 Levels of free and conjugated fatty acids in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside 71

Figure 3-5 Proposed pathway for the metabolism of sarsasapogenin β-D-glucoside to episarsasapogenin β-D-glucuronide 73

Figure 3-6 Levels of conjugated episarsasapogenins in bile samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin 79

Figure 3-7 Proposed pathway for the ovine metabolism of sarsasapogenin to episarsasapogenin via 3-keto-26- and/or 16-conjugates 85

Figure 3-8 Proposed pathway for C-20 and C-23 deuterium loss during ovine metabolism 86

Figure 3-9 Percentage contributions of deuterated sapogenin in rumen, jejunum, faeces and bile samples from sheep 2, dosed with 20,23,23-D_3-sarsasapogenin 88

Figure 3-10 Levels of free and conjugated sapogenins in rumen samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 98

Figure 3-11 Levels of free and conjugated sapogenins in jejunum samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 99

Figure 3-12 Levels of free and conjugated sapogenins in faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 100

Figure 3-13 Levels of conjugated episarsasapogenins in bile samples from sheep 1 and 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin (data from Tables 3-16 and 3-17) 101

Figure 3-14 Total cholesterol levels in bile samples from sheep 1 and sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 102

Figure 3-15 Expected percentage contributions (A) and observed percentage contributions (B) for the *m/z* 139, 140, 141, 142 ions of epismilagenins in bile samples recovered from a sheep, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin (sample 1-5B) 104

Figure 3-16 Molecular weight changes in the conversion of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin to episarsasapogenins 108

Figure 3-17 Calculated *m/z* 139/140/141/142 ion contributions for a 1:1 mixture of sarsasapogenin and a metabolised specimen of 20,23,23-D_3-sarsasapogenin, exhibiting *m/z* 139, 140, 141 and 142 ion responses in the ratio 19:69:11:1 109

Figure 3-18 Contributions to episarsasapogenins from sarsasapogenin β-D-glucoside (sar-glu) and 20,23,23-D_3-sarsasapogenin (D-sar) in the bile samples from sheep 1 (**A**) and sheep 2 (**B**) 114

Figure 3-19 Diagrammatic representation of *m/z* 139-142 ion ratios, and proposed metabolic pathways, for free and conjugated sapogenins and saponin during ovine ruminal metabolism 126

Figure 3-20 Non-D, D-, D_2- and D_3-sapogenin (free form) and saponin (conjugated form) contributions to selected rumen, jejunum, faeces and bile samples 127

Figure 3-21 Proposed structures of *m/z* 139, 284, 285, 344 and 345 fragment ions appearing in the mass spectra of smilagenin acetate and its 3β-D-analogue 130

Figure 3-22 Levels of free and conjugated sapogenins in rumen samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 133

Figure 3-23 Levels of free and conjugated sapogenins in jejunum samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 134

Figure 3-24 Levels of free and conjugated sapogenins in faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 135

Figure 3-25 Conversion of conjugated 3β-D-epismilagenin to epismilagenin and smilagenin and their conjugates 135

Figure 3-26 Percentage contributions (%) of free and conjugated smilagenins and epismilagenins in rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 138

Figure 3-27 Levels of conjugated epismilagenins in bile samples from sheep 3 and 4, dosed with 3β-D-epismilagenin β-D-glucoside 140

Figure 3-28 Levels and *m/z* 285/284 ion ratios of epismilagenin conjugates identified in bile samples from sheep 3 (A) and sheep 4 (B), dosed with 3β-D-epismilagenin β-D-glucoside 141

Figure 3-29 Levels of conjugated cholesterols in the bile samples from sheep 3 (A) and sheep 4 (B), dosed with 3β-D-epismilagenin β-D-glucoside 143

Figure 3-30 Ratios of *m/z* 285/284 ions in the free and conjugated epismilagenins in rumen samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 144

Figure 3-31 Proposed metabolic pools (conjugated pool and free pool) in the rumen of sheep 145

Figure 3-32 Time variation of *m/z* 285/284 ion ratios for free and conjugated epismilagenins in jejunum samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 146

Figure 3-33 Time variation of *m/z* 285/284 ion ratios for free and conjugated epismilagenins in faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 146

Figure 3-34 Ratios of *m/z* 285/284 ions in free and conjugated epismilagenins identified in extracts from bile, rumen and jejunum samples of sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 147

Figure 3-35 Plots of log *m/z* 285/284 ion ratio versus time (0-96 hours) determined for rumen, jejunum and bile samples 149

Figure 3-36 Plots of log *m/z* 285/284 ion ratio versus time (1-24 hours) determined for rumen, jejunum and bile samples, with exponential curve fitting of the form $y = a * 10^{-bx}$ 150

Figure 3-37 Enterohepatic circulation in sheep as related to bilirubin excretion (from Kaneko and Cornelius, 1970) 152

Figure 3-38 The process of active absorption of conjugated bile acids (from Davenport, 1978) 152

Chapter Four

Figure 4-1 Structures of narthecin, xylosin and narthecioside, as reported by Ceh and Hauge (1981) 155

Figure 4-2 Structures of saponins isolated from *N. asiaticum* by Kobayashi et al. (1993) and Inoue et al. (1995) 156

Figure 4-3 Structures of faroecin (**4-22**), asiaticioside (**4-23**), narthecioside (**4-24**) and 22-*O*-methyl-narthecioside (**4-17**) isolated from Norwegian *N. ossifragum* 159

Figure 4-4 Selected ROESY (^1H-^1H) and HMBC (^1H-^{13}C) correlations observed for faroecin (**4-22**) 165

Figure 4-5 Selected ROESY (^1H-^1H) and HMBC (^1H-^{13}C) correlations observed for narthecioside (**4-24**) 168

Figure 4-6 Selected ROESY (^1H-^1H) and HMBC (^1H-^{13}C) correlations observed for asiaticioside (**4-23**) 170

Figure 4-7 Structures of dichotomin (Munday et al., 1993b) and coloratin (Wilkins, pers commun) 173

Figure 4-8 Selected ROESY (^1H-^1H) and HMBC (^1H-^{13}C) correlations observed for saponin **4-25** 178

Figure 4-9 Temperature dependency of the chemical shifts of selected ^1H NMR signals of **4-25** 180

Figure 4-10 Structure of saponin **4-26** isolated from *P. miliaceum* 182

Figure 4-11 Structures of saponins isolated from *N. recurvata* by Takaashi et al. (1995) and Mimaki et al. (1996) 188

Figure 4-12 Structures of ruscogenin (**4-11**), neoruscogenin (**4-12**), $\Delta^{25(27)}$-ruscogenin (**4-40**), tigogenin (**4-7**), neotigogenin (**4-8**), gitogenin (**4-9**) and neogitogenin (**4-10**) 189

Figure 4-13 Selected HMBC (^{1}H-^{13}C) correlations observed for $\Delta^{25(27)}$-ruscogenin 192

Figure 4-14 Structures of saponin (**4-41**), saponin (**4-42**) and saponin (**4-43**) characterised from *N. texana* 197

Figure 4-15 ^{1}H NMR spectra of fraction Ic determined at 27°C and 45°C 199

Figure 4-16 Correlations of HMBC (^{1}H-^{13}C) and ROESY (^{1}H-^{1}H) observed for spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-fucopyranoside (**4-41**) 200

Figure 4-17 GC-MS *m/z* 139, 344 and 282 ion profiles of determined for sapogenins in the hydrolysed conjugated extract of an Iranian collection of *T. terrestris* (sample 4) 206

Figure 4-18 Levels of conjugated sapogenins in *B. decumbens* samples from the research plots in PNG 217

Figure 4-19 Levels of conjugated sterols in *B. decumbens* samples from the research plots in PNG 218

LIST OF TABLES

Chapter Two

Table 2-1 NMR data (^{1}H, ^{13}C, COSY, and NOE correlations) determined for 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**2-4**) (ppm in $CDCl_3$) 31

Table 2-2 ^{1}H and ^{13}C NMR assignments and some COSY correlations determined for sarsasapogenin β-D-glucoside tetraacetate (**2-5**) (ppm in $CDCl_3$) 33

Table 2-3 Selected HMBC correlations observed for sarsasapogenin β-D-glucoside (**2-2**) (ppm in DMSO-D_6) 35

Table 2-4 ^{1}H and ^{13}C NMR assignments and selected COSY correlations determined for sarsasapogenin β-D-glucoside (**2-2**) (ppm in DMSO-D_6) 36

Table 2-5 ^{13}C NMR assignments for smilagenin (**2-9**), smilagenone (**2-10**), 3β-D-epismilagenin (**2-11**) and epismilagenin (**2-14**) (ppm in $CDCl_3$) 41

Table 2-6 ^{1}H and ^{13}C NMR assignments and selected COSY correlations determined for 3β-D-epismilagenin-β-D-glucoside tetraacetate (**2-12**) (ppm in $CDCl_3$) 43

Table 2-7 Selected HMBC correlations observed for 3β-D-epismilagenin β-D-glucoside (**2-8**) (ppm in DMSO-D_6) 45

Table 2-8 ^{1}H and ^{13}C NMR assignments and COSY correlations determined for 3β-D-epismilagenin β-D-glucoside (**2-8**) (ppm in DMSO-D_6) 46

Table 2-9 Ratios (%) of non-deuterated, mono-deuterated, di-deuterated and tri-deuterated sarsasapogenins in the reaction mixtures after refluxing 50

Table 2-10 Percentage *m/z* 139-142 ion contributions (%) of deuterated sarsasapogenins in buffer solutions and acetic acids at 37°C, or at room temperature 56

Chapter Three

Table 3-1	Parameters of the sheep dosing experiments	63
Table 3-2	Levels (mg/kg) of free and conjugated saponins, sterols and fatty acids in a freeze-dried rumen sample from sheep A, 30 hours after dosing with sarsasapogenin β-D-glucoside	66
Table 3-3	Levels (mg/kg) of free and conjugated sapogenins, steroids and fatty acids in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside	67
Table 3-4	Ion ratio data for episarsasapogenin acetates detected in acetylated bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside	70
Table 3-5	Wet weight, freeze-dried weight, and percentage (%) dry matter content of rumen, jejunum and faeces samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin	76
Table 3-6	Levels (mg/kg) of conjugated sapogenins in bile samples from sheep 2, dosed with 20,23,23-D_3-sarsasapogenin	77
Table 3-7	Levels (mg/kg) of conjugated sapogenins in bile samples from sheep 3, dosed with 20,23,23-D_3-sarsasapogenin	77
Table 3-8	Levels (mg/kg) of free and conjugated sapogenins in rumen, jejunum and faeces samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin	78
Table 3-9	Percentages (%) of conjugated episarsasapogenin (*m/z* 139), D-episarsasa-pogenin (*m/z* 140), D_2-episarsasapogenin (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) identified in bile samples from sheep 2, dosed with 20,23,23-D_3-sarsasapogenin	80
Table 3-10	Percentages (%) of conjugated episarsasapogenin (*m/z* 139), D-episarsasa-pogenin (*m/z* 140), D_2-episarsasapogenin (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) identified in bile samples from sheep 3, dosed with 20,23,23-D_3-sarsasapogenin	81
Table 3-11	Percentage contributions (%) of episarsasapogenin (*m/z* 139), D-episarsasapogenin (*m/z* 140), D_2-episarsasapogenin	83
Table 3-12	Wet weight, freeze-dried weight and percentages (%) of dry matter contents of rumen, jejunum and faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin	92

Table 3-13 Levels (mg/kg, dry matter) of free and conjugated sapogenins in rumen samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 93

Table 3-14 Levels (mg/kg, dry matter) of free and conjugated sapogenins in jejunum samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 94

Table 3-15 Levels (mg/kg, dry matter) of free and conjugated sapogenins in faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 95

Table 3-16 Levels (mg/kg) of free and conjugated sapogenins and cholesterols in bile samples from sheep 1, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 96

Table 3-17 Levels (mg/kg) of free and conjugated sapogenins and cholesterols in bile samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 97

Table 3-18 The percentage contributions (%) of non-D (*m/z* 139), D- (*m/z* 140), D_2- (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) determined in bile samples from sheep 1, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 105

Table 3-19 The percentage contributions (%) of non-D (*m/z* 139), D- (*m/z* 140), D_2- (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) determined in bile samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 105

Table 3-20 Contributions (mg/kg) to episarsasapogenins from sarsasapogenin β-D-glucoside (Y) and 20,23,23-D_3-sarsasapogenin (X) and the ratio ($R_{(Y)140/139}$) in bile samples from sheep 1 and 2 determined using *m/z* 141 data from Trial 4 (sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin) (Method 1) 112

Table 3-21 Normalised contributions (Method 1) of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin to episarsasapogenin conjugates identified in bile samples from sheep 1 and 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin 116

Table 3-22	Contributions (mg/kg) to bile episarsasapogenins from sarsasapogenin β-D-glucoside (Y) and 20,23,23-D_3-sarsasapogenin (X) in bile samples from sheep 1 and 2 determined using *m/z* 139/140 ion ratio data from Trial 4 (sheep 2 and 3) (Method 2)	119
Table 3-23	Normalised contributions (Method 2) of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin to episarsasapogenin conjugates identified in bile samples from sheep 1 and 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin	120
Table 3-24	Percentages (%) of free and conjugated sapogenins in rumen samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin	122
Table 3-25	Percentages (%) of free and conjugated sapogenins in jejunum samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin	123
Table 3-26	Percentages (%) of free and conjugated sapogenins in faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin	124
Table 3-27	Wet weight, dried weight and % dry matter contents of rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside	131
Table 3-28	Levels (mg/kg, dry matter) of smilagenins, epismilagenins and smilagenones and *m/z* 285/284 ion ratios of epismilagenins in rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside	132
Table 3-29	Percentage contributions (%) of free and conjugated smilagenins and epismilagenins identified in rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside	136
Table 3-30	Levels (mg/kg) of free and conjugated epismilagenins and *m/z* 285/284 and *m/z* 345/344 ion ratios of conjugated epismilagenin in bile samples from sheep 3, dosed with 3β-D-epismilagenin β-D-glucoside	139
Table 3-31	Levels (mg/kg) of free and conjugated epismilagenins and *m/z* 285/284 and *m/z* 345/344 ion ratios of conjugated epismilagenin	

in bile samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside 139

Table 3-32 Levels (mg/kg) of free and conjugated cholesterols in bile samples from sheep 3 and 4, dosed with 3β-D-epismilagenin β-D-glucoside 142

Chapter Four

Table 4-1 Levels (mg/kg) of free and conjugated sapogenins and sterols identified in Norwegian *N. ossifragum* samples 157

Table 4-2 ^{13}C NMR assignments (ppm in C_5D_5N) for the aglycone atoms of faroecin (**4-22**), asiaticin (**4-15**), asiaticioside (**4-23**), narthecin (**4-13**), narthecioside (**4-24**) and 22-*O*-methyl-narthecioside (**4-17**) 161

Table 4-3 ^{1}H and ^{13}C NMR assignments (ppm in C_5D_5N) for the glycoside atoms of faroecin (**4-22**), asiaticioside (**4-23**), narthecioside (**4-24**), 22-*O*-methyl narthecioside (**4-17**), narthecin (**4-13**) and asiaticin (**4-15**) 162

Table 4-4 Selected ROESY correlations observed for faroecin (**4-22**) (ppm in C_5D_5N) 165

Table 4-5 Selected HMBC correlations observed for faroecin (**4-22**) (ppm in C_5D_5N) 166

Table 4-6 Selected ROESY correlations observed for narthecioside (**4-24**) (ppm in C_5D_5N) 168

Table 4-7 Selected HMBC correlations observed for narthecioside (**4-24**) (ppm in C_5D_5N) 168

Table 4-8 Selected HOHAHA correlations observed for narthecioside (**4-24**) (ppm in C_5D_5N) 169

Table 4-9 Selected ROESY correlations observed for asiaticioside (**4-23**) (ppm in C_5D_5N) 171

Table 4-10 Selected HMBC correlations observed for asiaticioside (**4-23**) (ppm in C_5D_5N) 171

Table 4-11 Selected HOHAHA correlations observed for asiaticioside (**4-23**) (ppm in C_5D_5N) 171

Table 4-12 Levels (mg/kg) of sapogenins and sterols in the free extracts and the conjugated extracts from USA *P. virgatum* samples 174

Table 4-13 ^{13}C NMR chemical shifts (ppm in C_5D_5N) for the aglycone atoms of saponin **4-25** isolated from *P. virgatum* 176

Table 4-14	^{13}C and ^{1}H chemical shifts (ppm in C_5D_5N) for the glycoside atoms of saponin **4-25** isolated from *P. virgatum*	177
Table 4-15	Selected HMBC correlations observed for saponin **4-25** (ppm in C_5D_5N)	178
Table 4-16	Selected ROESY correlations observed for **4-25** (ppm in C_5D_5N)	179
Table 4-17	Levels (mg/kg) of sapogenins and sterols in the free and conjugated extracts from New Zealand *P. miliaceum* samples	182
Table 4-18	^{13}C NMR chemical shifts (ppm in C_5D_5N) for the aglycone atoms of saponins **4-25** and **4-26** isolated from *P. miliaceum*	184
Table 4-19	^{13}C and ^{1}H chemical shifts (ppm in C_5D_5N) for the glycoside atoms of saponins **4-25** and **4-26** isolated from *P. miliaceum*	185
Table 4-20	Selected HMBC correlations observed for $\Delta^{25(27)}$-ruscogenin (**4-40**) (ppm in $CDCl_3$)	193
Table 4-21	^{13}C NMR spectral data of ruscogenin (**4-11**), neoruscogenin (**4-12**) and $\Delta^{25(27)}$-ruscogenin (**4-40**) determined in C_5D_5N and $CDCl_3$ (ppm)	194
Table 4-22	Levels (mg/kg) of free and conjugated sapogenins and sterols in the leaves, stems, blossoms of USA *N. texana*	195
Table 4-23	Selected HMBC correlations observed for saponin **4-41** (ppm in C_5D_5N)	200
Table 4-24	^{1}H and ^{13}C NMR assignments (ppm in C_5D_5N) for spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-fucopyranoside (**4-41**), spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-α-L-arabinopyranoside (**4-42**) and spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-galactopyranoside (**4-43**)	202
Table 4-25	Selected HMBC correlations observed for saponin **4-42** (ppm in C_5D_5N)	203
Table 4-26	Levels (mg/kg) of sapogenins and sterols in the free and conjugated extracts from Iranian *T. terrestris* samples	207
Table 4-27	Levels (mg/kg) of sapogenins and sterols in the free extracts and conjugated extracts from PNG *B. decumbens* samples	212

ABBREVIATIONS

%	percent
α	lower face
β	upper face
δ	chemical shift
μg	micrograms
μL	microlitre
^{1}J	One bond NMR coupling constant
^{2}J	two bond NMR coupling constant
^{3}J	three bond NMR coupling constant
^{4}J	four bond NMR coupling constant
2D	two-dimensional
A. lecheguilla	*Agave lecheguilla*
Ac	acetate functional group
AR	analysis grade solvent
br	broad
B. decumbens	*Brachiaria decumbens*
BIRD	birotational decoupler
°C	degrees Celsius
C_5D_5N	deuteropyridine
ca	approximately
camp	campesterol
$CDCl_3$	deuterochloroform
cf.	compared to
chol	cholesterol
cm	centimetre
conj	conjugated fraction
COSY	correlated spectroscopy
d	doublet NMR signal
D	dextrorotatory
D1	repetition delay
Da	dalton
DEPT	distortionless enhancement by polarization transfer

dio	diosgenin
DM	dry matter
DMSO-d_6	deuterodimethylsulfoxide
DQF	double-quantum filtered
Dr	doctor
EI	electron impact ionization
episar	episarsasapogenin
epismil	epismilagenin
ES-MS	electrospray mass spectrometry
et al.	and others
Et	ethyl
eV	electron volts
F1	frequency dimension one
F2	frequency dimension two
FID	free induction decay
FT	Fourier transformation
fuc	fucose
g	grams
gal	galactose
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
gito	gitogenin
glu	glucose
h	hours
heco	hecogenin
HMBC	heteronuclear multiple bond correlation
HMQC	heteronuclear multiple quantum coherence
HOHAHA	Homonuclear Hartmann-Hahn
HPLC	high performance liquid chromatography
HSQC	heteronuclear single quantum coherence
Hz	hertz
id	internal diameter
J	coupling constant
kg	kilogram
L	Laevorotatory
L	litre
LB	line broading

lit	literature
M	molar
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
MC	mode of 2D transformation
Me	methyl
mg	milligrams
mg/kg	milligrams per kilogram
MHz	megahertz
min	minute
mL	millilitre
mm	millimetre
MS	mass spectrometer
msec	millisecond
MWt	molecular weight
N. asiaticum	*Narthecium asiaticum*
N. ossifragum	*Narthecium ossifragum*
N. microcarpa	*Nolina microcarpa*
N. recurvata	*Nolina recurvata*
N. texana	*Nolina texana*
neogito	neogitogenin
neoheco	neohecogenin
neorusco	neoruscogenin
neotigo	neotigogenin
NMR	nuclear magnetic resonance
NOE	nuclear overhauser effect
NOESY	nuclear overhauser effect spectroscopy
NZ	New Zealand
P	power mode
P. coloratum	*Panicum coloratum*
P. dichotomiflorum	*Panicum dichotomiflorum*
P. miliaceum	*Panicum miliaceum*
P. schinzii	*Panicum schinzii*
P. virgatum	*Panicum virgatum*

pers commun	personal communication
PNG	Papua New Guinea
ppm	parts per million
Pr	propionate
Prof	Professor
q	quartet NMR signal
r	radius
R_F	response factor
R_f	radio frequency
rham	rhamnose
RI	refractive index
RMS	root mean square
ROESY	rotating frame Overhauser effect spectroscopy
RT	room temperature
rusco	ruscogenin
s	singlet NMR signal
sar	sarsasapogenin
sec	second
SIM	selected ion mode
sito	β-sitosterol
smil	smilagenin
spp	species
SSB	shifted sinebell value
Std	standard
stig	stigmasterol
SW	sweep width
T	temperature
t	triplet NMR signal
T. terrestris	*Tribulus terrestris*
TIC	total ion chromatogram
tigo	tigogenin
TLC	thin layer chromatography
TMS	tetramethylsilicane
TOCSY	total correlation spectroscopy
U.S.A.	United States of America
UV	ultra violet
V	volts

W	phase-sensitive mode
WDW	window type
Wt	weight
XHCORR	heteronuclear shift correlation spectroscopy
xyl	xylose
yam	yamogenin

Chapter One

INTRODUCTION

1.1 Photosensitization Diseases of Livestock

1.1.1 Introduction

Photosensitization diseases of animals are characterised by swelling, blistering and skin lesions on the face, ears, and other areas exposed to sunlight. Other visible symptoms include heat stress, loss of appetite, and debilitation. Depending on the severity of photosensitization, death may result.

Periodic outbreaks of photosensitization diseases are of great economic importance throughout the world. For example, the photosensitization disease known as geeldikopp, associated with the grazing of *Tribulus terrestris*, has been reported to kill as many as 500,000 sheep in a bad season in South Africa (Kellerman et al., 1994; Coetzer et al., 1983), while Tapia et al. (1994) have reported an outbreak of photosensitization affecting 40% of a flock of sheep grazing *Tribulus terrestris* in southern Buenos Aires province, Argentina.

In some years up to 50% of some flocks of Norwegian lambs suffer from alveld, a photosensitization disease associated with the grazing of *Narthecium ossifragum* (Flåøyen, 1996). In Australia, four outbreaks of photosensitization occurred in weaned lambs grazing *Panicum schinzii* in north eastern Victoria during the summers of 1985 and 1986, with attack rates of 7% to 43% and fatality rates of 60% to 71% (Button et al., 1987). Two outbreaks of photosensitivity disease, believed to be associated with the grazing of *T. terrestris*, were also noticed in weaned sheep in south-western New South Wales during early autumn 1982. In these outbreaks the prevalence rates for clinically diagnosable cases were 21% and 37%, while fatality rates approached 70% (Glastonbury et al., 1984)

Photosensitization outbreaks have also been frequently observed in cattle grazing *Brachiaria decumbens* in Brazil (Lemos et al., 1997). For example, an outbreak of photosensitization disease in cattle was found in the province of Mato Grosso do Sul in

1997. In a herd of 1600 Nelore calves, 70 were affected and 30 died 15 days after being introduced on to a pasture of *B. decumbens*.

Outbreaks of facial eczema disease, a mycotoxic hepatogenous photosensitization of ruminants, amongst New Zealand sheep, cattle and deer have been variously estimated to cause annual production losses ranging from NZ$30 million (Smith and Towers, 1985) for all species (1981 costs), to NZ$69 million (1989 costs) for the sheep industry alone (Anonymous, 1990). Photosensitization has also been noticed in sheep grazing *Panicum miliaceum* (Clare, 1955), and in sheep, goats and cattle grazing *Panicum dichotomiflorum* (Holland et al., 1991).

1.1.2 Types of Photosensitizations

Photosensitization of livestock is classified as either primary or secondary photosensitization (Camp et al., 1988).

Primary photosensitization

In primary photosensitization, the photodynamic agent is present in the food of the animal, and is absorbed unchanged by the animal. The toxic agent enters the circulatory system. When it reaches the skin, it may absorb sunlight, transferring the absorbed energy to other molecules which react with skin and surrounding tissue to cause pain and skin lesions. Plants that contain primary photosensitizers include *Hypericum perforatum* (St John's wort), *Fagopyrum esculentum* (buckwheat), *Cymopterus watsoni* (spring parsley), and *Ammi majus* (bishop's weed) (Camp et al., 1988; Familton, 1990).

Secondary (hepatogenous) photosensitization

In secondary (or hepatogenous) photosensitization, ingested toxins cause liver damage. This liver damage prevents excretion of phylloerythrin, a photosensitizing porphyrin pigment resulting from the intraruminal breakdown of chlorophyll. Consequently, the concentration of phylloerythrin in the blood increases, and when this compound reaches the skin it absorbs sunlight and transfers the absorbed energy to other molecules and surrounding tissue to cause skin lesions (Camp et al., 1988; Familton, 1990). Saponins are a group of naturally occurring molecules (see Section 1.3) implicated in hepatogenous photosensitization outbreaks.

Examples of saponin-associated hepatogenous photosensitization diseases of ruminants include *Agave lecheguilla* toxicity in USA (Mathews, 1937; Wall et al., 1962; Camp et al., 1988), *B. decumbens* toxicity in Australia, Malaysia, Indonesia, Nigeria, Brazil,

Papua New Guinea (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), alveld in Norway (caused by *N. ossifragum*) (Ender, 1955; Flåøyen, 1993; Flåøyen et al., 1994), sacahuiste poisoning (swellhead or fevered) in south-western United States and northern Mexico (*Nolina texana*) (Tunnicliff, 1929; Mathews, 1937; Mathews, 1940; Hershey, 1945; Rankins et al., 1986; Rankins et al., 1993), *Panicum* toxicity in New Zealand and Australia (*Panicum* spp) (Clare, 1952; Holland et al., 1991; Miles et al., 1991), dikoor in South Africa (*Panicum* spp.) (Kellerman et al., 1980), and geeldikopp in South Africa (*T. terrestris*) (Kellerman et al., 1980; Coetzer et al., 1983).

Other plants implicated in hepatogenous photosensitization diseases of ruminants include *Brassica napus*, *Kocia scoparia*, *Lantana* spp, *Medicago* spp, *Senecio* spp, *Tetradymia glabrata* and *T. canescens* (Clare, 1955).

The most common form of secondary photosensitization, facial eczema, experienced by New Zealand ruminants arises from the production of the mycotoxin sporidesmin by the saprophytic fungus *Pithomyces chartarum* (Mortimer et al., 1978; Munday et al., 1993a). Elevated levels of this fungus are periodically found on northern New Zealand pastures during autumn, and on the pastures of some other warm temperate countries. The external clinical symptoms of sporidesmin-induced photosensitization are similar to those of saponin-associated photosensitization diseases.

1.2 Biliary Crystals

Pathological examination of animals affected by some plant-associated photosensitization diseases reveals severe liver damage, and birefringent crystals may be present in the bile ducts (Mathews, 1940; Glastonbury et al., 1984; Glastonbury et al., 1985; Kellerman et al., 1980; Kellerman et al., 1991; Coetzer et al., 1983; Button et al., 1987; Collett and Spickett, 1989; Graydon et al., 1991; Tapia et al., 1994; Lemos et al., 1997). It is not known, however, if the crystalloid material is the primary cause of the liver lesions, or a secondary response to some other factor.

Several researchers have suggested that plant saponins are the causative agents of these hepatotoxic photosensitization diseases (Mathews, 1937; Henrici, 1952; Ender, 1955; Ceh and Hauge, 1981; Abdelkader et al., 1984), although others have debated whether

saponins or sapogenins are the sole cause of hepatogenous photosensitization diseases (Smith and Miles, 1993; Flåøyen et al., 1991; Flåøyen, 1993).

An early view was that because apparently identical birefringent crystalloid material had been observed in hepatogenously photosensitized sheep and goats grazing five plant species from three different families [*Agave lecheguilla* (Mathews, 1937), *Nolina texana* (Mathews, 1940), *Tribulus terrestris* (Henrici, 1952), *Panicum* spp (Clare, 1952) or *Narthecium ossifragum* (Ender, 1955)], a botanical origin for the biliary crystalline material seemed less likely than a common disturbance of bile acid metabolism (Griem et al., 1975).

The composition of the biliary crystals was not known until 1991. Prior to 1991 it was known that the crystals were not composed of common bile compounds, such as cholesterol, cholic acid or sodium glycocholate (Coetzer et al., 1983).

In 1988, Camp et al. concluded that the biliary crystalloid material from a sheep photosensitized by dosing of *A. lecheguilla* contained smilagenin (**1-1**). Three years later Lancaster et al. (1991) also reported that the mass spectrum (MS) of an extract of the biliary crystals recovered from photosensitized Australian lambs grazing *P. schinzii* was similar to that of smilagenin (**1-1**).

Holland et al. (1991) showed, using ^{1}H and ^{13}C NMR spectroscopy, that the sapogenin obtained by acid hydrolysis of the biliary crystalline material recovered from a New Zealand sheep affected by *P. dichotomiflorum* toxicosis was epismilagenin (**1-2**) [(25*R*)-5β-spirostan-3α-ol].

Epismilagenin (**1-2**) was observed to have a TLC R_F and a mass spectrum similar to those of smilagenin (**1-1**) [(25*R*)-5β-spirostan-3β-ol]. These compounds differ only in their configuration at C-3 (see Figure 1-1), thereby accounting for the difficulty in differentiating **1-1** from **1-2** by TLC or MS alone.

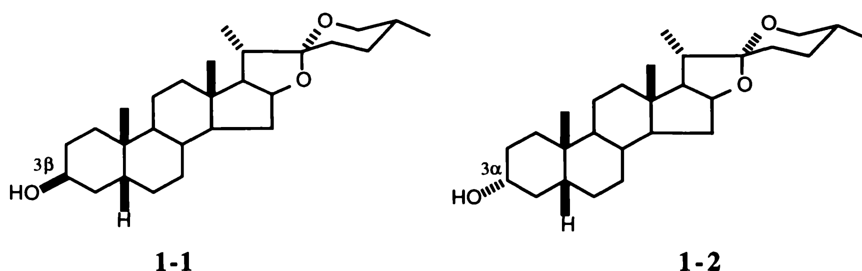


Figure 1-1. Structures of smilagenin (**1-1**) and epismilagenin (**1-2**).

Further mass spectral analysis of the crystalline material isolated by Lancaster et al. (1991) showed it to be comprised mainly of the insoluble salts of epismilagenin glycuronide (probably the glucuronide) (Miles et al., 1992a) rather than free smilagenin as original proposed (Lancaster et al., 1991).

Subsequently, insoluble calcium salts of the β-D-glucuronides of epismilagenin (**1-3**) and/or episarsasapogenin (**1-4**) (the 25*S*-isomer of **1-3**) (Figure 1-2) were identified in the biliary crystalloid material recovered from livestock grazing *P. dichotomiflorum* in New Zealand (Miles et al., 1992b), *P. schinzii* (*Panicum* toxicosis) in Australia (Miles et al., 1992a) and *T. terrestris* (geeldikopp) in South Africa (Miles et al., 1994a). Insoluble salts of the β-D-glucuronides of episarsasapogenin and epismilagenin were also identified in the bile of lambs grazing *N. ossifragum* (alveld) in Norway (Miles et al., 1993).

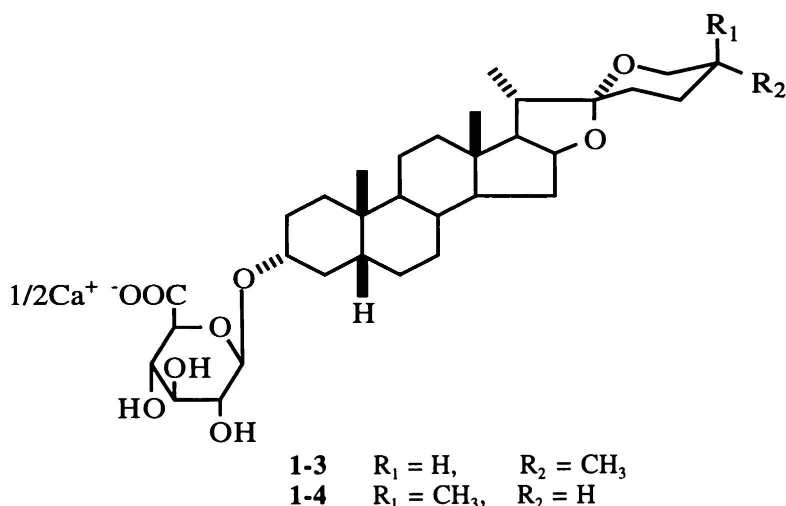


Figure 1-2. Structures of calcium salts of the β-D-glucuronides of epismilagenin (**1-3**) and episarsasapogenin (**1-4**).

Careful comparison of the mass spectra, reported by Camp et al. (1988) for a specimen of smilagenin (**1-1**) isolated from *A. lecheguilla,* and the crystalline material recovered from the sheep dosed with *A. lecheguilla*, reveals differences in the intensities of the *m/z* 273 and 255 fragment ions which appear to be consistent with the identification of the crystalline material as epismilagenin (**1-2**). Blunden et al. (1980) have reported that the ratios of mass spectral fragment ions (in the region *m/z* 255 to *m/z* 344) can be used to identify the C-3, C-5 and C-25 configurations of spirostanol isomers.

Wilkins et al. (1994) have reported a selected ion mode GC-MS procedure for the identification and quantification of acetylated sapogenins (including hydrolysed sapogenins derived from saponins) in plant and animal samples. This procedure made it

possible to undertake detailed studies of the metabolism of saponins and sapogenins in ruminants, a knowledge of which is required to define the metabolic processes leading to the formation and deposition of episapogenins in biliary crystalloid material. The GC-MS methodology of Wilkins et al. (1994) was also employed to show that although hepatogenous photosensitizations of sheep are associated with five species from three different families of plants (*N. ossifragum*, *P. dichotomiflorum*, *P. schinzii*, *T. terrestris* and *P. miliaceum*), biliary crystalline materials from photosensitized sheep were, in each case, insoluble calcium salts of epismilagenin and/or episarsasapogenin β-D-glucuronides (**1-3** and/or **1-4**) (Miles et al., 1991; Miles et al., 1992b; Miles et al., 1993; Miles et al., 1994a; Miles et al., 1994b; Wilkins et al., 1994).

The demonstration that biliary crystalloid deposits are insoluble salts of epismilagenin and/or episarsasapogenin β-D-glucuronides shows that plant saponins have a significant role in hepatogenous photosensitization diseases.

1.3 Saponins

Saponins are a naturally occurring compounds distributed in a wide variety of foods, forage plants and a few marine animals. Saponins (= soap forming compounds) derive their class name from their characteristically strong foaming power in aqueous solutions.

1.3.1 Chemical Structures

Saponins are composed of two parts, a carbohydrate part (sugar) and a non-carbohydrate (genin) part (Cheeke, 1971). The carbohydrate part is made up of sugar units, linked together either in linear, or branched, chains. The non-carbohydrate part may be either steroidal or triterpenoid in nature (Figure 1-3).

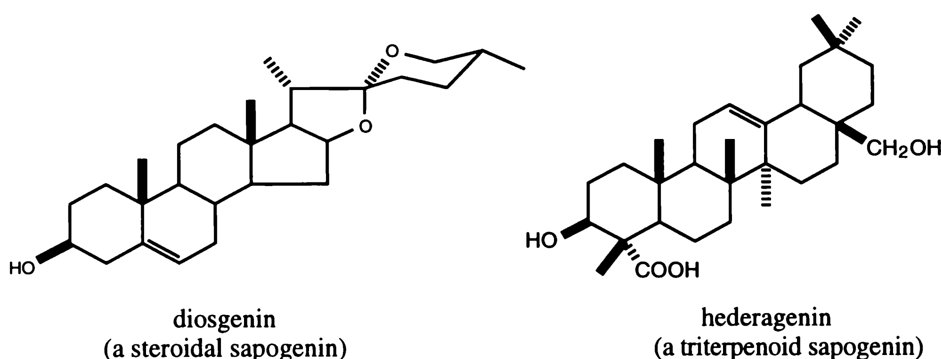


Figure 1-3. Structures of diosgenin (**1-5**) (a steroidal sapogenin) and hederagenin (**1-6**) (a triterpenoid sapogenin).

Steroidal sapogenins are typically 27-carbon spirostanes 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). A hydroxyl group is present at C-3 in most sapogenins. Tigogenin (**1-7**) and sarsasapogenin (**1-8**) are typical examples of 25*R*-5α- and 25*S*-5β-sapogenins respectively (Figure 1-4).

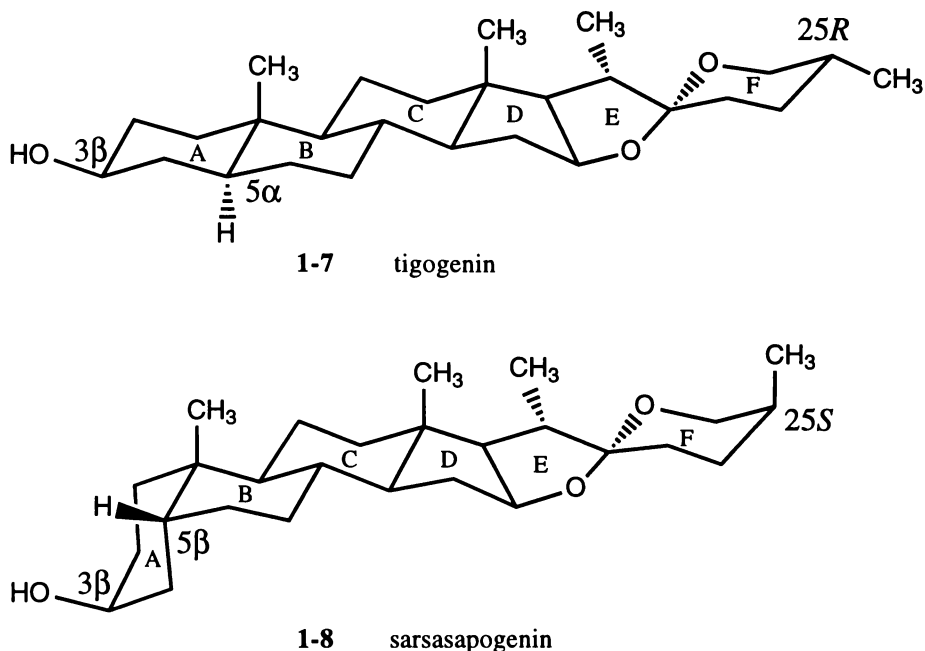


Figure 1-4. Structures of tigogenin (**1-7**) and sarsasapogenin (**1-8**).

The presence of a 5α-proton (H-5α) results in a *trans* configuration across the ring A/B junction and an equatorial orientation for a 3β-OH group, such as is the case for tigogenin (**1-7**). On the other hand, the presence of a 5β-proton (H-5β) results in a *cis* configuration across the ring A/B junction and an axial orientation for a 3β-OH group (with respect to ring A), such as is the case for sarsasapogenin (**1-8**). The absolute configuration of C-25 in tigogenin (**1-7**), in which the C-25 methyl group is equatorially oriented, is *R*, while the absolute configuration of C-25 in sarsasapogenin (**1-8**), in which the C-25 methyl group is axially oriented, is *S* (see Figure 1-4).

Steroidal sapogenins are typically hexacyclic (rings A-F) (spirostanol type), or pentacyclic (rings A-E) (furostanol type). Furostanol saponins are open ring F analogues of spirostanol saponins, and often have a glucosyl group attached to 26-OH of the genin (Figure 1-5) (Munday et al., 1993b; Wilkins et al., 1996; Meagher et al., 1996).

Sapogenins are biosynthesised by plants from cholesterol (Heftmann, 1967; Heftmann, 1968) by the way of intermediates in which the sterol-type side chain is kept open (protected) by attachment of a glycosyl residue (Bennett et al., 1970). Upon hydrolysis, furostanol 26-glycosides afford the corresponding closed ring F spirostanol isomers. The co-occurrence of spirostanol and furostanol saponins in plants associated with outbreaks of hepatogenous photosensitization diseases has been reported (Miles et al., 1993; Munday et al., 1993b; Meagher et al., 1996; Wilkins et al., 1996).

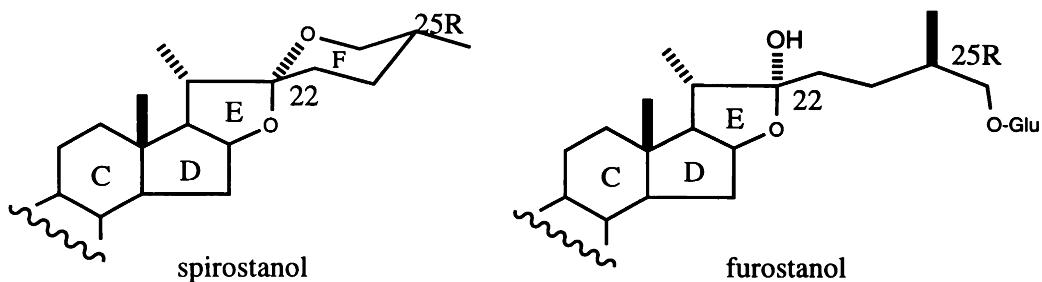


Figure 1-5. Partial structures of spirostanol and furostanol saponins.

In plants, sapogenins often occur in the form of their glycosides, i.e. as saponins. Typically the C-3 hydroxyl group is glycosylated, however saponins have been identified that glycosyl units attached to other positions in the genin, for example, at C-1 (Wilkins et al., 1996; Takaashi et al., 1995; Miamki et al., 1996), or C-23 (Takaashi et al., 1995).

The carbohydrate portion of a saponin may consist of one or more glycosyl units. Often saponins from a particular plant differ only in the number of glycosyl residues in the saponin, and in their point(s) of attachment. The sugars (glycosyl residues) frequently encountered in saponins include glucose, galactose, rhamnose, xylose, arabinose, and fucose (see Figure 1-6) (Stabursvik, 1959; Robinson, 1963; Ceh and Hauge, 1981; Agrawal et al., 1985; Munday et al., 1993b; Meagher et al., 1996; Wilkins et al., 1996).

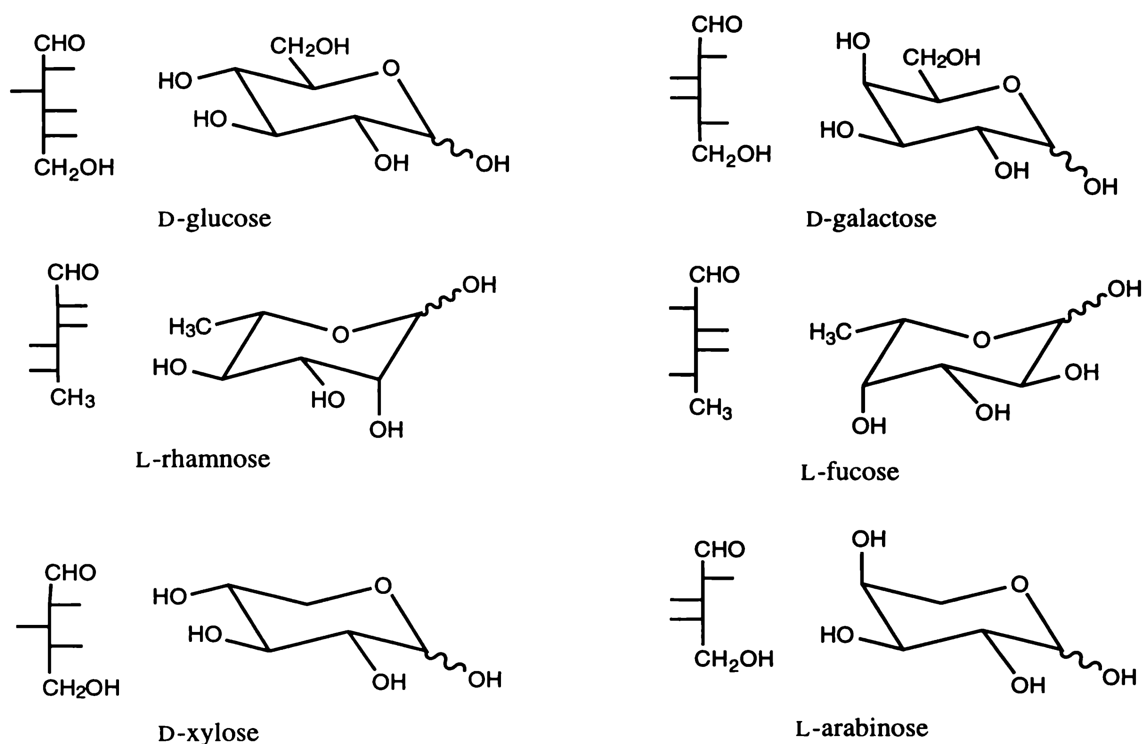


Figure 1-6. Structures of some sugars often found in saponins.

The NMR spectra of steroidal sapogenins and saponins have been reviewed on several occasions by Agrawal et al. (1985, 1995) and Agrawal (1992).

1.3.2 Biological Characteristics

1.2.3.1 General

Saponins possess a number of biological activities towards human and animals. Saponins have been shown to be haemolytic, and may be toxic if given intravenously, but not orally (Sollman, 1957). Ewart (1931) determined the relative susceptibility of many species toward saponin induced hemolysis as follows:

guinea pig ≅ horse > dog ≅ rat ≅ rabbit > man ≅ pig > sheep ≅ goat ≅ cattle.

Saponins have been investigated as ruminant bloat promoters due to their foaming properties and their presence in forage plants (Lindahl et al., 1957). Ingested saponins have also been observed to influence animal performance and metabolism in many ways (other than hemolysis and bloat), including reduced growth, inhibition of smooth muscle

activity, enzyme inhibition, changes in blood and liver cholesterol levels, and reduced nutrient absorption (Lindahl et al., 1957).

The deadly effect of saponins on cold blooded animals, especially fish and snails is well documented in Australian history due to their use by the Aboriginal culture as fish poisons (Milgate and Roberts, 1995). Saponins have also been found to inhibit the growth of mould and their presence in the shoots and bark of trees reinforces the view that they help protect the plant from insect attack (Milgate and Roberts, 1995).

1.3.2.2 Role of Saponins in Photosensitizations

In 1937, Mathews suggested that plant saponins may cause photosensitization disease. Mathews based his proposal on his findings that rats fed alcoholic extracts from *A. lecheguilla* were photosensitized and that the extracts used in the feeding experiment were shown to contain a saponin-like substance. More recently Allison (1991) has suggested that saponins might be implicated in the development of disease outbreaks amongst animal grazing *Nolina* species.

Mathews' view that saponins and/or sapogenins were implicated in photosensitization outbreaks was supported by the observation of Henrici (1952) that higher levels of sapogenins occurred in *T. terrestris* from areas where crystal-associated photosensitization (geeldikopp) occurred among South African sheep, while lower levels were present in *T. terrestris* specimens from areas where the disease was not seen. Enslin and Well (1956) isolated crude saponins, in 0.5-2.0% yield dry mass, from South African *T. terrestris*. Subsequently, De Kock and Enslin (1958) characterised four sapogenins from crude *T. terrestris* extracts. Since dosing of 20 g of crude *T. terrestris* saponins failed to induce photosensitization in a South African sheep, Brown (1968) questioned the involvement of saponins in disease development.

Although not able to induce photosensitization in Norwegian lambs by dosing with *N. ossifragum*, Ender (1955) was able to induce photosensitization by feeding lambs large doses of crude saponins obtained from the plant. Abdelkadar et al. (1984) have also reported the induction of photosensitization of sheep by dosing crude saponin-containing extracts from *N. ossifragum.*

In New Zealand, crystal-associated hepatogenous photosensitization has been noticed in sheep grazing *P. miliaceum* (Clare, 1955), and in sheep, goats and cattle grazing *P. dichotomiflorum* (Holland et al., 1991).

Interest in the role saponins played in the development of photosensitization diseases rose when Camp et al. (1988) reported the isolation of a sapogenin from bile crystals recovered from a sheep fed *A. lecheguilla.*

The identification of the biliary crystals which characterised *Panicum* toxicoses, alveld and geeldikopp as insoluble salts of episapogenin glucuronides (Holland et al., 1991; Miles et al., 1991; Miles et al., 1992a; Miles et al., 1992b; Miles et al., 1993; Miles et al., 1994a; Miles et al., 1994b; Wilkins et al., 1994) showed that, even if they were not primary causative agents, saponins were implicated in the development of the pathology of hepatogenous photosensitization diseases by their contribution to the biliary crystalloid material.

It has been now well established that a number of plants world-wide are associated with occasional outbreaks of hepatogenous photosensitization of ruminants, including *A. lecheguilla* (Mathews, 1937; Wall et al., 1962; Camp et al., 1988), *B. decumbens* (Abdullah et al., 1992; Meagher et al., 1996). *N. ossifragum* (Ceh and Hauge, 1981; Abdelkader et al., 1984; Flåøyen et al., 1995; Flåøyen and Wilkins, 1997), *N. microcarpa* (Norris and Valentine, 1954; Samford, 1985; Abdullah et al., 1988; Allison, 1991; Rankins et al., 1993), *N. texana* (Mathews, 1937; Mathews, 1940; Norris and Valentine, 1954; Abdullah et al., 1988; Allison, 1991; Rankins et al., 1986; Rankins et al., 1993), *P. dichotomiflorum* (Holland et al., 1991; Miles et al., 1991; Miles et al., 1992b; Munday et al., 1993b), *P. miliaceum* (Miles et al., 1993), *P. schinzii* (Button et al., 1987; Miles et al., 1992a; Miles et al., 1992b), *T. terrestris* (Coetzer et al., 1983; Kellerman et al., 1980; Kellerman et al., 1991; Kellerman et al., 1994; Miles et al., 1994a; Miles et al., 1994b).

The occurrence of steroidal sapogenin and saponins in the plants implicated in photosensitization outbreaks is reviewed in Section 1.3.3.

1.4 Plants

Saponins are widely distributed among plants. They occur in the *Liliaceae* in *Yucca, Trillium, Chlorogalum, Smilax, Agave, Nolina, Agapanthus* and *Narthecium*, in the *Amaryllidaceae* in *Manfreda*; in the *Dioscoreaceae* in *Dioscorea*; in the *Scrophulariaceae* in *Digitalis*; in the *Solanaceae* in *Solanum, Lycopersicon,* and *Cestrum* (Miller, 1973; Skerman and Riveros, 1990), in the *Poaceae* in *Brachiaria* and *Panicum* (Skerman and Riveros, 1990), and in the *Zygophyllaceae* in *Tribulus* (Kellerman and Coetzer, 1984).

Agave lecheguilla

A. lecheguilla is a conspicuous, long-lived perennial from a thick fibrous toothed crown bearing a cluster of thick, fleshy basal leaves and a tall flower stalk (Mathews, 1937). This plant grows thickly over a wide area of south western Texas, USA where it is reported to cause photosensitization of sheep and goats (Mathews, 1937; Wall et al., 1962).

Smilagenin (**1-1**) has been reported to be the sole sapogenin constituent of this plant. (Wall et al., 1962; Camp et al., 1988). A steroidal sapogenin, tentatively identified as smilagenin (**1-1**), was identified as the genin component of crystals from the bile of sheep fed *A. lecheguilla* (Camp et al., 1988). However critical inspection of the published mass spectrum suggests the genin to be epismilagenin (**1-2**) (see Section 1.2).

Brachiaria decumbens

B. decumbens, commonly known as Signal grass (Australia), Suriname grass (Jamaica), or Kenya sheep grass, is an important species of tropical pasture grass due to its aggressive growth, efficient nitrogen utilisation, and ability to withstand heavy grazing, drought, pests and diseases. It is native to Africa, but is now widespread in the tropical and subtropical countries (Skerman and Riveros, 1989).

Photosensitization of sheep, goats and cattle grazing this plant occurs in many countries including Australia, Malaysia, Indonesia, Nigeria, Brazil, Papua New Guinea (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).

Diosgenin (**1-5**) and yamogenin (**1-9**) sapogenins and saponins have been reported to be present in *B. decumbens* (Smith and Miles, 1993; Wilkins et al., 1994), with much higher levels present in young plants than in mature plants (ca 5:1) (Wilkins et al., 1994). Analyses of grass samples collected from a South American pasture during a photosensitization outbreak revealed **1-5** and **1-9** to be present in the plant primarily in conjugated forms, and not free forms (Meagher et al., 1996). Berndt (1997) has found the dominant *B. decumbens* saponins to be the furostenol saponins, protodioscin (**1-10**) and protoyamoscin (**1-11**) (Figure 1-7).

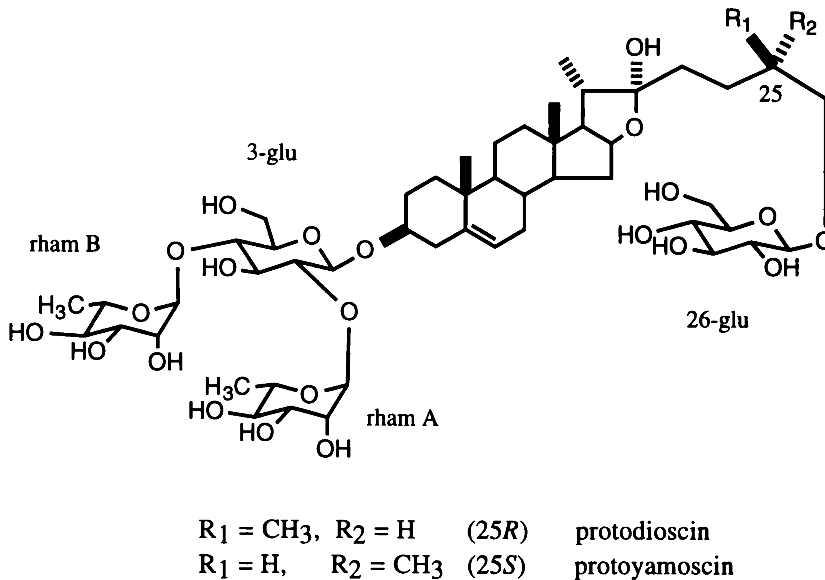


Figure 1-7. Structures of protodioscin (**1-10**) and protoyamoscin (**1-11**) (Berndt, 1997).

Episarsasapogenin (**1-4**) and epismilagenin (**1-2**) have been identified in the ruminal contents of a sheep suffering from *B. decumbens* intoxication (Lajis et al., 1993).

Narthecium ossifragum

N. ossifragum, the western European lily, is a loosely to densely clonal perennial herb with a creeping rhizome. The plant grows on oligotrophic, mesotrophic and eutrophic peat deposits in Scandinavia, the British Isles, the Netherlands, Belgium, north west Germany, western and central France, northern Spain and western Portugal (Summerfield, 1974). Alveld, a photosensitization of lambs grazing *N. ossifragum* has been reported from Norway (Ender, 1955; Ender, 1964; Flåøyen, 1993; Flåøyen et al., 1994). Ford (1964) has also reported the photosensitization of Scottish sheep grazing this plant.

The presence of sarsasapogenin glycosides in extracts of *N. ossifragum* was first demonstrated by Stabursvik (1954, 1959). Subsequently, Ceh and Hauge (1981) reported the occurrence in *N. ossifragum* extracts of two sapogenin trisaccharides, narthecin (**1-12**) and xylosin (**1-13**), and a sapogenin tetrasaccharide (**1-14**), which on acid hydrolysis afforded sarsasapogenin (**1-8**) and glucose, galactose and arabinose, or glucose, galactose and xylose, respectively. Miles et al. (1993) have found collections of *N. ossifragum* to contain saponins, the genin components of which were identified as sarsasapogenin (**1-8**), smilagenin (**1-1**), yamogenin (**1-9**) and another spirostanol,

tentatively identified as neotigogenin (**1-15**). These sapogenins were present in the ratio of 82:9:5:4 (Miles et al., 1993).

Bile crystals from a sheep affected by photosensitization after grazing *N. ossifragum* have been found to be the insoluble salts of a 4:1 mixture of epismilagenin β-D-glucuronide (**1-3**) and episarsasapogenin β-D-glucuronide (**1-4**) (Miles et al., 1993).

Inoue et al. (1995) have reported the isolation and structure elucidation of four saponins (**1-12, 1-13, 1-16** and **1-17**) (Figure 1-8) from a Japanese collection of *Narthecium asiaticum.* Previously, Kobayashi et al. (1993) had reported the isolation of some furostanol (**1-18**) and spirostanol saponins from *N. asiaticum* and suggested that two of the saponins were responsible for an outbreak of nephrotoxicosis in Japanese cattle.

N. ossifragum has also been reported to cause nephrotoxicity (kidney damage and associated symptoms) in Norwegian cattle (Malone et al., 1992; Flåøyen et al., 1995a; Flåøyen et al., 1995b), sheep (Flåøyen et al., 1995c), goats (Flåøyen et al., 1997a), moose, red deer and reindeer (Flåøyen et al., 1997b). There is, however, evidence (Flåøyen and Wilkins, pers commun) that a non-steroidal compound, present in both *N. asiaticum* and *N. ossifragum,* is responsible for the nephrotoxicity of *N. asiaticum* and *N. ossifragum,* rather than sapogenins or saponins.

Saponins **1-12, 1-13** and **1-16**, isolated from a Norwegian collection of *N. ossifragum,* were shown by Berndt (1997) to correspond to specimens of narthecin (**1-12**), xylosin (**1-13**) and the disaccharide (**1-16**) from *N. asiaticum,* respectively.

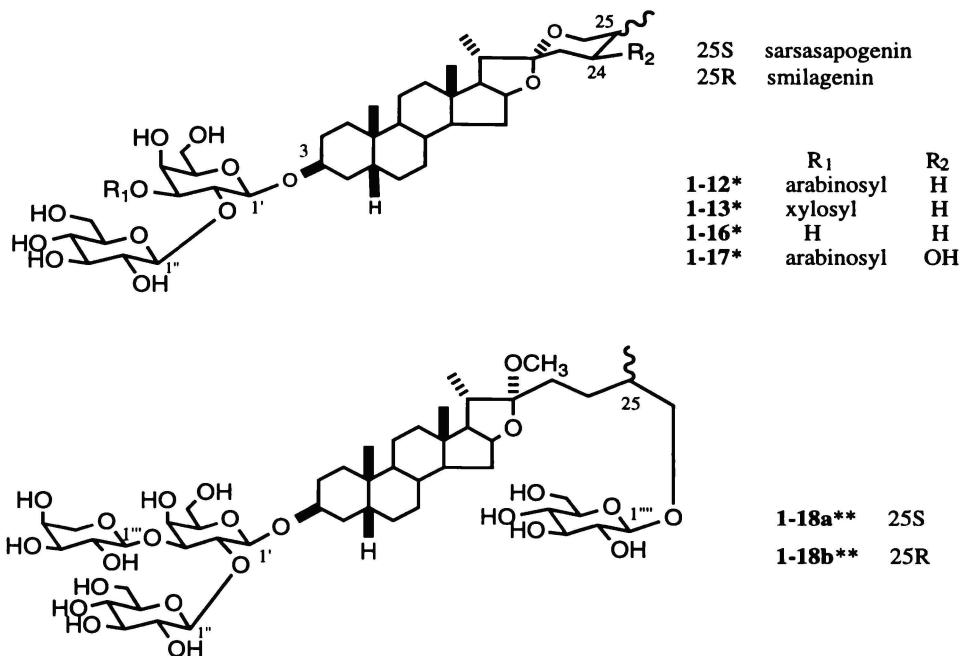


Figure 1-8. Structures of saponins isolated from *N. asiaticum* by Inoue et al. (1995) (*) and Kobayashi et al. (1993) (**).

Nolina texana

N. texana (sacahuiste), a member of lily family, is a perennial plant with a thick, short, caudex usually 15 cm tall or less, most of which is underground. The plant grows over a wide area of the south western United States and northern Mexico (Mathews, 1940).

The buds, blossoms and seeds of the plant have been reported to be highly poisonous to sheep and goats (Tunnicliff, 1929; Mathews, 1937; Mathews, 1940; Hershey, 1945; Rankins et al., 1986; Rankins et al., 1993). Sacahuiste poisoning, characterised by photosensitization, is also referred to as "swellhead" or "fevered".

In 1940, Mathews suggested the possibility that saponins were involved in the development of photosensitization diseases associated with the ingestion of *N. texana*, however no accounts of the saponin or sapogenin constituents of *N. texana* have appeared in the literature.

A series of saponins (Figure 1-9) have been reported as constituents of a related species, *N. recurvata*, by Takasshi et al. (1995) and Mimaki et al. (1996).

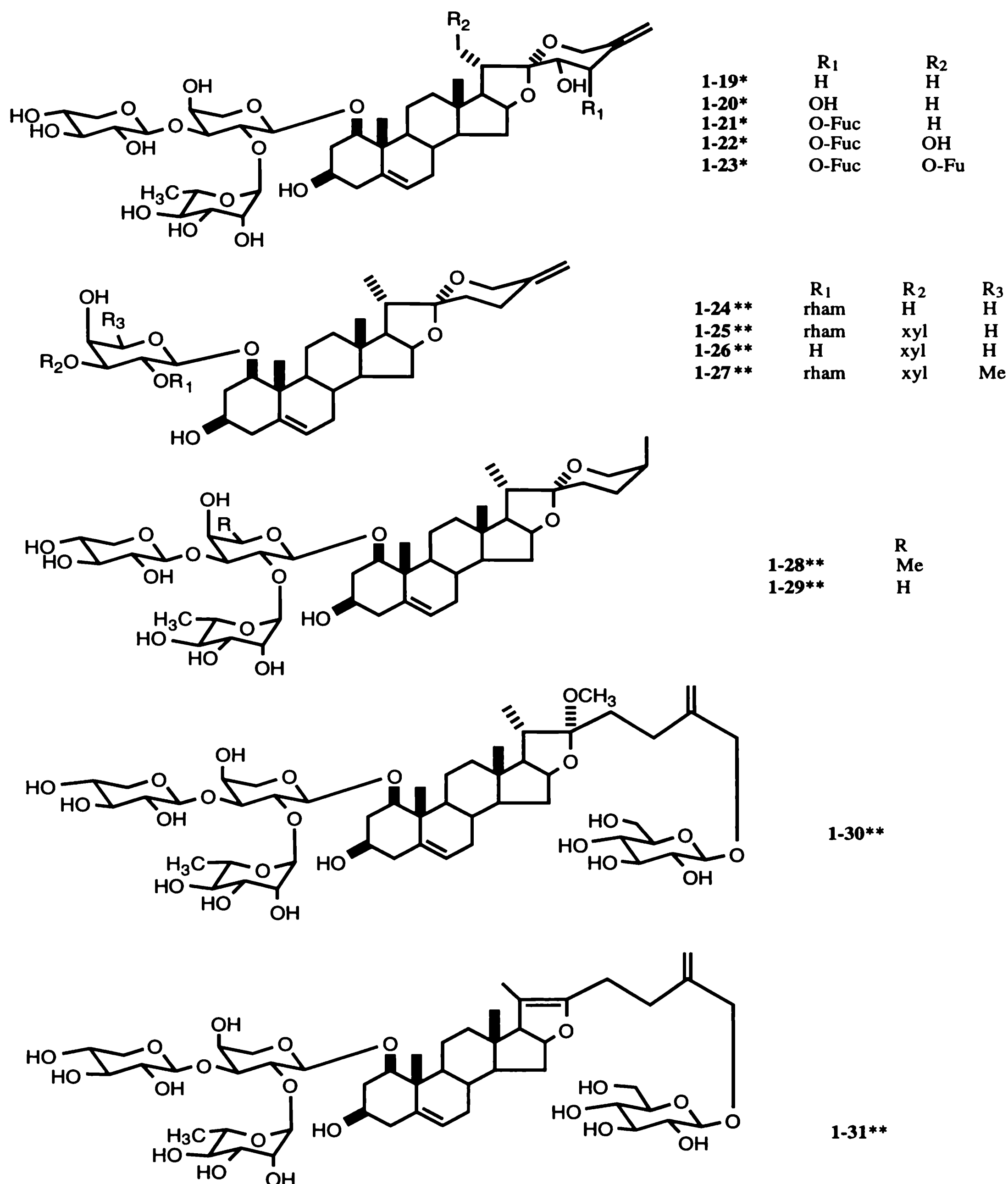


Figure 1-9. Structures of saponins isolated from *N. recurvata* by Takaashi et al. (1995) (*) and Mimaki et al. (1996) (**).

Panicum dichotomiflorum

P. dichotomiflorum (smooth witch grass) is a warm zone annual grass weed that is spreading in the northern districts of New Zealand (Holland et al., 1991). This plant is thought to have been introduced accidentally to New Zealand via aircraft transhipping of crates at the Te Rapa Airbase, Hamilton, during the Second World War (Herly, 1946).

Photosensitization of sheep grazing *P. dichotomiflorum* has been reported in New Zealand (Clare, 1952; Holland et al., 1991; Miles et al., 1991). As well as causing photosensitization in sheep this plant is known to cause photosensitization in cattle and goats (Holland et al., 1991). The only sapogenin present in hydrolysed *P. dichotomiflorum* extracts was diosgenin (Miles et al., 1991; 1992b), while biliary crystals from photosensitized sheep were identified as the calcium salt of epismilagenin β-D-glucuronide (Holland et al., 1991; Miles et al., 1991; 1992b).

Munday et al. (1993) have reported the isolation and structure elucidation of the diosgenin-derived furostanol saponin, dichotomin (**1-32**) (Figure 1-10), from *P. dichotomiflorum.*

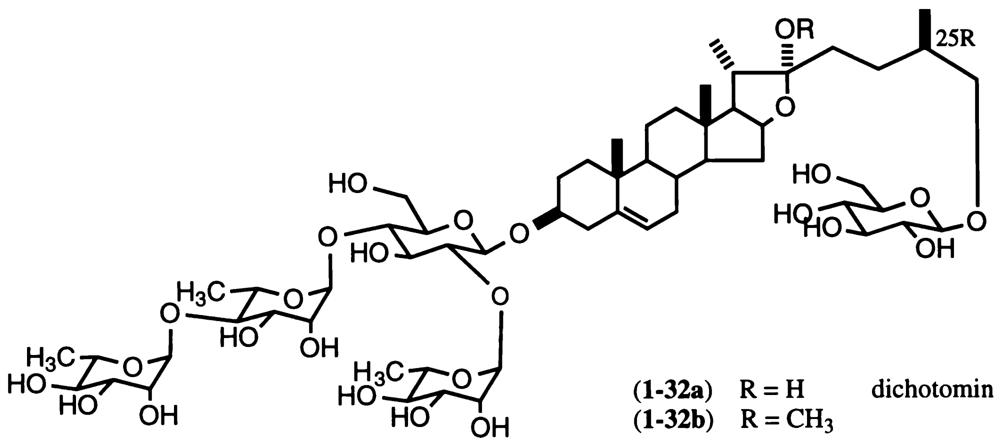


Figure 1-10. Structures of dichotomin and its 22-methoxy analogue isolated from *P. dichotomiflorum* by Munday et al. (1993).

Panicum coloratum

P. coloratum (Kleingrass) is a summer growing perennial, the height of which can be highly variable (8 to 100 cm). It originated from tropical regions of Africa, and was introduced to the United States in 1952 because it was considered to produce good quality of forage for sheep (Patamalai et al., 1990).

P. coloratum has been reported to cause dikoor, a hepatogenous photosensitization disease of sheep in South Africa (Kellerman and Coetzer, 1984) and to also be associated with photosensitization outbreaks of sheep in the United States (Bridges et al., 1987; Patamalai et al., 1990) and in Australia (Regnault, 1990).

Diosgenin (**1-5**) and yamogenin (**1-9**) are the principle sapogenin constituents of hydrolysed *P. coloratum* (Patamalai et al., 1990). A 25*S* furost-5-en-3β-ol saponin, designated as coloratin (**1-33**) (Figure 1-11), has been isolated from an Australian collection of *P. coloratum* and its structure elucidated by a combination of mass spectrometry and one- and two-dimensional NMR techniques (Wilkins, pers commun). No reports of the composition of biliary crystals from sheep photosensitized by ingestion of *P. coloratum* have appeared.

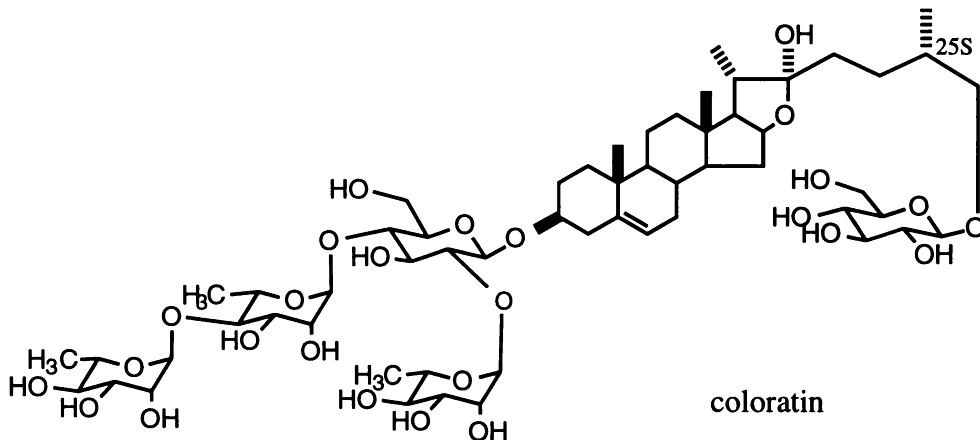


Figure 1-11. Structure of coloratin, as proposed by Wilkins (pers commun).

Panicum miliaceum

P. miliaceum (white French millet) is a grass that has its primary centre in northern China, where it has been used as a cereal (Zeven and Zhukovsky, 1975). This plant has been reported to cause photosensitization of sheep in New Zealand (Clare, 1955; Holland et al., 1991; Miles et al., 1991; Miles et al., 1993).

Miles et al. (1993) have demonstrated that the principal sapogenins in hydrolysed *P. miliaceum* extracts are diosgenin (**1-5**) and yamogenin (**1-9**) in a ratio of 4:1. No reports of the saponins (conjugated sapogenins) present in the plant, nor the composition of bile crystals from sheep photosensitized by ingestion of *P. miliaceum*, have appeared in the literature.

Panicum schinzii

P. schinzii (sweet grass) is a summer-growing annual grass that can be a major component of pasture in south-eastern Australia, provided summer rain fall is adequate.

P. schinzii-associated photosensitization of Australian lambs has been reported (Button et al., 1987; Lancaster et al., 1991; Miles et al., 1992a; Miles et al., 1992b).

Diosgenin (**1-5**) is the only sapogenin that has been reported in hydrolysed *P schinzii* extracts (Miles et al., 1992b). Bile crystals recovered from affected sheep have been identified as the calcium salt of epismilagenin β-D-glucuronide (**1-3**) (Miles et al., 1992b), as was also the case for *P. dichotomiflorum* (Miles et al., 1992b)

Panicum virgatum

P virgatum (switchgrass) is a warm-season perennial grass used for pasture and hay production in many regions of the United States. This plant has been reported to be implicated in the photosensitization of sheep in the United States (Puoli et al., 1992).

In a preliminary investigation, Berndt (1997) concluded that diosgenin and yamogenin saponins (ca 10:1) were present in *P. virgatum*, and that the molecular weight of these saponins was 1194 daltons. Berndt (1997) also noted that this molecular weight corresponded to that of the *25R*- and 25*S*-furostanol saponins previously identified as constituents of *P. dichotomiflorum* and *P. coloratum*, respectively (Munday et al., 1993b; Wilkins, pers commun). No accounts of the existence of biliary crystals from sheep suffering photosensitization after ingestion of this plant have appeared in the literature.

Tribulus terrestris

T. terrestris is an annual herb, native to Europe and Africa, and widespread in many parts of the world. Under some climatic conditions, particularly in late summer and early autumn when periods of drought are followed by heavy rain and hot sunny days, *T. terrestris* grows vigorously and can become almost the only forage plant available to sheep and cattle (Everist, 1981; Tapia et al., 1994).

This plant has been reported to cause photosensitization of ruminants in South Africa, where the disease is known as geeldikopp (Theiler, 1918; Coetzer et al., 1983; Miles et al., 1993; Miles et al., 1994a; Miles et al., 1994b; Wilkins et al., 1994; Wilkins et al., 1996). Outbreaks of photosensitization diseases amongst sheep grazing *T. terrestris* containing pastures have also been observed in Australia (Glastonbury et al., 1984), the United States (Camp et al., 1988), Argentina (Tapia et al., 1994) and Iran (Amjadi et al., 1977).

Collections of *T. terrestris* have been found to contain saponins which on hydrolysis afford a variety of steroidal sapogenins, including diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin, neogitogenin, and ruscogenin (Figure 1-12) (Miles et al., 1994b; Wilkins et al., 1994; Wilkins et al., 1996).

A number of saponins, including tribulosin and dioscin, have been isolated from *T. terrestris* (Mahato et al., 1982). Wilkins et al. (1996) have reported the structure elucidation of a ruscogenin saponin (saponin C) isolated from the β-glucosidase treated ethanol-water extracts of a South African collection of *T. terrestris* (Figure 1-12).

Bile crystals from a sheep photosensitized after ingestion of *T. terrestris* have been shown to be composed of calcium salts of epismilagenin β-D-glucuronide and episarsasapogenin β-D-glucuronide in the ratio of 6:1 (Miles et al., 1994b).

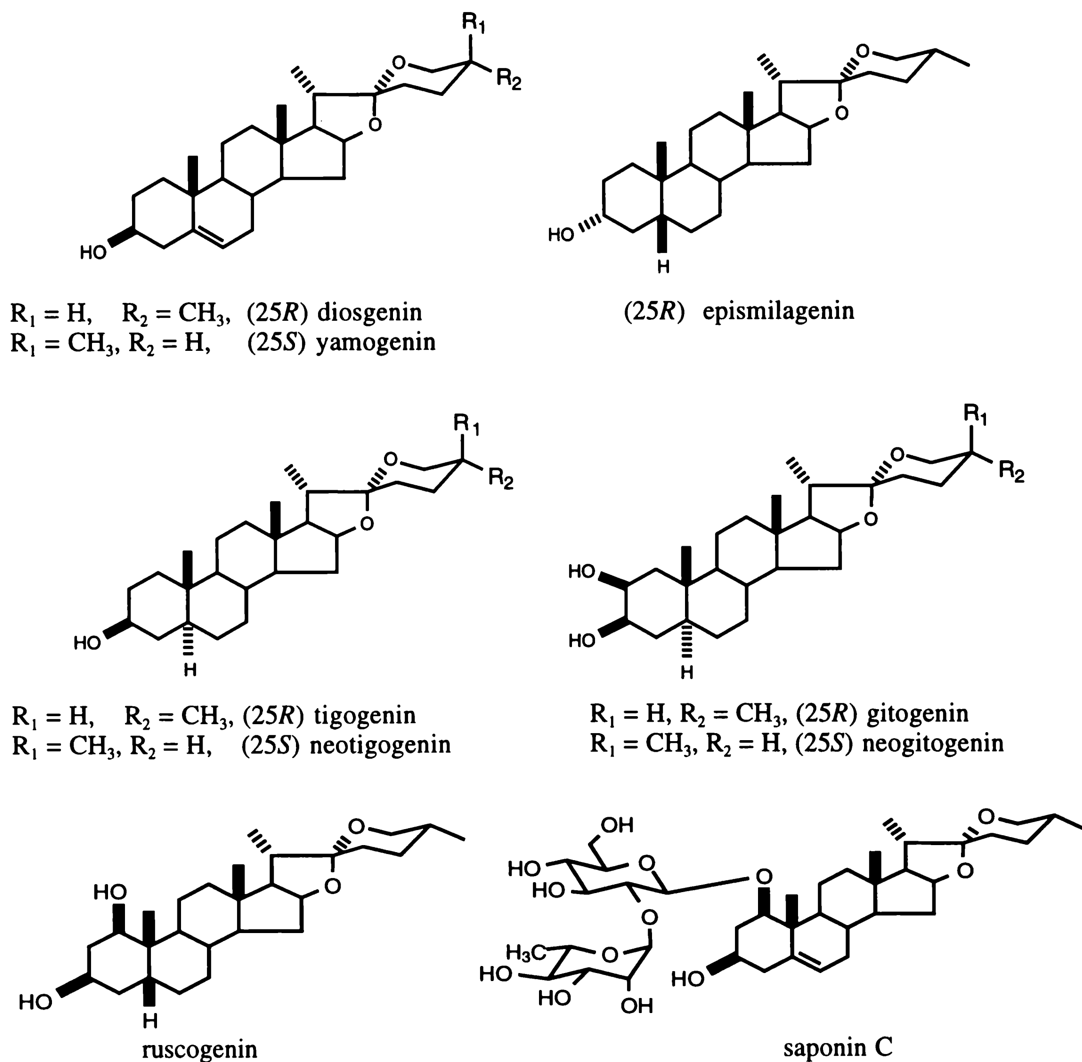


Figure 1-12. Structures of sapogenins and saponins isolated from *T. terrestris*.

1.5 Ovine Saponin Metabolism

Notwithstanding the wide variety of saponins and sapogenins identified in plants implicated in the hepatogenous photosensitization of livestock, biliary crystals from affected animals have always been found to be comprised of calcium salts of 5β-spirostan-3α-ol β-D-glucuronides (Miles et al., 1991; Miles et al., 1992a; Miles et al., 1992b; Miles et al., 1993; Miles et al., 1994a; Miles et al., 1994b; Wilkins et al., 1994).

Miles et al. (1994b) and Wilkins et al. (1996) have suggested that some saponins are lithogenic (crystal forming), and that other are not lithogenic. In particular, diosgenin, yamogenin, smilagenin, sarsasapogenin, epismilagenin and episarsasapogenin are considered to be lithogenic sapogenins (capable of contributing to bile crystal formation) whereas tigogenin, neotigogenin and dihydroxylated genins appear to not be lithogenic.

Miles et al. (1993, 1994b) have proposed that the ruminal metabolism of saponins containing diosgenin, yamogenin, smilagenin and sarsasapogenin proceeds to afford epismilagenin (25*R*-genins) and episarsasapogenin (25*S*-genins).

Ruminant metabolism of diosgenin saponins is envisaged as involving removal of sugar residues by hydrolysis, followed by reduction of the 5(6)-double bond to afford a mixture of 5α- and 5β-sapogenins (tigogenin (**1-7**) and smilagenin (**1-1**) respectively). Thereafter oxidation and reduction of smilagenin (**1-1**) (3β-OH), via smilagenone (**1-34**) (3-keto-smilagenin), affords epismilagenin (**1-2**) (3α-OH), which reconjugates with glucuronic acid. Subsequent deposition of the glucuronide from the bile, predominantly as the calcium salt of the glucuronide, is believed to afford the characteristic biliary crystalloid material (Miles et al., 1993; Miles et al., 1994b).

The proposed metabolic pathway is presented in Figure 1-13. This pathway presumes the saturation of the 5(6)-double bond occurs before epimerisation at C-3. Saturation and epimerisation could, however, occur in either order. The detection, in metabolic studies of low levels of smilagenone, but not of 3-keto-spirost-5-ene intermediates, can be interpreted as supporting the pathway depicted in Figure 1-13. The possibility that both smilagenone and the 3-keto-spirost-5-ene are metabolic intermediates, and that the latter ketone is reduced much more quickly than the former ketone, can not be excluded.

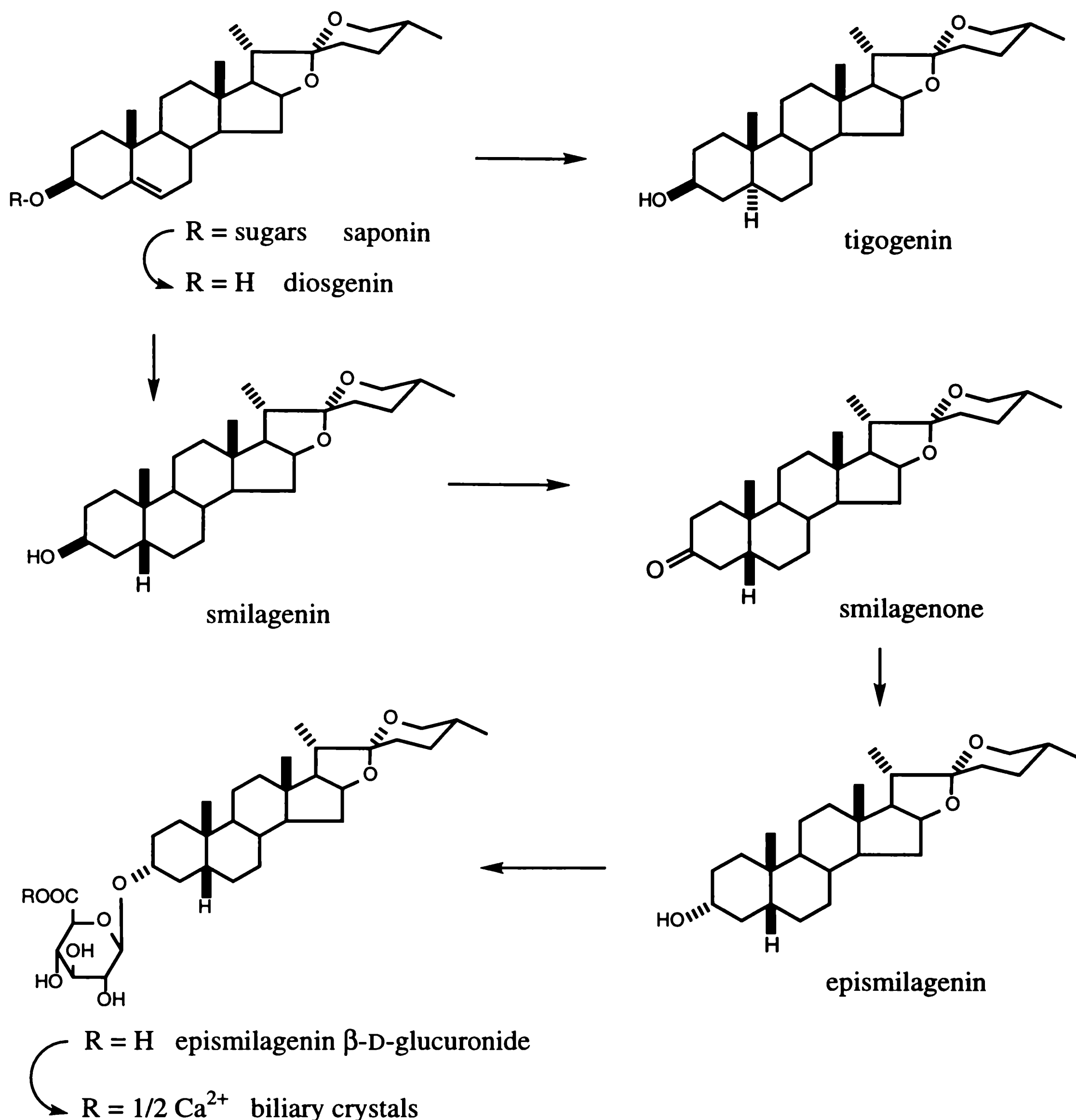


Figure 1-13. Proposed pathway for the ruminal metabolism of diosgenin-containing saponins to calcium salt of the β-D-glucuronide of epismilagenin (biliary crystals) (after Miles et al., 1994b).

Likewise metabolism of yamogenin (**1-9**) and sarsasapogenin (**1-8**) (25*S*-isomers of diosgenin (**1-5**) and smilagenin (**1-1**) respectively) is envisaged as proceeding to afford episarsasapogenin (**1-35**) and thence the calcium salt of the β-D-glucuronide of episarsasapogenin (**1-4**) (Miles et al., 1993).

The proposal of Miles et al. (1994b) that some saponins and sapogenins are not lithogenic is supported by the results of dosing experiments. In an experiment in which a crude diosgenin containing saponin was administered to sheep (Meagher, 1996), rumen metabolism was found to afford a mixture of smilagenin (**1-1**), epismilagenin (**1-2**) (5β-

spirostanols) and tigogenin (**1-7**) (a 5α-spirostanol), however only epismilagenin conjugates were identified in the bile of the dosed sheep.

In another dosing experiment, in which a *T. terrestris* extract containing diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin and neogitogenin saponins was administered to a South African sheep (Miles et al., 1994b), only epismilagenin and episarsasapogenin conjugates were identified in the bile crystalloid material, whereas free and conjugate forms of diosgenin, yamogenin, smilagenin, epismilagenin, episarsasapogenin, tigogenin, neotigogenin, gitogenin and neogitogenin (Figure 1-14) were detected in bile supernatant. It is not yet known why only the calcium salts of epismilagenin and episarsasapogenin glucuronides contribute to the biliary crystalline deposits.

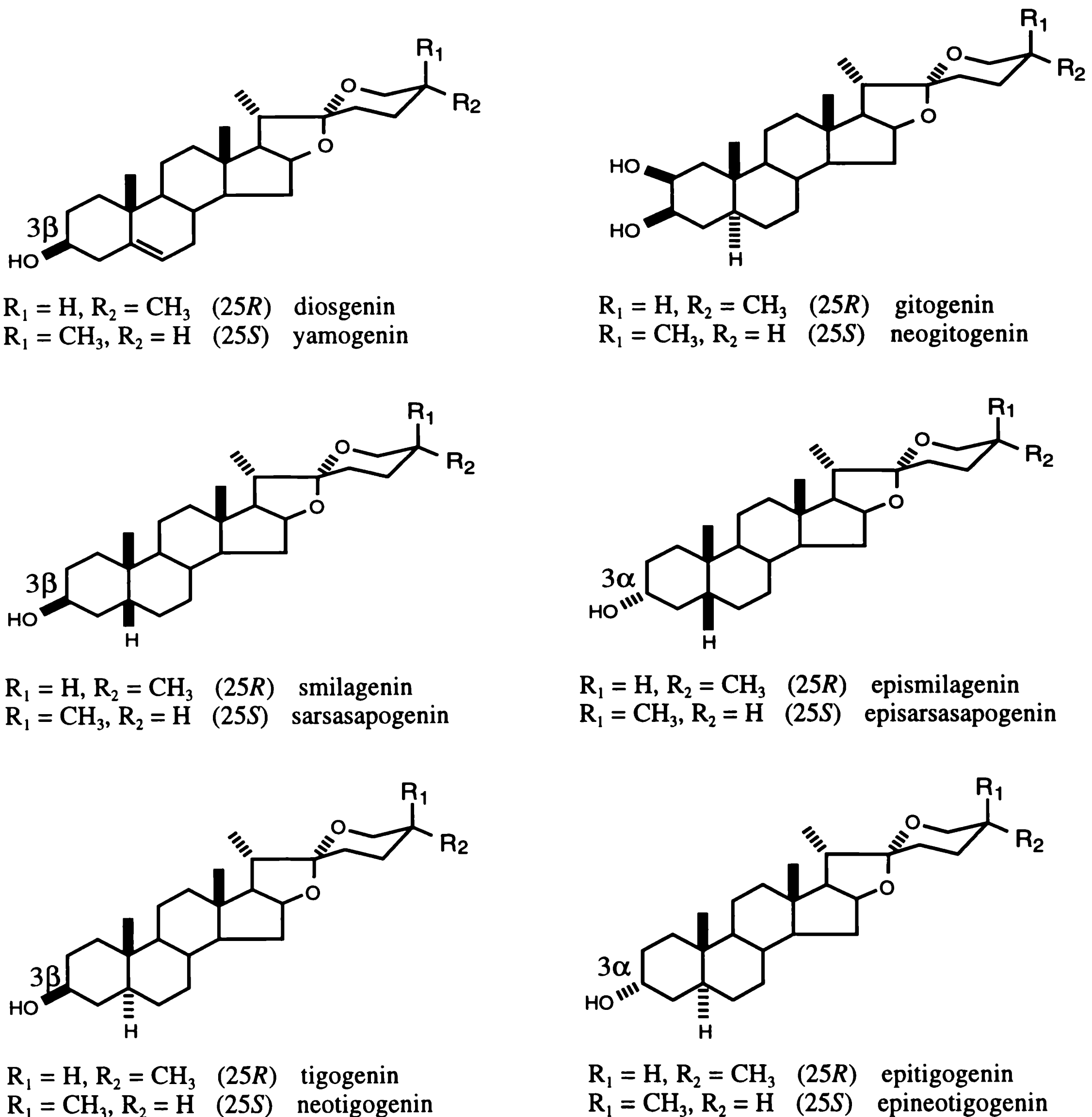


Figure 1-14. Structures of the mono- and di-hydroxylated sapogenins mentioned in the text.

Recently, Flåøyen and Wilkins (1997) have reported the results of an experiment in which *N. ossifragum* was dosed to a Norwegian sheep. This investigation identified three distinct regions of metabolic activity (Figure 1-15).

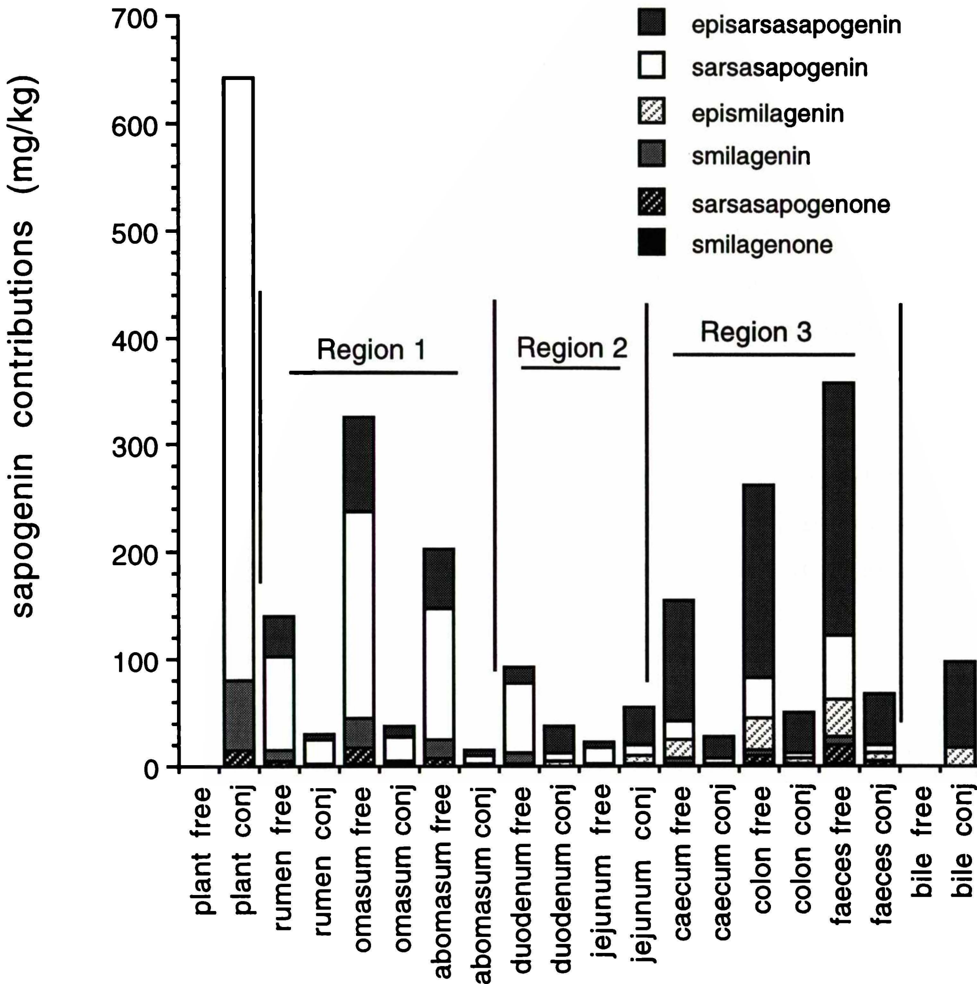


Figure 1-15. Levels (mg/kg dry matter) of free and conjugated sapogenins identified in three metabolic regions and in the *N. ossifragum* sample (Flåøyen and Wilkins, 1997).

In the rumen and omasum (the first metabolic region), the ingested plant saponins (predominantly sarsasapogenin saponins) were hydrolysed to the parent sapogenins, oxidised at C-3, and reduced to afford epi-analogues of the ingested sapogenins (Flåøyen and Wilkins, 1997).

The second metabolic region was found to consist of the duodenum, jejunum, liver and associated ducts. It was proposed the free sapogenins were absorbed in the jejunum and transported via the portal vein to the liver where 3α-OH-5β-H-sapogenins (epismilagenin and episarsasapogenin), but not 3β-OH-5β-H-sapogenins (smilagenin and

sarsasapogenin) were conjugated with glucuronic acid and excreted into the bile as epismilagenin and episarsasapogenin conjugates (Flåøyen and Wilkins, 1997).

The third metabolic region was comprised of the lower intestines (the colon and caecum). In this region, epi-sapogenin conjugates arriving from jejunum were hydrolysed to free epi-sapogenins. Only small amount of sapogenins present in the lower intestine revealed that most of sapogenins/saponins were absorbed in the jejunum and metabolised to epi-sapogenins in the liver (Flåøyen and Wilkins, 1997).

Sapogenins were not detected in a urine sample collected 24 hours after dosing commenced. This observation indicated that sapogenins and their metabolites do not appear to have been transported from the liver with the blood and subsequently excreted into the urine (Flåøyen and Wilkins, 1997).

The results of the dosing experiment (Flåøyen and Wilkins, 1997) were in accord with the proposal of Miles et al. (1993) that ruminal metabolism of sarsasapogenin saponins (present in the dosed *N. ossifragum* plant material) proceeds to afford episarsasapogenin.

1.6 Aims and Objectives of the Present Investigation.

The principle aims and objectives of the present investigation were:

(i) to establish synthetic procedures for the preparation of deuterium labelled sapogenins and saponins, to be utilised in dosing trials.

(ii) to explore the ruminal metabolism of deuterium labelled sapogenins and saponins. It was anticipated that this would afford information in respect of the relative uptake rates of free and conjugated sapogenins.

(iii) to ascertain the time course of the uptake, and subsequent purging from the rumen, jejunum and bile of free and conjugated sapogenins and metabolised episapogenins.

(iv) to determine the levels of sapogenins and saponins in collections of *N. ossifragum*, *P. virgatum*, *P. miliaceum*, *N. texana*, *B. decumbens* and *T.*

terrestris (plants implicated as causative agents in photosensitization outbreaks) using GC-MS method.

(v) to determine the structures of saponins present in some collections of *N. ossifragum*, *P. virgatum*, *P. miliaceum* and *N. texana*, using a combination of one- and two-dimensional NMR, ES-MS and GC-MS methods.

Chapter Two

SYNTHESES

2.1 Introduction

The biliary crystals, from sheep suffering from several photosensitization diseases, including geeldikkop in South Africa, alveld in Norway and *Panicum* toxicoses in New Zealand and Australia, have been found to be composed mainly of the insoluble salts of epismilagenin and/or episarsasapogenin β-D-glucuronides (Miles et al., 1992a; Miles et al., 1992b; Miles et al., 1993; Miles et al., 1994a; Miles et al., 1994b; Wilkins et al., 1994). The sources of lithogenic (crystal forming) episapogenins are thought to be saponins in the ingested plant materials (Munday et al., 1993b; Miles et al., 1994a; Miles et al., 1994b; Wilkins et al., 1994; Flåøyen and Wilkins, 1997).

Dosing experiments have shown that the ruminal metabolism of crude saponin extracts from implicated plants predominantly affords potentially lithogenic episapogenin conjugates of the dosed sapogenin glycosides (saponin) (Miles et al., 1994b). On the other hand, when free sapogenins (e.g. sarsasapogenin), or sapogenins surface absorbed onto cellulose were dosed, only moderate metabolism (typically <10-15% conversion) to potentially lithogenic substances occurred (Meagher et al., 1996).

Efforts to reproduce disease symptoms in saponin, or sapogenin, dosing experiments have only occasionally been successful (Kellerman et al., 1991, Miles et al., 1994a; Camp et al., 1988). A possible implication of these findings is that an as yet unidentified cofactor in the crude plant saponin extracts may influence the ruminal metabolism of ingested saponins to potentially lithogenic substances. Variations in rumen micro-flora activity of sheep used in dosing trials may also be a significant factor in the apparently low conversion of free sapogenins to episapogenin conjugates.

In order that the ruminal metabolism of sapogenins and saponins implicated in hepatogenous photosensitization diseases might be further explored, the synthesis of some sapogenin glucosides (saponins) and deuterium labelled sapogenins and glucoside adducts was undertaken.

2.2 Synthesis of Sarsasapogenin β-D-Glucoside

2.2.1 Introduction

Sarsasapogenin (**2-1**) is a typical genin component of the plant saponins associated with the development of ovine hepatogenous photosensitization diseases (Miles et al., 1994b; Wilkins et al., 1994; Flåøyen and Wilkins, 1997). It can be proposed that glucosides of sarsasapogenin, and those of other sapogenins might be more bio-available in ovine digestive tracts than is the case for the corresponding free sapogenins (e.g. sarsasapogenin). In order that this hypothesis might be examined in a dosing trial, a bulk specimen of sarsasapogenin β-D-glucoside (**2-2**) was prepared.

A variety of methods for the preparation of β-D-glucosides have been reported. The formation of the glucoside bond is usually accomplished by reacting a α-D-acetoglycopyranosyl halide (normally the bromide or chloride) with a hydroxylic substance (e.g. a sapogenin) (Figure 2-1), in the presence of various catalysts.

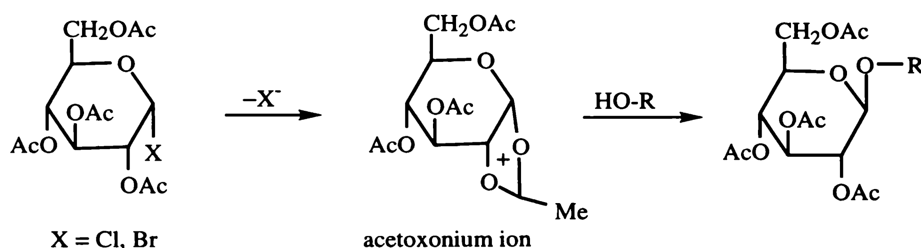


Figure 2-1. Reaction of α-glucosyl halides with an alcohol to form β-glucosides.

Insoluble silver catalysts, such as Ag_2O and Ag_2CO_3, have been used to promote glycoside formation via a S_N2-like mechanism. This synthetic pathway, in which silver carbonate or silver oxide is utilised to neutralise the hydrohalogenic acid formed by reaction of *O*-acetylglucosyl halide with the hydroxylic reagent, is the oldest known variant of the Koenig-Knorr synthesis (Koenig and Knorr, 1901). Subsequently, higher yielding variants of the reaction have been reported (Robertson and Water, 1930; Noller and Rockwell, 1938; Karjala and Link, 1940; McCloskey et al., 1944; Bollenback et al., 1955; Wotiz et al., 1959; Nambara and Imai, 1967; Schneider and Bjacca, 1969). Recently, Takechi et al. (1991, 1992a) have reported the synthesis of a number of diosgenyl monoglycosides and diglycosides in the presence of an insoluble silver catalyst. A disadvantage of the use of insoluble silver catalysts is that water is formed in the course of the reaction, making it

necessary to add drying agents such as calcium sulfate. Since yields are also relatively low, this approach is often considered to be less suitable (Paulsen, 1982; Paulsen, 1984).

Mercuric salts, such as mercuric acetate, mercuric bromide, mercuric oxide and mercuric cyanide (the Helferich catalyst), have also been identified as catalysts which promote glycoside bond formation (Linstead, 1940; Flowers and Jeanloz, 1963; Schroeder and Green, 1966; Lemieux and Driguez, 1975; Lemieux et al., 1975; Rodriguez et al., 1990). Since the reaction is believed to proceed via an intermediate acetoxonium ion (Figure 2-1), it is important to use a moderate polarity solvent to facilitate the formation of the acetoxonium ion. If excessively polar solvents are used, the glycosyl halide tends to decompose. A mixture of benzene and nitromethane is often used as the solvent for the reaction. The ratio of benzene to nitromethane is a critical factor which can influence selectivity and yield, and must be carefully optimised in each case (Paulsen, 1982). Products may be contaminated with organomercury complexes which are difficult to remove. Paulsen (1982) has reported that the yield of glycosides, formed in the presence of mercuric salts, typically varied from 25-90%, often with poor repeatability, depending on the structure of the hydroxylic compound.

Reaction of acetobromoglucose with the sodium salt of a hydroxylic reagent in the non-polar solvents typically affords a 55% yield of the corresponding glucosides (Lubineau and Queneau, 1987; Lorimer et al., 1995).

Cadmium carbonate has also been found to be a suitable catalyst for glucosylation, with yields of 46-71% (Conrow and Bernstein, 1971). In the examples which Conrow and Bernstein (1971) investigated, yields using cadmium carbonate were higher than those for reactions performed under the same conditions, using silver carbonate or mercury salts as catalysts in the standard Koenig-Knorr reaction. The isolation of comparatively polar glycosides from low yield reactions often necessitates tedious crystallisation and counter-current or chromatographic procedures. Organomercury complexes are also difficult to remove. On the other hand, the work up of mixtures containing cadmium salts is relatively straightforward.

Recently Zill et al. (1990) have reported that potassium carbonate can be utilised to avoid the disadvantages of heavy-metal salts, however yields are lower (e.g. 18%).

Phase-transfer conditions for glucosylation reactions, using Bu_4NBr, in the absence of heavy-metal salts, have been reported by Grabley et al. (1992), who obtained essentially quantitative yields of some glycosides.

Besides halides (e.g. chlorine or bromine), a trichloroacetimidate-oxyl group can be employed as the leaving group. Reactions of a series of 1-*O*-glucosyl trichloroacetimidates with some alcohols have given the corresponding glucosides in yields of 32-90% (Schmidt, 1986).

2.2.2 Results and Discussion

The reactions involved in the synthesis of sarsasapogenin β-D-glucoside (**2-2**) are outlined in Figure 2-2.

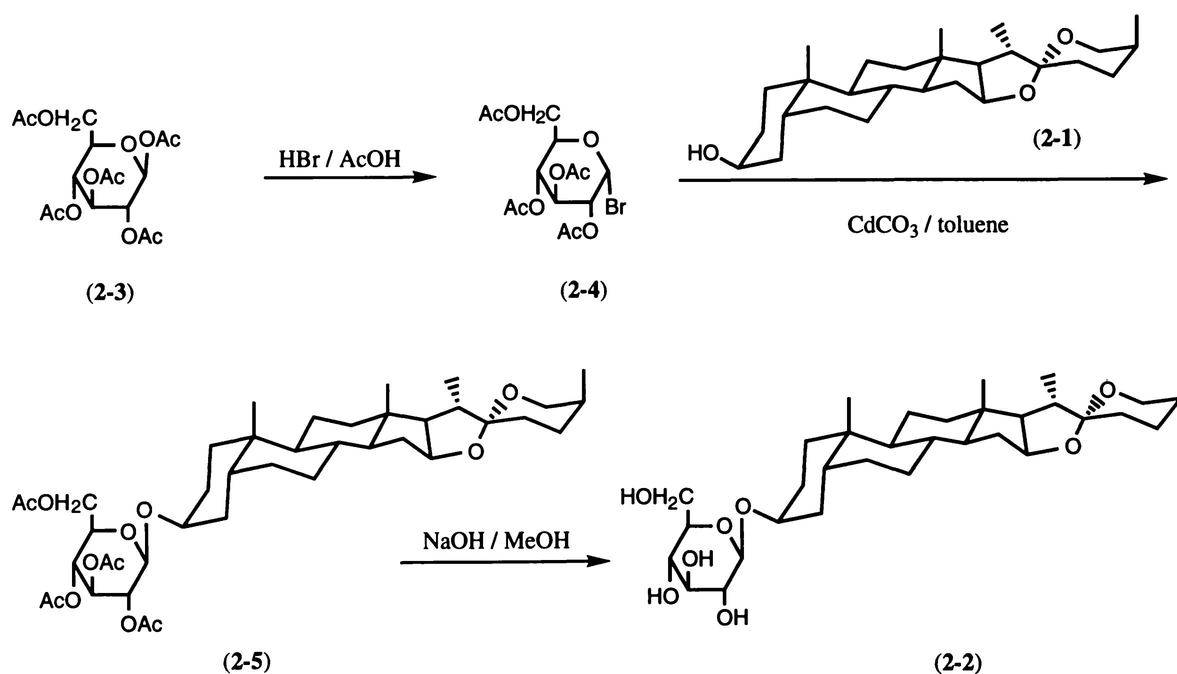


Figure 2-2. Reactions utilised in the synthesis of sarsasapogenin β-D-glucoside (**2-2**).

1,2,3,4,6-Penta-*O*-acetyl-β-D-glucopyranose (**2-3**), when reacted with hydrobromic acid in glacial acetic acid solution at 5°C for 24 hours, gave 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**2-4**) in an yield of 96%. The reaction temperature was found to be a critical factor, influencing both the configuration of the glycoside linkage and the yield of the desired glucoside. For example, TLC and NMR analyses showed that reaction at room temperature (20°C) afforded a mixture consisting of 1,2,3,4,6-penta-*O*-acetyl-α-D-

glucopyranose (**2-6**) and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide (**2-7**) as major products, and minor amounts of **2-3** and **2-4**.

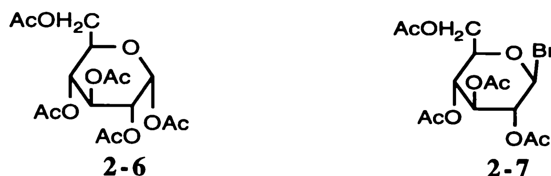


Figure 2-3. Structures of by-products **2-6** and **2-7**.

The configuration of the anomeric carbon of **2-4** was revealed by the NMR coupling constant of the well defined H-1 doublet (J = 4.0 Hz) which occurred at 5.40 ppm. This coupling constant showed H-1 was equatorially oriented (Agrawal, 1992) and that the bromine atom was therefore axially oriented, hence the replacement of the anomeric acetoxyl group of **2-3** by bromine had proceeded with inversion at C-1.

In accord with the conclusion that H-1 was equatorially inclined, no correlation was observed between H-1 and H-3 or between H-1 and H-5 in a NOE-difference spectrum of **2-4** (Table 2-1) when H-1 was irradiated. The α- (axial) orientation of the bromine group is that required for the formation of β-glucoside, via the S_N2 like mechanism of Koenig-Knorr type reactions. A complete assignment of the ^1H and ^{13}C NMR signals of **2-4** is presented in Table 2-1. The β-bromo-acetylglucoside (**2-4**) made by this method was stable at 5°C and could be kept for several months.

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

	^1H*	^{13}C*	COSY	NOE
1	6.55 (d, J = 4.0 Hz)	86.6	H-2	
2	4.78 (dd, J = 6.0, 4.0 Hz)	70.6	H-1, H-3	H-4
3	5.49 (t, J = 9.7, 9.7 Hz)	70.1	H-2, H-4	H-5
4	5.10 (t, J = 10.1, 9.8 Hz)	67.2	H-3, H-5	H-2, H-6a, H-6b
5	4.25 (m)	72.2	H-4,H-6a,H-6b	H-3
6	4.29 (m);	61.0	H-5, H-6b	
	4.23 (br d)		H-5, H-6a	
$COCH_3$	1.98 (s)	20.5		
	1.99 (s)	20.5		
	2.04 (s)	20.6		
	2.05 (s)	20.6		
$COCH_3$		169.4		
		169.7		
		169.8		
		170.4		

* ^1J $_{^1H-^{13}C}$ correlations established in a XHCORR experiment

Coupling of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**2-4**) and sarsasapogenin (**2-1**) was carried out in toluene at reflux temperature and using cadmium carbonate as catalyst, according to the method of Conrow and Bernstein (1971). When TLC analyses showed complete reaction (24 hours), cadmium salts were removed by filtration of the hot reaction mixture and, after cooling, the solvent was removed using a rotary evaporator. Recrystallisation of the residue from diethyl ether gave sarsasapogenin β-D-glucoside tetraacetate (**2-5**). TLC analyses of a trial reaction mixture indicated that the use of potassium carbonate in place of cadmium carbonate did not afford a coupled (glucosidic) product.

The ^{1}H NMR spectrum of **2-5** (in $CDCl_3$) included four angular methyl signals at 0.75 (3H, s, H-18), 0.92 (3H, s, H-19), 0.98 (3H, d, J = 6.6 Hz, H-21) and 1.07 ppm (3H, d, J = 7.1 Hz, H-27), four acetyl methyl signals in the region 2.00-2.06 ppm (4 × 3H, s) and glucosyl proton signals in the lowfield 3.6-5.2 ppm.

The ^{13}C and DEPT NMR spectra of **2-5** showed a total of 41 carbon signals, amongst which 27 signals arose from the genin, 6 signals from the glucosyl residue and 8 signals from the four acetyl groups.

The H-1' to H-6' resonances of **2-5** were identified from correlated peaks appearing in the COSY spectrum. These assignments were used to define the ^{13}C resonances of the corresponding carbons, via correlations observed in the HSQC and HMBC spectra of **2-5**. Other ^{1}H and ^{13}C NMR data for **2-5** are presented in Table 2-2.

The H-1' NMR signal of the glucoside (**2-5**) appeared as a sharp doublet (4.54 ppm, J = 8.0 Hz) in the ^{1}H NMR spectrum. This coupling constant demonstrates that H-1' is 1,2-*trans*-diaxially oriented with respect to H-2' (Agrawal, 1992), and that the glucoside linkage is therefore β- (equatorially) oriented.

The positive ion ES-MS spectrum of **2-5** in MeCN-H_2O (1:1) at +40 V showed pseudomolecular ions at *m/z* 764.2 and 769.4, attributable to $(M+NH_4)^+$ and $(M+Na)^+$ ions respectively, arising from the addition of NH_4^+ and Na^+ to a glucoside of molecular weight 746 daltons.

Table 2-2. ^1H and ^{13}C NMR assignments and some COSY correlations determined for sarsasapogenin β-D-glucoside tetraacetate (**2-5**) (ppm in $CDCl_3$).

Atom	^1H	^{13}C*	COSY
1	1.85, 1.56	29.7	
2	1.81, 1.52	26.7	
3	3.99 (br s)	68.7	H-2α, H-2β, H-4
4	1.62	30.2	
5	1.87	36.8	
6	1.51, 1.12	26.6	
7	1.63, 1.40	26.4	
8	1.50	35.3	
9	1.35	40.1	
10	-	35.0	
11	1.37, 1.16	20.9	
12	1.69, 1.12	40.3	
13	-	40.7	
14	1.19	56.5	
15	1.99, 1.18	31.8	
16	4.39 (q, *J* = 7.3, 6.6, 7.8 Hz)	81.0	H-15α, H-15β, H-17
17	1.75	62.2	
18	0.75 (s)	16.5	
19	0.92 (s)	24.0	
20	1.85	42.1	
21	1.07 (d, *J* = 7.1 Hz)	14.4	
22	-	109.8	
23	1.37	26.0	
24	1.42, 1.11	25.8	
25	1.75	27.1	
26	3.94 (dd, *J* = 2.7, 8.5, 2.5 Hz)	65.7	H-26α, H-25
	3.29 (br d, *J* = 11.0 Hz)		H-26β, H-25
27	0.98 (d, *J* = 6.6 Hz)	16.0	
1'	4.54 (d, *J* = 7.9 Hz)	98.6	H-2'
2'	4.99 (dd, *J* = 8.0, 1.6 Hz)	71.7	H-1', H-3'
3'	5.21 (t, *J* = 9.5, 9.5 Hz)	74.4	H-2', H-4'
4'	5.07 (t, *J* = 9.7, 9.6 Hz)	73.0	H-3', H-5'
5'	3.66 (m)	71.5	H-4', H-6'a, H-6'b
6'	4.25 (dd, *J* = 4.9, 7.3, 4.9 Hz);	62.2	H-5', H-6'
	4.10 (dd, *J* = 2.3, 9.9, 2.4 Hz)		H-5', H-6'
$OCOCH_3$	2.00 (s)	20.6	
	2.01 (s)	20.7	
	2.02 (s)	20.7	
	2.06 (s)	20.8	
$OCOCH_3$		169.1	
		169.4	
		170.4	
		170.7	

* $^1J_{^1H-^{13}C}$ correlations established in a XHCORR experiment

Hydrolysis of sarsasapogenin β-D-glucoside tetraacetate (**2-5**) in a 0.25 M methanolic sodium hydroxide solution at 50-60°C for 1.5 hours, followed by extraction with 1-butanol and recrystallisation from 70% aqueous acetone, gave sarsasapogenin β-D-glucoside (**2-2**) with a yield of 89.3%. Hydrolysis was routinely performed without recrystallisation of the initially formed acetylated glycoside (**2-5**).

The ^{1}H NMR spectrum (in DMSO-D_6) of **2-2** showed four angular methyl group resonances, typical of sapogenins, at 0.81 (3H, s, H-18), 1.00 (3H, s, H-19), 1.02 (3H, d, J = 7.0 Hz, H-21) and 1.10 ppm (3H, d, J = 7.1 Hz, H-27). No acetoxyl signals were detected in the region 1.9-2.2 ppm. This observation is consistent with hydrolysis of the acetyl groups.

The ^{13}C NMR spectrum (in DMSO-D_6) exhibited a total of 33 signals, amongst which 27 signals arose from the genin and 6 signals from the sugar unit. A complete assignment of the ^{1}H and ^{13}C NMR signals of **2-2** is presented in Table 2-4.

The COSY spectrum of **2-2** (in DMSO-D_6) (Table 2-4) identified the following correlation pathway for the sugar protons: 4.23 ppm (H-1') ↔ 3.02 ppm (H-2') ↔ 3.22 ppm (H-3') ↔ 3.14 ppm (H-4', H-5') ↔ 3.51 ppm (H-6'b) ↔ 3.73 ppm (H-6'a). Two overlapping proton resonances (H-4' and H-5') at 3.14 ppm were assigned, according to correlations observed in the HMQC spectrum, to 74.1 (C-4') and 80.6 (C-5') ppm respectively.

The angular methyl group resonances were identified on the basis of correlations observed in the HMBC spectrum of **2-2**. Two of the methyl group signals (0.81 ppm, s, and 1.02 ppm, d, J = 7.0 Hz) exhibited correlations to C-17 (65.8 ppm) (Table 2-3), thereby identifying the H-18 (s) and H-21 (d, J = 7.0 Hz) resonances, respectively. It follows that the remaining methyl resonances arise from H-19 (1.00 ppm, s) and H-27 (1.10 ppm, d, J = 7.1 Hz). Other HMBC correlations observed for **2-2** were consistent with the assignments presented in Figure 2-4 and Table 2-3.

Correlations observed in the HMBC spectrum of **2-2** (Table 2-3) between H-1' (4.23 ppm) and C-3 (76.5 ppm), and between H-3 (4.01 ppm) and C-1' (104.9 ppm), confirmed the presence of a 3-*O*-glucoside linkage. The coupling constant of H-1' (J = 7.8 Hz) showed that the anomeric glucosyl proton was axially oriented and the glucoside linkage was therefore equatorially oriented (β-linkage).

The negative ion ES-MS spectrum of **2-2** in MeCN-H_2O (1:1) at -100 V showed a pseudomolecular ion at *m/z* 577.5 $(M-H)^-$ corresponding to the loss of H^+ from a compound of molecular weight 578 daltons.

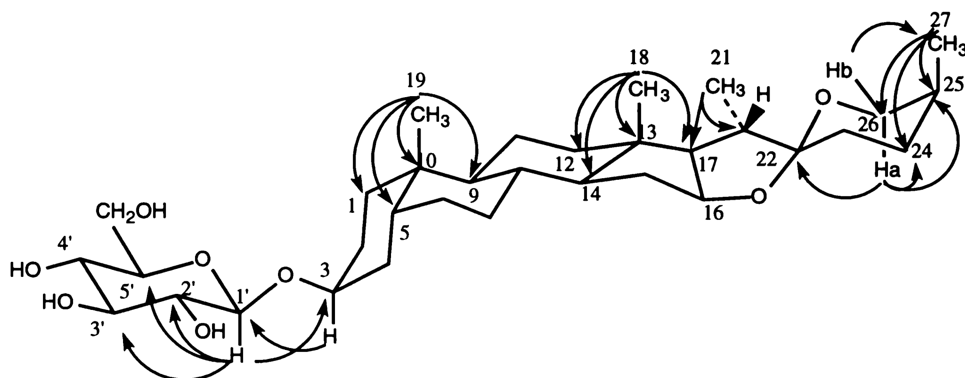


Figure 2-4. Selected HMBC correlations observed for sarsasapogenin β-D-glucoside (**2-2**).

Table 2-3. Selected HMBC correlations observed for sarsasapogenin β-D-glucoside (**2-2**) (ppm in DMSO-D_6).

^1H signal	correlated ^{13}C signal(s)
4.38 (H-16)	44.2 (C-13)
4.23 (H-1')	76.5 (C-3), 77.5 (C-2'), 80.6 (C-5'), 80.8 (C-3')
4.01 (H-3)	104.9 (C-1')
3.88 (H-26b)	19.9 (C-27)
3.31 (H-26a)	29.3 (C-24), 30.4 (C-25), 112.8 (C-22)
3.22 (H-3')	74.1 (C-4'), 77.5 (C-2'), 80.6 (C-5')
3.14 (H-4')	65.0 (C-6'), 80.6 (C-5'), 80.8 (C-3')
3.02 (H-2')	80.8 (C-3'), 104.9 (C-1')
1.10 (H-27)	29.3 (C-24), 30.4 (C-25), 68.2 (C-26)
1.02 (H-21)	45.5 (C-20), 65.8 (C-17), 112.8 (C-22)
1.00 (H-19)	33.4 (C-1), 38.8 (C-10), 39.8 (C-5), 43.7 (C-9)
0.81 (H-18)	43.6 (C-12), 44.2 (C-13), 59.6 (C-14), 65.8 (C-17)

Table 2-4. ^{1}H and ^{13}C NMR assignments and selected COSY correlations determined for sarsasapogenin β-D-glucoside (**2-2**) (ppm in DMSO-D_6).

	^{1}H*	^{13}C*	COSY
1	1.82, 1.50	33.4	
2	1.90, 1.19	30.3	
3	4.01 (s)	76.5	H-4α, H-4β, H-2α, H-2β
4	1.47	34.1	
5	1.81	39.8	
6	1.46, 1.12	30.1	
7	1.62, 1.50	30.0	
8	1.61	38.8	
9	1.44	43.7	
10	-	38.5	
11	1.43, 127	24.4	
12	1.76, 1.23	43.6	
13	-	44.2	
14	1.24	59.6	
15	1.99, 1.24	35.3	
16	4.38 (m)	84.3	H-15α, H-15β, H-17
17	1.76	65.8	
18	0.81 (s)	20.1	
19	1.00 (s)	27.5	
20	1.85	45.5	
21	1.02 (d, J = 7.0 Hz)	18.4	
22	-	112.8	
23	1.37	29.5	
24	1.93, 1.43	29.3	
25	1.73	30.4	
26	3.88 (dd, J = 2.8, 8.3, 2.6 Hz)	68.2	H-26b, H-25
	3.31 (d, J = 11.1 Hz)		H-26a, H-25
27	1.10 (d, J = 7.1 Hz)	19.9	
1'	4.23 (d, J = 7.8 Hz)	104.9	H-2'
2'	3.02 (t, J = 8.2, 8.5 Hz)	77.5	H-1', H-3'
3'	3.22 (t, J = 8.7, 8.9 Hz)	80.8	H-2', H-4'
4'	3.14 (m)	74.1	H-3', H-5'
5'	3.14 (m)	80.6	H-4', H-6'a, H-6'b
6'	3.51 (br d, J = 8.2 Hz)	65.0	H-5', H-6'b
	3.73 (d, J = 11.3 Hz)		H-5', H-6'a

* $^{1}J_{^{1}H-^{13}C}$ correlations established in a HMQC experiment

2.3 Synthesis of 3β-D-Epismilagenin β-D-Glucoside

2.3.1 Introduction

Miles et al. (1992b, 1993, 1994b) and Wilkins et al. (1994) have proposed that the metabolic pathway whereby plant saponins are converted to insoluble biliary crystalline material is via hydrolysis of glycoside bonds to give free sapogenins, oxidation of the 3β-hydroxyl group to a 3-keto group, reduction of the keto group to afford a 3α-hydroxyl group, and combination with D-glucuronic acid to form an episapogenin glucuronides (Figure 1-12).

To test the validity of the proposed pathway, a C-3 deuterium labelled compound was prepared, and its uptake and metabolism were followed using well established GC-MS analysis methods (Wilkins et al., 1994). If, in ovine metabolism, the reduction of a 3-keto group to afford a 3α-hydroxyl group, as proposed by Miles et al. (1992b, 1993, 1994b) is a reversible process, dosing of a 3β-D labelled 3α-OH sapogenin would result in the progressive loss of the 3β-D (Figure 2-5).

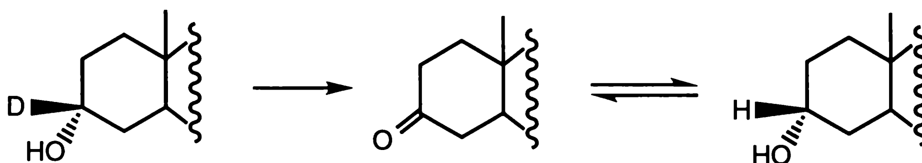


Figure 2-5. Proposed pathway for the loss of the 3-deuterium atom of episapogenin during ovine metabolism.

Since experience in our laboratory is that the uptake of saponins in dosing trials is generally greater than that of the corresponding sapogenins (i.e. glycosylated sapogenins appear to be more bio-available in ovine metabolism than is the cases for free sapogenins), 3β-D-epismilagenin β-D-glucoside became a target compound for synthesis, and use in a dosing experiment.

2.3.2 Results and Discussion

Sarsasapogenin (**2-1**), a relatively cheap and readily available substance, was used as the starting material to prepare the target compound, 3β-D-epismilagenin β-D-glucoside (**2-8**). The reactions involved in the synthesis of **2-8** are outlined in Figure 2-6.

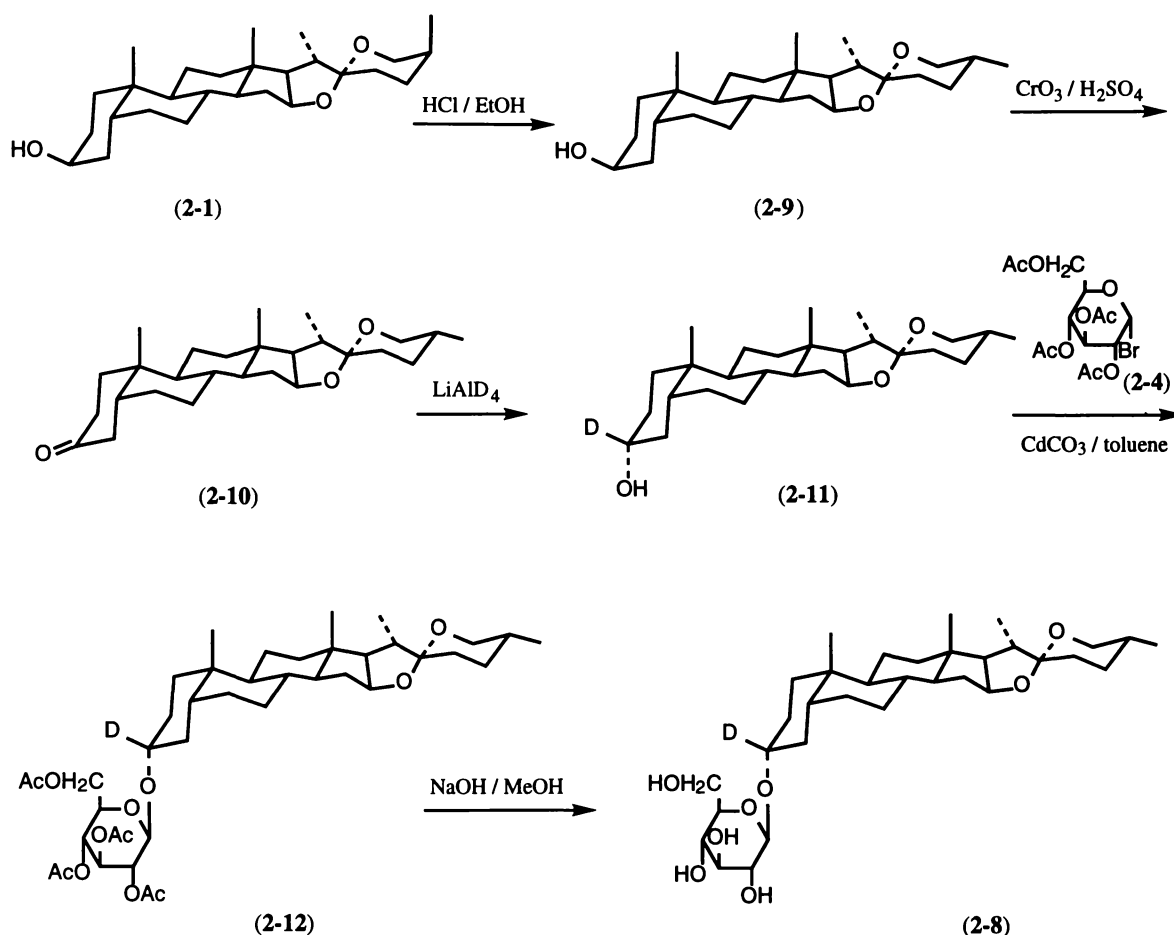


Figure 2-6. Reactions involved in the synthesis of 3β-D-epismilagenin β-D-glucoside (**2-8**).

The isomerisation of sarsasapogenin (**2-1**) to smilagenin (**2-9**) (the 25*R*-analogue of **2-1**) was performed in hydrochloric acid-ethanol solution. The procedure was based on those of Marker and Rohrmann (1939) and Wall et al. (1955b), in which sapogenin was refluxed for 96 hours with concentrated hydrochloric acid. Thereafter additional concentrated hydrochloric acid was added and the mixture refluxed for a further 24 hours.

Wall et al. (1955b) detected four sapogenins in the reaction mixture using column chromatography. These sapogenins were identified as sarsasapogenin (**2-1**), smilagenin

(**2-9**), and dehydrated analogues of sarsasapogenin and smilagenin (probably 2-enes). Of these, smilagenin (**2-9**) and dehydrated smilagenin were major constituents and sarsasapogenin (**2-1**) and dehydrated sarsasapogenin were minor constituents.

In this study, it was found that the second addition of concentrated hydrochloric acid increased the level of dehydrated compounds. Accordingly isomerisation reactions were routinely carried out using only the initial addition of hydrochloric acid. The 25*R*:25*S* isomer ratio of the reaction products was determined by SIM GC-MS analysis to be 88.6:11.4. GC-MS analysis also indicated the presence of moderate levels of dehydrated compounds.

Recrystallisation of the product mixture from acetone progressively enhanced the 25*R*:25*S* ratio of the product mixture from 88.6:11.4 (first) to 93.6:6.4 (second) and then 96.3:3.7 (third recrystallisation). ^{1}H NMR readily distinguished the 25*R*:25*S* isomers. The H-26a and H-26b resonances of sarsasapogenin (**2-1**, 25*S*) occurred at 3.28 and 3.94 ppm while those of smilagenin (**2-9**, 25*R*) occurred at 3.36 and 3.46 ppm, respectively. The ^{13}C NMR data determined for **2-9** are presented in Table 2-5.

Smilagenin (**2-9**) was oxidised to smilagen-3-one (**2-10**), according to the method of Djerassi et al. (1956), using CrO_3/H_2SO_4 in acetone at room temperature for 2 hours. Comparison of ^{1}H NMR spectral data determined for **2-10** with that determined for **2-9** showed the absence in **2-10** of a signal attributable to the H-3 resonance of **2-9** (4.10 ppm) while a **CHOH** signal at 67.1 ppm in the ^{13}C NMR spectrum of **2-9** had been replaced by a **C=O** signal at 213.2 ppm in the ^{13}C NMR spectrum of **2-10** (Table 2-5).

Smilagen-3-one (**2-10**) was reduced with $LiAlD_4$ to afford 3β-D-epismilagenin (**2-11**). The product mixture was shown by GC-MS to be comprised of 81.1% of 3β-D-epismilagenin, 13.6% of 3α-D-smilagenin and 5.3% of 3β-D-episarsasapogenin.

Shoppee and Summers (1950) have examined the stereochemical selectivity of lithium aluminium hydride reduction reactions of 3-keto-5α- and 3-keto-5β-steroids (Figure 2-7). In 5β-spirostanes, the steric hindrance of the α-face (lower face) is such that hydride (or deuteride) attachment occurs preferentially on the β-face (upper face). Thus reduction of smilagen-3-one with lithium aluminium deuteride affords predominantly 3β-D-epismilagenin. The minor 3β-D-episarsasapogenin contribution to the reduction products can be attributed to the presence of a residual level of sarsasapogenone (typically 3-5%) in the starting material.

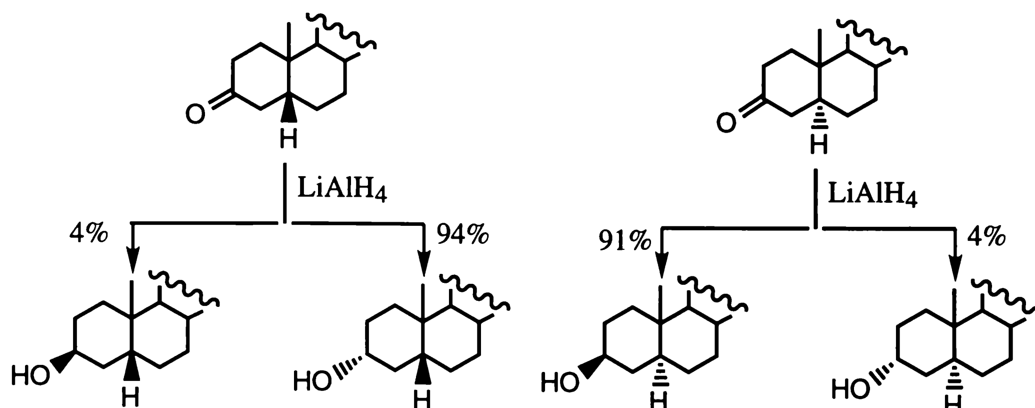


Figure 2-7. Stereochemical outcomes of lithium aluminium hydride reduction of 3-keto-5α- and 3-keto-5β-steroids (from Shoppee and Summers, 1950).

A comparison of the ^{13}C NMR spectra of smilagenin (**2-9**) and of 3β-D-epismilagenin (**2-11**) revealed the absence in the latter spectrum of a signal attributable to the C-3 signal of 3β-D-epismilagenin (**2-11**) (Table 2-5). This can be attributed to a combination of four factors, the overall consequence of which is to reduce the signal of the deuterated C-3 to a level such that it was not observed.

Firstly, ^{2}D, which has I = 1, is not decoupled on ^{1}H irradiation, thus deuterated carbons experience ^{13}C-^{2}D coupling (typically a 1:1:1 multiplet for a carbon atom carrying one deuterium). Secondly, deuterated carbons do not exhibit NOE enhancements on ^{1}H irradiation (thereby reducing their intensities by a factor of ca 2-3 fold compared to protonated carbons). Thirdly, the relaxation time of a deuterated carbon is typically greater than that of a non-deuterated carbon. Fourthly, since deuterium is a quadrupolar nucleus, lines are significantly broadened compared to the corresponding lines in the non-deuterated analogue. The net effect of these factors is to dramatically reduce the observability of the deuterated ^{13}C signal.

Table 2-5. ^{13}C NMR assignments for smilagenin (**2-9**), smilagenone (**2-10**), 3β-D-epismilagenin (**2-11**) and epismilagenin (**2-14**) (ppm in $CDCl_3$)

Carbon	**2-9***	**2-14***	**2-9**	**2-10**	**2-11**
1	29.9	35.5	30.0	37.2	35.4
2	27.8	30.5	27.8	37.0	30.4
3	66.9	71.8	67.1	213.2	-
4	33.5	36.5	33.6	42.4	36.4
5	36.5	42.1	36.6	44.3	42.1
6	26.6	27.1	26.6	26.5	27.1
7	26.6	26.7	26.6	26.0	26.7
8	35.3	35.5	35.3	35.2	35.5
9	40.3	40.6	39.9	40.9	40.6
10	35.3	34.7	35.3	35.1	34.7
11	20.9	20.6	20.9	21.1	20.6
12	39.9	40.3	40.3	40.2	40.3
13	40.7	40.6	40.7	40.7	40.6
14	56.5	56.4	56.5	56.3	56.4
15	31.7	31.8	31.8	31.8	31.8
16	80.9	80.9	80.9	80.8	80.9
17	62.3	62.3	62.3	62.3	62.3
18	16.4	16.5	16.5	16.5	16.5
19	23.9	23.4	23.9	22.7	23.4
20	41.6	41.6	41.6	41.7	41.7
21	14.5	14.5	14.5	14.5	14.5
22	109.2	109.2	109.2	109.3	109.3
23	31.4	31.4	31.4	31.4	31.4
24	28.8	28.8	28.8	28.8	28.8
25	30.3	30.3	30.3	30.3	30.3
26	66.8	66.8	66.8	66.9	66.9
27	17.1	17.1	17.2	17.2	17.2

* reported by Agrawal et al. (1985)

The glucosylation of 3β-D-epismilagenin (**2-11**) was carried out using 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**2-4**) to produce 3β-D-epismilagenin β-D-glucoside tetraacetate (**2-12**). The same methodology as that used in the preparation of sarsasapogenin β-D-glucoside tetraacetate (**2-5**) was utilised in this synthesis.

The ^{1}H NMR spectrum (in $CDCl_3$) of **2-12** included four angular methyl signals at 0.73 (3H, s, H-18), 0.77 (3H, d, J = 6.1 Hz, H-27), 0.91 (3H, s, H-19) and 0.94 ppm (3H, d, J = 6.8 Hz, H-21), four acetyl group signals at 1.99, 2.01, 2.02 and 2.06 ppm (4 × 3H, s, four $-COCH_3$, respectively), and lowfield genin signals at 3.36 (1H, t, J = 10.6 and 10.9 Hz, H-26b), 3.46 (1H, m, H-26a) and 4.57 ppm (1H, d, J = 7.7 Hz, H-1').

The ^{13}C and DEPT NMR spectra showed **2-12** to contain 8 methyl, 11 methylene, 13 methine and 7 quaternary carbons. The C-3 signal was not found in the ^{13}C spectrum due to its intensity being reduced by deuteration (see preceding discussion).

The COSY spectrum of **2-12** identified the following glucosyl proton correlations: 4.57 ppm (H-1') ↔ 4.92 ppm (H-2') ↔ 5.18 ppm (H-3') ↔ 5.05 ppm (H-4') ↔ 3.67 ppm (H-5') ↔ 4.09 ppm (H-6'a) ↔ 4.24 ppm (H-6'b). ^{1}H, ^{13}C and COSY NMR data (in $CDCl_3$) determined for **2-12** is given in Table 2-6.

The positive ion ES-MS of **2-12** at +100 V and using acetonitrile-water (1:1) as solvent showed pseudomolecular ions at *m/z* 770.4 $(M+Na)^+$ (addition of sodium to a compound of molecular weight 747 daltons), *m/z* 765.5 $(M+NH_4)^+$ and *m/z* 748.2 $(M+H)^+$.

Table 2-6. ^{1}H and ^{13}C NMR assignments and selected COSY correlations determined for 3β-D-epismilagenin-β-D-glucoside tetraacetate (**2-12**) (ppm in $CDCl_3$)

Atom	^{1}H	^{13}C	COSY
1	1.84, 1.56	34.1	
2	1.79, 1.34	29.7	
3		-	
4	1.80, 0.91	35.2	
5	1.35	42.2	
6	1.84, 1.25	26.6[a]	
7	1.40, 1.12	26.4[a]	
8	1.50	35.5	
9	1.36	40.1	
10	-	35.0	
11	1.37, 1.18	20.9	
12	1.69, 1.12	40.3	
13	-	40.7	
14	1.19	56.2	
15	1.97, 1.19	31.8	
16	4.39 (m)	81.0	H-15α, H-15β, H-17
17	1.75	62.3	
18	0.73 (s)	16.5	
19	0.91 (s)	23.4	
20	1.85	41.6	
21	0.94 (d, 6.8 Hz)	14.5	
22	-	109.2	
23	1.63, 1.25	31.4[b]	
24	1.63, 1.25	28.8[b]	
25	1.45	30.2	
26	3.46 (m),3.36 (t, 10.6, 10.9 Hz)	68.7	H-26a, H-25, H-26b
27	0.77 (d, 6.1 Hz)	17.1	
1'	4.57 (d, 7.7 Hz)	99.9	H-2'
2'	4.92 (t, 8.0, 7.9 Hz)	71.7	H-1', H-3'
3'	5.18 (t, 7.8, 7.8 Hz)	72.9	H-2', H-4'
4'	5.05 (t, 9.7, 9.4 Hz)	68.7	H-3', H-5'
5'	3.67 (m)	71.7	H-4', H-6', H-6"
6'	4.24 (dd, 4.8, 7.3, 4.9 Hz);	62.2	H-5', H-6'b
	4.09 (br d, 11.9 Hz)		H-5', H-6'a
OCOCH3	1.99 (s)	20.6	
	2.01 (s)	20.7	
	2.02 (s)	20.8	
	2.06 (s)	20.8	
OCOCH3		169.1	
		169.4	
		170.4	
		170.7	

[a, b] interchanged

Hydrolysis of **2-12** in a 2.5 M methanolic sodium hydroxide solution at 50-60°C for 1.5 hours afforded 3β-D-epismilagenin β-D-glucoside (**2-8**) in a yield of 92.7%. The ^{1}H NMR spectrum (in DMSO-D_6) of **2-8** showed signals at 0.78 (3H, s, H-18), 0.81 (3H, d, J = 6.3 Hz, H-27), 0.97 (3 H, s, H-19), 0.98 (3H, d, J = 6.2 Hz, H-21) and 4.29 ppm (1H, d, J = 7.7 Hz, H-1'). The disappearance of four singlet signals in the region 2.0-2.1 ppm is consistent with the hydrolysis of the four acetyl groups. The coupling constant of the H-1' glucosyl proton (4.29 ppm, J = 7.7 Hz) revealed that this proton was axially (α-) oriented, and 1,2-*trans* coupled with H-2' (Agrawal, 1992), hence the glucoside linkage was equatorially inclined (i.e. a β-glucoside).

The ^{13}C spectrum exhibited a total of 32 signals, amongst which 26 signals arose from the genin and 6 signals from the sugar unit. No ^{13}C signals attributable to acetoxyl groups were observed.

The COSY spectrum of **2-8** revealed the following correlation pathways: 4.29 ppm (H-1') ↔ 2.96 ppm (H-2') ↔ 3.20 ppm (H-3') ↔ 3.12 ppm (H-4', H-5') ↔ 3.48 ppm (H-6'a) ↔ 3.71 ppm (H-6'b); 3.49 ppm (H-26a) ↔ 3.29 ppm (H-26b) ↔ 1.60 ppm (H-25); and 4.38 ppm (H-16) ↔ 1.99 ppm (H-15α) ↔ 1.77 ppm (H-17) ↔ 1.24 ppm (H-15β). Two overlapping proton resonances (H-4' and H-5') at 3.12 ppm were assigned, according to correlations observed in the HMQC spectrum, to 74.1 (C-4') and 80.6 (C-5') ppm respectively.

A combination of ^{1}H, ^{13}C, COSY, HMBC (Table 2-7) and HMQC spectral data led to the assignments of the ^{1}H and ^{13}C signals of **2-8** presented in Table 2-8.

Table 2-7. Selected HMBC correlations observed for 3β-D-epismilagenin β-D-glucoside (**2-8**) (ppm in DMSO-D_6).

^{1}H signal	correlated ^{13}C signal(s)
4.29 (H-1')	77.5 (C-2')
3.49 (H-26b)	33.8 (C-25), 21.0 (C-27)
3.29 (H-26a)	112.3 (C-22), 33.8 (C-25), 32.4 (C-24), 21.0 (C-27)
3.20 (H-3')	74.1 (C-4')
1.99 (H-15α)	84.2 (C-16), 65.8 (C-17), 59.6 (C-14), 44.0 (C-13)
1.90 (C-20)	112.3 (C-22), 65.8 (C-17), 44.0 (C-13), 18.6 (C-21)
1.24 (H-15β)	84.2 (C-16), 59.6 (C-14), 44.0 (C-13), 20.1 (C-18)
0.98 (H-21)	112.3 (C-22), 65.8 (C-17), 45.0 (C-20)
0.97 (H-19)	45.4 (C-5), 43.7 (C-9), 38.3 (C-10), 37.6 (C-1)
0.81 (H-27)	69.8 (C-26), 65.8 (C-17), 33.8 (C-25), 32.4 (C-24)
0.78 (H-18)	65.8 (C-17), 59.6 (C-14), 44.0 (C-13), 43.6 (C-12)

The negative ion ES-MS spectrum of **2-8** in MeCN-H_2O (1:1) at -100 V showed a pseudomolecular ion at *m/z* 578.2 $(M-H)^-$, which corresponds to the loss of H^+ from a compound of molecular weight 579 daltons.

^{1}H NMR analyses showed that in addition to the expected 3β-D-epismilagenin β-D-glucoside (**2-8**) (H-1' resonance at 4.29 ppm, d, J = 7.7 Hz), a moderate level of 3α-D-smilagenin β-D-glucoside (H-1' resonance at 4.21 ppm, d, J = 7.7 Hz) was also present in the final product.

The HMQC spectrum of the product confirmed the presence of a major and a minor glucoside. In particular, correlations arising from the H-19 signals of 3β-D-epismilagenin β-D-glucoside and 3α-D-smilagenin β-D-glucoside respectively (with C-1, C-5, C-9, C-13) and the H-21 signals of 3β-D-epismilagenin β-D-glucoside and 3α-D-smilagenin β-D-glucoside respectively (with C-17, C-20, C-22) were observed.

GC-MS analyses of the hydrolysed **2-8** (*m/z* 139 selected ion profile) established the presence of 3β-D-epismilagenin (**2-10**) (77.9%) and 3α-D-smilagenin (14.5%). Further selected ion analyses (*m/z* 284, 285, 344, 345, 458 and 459) (Wilkins et al., 1994) afforded confirmatory ion ratio data for the respective isomers, *m/z* 285/*m/z* 345 = 8.9, *m/z* 345/*m/z* 459 = 1.7 for 3β-D-epismilagenin (**2-10**) and *m/z* 285/*m/z* 345 = 2.6, *m/z* 345/*m/z* 459 = 0.6 for 3α-D-smilagenin.

Table 2-8. ^{1}H and ^{13}C NMR assignments and COSY correlations determined for 3β-D-epismilagenin β-D-glucoside (**2-8**) (ppm in DMSO-D_6).

Atom	^{1}H*	^{13}C *	COSY
1	1.82, 1.55	37.6	
2	1.91, 1.29	30.6[a]	
3	-	-	
4	1.81, 0.94	38.7	
5	1.43	45.4	
6	1.81, 1.14	30.1[a]	
7	1.48, 1.18	30.3[a]	
8	1.62	38.9	
9	1.48	43.7	
10	-	38.3	
11	1.44, 1.26	24.1	
12	1.77,1.25	43.6	
13	-	44.0	
14	1.23	59.6	
15	1.99, 1.24	35.4	
16	4.38 (m)	84.2	H-15α, H-15β, H-17
17	1.77	65.8	
18	0.78 (s)	20.1	
19	0.97 (s)	27.1	
20	1.90	45.0	
21	0.98 (d, 6.2 Hz)	18.6	
22	-	112.3	
23	1.70, 1.57	34.9	
24	1.66, 1.40	32.4	
25	1.60	33.8	
26	3.49 (m),3.29 (d, 10.8 Hz)	69.8	H-26b, H-26a, H-25
27	0.81 (d, 4.3 Hz)	21.0	
1'	4.29 (d, 7.7 Hz)	104.3	H-2'
2'	2.96 (t, 8.2, 8.1 Hz)	77.5	H-1', H-3'
3'	3.20 (t, 8.1, 9.0 Hz)	80.7	H-2', H-4'
4'	3.12 (m)	74.1	H-3', H-5'
5'	3.12 (m)	80.6	H-4', H-6'a, H-6'b
6'	3.48 (br d, 10.5 Hz)	65.1	H-5', H-6'b
	3.71 (br d, 11.0 Hz)		H-5', H-6'a
2'-OH	4.84 (br s)		
3'-OH	4.92 (br s)		
4'-OH	4.89 (br s)		
6'-OH	4.47 (br s)		

* ^{1}J ^{1}H-^{13}C correlations established in a XHCORR experiment
[a] interchanged

2.4 Preparation of 20,23,23-$^{2}D_3$-Sarsasapogenin

2.4.1 Introduction

Of interest in dosing experiments is the possibility that an assessment of the levels of free and conjugated deuterated and non-deuterated sapogenins in faeces samples randomly gathered from a sheep grazing pasture plants containing saponins, and dosed once daily with a deuterated sapogenin, could be used to calculate the daily intake of natural plant saponins under normal grazing conditions.

Sapogenins can be easily deuterated at C-2 and C-4, and/or at C-23 and C-20, and some other positions (Shapiro et al., 1964; Faul et al., 1970). Deuteration at C-2 and C-4 was performed via a 3-keto intermediate. The sequence of reactions used comprised oxidation of the 3-hydroxyl to a 3-keto group, catalytic deuteration with basic deuteriomethanol-deuterium oxide, followed by reduction of the 3-keto group to a 3-hydroxyl group (Figure 2-8 A).

C-23 and C-20 were deuterated by refluxing the sapogenin in AcOD or DCl-EtOD (Figure 2-8 B). Deuteration can be envisaged as proceeding via a ring opened 22-keto intermediate.

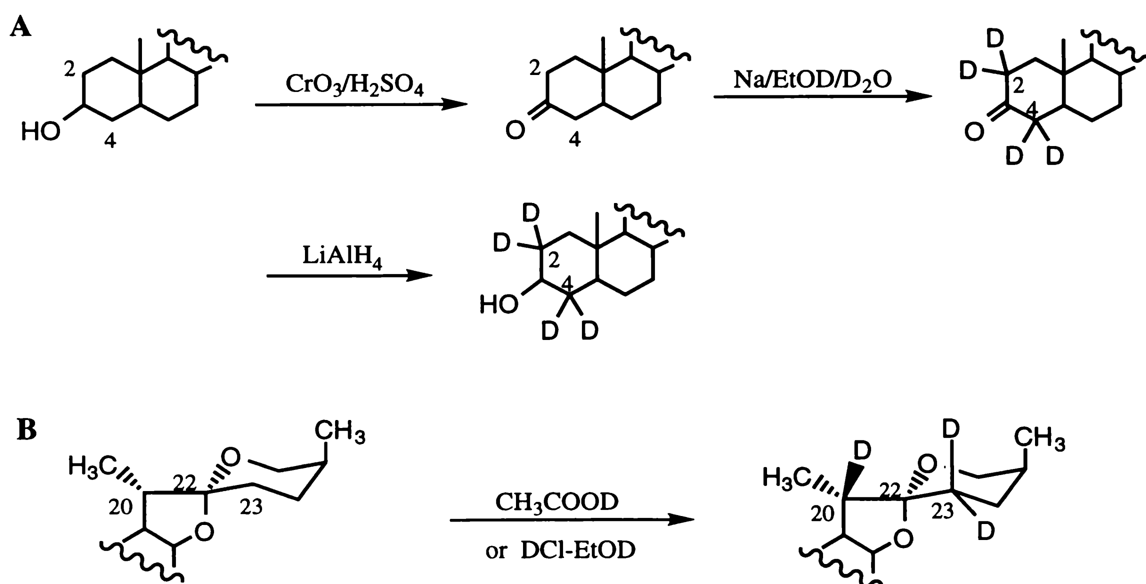


Figure 2-8. Deuterium labelling at ring A or ring E/F of sapogenins.

Wilkins et al. (1994) have reported a GC-MS procedure for the quantitative analyses of sapogenins using selection detection (*m/z* 139 ion profile). The *m/z* 139 fragment, arising from ring E/F (Figure 2-9), is normally the base peak in the mass spectra of sapogenins.

When deuterium atoms are present in ring F of sarsasapogenin, the corresponding fragment ions occur at *m/z* 140, 141 and 142 in D-, D_2- and D_3-substituted sarsasapogenin respectively. The selected ion mode GC-MS procedure of Wilkins et al. (1994) was therefore modified to include *m/z* 139, 140, 141 and 142 ion profiles.

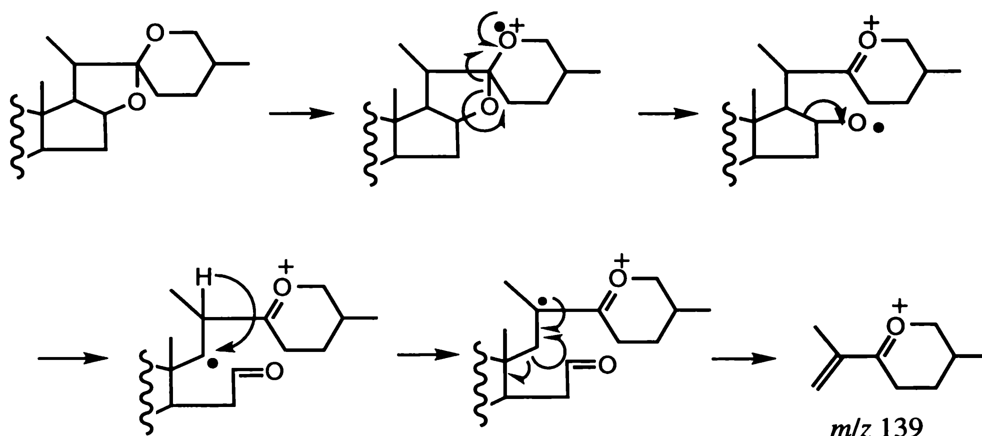


Figure 2-9. Structure of the *m/z* 139 fragment ion of sapogenins.

2.4.2 Results and Discussion

Deuteration at C-20 and C-23 was achieved by refluxing sarsasapogenin in deuterioacetic acid (CH_3COOD) (prepared by reacting acetic anhydride with D_2O). This afforded a mixture of non-D-, D-, D_2- and D_3-sarsasapogenin (Figure 2-10).

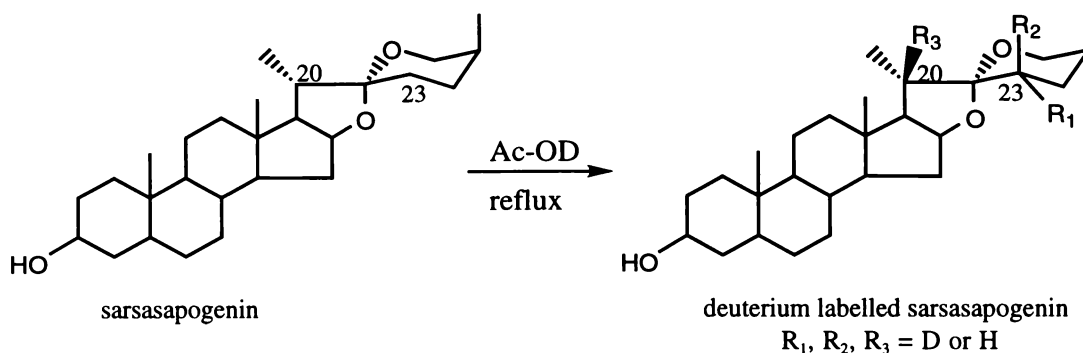


Figure 2-10. Deuterium-labelling reaction of sarsasapogenin at C-20 and C-23.

Faul et al. (1970) have reported that two protons (H-23a, H-23b) of (25*R*)-5α-spirostane were easily substituted by deuterium when the (25*R*)-5α-spirostane was refluxed for 1 hour in AcOD, and that H-20 was only significantly replaced by deuterium upon additional refluxing. Time seems to be a very important factor in determining the extent to which deuteriums (one to three) are introduced into the genin.

To assess the significance of reaction (exchange) time, a series of trial reactions were performed using sarsasapogenin and freshly prepared deuterioacetic acid (CH_3COOD). Subsamples were collected after 15, 30 and 45 minutes, and 1, 2, 3, 4, 5, 6, 10, 12, 24, 48, 72, 96, 120, 144 and 168 hours refluxing. After acetylation with acetic anhydride in pyridine the samples were analysed using the selected ion mode GC-MS protocol of Wilkins et al. (1994), modified to include *m/z* 139, 140, 141 and 142 profiles for non-deuterated, mono-deuterated, di-deuterated and tri-deuterated sarsasapogenins. Results are presented in Table 2-9 and Figure 2-11.

Table 2-9. Ratios (%) of non-deuterated, mono-deuterated, di-deuterated and tri-deuterated sarsasapogenins in the reaction mixtures after refluxing.

Time (h)	sarsasapogenin *m/z* 139	D-sarsasapogenin *m/z* 140	D_2-sarsasapogenin *m/z* 141	D_3-sarsasapogenin *m/z* 142
0	83.2	13.4	2.2	1.2
0.25	3.6	22.6	60.3	13.5
0.5	3.8	21.5	59.1	15.7
0.75	3.0	20.4	58.1	18.5
1	3.4	20.3	55.5	20.8
2	3.0	19.6	48.4	29.0
3	3.1	18.6	46.1	32.2
4	3.1	18.7	42.1	36.1
5	3.1	17.5	40.1	39.3
6	3.2	17.8	38.1	40.9
10	3.3	13.4	33.5	49.8
12	3.0	13.7	34.9	48.5
24	2.8	12.9	31.9	52.4
48	3.0	12.9	33.7	50.4
72	2.9	13.6	34.8	48.7
96	2.9	12.3	34.9	49.9
120	2.9	13.1	34.5	49.5
144	2.8	12.5	34.4	50.3
168	2.7	12.1	34.7	50.4

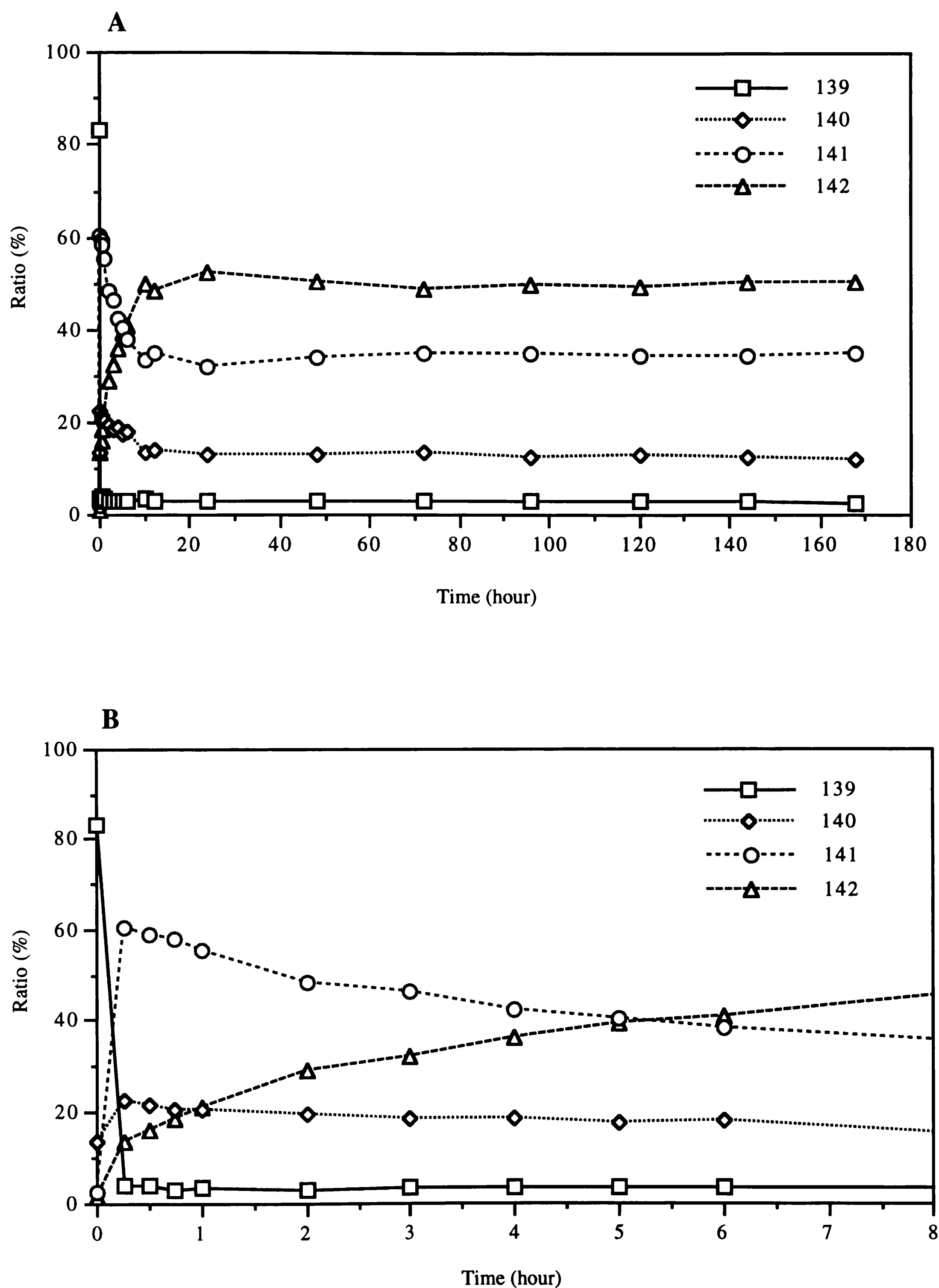


Figure 2-11. Ratio (%) of non-deuterated (*m/z* 139), mono-deuterated (*m/z* 140), di-deuterated (*m/z* 141) and tri-deuterated (*m/z* 142) sarsasapogenins in the reaction mixtures after refluxing. **A**: 0-168 hours. **B**: expansion of the 0-8 hours region of **A**.

The results, presented in Table 2-9 and depicted Figure 2-11 A and B, revealed the level of non-deuterated sarsasapogenin (*m/z* 139) changed sharply after being refluxed in acetic acid-OD. It decreased rapidly from 83% to 4% during the first 15 minutes after refluxing, and then stayed approximately constant at the 4% level for 168 hours.

The level of di-deuterated sarsasapogenin (D_2-sarsasapogenin) (*m/z* 141) increased quickly, rising from 2% to 60% during the first 15 minutes of refluxing. After that, the level slowly decreased to ca 33% after 15 hours, and remained at this level for the rest of the reflux period (maximum 168 hours).

The level of tri-deuterated sarsasapogenin (D_3-sarsasapogenin) (*m/z* 142 ion profile) slowly increased from zero to ca 50% after 15 hours of refluxing, and then remained at this level during rest of the 168 hour investigation period. As the level of D_3-sarsasapogenin increased, the level of D_2-sarsasapogenin decreased. After approximately five hours the levels of both D_2-sarsasapogenin and D_3-sarsasapogenin were the same (40%).

These changes in the relative levels of D_2-sarsasapogenin and D_3-sarsasapogenin revealed the comparatively rapid introduction of two deuteriums (D-23a and D-23b), followed by the slow introduction of the third deuterium (D-20). These observations are consistent with Faul et al's (1970) and Shapiro et al's (1964) conclusion that two of the ring protons (H-23a and H-23b) of sarsasapogenin can be easily substituted by deuterium. The H-23a and H-23b protons are less sterically hindered than the H-20 proton which is adjacent to a methyl group (Figure 2-11).

The level of mono-deuterated sarsasapogenin (D-sarsasapogenin) (*m/z* 140 ion profile) increased from 13% to 23% during the first 15 minutes after refluxing, and slowly decreased to 13% during a 15 hour period, and then stayed at this level for the rest of the 168 hour investigation period.

The results, presented in Figure 2-11, showed that equilibrium levels of non, mono-, di- and tri-deuterated sarsasapogenins are achieved after ca 20 hours refluxing, and that levels remain stable during the rest of the 168 hour investigation period.

When sarsasapogenin 3-acetate was substituted for sarsasapogenin, similar deuteration results were obtained. After deuteration, hydrolysis of the 3-acetoxyl group, using sodium hydroxide in methanol, afforded deuterated sarsasapogenin.

The ^{1}H NMR spectrum of 20,23,23-D_3-sarsasapogenin (in $CDCl_3$) included three singlet methyl signals at 0.76 (3H, s, H-18), 0.97 (3H, s, H-19) and 0.98 ppm (3H, s, H-21), and a doublet methyl signal at 1.07 ppm (3H, d, J = 7.0 Hz, H-27). On the other hand, two doublet and two singlet methyl group signals appeared in the ^{1}H NMR spectrum of sarsasapogenin. The appearance of a broadened H-21 methyl group signal in 20,23,23-D_3-sarsasapogenin, compared to the H-21 methyl group signal of sarsasapogenin as a well defined doublet (J = 6.5 Hz), is consistent with the replacement of H-20 by D-20, since although ^{2}D has I = 1, the $^3J_{H\text{-}C\text{-}C\text{-}D}$ coupling between D-20 and H-21 is smaller than the line width of the H-21 signal.

The H-26a and H-26b resonances of the deuterated sarsasapogenin (3.29 and 3.94 ppm respectively) corresponded with those observed for sarsasapogenin, rather than for smilagenin. This correspondence demonstrated that the configuration of C-25 had not been epimerised from 25*S* to 25*R* when refluxed in deuterioacetic acid (CH_3COOD) for 168 hours.

The ^{13}C NMR spectrum of 20,23,23-D_3-sarsasapogenin, when compared to that of sarsasapogenin showed the absence of C-20 and C-23 signals, due to the deuteration of these carbons. The suppression of the C-20 and C-23 signals can be attributed to a combination of four factors, (i) no decoupling on ^{1}H irradiation, (ii) no NOE enhancements, (iii) greater relaxation times and (iv) broadened lines. The overall consequence of these factors is to reduce the intensity of the deuterated C-20 and C-23 signals to a level such that they were not observed.

Given that equilibrium levels of the deuterated analogues were achieved after 20 hour reaction (see Table 2-9 and Figure 2-11), 24 hours was considered to be an appropriate reaction time for the deuteration reaction since this afforded the maximum level of D_3-sarsasapogenin.

A repetition of the deuterium exchange reaction in which a bulk sample of sarsasapogenin was refluxed in deuterioacetic acid (CH_3COOD) for 24 hour, afforded a product mixture comprised of 65%, 28%, 6% and 1% of D_3-, D_2-, D- and non-deuterated sarsasapogenins respectively. This material was subsequently used in sheep dosing experiments.

2.5 Stability Tests

2.5.1 Introduction

Results from sheep dosing experiments showed that deuterium atoms in 20,23,23-D_3-sarsasapogenin were progressively lost during the 96 hour time course of the dosing experiments (see Section 3.5). For example, a bile extract, recovered after dosing with 20,23,23-D_3-sarsasapogenin, was found to be comprised predominantly of conjugated episarsasapogenins containing 1% of tri-deuterated episarsasapogenin and 40% of mono-deuterated episarsasapogenin, compared to 65% tri- and 6% mono-deuteration in the dosed substrate.

Flåøyen and Wilkins (pers commun) have also observed extensive deuterium loss (> 99%) in an experiment in which a homogenised water-acetic acid solution of 20,23,23-D_3-sarsasapogenin was dosed to a Norwegian sheep.

In order that a better understanding of the factors which contributable to the loss of deuterium atoms from 20,23,23-D_3-sarsasapogenin might be obtained, a series of buffer solutions were prepared and the stability (exchangeability) of the deuterium atoms at 37°C (normal metabolic temperature) was investigated.

2.5.2 Results and Discussion

The stability tests were performed by suspending 20,23,23-D_3-sarsasapogenin in pH 1, 2, 4, 6, and 8 buffer solutions and acetic acid solutions, respectively, at 37°C. The pH of the buffer solutions covered the range of pHs exhibited by ovine digestive fluids, such as those of rumen, intestine and bile fluids (Spector, 1956). Results were compared with those determined for a reference solution of labelled sarsasapogenin in acetic acid at room temperature (ca 17°C). The subsamples were recovered from each of the solutions after 0.25, 0.5, 1, 2, 3, 4, 6, 12, 24, 48 and 72 hours.

Recovered subsamples were acetylated with acetic anhydride in pyridine, prior to selected ion mode (SIM) GC-MS analysis (*m/z* 139, 140, 141 and 142 ion profiles). Results are presented in Table 2-10.

No significant changes were observed in the deuteration levels of labelled sarsasapogenin suspended in any of the buffer solutions (pH 1 to 8) or in acetic acid at room temperature

(17°C) (Table 2-10 and Figure 2-12). In each case, the ratios of the *m/z* 139, 140, 141 and 142 ions were approximately constant over the 72 hour test period.

Significant loss (exchange) of deuterium, however, occurred when the temperature of the acetic acid solution was raised to 37°C. After 72 hours the level of D_3-sarsasapogenin had decreased from 65% to 35%, while the levels of D_2- and D-sarsasapogenin rose from ca 30% and 8% to 41% and 20% respectively (Figure 2-12).

The stability tests showed that 20,23,23-D_3-sarsasapogenin was stable at pHs in the range 1 to 8 at 37°C, in acetic acid at room temperature, and under the subsequent GC-MS analysis conditions. It can therefore be concluded that the deuterium loss observed during dosing experiments (see Sections 3.4 and 3.5) was not likely to have occurred during sample work up and GC-MS analyses.

Table 2-10. Percentage *m/z* 139-142 ion contributions (%) of deuterated sarsasapogenins in buffer solutions and acetic acids at 37°C, or at room temperature.

Soln	Temp	Time (h)	sar *m/z* 139	D-sar *m/z* 140	D_2-sar *m/z* 141	D_3-sar *m/z* 142
		0	1.3	6.1	27.9	64.8
pH 1	37°C	0.25	1.0	5.7	29.1	64.2
		0.5	1.0	5.5	28.2	65.3
		1	1.0	5.5	28.9	64.6
		3	1.0	5.7	28.6	64.7
		6	0.9	5.6	28.3	65.1
		12	0.9	5.4	28.2	65.5
		24	1.0	5.6	28.9	64.5
		48	1.0	5.6	28.1	65.3
		72	1.2	6.8	28.8	63.2
pH 2	37°C	0.25	1.3	6.5	28.2	64.0
		0.5	1.3	6.3	28.2	64.3
		1	1.1	6.6	28.5	63.8
		3	1.3	6.6	28.6	63.4
		6	1.2	6.8	28.5	63.5
		12	1.4	6.6	28.6	63.5
		24	1.1	6.4	27.9	64.5
		48	1.4	7.7	29.5	61.4
		72	1.4	8.0	29.7	60.9
pH 4	37°C	0.25	1.4	8.4	30.1	60.1
		0.5	1.3	7.7	29.6	61.4
		1	1.3	7.2	28.8	62.7
		3	1.7	8.1	30.5	59.7
		6	1.5	7.6	30.0	60.9
		12	1.4	7.7	29.7	61.2
		24	1.5	8.4	30.2	59.9
		48	1.5	8.2	30.3	59.9
		72	1.5	8.5	30.5	59.5
pH 6	37°C	0.25	1.4	8.1	30.0	60.4
		0.5	1.5	7.5	29.2	61.8
		1	1.5	8.2	30.1	60.2
		3	1.5	8.2	30.3	60.0
		6	1.3	7.6	29.5	61.6

		12	1.4	6.9	28.4	63.3
		24	1.4	8.2	29.9	60.5
		48	1.5	7.7	29.4	61.4
		72	1.6	9.2	31.0	58.2
pH 8	37°C	0.25	1.5	7.3	29.1	62.1
		0.5	1.7	10.0	31.9	56.4
		1	1.7	8.8	31.0	58.5
		3	1.4	7.3	29.5	61.8
		6	1.8	9.1	31.1	58.1
		12	1.5	8.1	30.4	59.9
		24	1.6	8.1	30.6	59.7
		48	1.7	10.6	32.7	55.1
		72	1.5	6.1	30.2	62.2
AcOH	37°C	0.25	1.5	7.9	30.1	60.4
		0.5	1.5	7.7	29.9	61.0
		1	1.6	8.2	27.2	63.0
		3	1.9	9.7	31.9	56.5
		6	1.7	9.1	31.7	57.4
		12	2.5	11.1	35.3	51.1
		24	2.2	11.5	35.1	51.2
		48	3.2	15.6	38.9	42.3
		72	4.2	19.7	40.7	35.4
AcOH	RT (17°C)	0.25	1.0	5.5	26.3	67.3
		0.5	1.0	5.4	26.2	67.4
		1	1.0	5.3	26.3	67.4
		3	1.0	5.5	26.3	67.2
		6	1.0	5.6	26.7	66.7
		12	1.0	5.7	26.8	66.4
		24	1.1	5.8	27.1	66.0
		48	1.1	6.6	28.5	63.8
		72	1.0	5.6	26.6	66.8

Soln = solution, Temp = temperature, sar = sarsasapogenin, D-sar = D-sarsasapogenin,

D_2-sar = D_2-sarsasapogenin, D_3-sar = D_3-sarsasapogenin

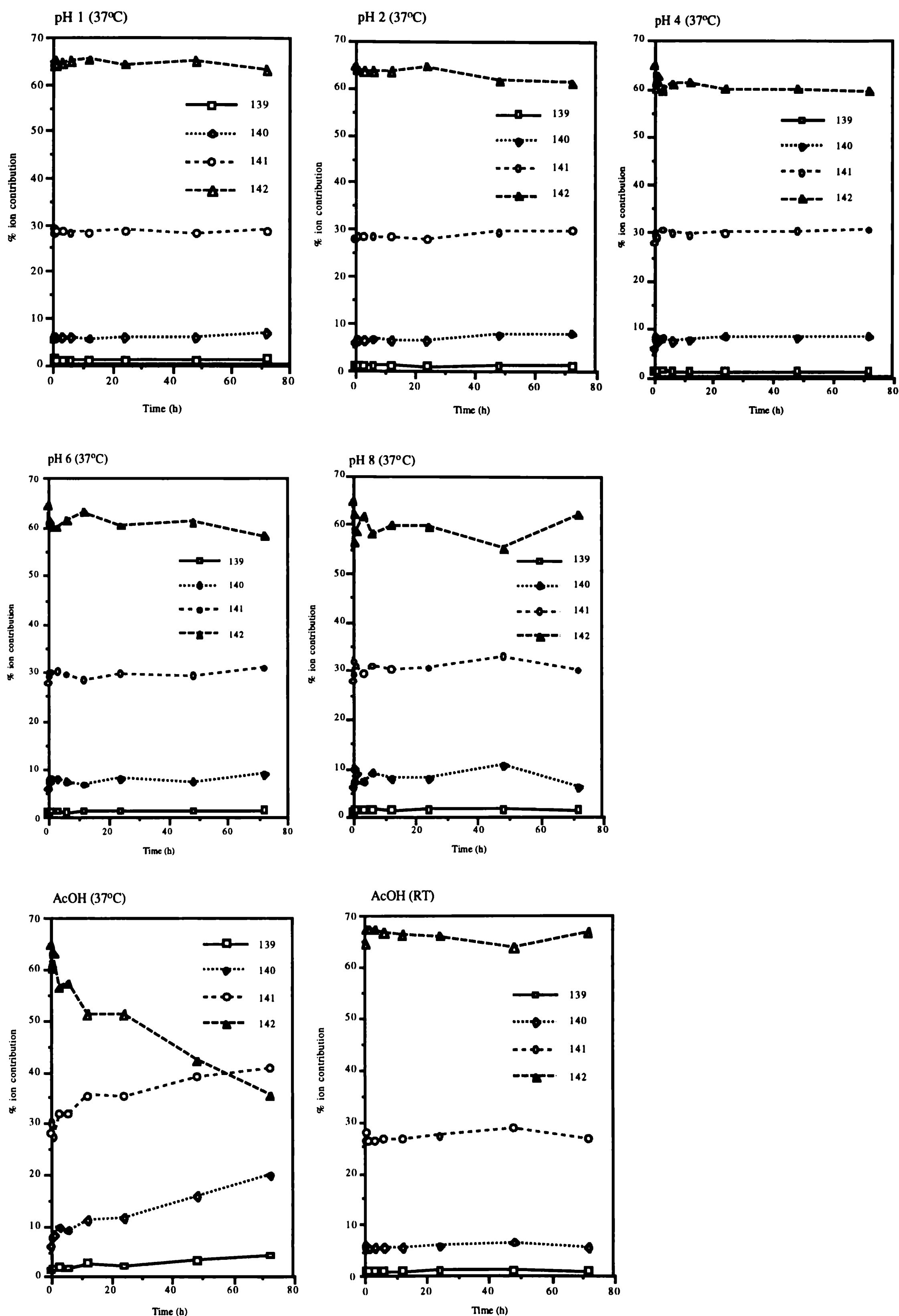


Figure 2-12. Percentage *m/z* 139-142 ion contributions (%) of deuterated sarsasapogenin samples in buffer solutions and acetic acids at 37°C, or at room temperature.

2.6 Summary

Sarsasapogenin β-D-glucoside (**2-2**) was synthesised by coupling of sarsasapogenin (**2-1**) and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide (**2-7**) using cadmium carbonate as catalyst to promote glucoside formation. The purity and structures of intermediates and the target compound were established by analyses of GC-MS, ES-MS, and one- and two-dimensional NMR spectral data.

3β-D-Epismilagenin β-D-glucoside (**2-8**) was synthesised through isomerisation of sarsasapogenin to smilagenin, oxidation of 3-OH group of smilagenin to a 3-keto group, reduction using $LiAlD_4$ to afford 3β-D-epismilagenin (**2-11**), and glucosylation by coupling of 3β-D-epismilagenin (**2-11**) and 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl bromide (**2-7**) using cadmium carbonate as catalyst. Structures of intermediates and the target compound were established by analyses of GC-MS, ES-MS, and one- and two-dimensional NMR spectral data.

20,23,23-D_3-sarsasapogenin was prepared by refluxing sarsasapogenin in deuterioacetic acid (CH_3COOD) for 24 hours. GC-MS analysis showed the product material to be a mixture of non-, mono-, di- and tri-deuterated sarsasapogenins in a percentage contribution of 1%, 6%, 28% and 65% respectively.

Stability (exchangeability) of the deuterated sarsasapogenin was assessed by suspending the material in a series of buffer solutions and in acetic acids. The results showed that D-20 and D-23 were stable in the buffer solutions at 37°C, and in acetic acid at room temperature. Significant loss of deuterium occurred in acetic acid at 37°C.

Chapter Three

ANIMAL TRIALS

3.1 Introduction

Animal dosing experiments using potentially toxic plant materials are a common and important procedure in etiologic studies of hepatogenous photosensitization diseases.

One of the first reported saponin dosing experiments was performed by Mathews (1937), who fed rats alcoholic extracts from *A. lecheguilla* and produced symptoms of photosensitization. *A. lecheguilla* is implicated in the development of hepatogenous photosensitization diseases of livestock in some parts of the USA. In 1940, Mathews also investigated *N. texana* induced photosensitization by dosing this plant to sheep and goats.

The oral administration of an ethanol-water extract from *T. terrestris*, containing diosgenin, yamogenin, tigogenin, neotigogenin, smilagenin, sarsasapogenin, gitogenin and neogitogenin saponins to a South African sheep, was shown by Miles et al. (1994b) to generate free and conjugated analogues of epismilagenin, episarsasapogenin, tigogenin, neotigogenin, smilagenin, sarsasapogenin, gitogenin and neogitogenin along with traces of smilagenone and sarsasapogenone. Biliary crystals, recovered from the sheep which exhibited photosensitization symptoms, were found to be comprised mainly of the calcium salts of epismilagenin β-D-glucuronide and episarsasapogenin β-D-glucuronide (Miles et al., 1994b).

A subsequent experiment in which a New Zealand sheep was dosed with diosgenin was shown to afford free and conjugated analogues of epismilagenin, smilagenin, tigogenin and traces of smilagenone (Wilkins et al., 1994), however, the sheep did not exhibit photosensitization symptoms (Flåøyen et al., 1993). A metabolic pathway for the conversion of diosgenin and yamogenin saponins to biliary glucuronides has been proposed (Miles et al., 1994b; Wilkins et al., 1994).

A quantitative analysis of free and conjugated sapogenin levels present in samples recovered from a Norwegian sheep dosed with *N. ossifragum* (a plant known to contain sarsasapogenin and smilagenin glycosides implicated in the development of the photosensitization disease known as alveld) identified three distinct regions of metabolic activity. Ingested plant saponins were found to be hydrolysed to free sapogenins, oxidised

at C-3 and reduced to episapogenins in the rumen and omasum. Free sapogenins and episapogenins were proposed to be absorbed in the jejunum and transported to the liver where oxidation and reduction of sapogenins afforded episapogenins which were conjugated with glucuronic acid and secreted into the bile. Conjugated sapogenins and episapogenins were hydrolysed to free sapogenins and episapogenins in the caecum and colon (Flåøyen and Wilkins, 1997).

The metabolic pathway, proposed by Flåøyen and Wilkins (1997) for the conversion of sarsasapogenin and smilagenin saponins to the calcium salts of episarsasapogenin and epismilagenin glucuronides, was consistent with that previously advanced for diosgenin and yamogenin saponins by Miles et al. (1994b).

Results to date suggest that the uptake of free sapogenins in animal dosing experiments is low compared to crude saponin containing extracts, or saponins in natural plant material. Given the poor uptake of free sapogenins, it is desirable that future dosing experiments be performed using sapogenin glycosides (saponins), preferably deuterium labelled variants. The synthesis of sarsasapogenin β-D-glucoside, 3β-^{2}D-epismilagenin β-D-glucoside and 20,23,23-$^{2}D_3$-sarsasapogenin, reported in Chapters Two and Five, made this possible.

In this chapter the results of four dosing experiments (trials) using five sheep are reported. The substrates dosed in the four experiments (trials) are listed below:

Trial 1	sarsasapogenin β-D-glucoside (sheep A)
Trial 2	sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin (sheep 1 and sheep 2)
Trial 3	3β-D-epismilagenin β-D-glucoside (sheep 3 and sheep 4)
Trial 4	20,23,23-D_3-sarsasapogenin (sheep 2 and sheep 3)

Trial 1 was a preliminary experiment performed in June 1996, while Trials 2-4 were performed in July 1997.

3.2 Methodology

3.2.1 Sheep Trials

Five 1 year old 20 kg Romney lambs (one in 1996 and four in 1997) (Figure 3-1) were used for the dosing trials, which were performed at the AgResearch, Ruakura, Hamilton, New Zealand. Details of the dosed materials, sample collecting points, sample intervals and other relevant parameters are given in Table 3-1.

Sampling locations (rumen, jejunum, faeces and bile) were selected according to previous dosing experiments (Flåøyen et al., 1993; Miles et al., 1994b; Meagher, 1996; Flåøyen and Wilkins, 1997). Urine samples were not collected since no urinary saponins or sapogenins were detected in previous studies.

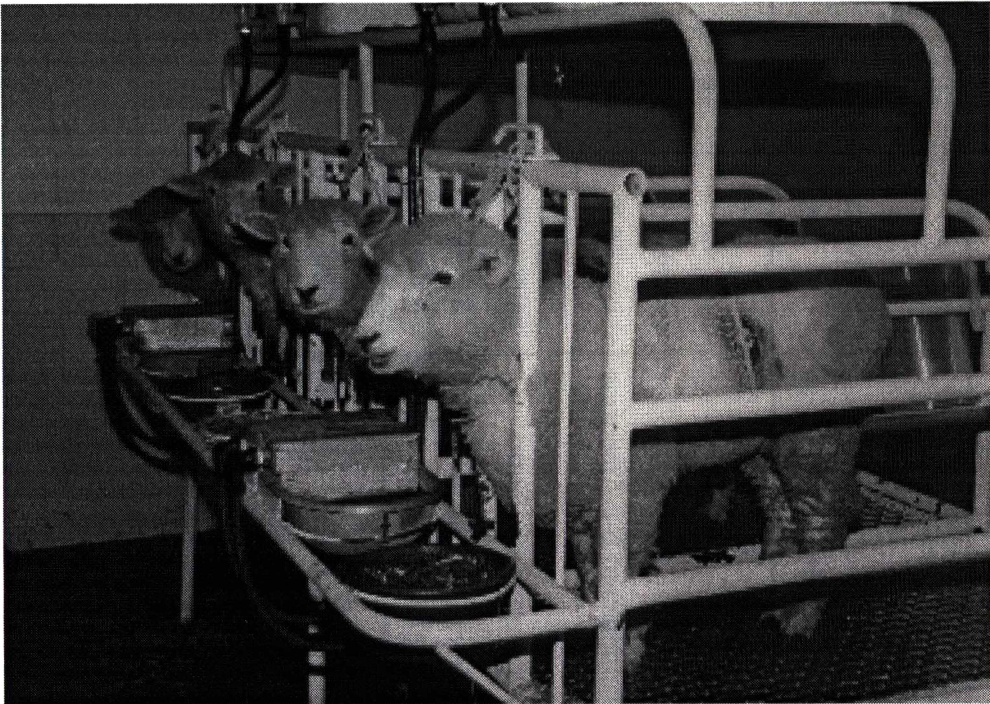


Figure 3-1. Sheep used in the dosing experiments (trials) in July 1997.

Table 3-1. Parameters of the sheep dosing experiments.

	date	dosed material	dosage	sheep code	age of sheep	weight of sheep	sample intervals (hour)	sample location
Trial 1	10:00 am 12-6-96	sarsasapogenin β-D-glucoside	2 g (single oral dose)	A	1 year	20 kg	0, 0.5, 1, 2, 3, 5, 6, 12, 14, 24, 27, 30	bile rumen
Trial 2	9:00 am 8-7-97	mixtures of sarsasapogenin β-D-glucoside and 20,23,23,-D_3-sarsasapogenin	2 g of sar-glu 2 g of D-sar (single oral dose)	1 2	1 year 1 year	20.5 kg 19.8 kg	0, 1, 3, 5, 7, 11, 16, 21, 29, 49, 54, 62, 74, 79, 97	bile rumen jejunum faeces
Trial 3	9:10 am 8-7-97	3β-D-epismilagenin β-D-glucoside	2 g (single oral dose)	3 4	1 year 1 year	19.5 kg 15.5 kg	0, 1, 3, 5, 7, 11, 16, 21, 29, 49, 54, 62, 74, 79, 97	bile rumen jejunum faeces
Trial 4	1:30 pm 22-7-97	20,23,23,-D_3-sarsasapogenin	2 g (single oral dose)	2 3	1 year 1 year	19.8 kg 19.5 kg	0, 3, 20, 27, 44, 51, 69	bile rumen jejunum faeces

3.2.2 Sample Preparation

Rumen, jejunum and faeces samples were stored at -5°C, prior to being freeze-dried for 24 hours, and sequentially extracted in a Soxhlet apparatus with hexane and ethanol-water to afford free and conjugated sapogenin extracts respectively. Free sapogenin extracts were concentrated using a rotary evaporator, methylated with diazomethane to convert acids to methyl esters, and acetylated with acetic anhydride and pyridine to convert hydroxyl groups to acetates. Conjugated sapogenin extracts were hydrolysed using 1 M HCl (2 hours at 80-85°C), extracted with chloroform, methylated with diazomethane to convert acids to methyl esters, and acetylated with acetic anhydride and pyridine. The free and conjugated extracts were analysed using selected ion mode GC-MS.

Bile samples were stored at -5°C, prior to being extracted. A preliminary evaluation of the methodology applied to bile samples established that free sapogenins were more readily extracted using chloroform (protocol B) than was the case using hexane (protocol A) (see Section 5.3.1). This can be attributed to the greater solubility of free sapogenins in chloroform, compared to hexane. While hexane adequately extracts sapogenins from solid samples using a continuous liquid-liquid extractor, it is not an appropriate solvent for the extraction of liquid samples under simple partition (separation funnel-type) conditions. Details of the extraction protocols are given in the experimental section (Section 5.3.1).

3.2.3 GC-MS Analysis

Quantitative analyses of sapogenins, steroids and fatty acids in the rumen, jejunum, faeces and bile samples were performed using of modifications of the selected ion mode GC-MS protocol reported by Wilkins et al. (1994). This protocol, when applied to acetylated sapogenin samples, utilises the intense *m/z* 139 ion arising from cleavage across ring E to afford a ring F fragment (Figure 2-9). Typically this fragment is the base peak in mass spectrum of a sapogenin lacking a ring E/F substituent group.

For sapogenins deuterated in rings E/F, e.g. 20,23,23-D_3-sarsasapogenin, the presence of one, two or three deuterium atoms at C-20, and/or C-23 affords *m/z* 140, 141, and 142 fragment ions, respectively. These ions were therefore included in the analytical protocol.

For ring A deuterium labelled sapogenins, e.g. 3β-D-epismilagenin β-D-glucoside, the presence of a deuterium atom at C-3 affords *m/z* 285 and 345 fragment ions corresponding

to the *m/z* 284 and 344 fragment ions of the C-3 protonated analogue (Figure 3-21). These ions were also included in the analytical protocol.

Other ions included in the GC-MS protocol were *m/z* 368, 382, 394 and 396 (M-HOAc fragment ions from cholesterol acetate, campesterol acetate, stigmasterol acetate and β-sitosterol acetate respectively), *m/z* 74 and 79 (for saturated and unsaturated methylated fatty acids respectively), and *m/z* 313 (for cholic acid methyl esters).

The *m/z* 74 ion is typically the mass spectral base peak of saturated fatty acid methyl esters (but not unsaturated fatty acid methyl esters), while the *m/z* 79 ion has reasonable intensity in the mass spectra of both saturated and unsaturated fatty acid methyl esters.

The GC oven temperature was programmed from 200°C to 250°C at 35°C/min, then to 295°C at 3°C/min with an 18 minute final hold time. These conditions adequately resolved sapogenin acetate peaks, other than epismilagenin acetate (a 25*R*-sapogenin) and sarsasapogenin acetate (a 25*S*-sapogenin) (Wilkins et al., 1994). Ion ratio data (e.g. the ratio of the *m/z* 284 to *m/z* 344 ion intensities) was used to define the contributions of these acetates to a merged peak. In general, peak overlap did not cause difficulties in the dosing studies reported here since only 25*S* sapogenins or saponins were dosed in Trials 1, 2 and 4, while a 25*R* saponin was dosed in Trial 3.

Sapogenin acetates, steroid acetates and fatty acid methyl ester peaks were identified by comparison of the retention times of authentic standards. Peak identifications were also confirmed using adaptations of the selected ion ratio profiling technique reported by Wilkins et al. (1994). For example, sapogenin acetates were characterised by the presence of low intensity *m/z* 458 (M^+) ions and a *m/z* 284 to *m/z* 344 ion ratio of ca 3-4 for smilagenin and sarsasapogenin acetates, compared to 14-16 for episarsasapogenin and epismilagenin acetates. *M/z* 271 and 300 ion profiles facilitated the identification of peaks arising from smilagenone and sarsasapogenone (3-keto sapogenins).

3.3. Trial 1 - Dosing of Sarsasapogenin β-D-Glucoside

3.3.1 Introduction

An aqueous suspension of sarsasapogenin β-D-glucoside (2 g), prepared as described in Section 5.2.1, was dosed to sheep A. A series of bile samples and a rumen sample were recovered from this sheep.

The principle objectives of trial 1 were to determine:

i) if metabolism of a synthetic sarsasapogenin β-D-glucoside, dosed as an aqueous slurry, proceeded in a manner analogous to that previously determined for natural sarsasapogenin glycosides, dosed as dried plant material;

ii) the time profile of episapogenin uptake into bile samples;

iii) the levels of sterols, cholic acid, and fatty acids in bile and rumen samples.

3.3.2 Results and Discussion

The levels of free and conjugated sapogenins, steroids and fatty acids, identified in a rumen sample and a series of bile samples recovered from sheep A during a 30 hour period are presented in Table 3-2 and 3-3.

Table 3-2. Levels (mg/kg) of free and conjugated saponins, sterols and fatty acids in a freeze-dried rumen sample from sheep A, 30 hours after dosing with sarsasapogenin β-D-glucoside.

	sar	episar	sar-CO	chol	camp	stig	sito	16:0	18:0	18:1
conj	46.2	3.2	2.5	28.8	7.5	5.5	36.8	3360	942	3356
free	286	24.5	4.0	18.7	6.3	3.9	22.1	12290	9965	15160

conj = conjugated extract; free = free extract; sar = sarsasapogenin; episar = episarsasapogenin; sar-CO = sarsasapogen-3-one; chol = cholesterol; camp = campesterol; stig = stigmasterol; sito = β-sitosterol; 16:0 = palmitic acid; 18:0 = stearic acid; 18:1 = oleic acid.

Table 3-3. Levels (mg/kg) of free and conjugated sapogenins, steroids and fatty acids in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside.

No	sample		sar	episar	chol	ch acid	16:0	18:0	18:1
1	0 h	conjugate	0	0	3.3	13.1	84.0	27.7	30.3
		free	0	0	105	0	248	121	451
2	0.5 h	conjugate	0	0	7.5	143	78.2	25.4	40.1
		free	0	0	70.4	0	100	52.0	179
3	1 h	conjugate	0	0	7.8	118	51.1	14.9	22.2
		free	0	0	58.7	0	82.3	39.2	165
4	2 h	conjugate	0	0	2.1	32.1	15.1	5.7	6.8
		free	0	0	63.2	0	121	57.0	222
5	3 h	conjugate	0	0.5	7.7	50.4	85.6	30.0	22.8
		free	0	0	67.1	0	117	45.9	254
6	5 h	conjugate	0	1.1	21.8	72.8	139	59.2	57.7
		free	0	0	66.6	0	130	50.1	374
7	6 h	conjugate	0	1.9	26.5	102	145	56.0	66.0
		free	0	0	120	0	147	55.6	344
8	12 h	conjugate	0	2.4	12.6	98.7	30.6	9.2	15.2
		free	0	0.1	97.2	0	114	49.8	210
9	14 h	conjugate	0	2.9	14.3	88.5	19.2	5.8	10.1
		free	0	0.1	90.9	0	163	70.6	275
10	24 h	conjugate	0	2.4	2.7	88.8	75.0	25.7	24.8
		free	0	NQ	84.8	0	165	76.5	320
11	27 h	conjugate	0	2.5	10.6	68.4	177	42.2	47.9
		free	0	0.1	94.1	0	170	76.5	338
12	30 h	conjugate	0	3.1	27.0	70.5	76.8	19.4	70.5
		free	0	0.1	120	0	231	105	433

conjugate = conjugated fraction; free = free fraction; sar = sarsasapogenin; episar = episarsasapogenin; chol = cholesterol; ch acid = cholic acid; 16:0 = palmitic acid; 18:0 = stearic acid; 18:1 = oleic acid; NQ = not quantifiable; h = hours.

Rumen sample

A greater level of free sarsasapogenin (286 mg/kg) was found in the rumen sample compared to conjugated sarsasapogenin (46 mg/kg). Much lower levels of free and conjugated episarsasapogenin (3 and 24 mg/kg respectively) and free and conjugated sarsasapogenone (4 and 3 mg/kg respectively) were also found.

The apparent detection of a conjugated form of sarsasapogenone implies the presence of either conjugated furostanol analogues of sarsasapogenone (i.e. open ring F analogues, see Section 3.4.2.3), or the incomplete recovery of free sarsasapogenone prior to the conjugated extraction. The former explanation appears to be more probable, given the exhaustive nature of the Soxhlet extraction protocol used to obtain the free rumen extract.

The rumen sample results can be interpreted as indicating that, while the bulk of the dosed sarsasapogenin glucoside has been hydrolysed to afford free sarsasapogenin, only moderate conversion of sarsasapogenin to episarsasapogenin has occurred. Re-conjugation of episarsasapogenin is also apparent.

The levels of free and conjugated sapogenins found in the rumen sample are comparable with those found in another study, in which plant material containing sarsasapogenin glycosides were dosed to a Norwegian sheep (Wilkins and Flåøyen, pers commun). It is apparent that the metabolism of the synthetic sarsasapogenin β-D-glucoside sample utilised in this trial, and in trials 2, 3 and 4 (see Sections 3.4, 3.5 and 3.6) is comparable to that of natural sarsasapogenin glycosides.

Bile samples

Conjugated episarsasapogenin was first detected in bile samples 3 hours after dosing commenced. Thereafter the level of conjugated episarsasapogenin steadily increased to a maximum of ca 3 mg/kg after 12 hours. This level was then maintained until the end of the sampling period (30 hours) (Table 3-3 and Figure 3-2). It was not clear from curve fitting if, at 30 hours, bile episarsasapogenin conjugate levels were still rising (curve A), or if they were starting to decline (curve B) (Figure 3-2).

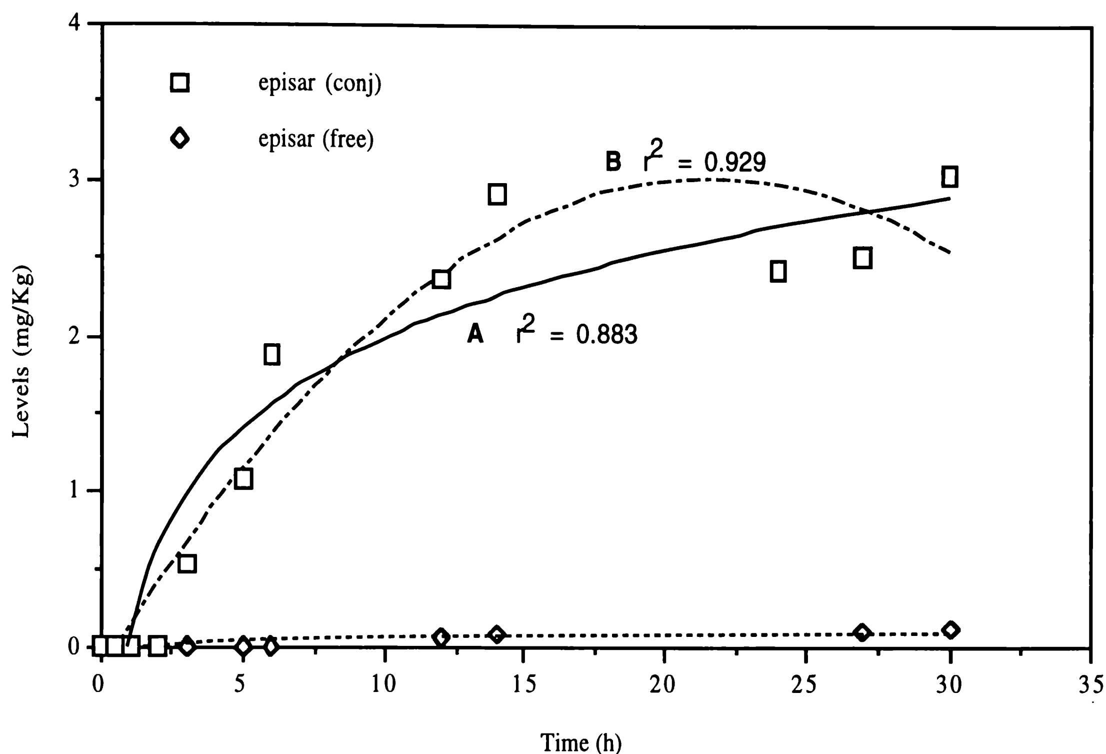


Figure 3-2. Levels of conjugated and free episarsasapogenin in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside.

Very low levels of free episarsasapogenin were detected after 12 hours. No free and conjugated sarsasapogenin were identified in the bile samples. Sarsasapogenone, the 3-keto sapogenin proposed as the metabolic intermediate in the conversion of sarsasapogenin to episarsasapogenin (Miles et al., 1994b), was not detected in any of the bile samples. It was, however, detected in the rumen sample.

GC-MS analysis of the acetylated bile sapogenins was complicated by a *m/z* 139 response, possibly arising from a methylated fatty acid, at a retention time similar to that of sarsasapogenin acetate. Ion ratio profiling (*m/z* 284, 344 and 458 ions) was used to explicitly establish the absence of sarsasapogenin acetate, and confirm the presence of episarsasapogenin acetate in the acetylated bile samples (Table 3-4).

Table 3-4. Ion ratio data for episarsasapogenin acetates detected in acetylated bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside.

Sample No	time	fraction	*m/z* 344/458	*m/z* 284/344
5	3 h	conjugate	0.6	15.1
6	5 h	conjugate	0.6	(21.5)
7	6 h	conjugate	0.5	14.7
8	12 h	conjugate	0.6	16.4
		free	0.7	16.1
9	14 h	conjugate	0.6	15.4
		free	0.6	15.7
10	24 h	conjugate	0.6	14.2
11	27 h	conjugate	0.5	14.7
		free	0.5	14.8
12	30 h	conjugate	0.5	15.2
		free	0.8	(11.0)

( ) indicates a value outside the expected range.

No crystalline material, attributable to the presence of the calcium salt of episarsasapogenin β-D-glucuronide (Miles et al., 1991; Miles et al., 1992a) was detected in any of the bile samples.

Because of the limited sampling time (30 hours) no estimate of the time required for episarsasapogenin conjugates to be purged from the bile could be made. Accordingly a longer sampling time was employed in subsequent dosing trials in order that this point might be addressed (see Section 3.4, 3.5 and 3.6).

The levels of sterols, cholic acid, and fatty acids identified in the bile samples (Table 3-3) are depicted in Figures 3-3 and 3-4. Possibly, the variation in levels observed during the dosing experiment may correlate with the time of day - i.e. rising after noon, until approximately sunset, then gradually falling overnight. No direct, or indirect, correlation between the level of episarsasapogenin and any of the sterols, cholic acid or fatty acids was found.

It is of note that cholic acid was predominantly present in the bile samples in conjugated forms, and that the conjugated cholic acid level (ca 40-100 mg/kg) was substantially greater than the maximum episarsasapogenin conjugate level (ca 3 mg/kg). Elevated levels of conjugated cholesterols and free and conjugated fatty acids were also identified in the bile samples.

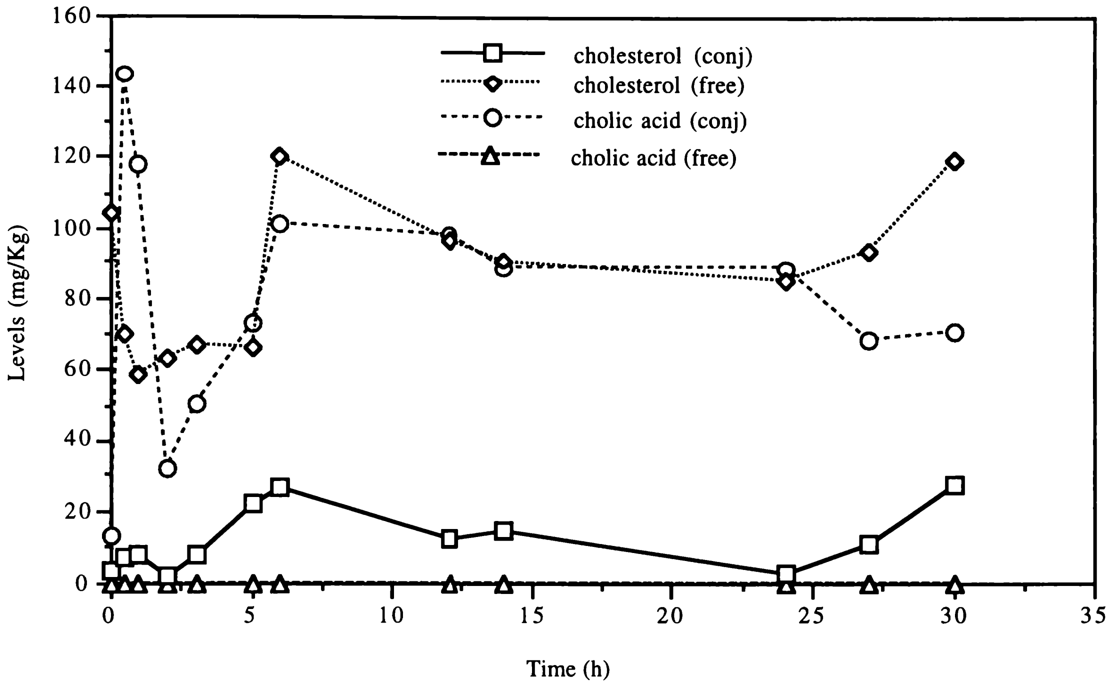


Figure 3-3. Levels of free and conjugated cholesterol and cholic acid identified in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside.

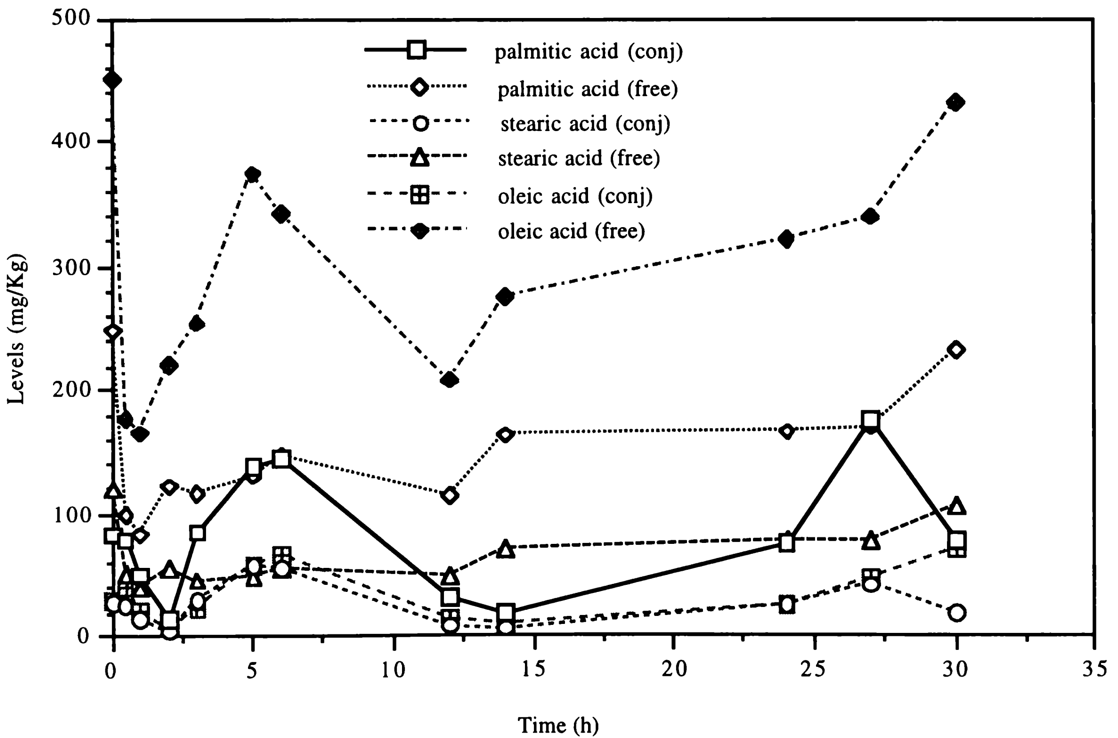


Figure 3-4. Levels of free and conjugated fatty acids in bile samples from sheep A, dosed with sarsasapogenin β-D-glucoside.

Metabolism

Both free and conjugated episarsasapogenins were found in the rumen sample. However only episarsasapogenin was found in the bile samples. This is consistent with the earlier findings of Miles et al. (1993, 1994b) that episarsasapogenin conjugates are the dominant sapogenin constituents of bile samples of animal ingested diosgenin, yamogenin, sarsasapogenin and/or smilagenin glycosides.

The absence of free and conjugated sarsasapogenins is consistent with the proposal (Flåøyen and Frøslie, 1997) that when sapogenins and/or episapogenin are absorbed into the portal vein and transported to the liver, sapogenins are oxidised and reduced at C-3 to afford episapogenins which are then conjugated with D-glucuronic acid and excreted into the bile. Glucuronide conjugates are common phase II liver metabolic products (Di Giulio et al., 1995).

Another possibility is that the small quantity of conjugated episarsasapogenin entering the small intestine region (duodenum, jejunum and ileum) is absorbed and transported to the liver (Figure 3-5), where de-conjugation and re-conjugation with D-glucuronic acid occurs. Further work is required to elucidate the chemical structures and metabolic fate of epismilagenin conjugates present in rumen, jejunum and bile extracts.

The proposed metabolic pathway whereby dosed synthetic sarsasapogenin β-D-glucoside is metabolised to episarsasapogenin β-D-glucuronide, is presented in Figure 3-5. This pathway is based on that previously proposed by Miles et al. (1994b) for the metabolism of natural saponins.

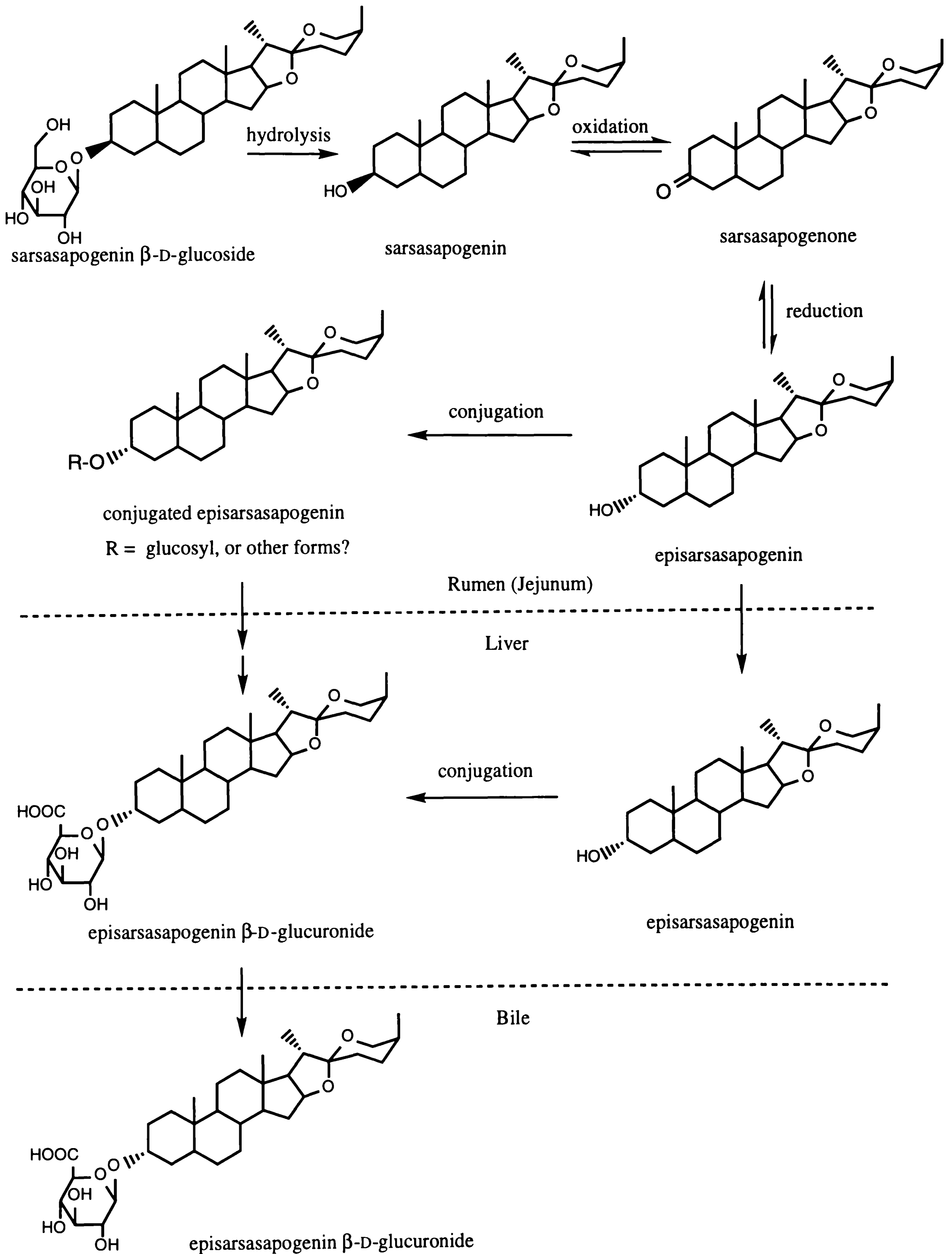


Figure 3-5. Proposed pathway for the metabolism of sarsasapogenin β-D-glucoside to episarsasapogenin β-D-glucuronide.

3.3.3 Conclusions

It is apparent from the results presented in Tables 3-2 and 3-3, and Figures 3-3, 3-4 and 3-5 that:

i) ruminal metabolism of synthetic sarsasapogenin β-D-glucoside proceeded in a manner analogous to that previously reported for natural sarsasapogenin glycosides;

ii) episapogenin conjugates were detectable in bile samples ca 3 hours after dosing commenced;

iii) bile episapogenin conjugate levels were highest ca 12-30 hours after dosing commenced;

iv) crystalline materials were not found in any of the bile samples;

v) no correlation between the level of episarsasapogenin and any of the sterols, cholic acid or fatty acids was found;

vi) a longer sampling time (> 30 hours) was required to determine the time required for episarsasapogenin conjugates to be purged from the bile.

3.4 Trial 4 - Dosing with 20,23,23-D_3-Sarsasapogenin

3.4.1 Introduction

An experiment in which a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin (see Section 3.5) was dosed to two sheep showed that the 20- and 23-deuterium atoms were progressively lost during ovine metabolism. Since a full analysis of results obtained in Section 3.5 requires a knowledge of the extent to which deuterium was lost during the dosing experiment, the dosing experiment was repeated using only 20,23,23-D_3-sarsasapogenin.

It was anticipated that this dosing experiment would afford a *m/z* 139-142 isotope ratio pattern which could then be compared with the isotope ratio pattern found in the mixed labelled/non-labelled dosing experiment (Section 3.5), thereby allowing the rate at which dosed free and conjugated sarsasapogenins appeared in the bile to be determined (see Section 3.5.2.2).

3.4.2 Results and Discussion

Aqueous suspensions of 20,23,23-D_3-sarsasapogenin (2 g) were dosed to two sheep (sheep 2 and sheep 3). A series of bile samples, and some rumen, jejunum and faeces samples were collected from these sheep.

3.4.2.1 Levels of Sapogenins in Rumen, Jejunum, Faeces and Bile Samples

Bile samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin, were frozen immediately after collection and stored at -5°C prior to the extraction. Rumen, jejunum and faeces samples from sheep 2 and 3 were frozen immediately after collection, and freeze-dried for 24 hours. The percentage dry matter content of these samples is given in Table 3-5.

Quantification of sapogenins in the bile, rumen, jejunum and faeces samples from sheep 2 and 3 were performed by the method of Wilkins et al. (1994), using SIM GC-MS profiles of *m/z* 139 for non-D sapogenins, *m/z* 140 for D-sapogenins, *m/z* 141 for D_2-sapogenins,

and *m/z* 142 for D_3-sapogenins, respectively. The levels of conjugated sapogenins in bile samples from sheep 2 and 3 are presented in Tables 3-6 and 3-7, while the levels of free and conjugated sapogenins in the rumen, jejunum and faeces samples are presented in Table 3-8.

Table 3-5. Wet weight, freeze-dried weight, and percentage (%) dry matter content of rumen, jejunum and faeces samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin.

No	Time	wet weight (g)	dried weight (g)	% dry matter
sheep 2				
S2-1R	3 h	25.81	3.11	12.1
S2-2R	20 h	18.50	2.82	15.2
S2-2J	20 h	23.82	4.62	19.4
S2-2F	19 h	15.42	4.54	29.4
sheep 3				
S3-2R	20 h	19.45	2.65	13.6

Time = interval time for collecting samples

Table 3-6. Levels (mg/kg) of conjugated sapogenins in bile samples from sheep 2, dosed with 20,23,23-D_3-sarsasapogenin.

No	Time (h)		episarsasapogenin 139	140	141	142	sum
S2-PB	0	conj	0	0	0	0	0
S2-1B	3	conj	2.3	7.2	1.1	0.2	10.7
S2-2B	20	conj	2.7	9.7	3.7	0.9	17.0
S2-3B	27	conj	1.6	5.6	2.0	0.5	9.6
S2-4B	44	conj	1.0	3.6	1.5	0.4	6.6
S2-5B	51	conj	1.0	2.9	1.2	0.3	5.3
S2-6B	69	conj	1.0	2.9	0.4	0	4.4

Time = interval time for collecting samples, conj = conjugated extract, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins

Table 3-7. Levels (mg/kg) of conjugated sapogenins in bile samples from sheep 3, dosed with 20,23,23-D_3-sarsasapogenin.

No	Time (h)		episarsasapogenin 139	140	141	142	sum
S3-PB	0	conj	0	0	0	0	0
S3-1B	3	conj	1.5	5.1	0.8	0.1	7.6
S3-2B	20	conj	3.4	11.0	1.8	0.3	16.4
S3-3B	27	conj	2.4	8.4	1.4	0.2	12.4
S3-4B	44	conj	1.3	5.2	0.9	0	7.5
S3-5B	51	conj	1.1	4.1	0.8	0	6.0
S3-6B	69	conj	0.7	2.2	0.3	0	3.1

Time = interval time for collecting samples, conj = conjugated extract, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins

Table 3-8. Levels (mg/kg) of free and conjugated sapogenins in rumen, jejunum and faeces samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin.

No	Time (h)		sarsasapogenin 139	140	141	142	sum	episarsasapogenin 139	140	141	142	sum	3-CO-sarsasapogenin 139	140	141	142	sum
sheep 2																	
rumen																	
S2-1R	3	conj	6.4	11.7	2.5	1.9	22.6	0	0	0	0	0	0	0	0	0	0
		free	7.3	20.9	62.3	51.9	143	0	0	0	0	0	0.2	1.4	1.8	1.5	4.9
S2-2R	20	conj	32.5	76.0	37.5	14.1	160	3.6	5.9	2.6	1.1	13.1	0	0	0	0	0
		free	49.2	182	314	303	848	14.4	26.4	10.0	3.2	54.0	0	1.8	3.2	2.5	7.4
jejunum																	
S2-2J	20	conj	41.9	86.4	25.3	6.1	160	6.1	12.1	4.9	2.0	25.1	0	0	0	0	0
		free	31.0	121	149	116	418	5.5	15.7	4.8	2.4	28.4	0	0.8	2.1	1.4	4.3
faeces																	
S2-2F	19	conj	11.4	19.5	7.0	2.7	40.6	12.5	24.8	8.3	2.9	48.6	0	0	0	0	0
		free	85.8	288	340	273	987	5.7	25.4	11.8	3.9	46.8	0.7	3.3	6.7	3.4	14.1
sheep 3																	
rumen																	
S3-2R	20	conj	10.2	23.5	6.8	3.3	43.8	0	0	0	0	0	0	0	0	0	0
		free	34.1	123	210	194	561	0	0	0	0	0	0	0	0	0	0

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins

The episarsasapogenin conjugate levels vs time profile determined for bile samples from sheep 2 and 3 were very similar (Figure 3-6). The levels of deuterated episarsasapogenins increased quickly from zero to a maximum of ca 16 mg/kg 20 hours after dosing, after which the levels progressively declined. Because of the absence of 4 to 20 hour (overnight) samples, it is possible that bile conjugate levels initially rose to values greater than those depicted in Figure 3-6. No free or conjugated sarsasapogenins, or free episarsasapogenins were detected in any of the bile samples from sheep 2 and sheep 3.

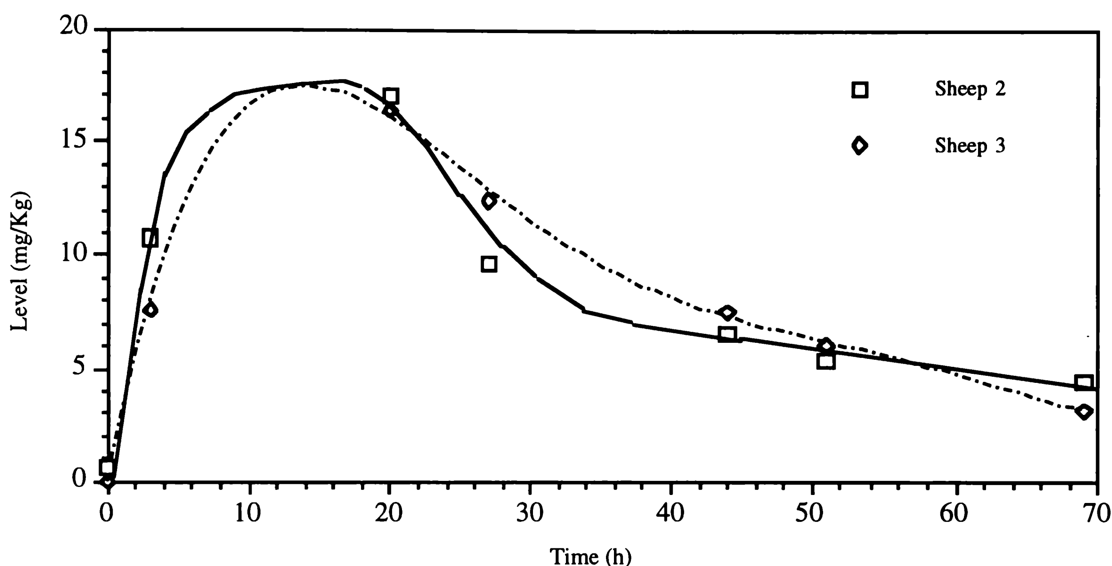


Figure 3-6. Levels of conjugated episarsasapogenins in bile samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin.

A high level of free sarsasapogenins (848 mg/kg) and a lower level of conjugated sarsasapogenins (160 mg/kg) were found in the rumen sample collected from sheep 2 after 20 hours. Low levels of free and conjugated episarsasapogenins (54 and 13 mg/kg) and free sarsasapogenones (3-CO-sarsasapogenins) (7 mg/kg) were also detected in this sample (Table 3-8).

Likewise, high levels of free sarsasapogenins (417 and 987 mg/kg for the jejunum and faeces samples respectively) and lower levels of conjugated sarsasapogenins (159 and 41 mg/kg respectively) were detected in the jejunum and faeces samples collected 20 hours after dosing started. Low levels of free episarsasapogenins (28 and 46 mg/kg respectively for the jejunum and faeces samples), conjugated episarsasapogenins (25 and 49 mg/kg respectively) and free sarsasapogenones (3-CO-sarsasapogenins) (4 and 14 mg/kg respectively) were detected in the corresponding jejunum and faeces samples. No

conjugated sarsasapogenones were found in the rumen, jejunum or faeces samples (Table 3-8).

The results, presented in Tables 3-6, 3-7 and 3-8, show that ruminal metabolism of 20,23,23-D_3-sarsasapogenin proceeds in a manner analogous to that observed when a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin was dosed (Trial 2, see Section 3.5).

3.4.2.2 Contributions to Bile Samples

The percentage contributions of episarsasapogenin (*m/z* 139), D-episarsasapogenin (*m/z* 140), D_2-episarsasapogenin (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) identified in bile samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin, are presented in Tables 3-9 and 3-10.

Table 3-9. Percentages (%) of conjugated episarsasapogenin (*m/z* 139), D-episarsasapogenin (*m/z* 140), D_2-episarsasapogenin (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) identified in bile samples from sheep 2, dosed with 20,23,23-D_3-sarsasapogenin.

No	Time (h)		episarsasapogenin 139	140	141	142
S2-PB	0	conj	0	0	0	0
S2-1B	3	conj	21.1	66.9	10.3	1.7
S2-2B	20	conj	15.8	57.3	21.6	5.2
S2-3B	27	conj	16.2	58.5	20.6	4.7
S2-4B	44	conj	15.8	55.6	22.8	5.8
S2-5B	51	conj	18.3	54.1	22.2	5.4
S2-6B	69	conj	23.6	66.4	10.0	0
		average %	18.5	59.8	17.9	3.8
		ratio (rel to 140)*	0.30	1.00	0.30	0.06
		ratio (rel to 141)**	1.03	3.34	1.00	0.21

* ratio relative to *m/z* 140
** ratio relative to *m/z* 141

Table 3-10. Percentages (%) of conjugated episarsasapogenin (*m/z* 139), D-episarsasapogenin (*m/z* 140), D_2-episarsasapogenin (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) identified in bile samples from sheep 3, dosed with 20,23,23-D_3-sarsasapogenin.

No	Time (h)		episarsasapogenin 139	140	141	142
S3-PB	0	conj	0	0	0	0
S3-1B	3	conj	19.6	67.6	11.2	1.6
S3-2B	20	conj	20.5	66.9	10.9	1.7
S3-3B	27	conj	19.2	67.6	11.4	1.8
S3-4B	44	conj	17.2	70.2	12.6	0
S3-5B	51	conj	18.3	68.6	13.1	0
S3-6B	69	conj	20.9	70.1	9.0	0
	average %		19.3	68.5	11.4	0.9
	ratio (rel to 140)*		0.28	1.00	0.17	0.01
	ratio (rel to 141)**		1.69	6.01	1.00	0.08

* ratio relative to *m/z* 140
** ratio relative to *m/z* 141

D_3-sarsasapogenin accounted for 65% of the dosing material 20,23,23-D_3-sarsasapogenin. However, only a small contribution of D_3-episarsasapogenin (3.8% for sheep 2 and 0.9% for sheep 3) was identified in the bile samples. D-episarsasapogenin was found to be the main component of the bile samples (59.8% for sheep 2 and 68.5% for sheep 3). Non-D and D_2-episarsasapogenin accounted for 18.5% and 17.9% respectively for sheep 2, and 19.3% and 11.4% respectively for sheep 3 (Tables 3-9 and 3-10).

Ion Ratios Relative to *m/z* 140 or *m/z* 141

The average *m/z* 139-142 ion contributions determined for conjugated episarsasapogenins in the bile samples (Tables 3-8 and 3-9), were used to determine the ratios of the *m/z* 139 to *m/z* 140, *m/z* 141 to *m/z* 140, and *m/z* 142 ion to *m/z* 140 responses, respectively (Tables 3-9 and 3-10):

sheep 2	sheep 3
m/z 139 / 140 = 18.5 / 59.8 = 0.30	*m/z* 139 / 140 = 19.3 / 68.5 = 0.28
m/z 140 / 140 = 59.8 / 59.8 = 1.00	*m/z* 140 / 140 = 68.5 / 68.5 = 1.00
m/z 141 / 140 = 17.9 / 59.8 = 0.30	*m/z* 141 / 140 = 11.4 / 68.5 = 0.17
m/z 142 / 140 = 3.8 / 59.8 = 0.06	*m/z* 142 / 140 = 0.9 / 68.5 = 0.01

Likewise, the ratios relative to the *m/z* 141 ion response were calculated (Tables 3-9 and 3-10):

sheep 2	sheep 3
m/z 139 / 141 = 18.5 / 17.9 = 1.03	*m/z* 139 / 141 = 19.3 / 11.4 = 1.69
m/z 140 / 141 = 59.8 / 17.9 = 3.34	*m/z* 140 / 141 = 68.5 / 11.4 = 6.01
m/z 141 / 141 = 17.9 / 17.9 = 1.00	*m/z* 141 / 141 = 11.4 / 11.4 =1.00
m/z 142 / 141 = 3.8 / 17.9 = 0.21	*m/z* 142 / 141 = 0.9 / 11.4 = 0.08

These ratios were used to interpret the results of experiments in which mixtures of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin were dosed (see Section 3.5).

3.4.2.3 Contributions to Rumen, Jejunum and Faeces Samples

D_2-sarsasapogenin and D_3-sarsasapogenin (ca 37% and 32% respectively) were the main genin contributors to free rumen, jejunum and faeces extracts, while non-D- and D-sarsasapogenins were minor contributors (ca 5% and 23% respectively) (Table 3-11). However, D-sarsasapogenin was the major contributor (ca 50%) to conjugated extracts, in comparison to a 25% contribution for non-deuterated sarsasapogenin, a 17% contribution for D_2-sarsasapogenin, and a 7% contribution for D_3-sarsasapogenin).

Free sarsasapogenones (3-CO-sarsasapogenins) in the rumen sample were found to be comprised of a trace of non-D-sarsasapogenone, 24% of D-sarsasapogenone, 42% of D_2-sarsasapogenone and 33% of D_3-sarsasapogenone. Similar deuterated and non-deuterated sarsasapogenone contributions were identified in jejunum and faeces samples (Table 3-11).

Table 3-11. Percentage contributions (%) of episarsasapogenin (*m/z* 139), D-episarsasapogenin (*m/z* 140), D_2-episarsasapogenin (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) to rumen, jejunum and faeces samples from sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin.

sample			sarsasapogenin				episarsasapogenin				3-CO-sarsasapogenin			
			139	140	141	142	139	140	141	142	139	140	141	142
sheep 2														
rumen														
S2-1R	3 h	conj	28.5	51.8	11.2	8.5	0	0	0	0	0	0	0	0
		free	5.1	14.7	43.7	36.4	0	0	0	0	3.5	28.6	36.6	31.3
S2-2R	20 h	conj	20.3	47.5	23.4	8.8	27.0	44.7	19.9	8.4	0	0	0	0
		free	5.8	21.5	37.0	35.7	26.7	48.9	18.6	5.9	0	23.9	42.7	33.3
jejunum														
S2-2J	20 h	conj	26.2	54.1	15.8	3.8	24.4	48.3	19.4	7.8	0	0	0	0
		free	7.4	29.1	35.7	27.9	19.4	55.2	16.8	8.6	0	19.1	48.6	32.3
faeces														
S2-2F	20 h	conj	28.0	48.2	17.2	6.6	25.8	51.1	17.2	5.9	0	0	0	0
		free	8.7	29.2	34.4	27.7	12.1	54.4	25.3	8.2	4.8	23.7	47.5	23.9
sheep 3														
rumen														
S3-2R	20 h	conj	23.4	53.7	15.5	7.5	0	0	0	0	0	0	0	0
		free	6.1	21.9	37.4	34.6	0	0	0	0	0	0	0	0

When deuterated sapogenin material, comprised mainly of D_3-sarsasapogenin (65%), was introduced into the rumen, one deuterium atom was lost comparatively rapidly (see Figure 3-9) as evidenced by the % *m/z* 139-142 ion contributions of free sarsasapogenin (Table 3-11). For example, after 3 hours, the initial D_3-sarsasapogenin contribution decreased from 65% to 36%, while the percentage contributions of D_2-sarsasapogenin, D-sarsasapogenin and non-D-sarsasapogenins increased from 28%, 6% and 1%, to 44%, 15% and 5%, respectively. Similar changes in percentage contributions were also found for free sarsasapogenone (3-CO-sarsasapogenin) (Table 3-11).

Greater loss of deuterium was observed for conjugated sarsasapogenins, as evidenced by increased percentage D-sarsasapogenin and non-D-sarsasapogenin (52% and 29%) and lower D_3-sarsasapogenin (9%) contributions respectively, determined for a rumen sample collected 3 hours after dosing commenced.

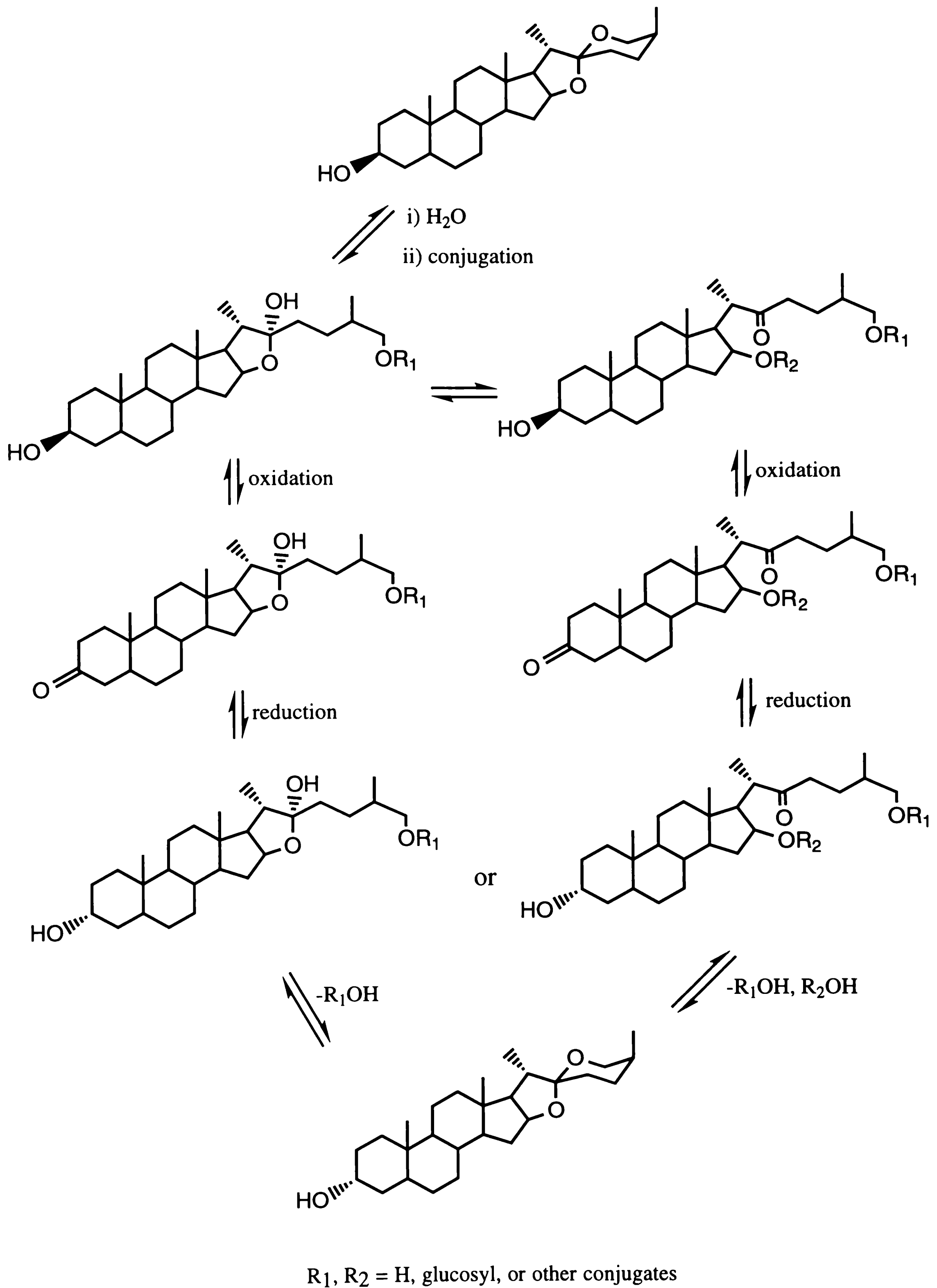
Conjugated 3-Keto-sapogenins

Interestingly, both free and conjugated episarsasapogenins were shown to have the same pattern of percentage ion contribution patterns as conjugated sarsasapogenins (Figure 3-9). This observation can be interpreted as indicating that free and conjugated episarsasapogenins originate from conjugated sarsasapogenin(s) via 'conjugated' sarsasapogenones.

It is not immediately apparent what constitutes a 'conjugated' sarsasapogenone, since there is no position in the genin structure (other than at C-3) at which a conjugate might be formed. Conjugated sarsasapogenones have however been detected in previous studies (Flåøyen and Wilkins, 1997). They were also detected in Trials 1 and 2 (see Section 3.3 and 3.5).

One possibility is the reversible opening of ring F, or both of rings E and F, to afford (for example) a glycosylated 26-hydroxy furostanone (Figure 3-7), which while extracted as a conjugated species, may subsequently undergo hydrolysis and ring F closure during work-up and derivativation prior to GC-MS analysis.

An alternative possibility is that a residual level of free sarsasapogenone, not extracted during the initial hexane extraction (free fraction), (possibility because it is strongly bound to rumen enzymes, etc.), is subsequently extracted using ethanol-water (conjugated fraction).



R_1, R_2 = H, glucosyl, or other conjugates

Figure 3-7. Proposed pathway for the ovine metabolism of sarsasapogenin to episarsasapogenin via 3-keto-26- and/or 16-conjugates.

Deuterium loss

The percentage *m/z* 139-142 ion contributions depicted in Figure 3-9 show that in rumen, jejunum and faeces samples, free sarsasapogenins were composed mainly of D_2- and D_3-sarsasapogenins, while conjugated (bound) sarsasapogenins were comprised mainly of D-sarsasapogenin. This data shows that deuterium atoms are lost more quickly from conjugated sarsasapogenin than from free sarsasapogenin.

Chemically, deuterium loss is only likely to occur at a significant rate when both of rings E and F are opened, to afford a 22-keto group and 26-OH and 16-OH groups (Figure 3-8). Reconjugation could then give 26- or 16-glycosides (or glucuronide), which would still be susceptible to facile exchange (loss) of deuterium at C-20 and C-23 (α-positions with respect to the keto group) via equilibrating enol forms (Figure 3-8).

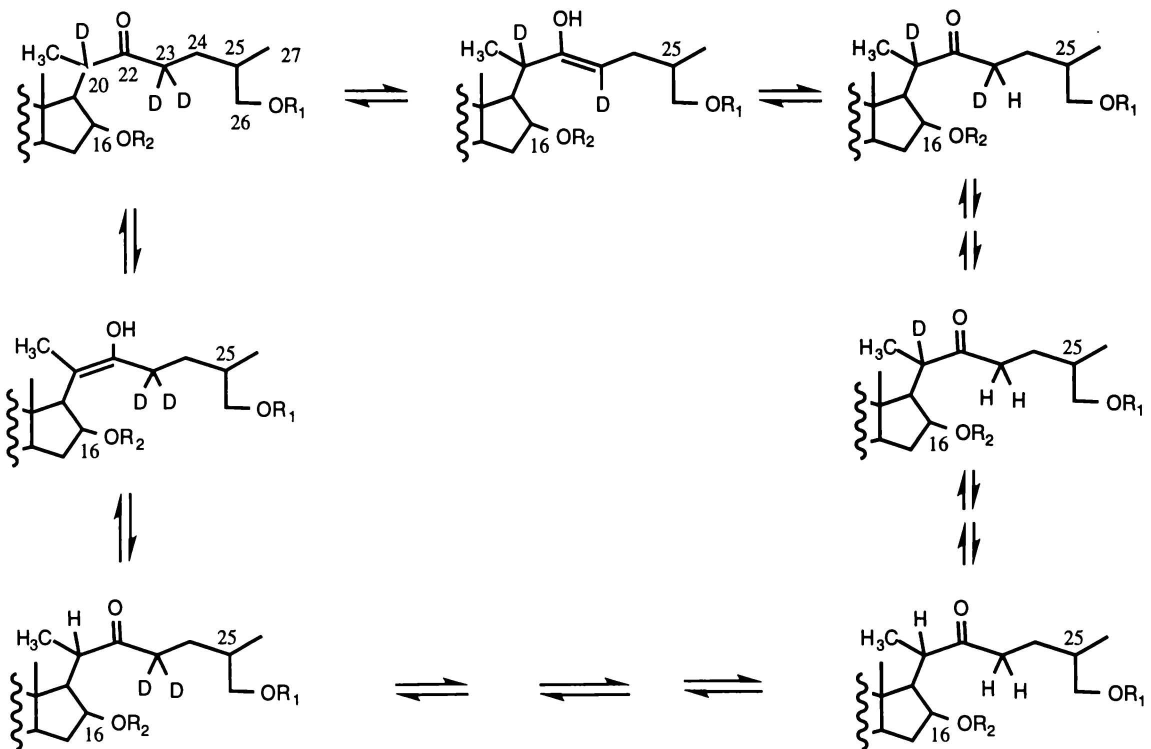


Figure 3-8. Proposed pathway for C-20 and C-23 deuterium loss during ovine metabolism.

3.4.2.4. Metabolism

Free episarsasapogenin exhibited the same pattern of *m/z* 139-142 ion contributions as conjugated sarsasapogenins (e.g. ca 50% mono-D contributions), rather than the patterns exhibited by free sarsasapogenone and free sarsasapogenin (ca 40% of D_2- and 32% of D_3-contributions) (Figure 3-9).

This suggests that, in the rumen, free episarsasapogenin does not primarily originate from free sarsasapogenin via reduction of free sarsasapogenone. Rather it appears to originate primarily from conjugated sarsasapogenin, via a conjugated ring E/F opened analogue of sarsasapogenone (may be of the type proposed in Figure 3-8). Reduction of the conjugated keto species would then afford a conjugated episarsasapogenin analogue, which on hydrolysis and ring E/F closure would give free episarsasapogenin.

Unfortunately the levels of conjugated sarsasapogenones were such that their percentage *m/z* 139-142 contributions could not be determined. It was anticipated that had the pattern been determinable it would have been similar to the patterns exhibited by conjugated sarsasapogenins and conjugated episarsasapogenins, rather than free sarsasapogenone. The presence of only very low levels of conjugated keto-sarsasapogenins suggests that once formed, they are rapidly reduced to conjugated forms of sarsasapogenin or episarsasapogenin.

The percentage *m/z* 139-142 ion contributions of free and conjugated sarsasapogenin and episarsasapogenins in jejunum and faeces samples were very similar to those found for free and conjugated sarsasapogenins and episarsasapogenins in rumen samples (Figure 3-9). This suggests that in these metabolic regions, free episarsasapogenin also arises predominantly from 'conjugated' forms of sarsasapogenone.

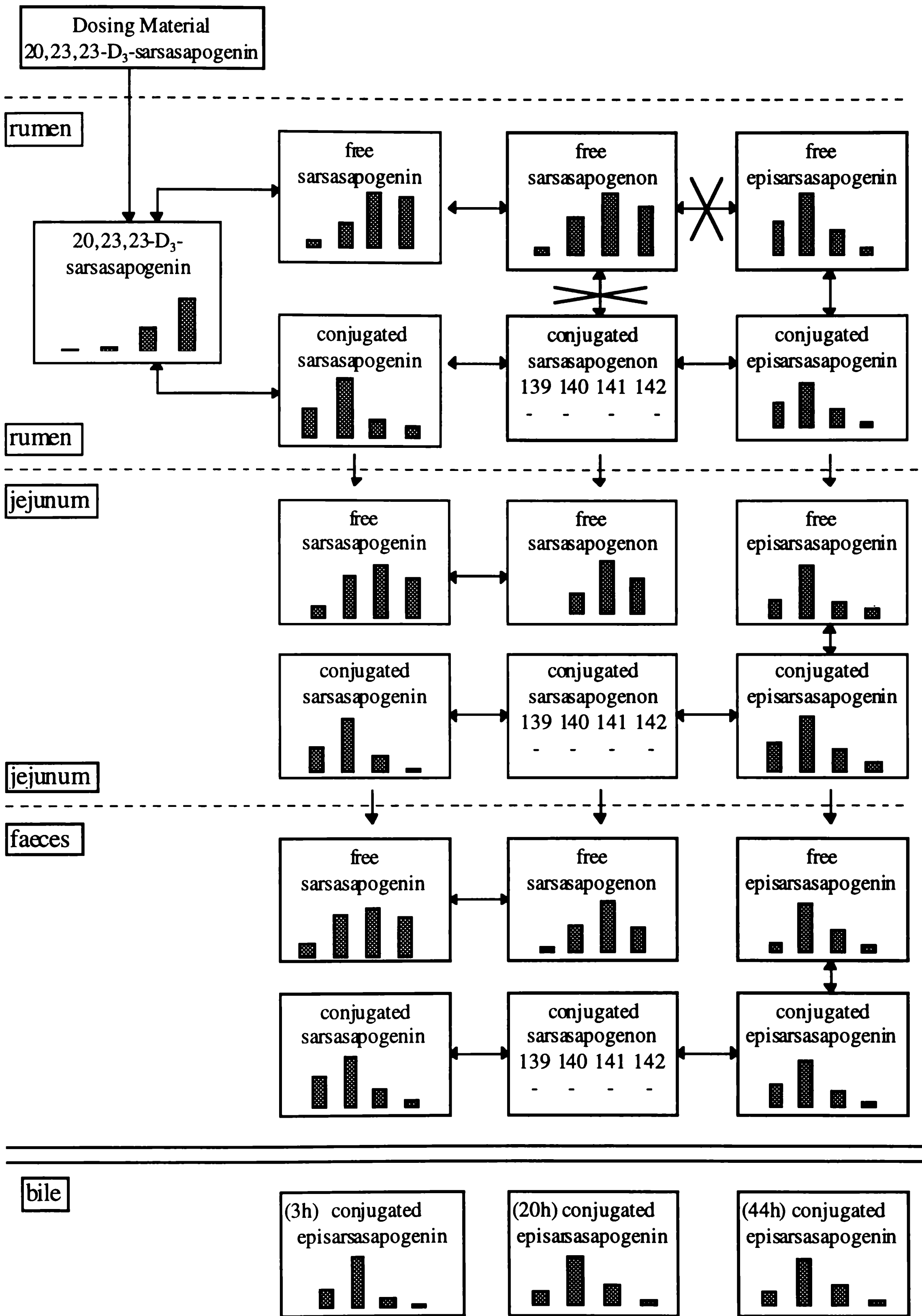


Figure 3-9. Percentage contributions of deuterated sapogenin in rumen, jejunum, faeces and bile samples from sheep 2, dosed with 20,23,23-D_3-sarsasapogenin.

The percentage *m/z* 139-142 ion contributions of free and conjugated episarsasapogenin in rumen, jejunum and faeces samples were also close to those of conjugated episarsasapogenins in the bile samples (Figure 3-9).

3.4.3 Summary and Conclusions

Mainly conjugated mono-D-episarsasapogenin was detected in bile samples after dosing of 20,23,23-D_3-sarsasapogenin (65% of D_3-sarsasapogenin), indicating that the loss of two deuterium atoms from ring F is a facile process during ovine metabolism.

GC-MS analyses revealed the ratios of non-D-, D-, D_2- and D_3-episarsasapogenin in biliary conjugates. These ratios assisted in the interpretation results obtained in experiments in which mixtures of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin were dosed to two sheep (see Section 3.5).

Free episarsasapogenin was found to originate primarily from conjugated episarsasapogenin, rather than conversion of free sarsasapogenin to free episarsasapogenin via sarsasapogenone.

There is evidence that the presence of conjugated forms of sarsasapogenone is necessary for the ruminal metabolism of a conjugated genin (saponin) to the corresponding free and conjugated episarsasapogenins. Differences in the levels and activities of rumen flora enzymes would be expected to contribute to oscillations in the levels of conjugated episapogenins.

3.5 Trial 2 - Dosing of a Mixture of Sarsasapogenin β-D-Glucoside and 20,23,23-D_3-Sarsasapogenin

3.5.1 Introduction

The previous experiment (Section 3.3) in which sarsasapogenin β-D-glucoside was dosed showed that a readily detectable level of conjugated episarsasapogenin (ca 2-3 mg/kg) appeared in the bile of the sheep 3 hours after sarsasapogenin β-D-glucoside was administered orally. A maximum level of conjugated episarsasapogenin was found 12-30 hours after dosing. Because of the limited time duration of the previous dosing experiment, no estimate of the time required for conjugated episarsasapogenins to be purged (eliminated) from the bile could be made. Accordingly the sampling time of a dosing trial in which a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin were administered was extended to 96 hours, in order that the time required for conjugated episarsasapogenin to be purged from the bile might be determined.

It was also anticipated that the simultaneous dosing of a free sapogenin (20,23,23-D_3-sarsasapogenin) and a conjugated sapogenin, saponin (sarsasapogenin β-D-glucoside) might enable differences in the rates of metabolism of sapogenins and saponins to be identified.

It has been suggested (Flåøyen and Wilkins, pers commun) that, in a farm environment, it might be possible to determine the average daily intake of natural pasture saponins by dosing sheep once a day with a known level of a deuterated sapogenin (or saponin) and determining the level of non-deuterated and deuterated free and conjugated sapogenins in faeces samples. For this approach to be viable, faeces levels need to be generally consistent over a 24 hour period after dosing, and the ratio of deuterated to non-deuterated sapogenins (saponins) dosed to the sheep needs to remain relatively constant over a 24-72 hour period. Excessive loss of deuterium, by exchange processes, would result in an over-estimation of the average daily intake of natural pasture saponins.

A dosing experiment performed in Norway (Flåøyen and Wilkins, pers commun) has shown that the daily dosing of a sheep with a known level of saponin containing plant material leads to a reasonably consistent level of total free and conjugated sapogenins in faeces samples.

The extent of deuterium exchange during ovine metabolism can be elucidated by dosing known amounts of deuterated and non deuterated sapogenins, or saponins, and comparing the ratio of deuterated to non-deuterated free and conjugated sapogenins recovered from rumen, jejunum, bile and faeces samples. For example, if the dosed ratio was 1:1, whereas the faeces ratio was 1:3, it follows that 50% of the dosed deuterium has been exchanged.

Aqueous suspensions of mixtures of sarsasapogenin β-D-glucoside (2 g) and 20,23,23-D_3-sarsasapogenin (2 g), prepared as described in Sections 5.2.1 and 5.2.3, were dosed to two sheep (sheep 1 and sheep 2). A series of bile samples were collected from sheep 1 and sheep 2, while rumen, jejunum and faeces samples were recovered from sheep 2, over a 96 hour period.

Prior to the experiment being performed it was anticipated that sarsasapogenin β-D-glucoside might be more soluble in an aqueous medium than was the case for 20,23,23-D_3-sarsasapogenin, however both of these substances proved to be essentially insoluble in the ethanol-water (1:4) solution (100 mL) used to prepare the dosing solution.

The principle objectives of this trial were:

i) to determine the persistence of free and conjugated sapogenins in bile, rumen, jejunum and faeces samples over a 5 day period;

ii) to determine differences in the bio-utilisation of a free sapogenin (20,23,23-D_3-sarsasapogenin) and a conjugated sapogenin (sarsasapogenin β-D-glucoside) in the same metabolic environment;

iii) to assess the extent of deuterium loss (exchange) during ovine metabolism;

iv) to determine free and conjugated cholesterol levels in bile samples and to ascertain if changes occurred in cholesterol levels during episapogenin uptake.

3.5.2 Results and Discussion

Rumen, jejunum and faeces samples from sheep 2 were frozen immediately after collection, and freeze-dried for 24 hours. The percentage of dry matter content of the samples is given in Table 3-12. The levels of sapogenins in rumen, jejunum and faeces samples from sheep 2 are given in Tables 3-13, 3-14 and 3-15.

Bile samples from sheep 1 and 2 were frozen immediately after collection, and stored at -5°C, prior to analysis. The levels of sapogenins and cholesterol in the bile samples are presented at Table 3-16 (sheep 1) and Table 3-17 (sheep 2).

Preliminary GC-FID and SIM GC-MS analyses showed that comparatively few peaks appeared in bile sample CC-MS profiles, whereas many peaks appeared in the rumen, jejunum and faeces sample GC-MS profiles.

Table 3-12. Wet weight, freeze-dried weight, and percentages (%) of dry matter contents of rumen, jejunum and faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)	wet weight (g)	dried weight (g)	% dry matter
rumen				
2-1R	1	14.2	1.8	12.5
2-3R	4	15.8	2.3	14.7
2-5R	7	20.1	3.3	16.2
2-6R	12	19.2	3.2	16.6
2-8R	22	18.1	2.7	14.7
2-12R	63	16.9	2.9	17.4
2-14R	80	14.8	2.3	15.5
jejunum				
2-3J	3	24.6	1.4	5.5
2-5J	8	23.4	2.0	8.6
2-6J	12	22.6	1.9	8.3
2-9J	29	22.1	0.8	3.6
2-10J	49	23.8	0.8	3.3
2-13J	74	15.9	1.7	11.0
faeces				
2-3F	3	21.6	2.9	13.3
2-5F	9	16.2	2.1	13.1
2-9F	25	18.8	2.7	14.5
2-14F	80	19.2	3.7	19.4

Table 3-13. Levels (mg/kg, dry matter) of free and conjugated sapogenins in rumen samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		sarsasapogenin					episarsasapogenin					3-CO-sarsasapogenin				
			139	140	141	142	sum	139	140	141	142	sum	139	140	141	142	sum
2-1R	1	conj	195	98.1	29.1	0	322	0	0	0	0	0	0	0	0	0	0
		free	1738	2676	3809	3672	11894	179	370	346	107	1001	42.7	17.2	24.6	32.1	117
2-3R	4	conj	200	99.1	26.2	5.5	331	0	0	0	0	0	8.7	tr	tr	0	8.7
		free	1387	2283	2809	2250	8729	178	301	221	46.7	747	66.0	54.8	45.4	27.4	194
2-5R	7	conj	180	74.0	16.4	4.9	276	49.7	22.6	4.7	tr	76.9	9.3	4.1	tr	0	13.4
		free	1087	1678	2142	1708	6615	341	213	157	41.5	752	58.9	46.0	40.6	25.3	171
2-6R	12	conj	173	67.4	18.8	5.7	265	65.7	30.0	5.2	tr	101	10.2	4.2	tr	0	14.4
		free	713	932	1453	1289	4388	330	131	118	75.5	655	51.5	27.8	29.3	23.1	132
2-8R	22	conj	145	56.7	9.3	2.8	214	78.8	40.0	5.2	tr	124	10.3	5.5	tr	0	15.8
		free	846	1082	1313	1185	4425	523	199	81.8	151	955	60.4	31.7	24.4	24.7	141
2-12R	63	conj	33.6	25.1	9.7	tr	68.3	32.9	20.6	tr	tr	53.4	0	0	0	0	0
		free	247	234	372	380	1233	249	110	123	83.4	565	23.0	10.0	11.9	12.8	57.8
2-14R	80	conj	24.5	20.3	tr	tr	44.7	20.8	12.6	tr	tr	33.4	0	0	0	0	0
		free	85.9	70.2	104	107	367	84.6	45.2	50.0	45.5	225	8.1	5.5	6.2	5.6	25.4

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins

Table 3-14. Levels (mg/kg, dry matter) of free and conjugated sapogenins in jejunum samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		sarsasapogenin					episarsasapogenin					3-CO-sarsasapogenin				
			139	140	141	142	sum	139	140	141	142	sum	139	140	141	142	sum
2-3J	3	conj	110	59.0	21.6	tr	191	128	101	22.2	tr	252	0	0	0	0	0
		free	2702	7262	7848	4518	22329	335	961	696	208	2200	47.5	111	81.1	43.3	283
2-5J	8	conj	132	56.1	18.6	6.2	213	273	187	31.0	6.6	498	5.5	tr	0	0	5.5
		free	2662	5650	8068	6985	23365	381	845	771	262	2258	72.3	76.2	71.8	63.8	284
2-6J	12	conj	181	54.4	17.3	7.1	260	300	179	29.9	7.0	516	5.4	tr	0	0	5.4
		free	1833	4414	5449	3914	15609	265	583	506	171	1526	40.1	74.6	75.3	44.7	235
2-9J	29	conj	132	153	37.1	tr	322	645	386	48.7	14.2	1094	0	0	0	0	0
		free	284	832	599	237	1952	65.1	66.0	47.9	tr	179	0	0	0	0	0
2-10J	49	conj	15.3	17.4	tr	tr	32.7	210	159	20.5	tr	389	0	0	0	0	0
		free	48.4	86.0	55.3	24.8	215	36.3	28.1	17.1	tr	81.6	0	0	0	0	0
2-13J	74	conj	15.5	10.0	tr	tr	25.6	65.9	60.1	10.9	tr	137	0	0	0	0	0
		free	111	180	169	91.6	551	76.4	58.4	48.9	16.5	200	7.5	6.1	tr	0	13.6

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins.

Table 3-15. Levels (mg/kg, dry matter) of free and conjugated sapogenins in faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		sarsasapogenin					episarsasapogenin					3-CO-sarsasapogenin				
			139	140	141	142	sum	139	140	141	142	sum	139	140	141	142	sum
2-3F	3	conj	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		free	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
2-5F	9	conj	113	69.8	24.5	11.2	218	111	80.2	16.7	tr	208	0	0	0	0	0
		free	2103	5470	6409	4096	18078	1375	893	670	372	3310	44.9	81.7	85.6	24.1	236
2-9F	25	conj	112	64.2	19.9	8.7	205	197	115	26.4	9.8	348	0	0	0	0	0
		free	1453	3283	3493	1998	10228	1765	1492	1011	373	4643	43.6	45.3	34.1	tr	123
2-14F	80	conj	18.5	15.5	tr	tr	34.0	108	73.4	18.0	tr	199	0	0	0	0	0
		free	141	246	231	135	753	643	395	320	208	1566	0	0	0	0	0

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins.

Table 3-16. Levels (mg/kg) of free and conjugated sapogenins and cholesterols in bile samples from sheep 1, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		chol	episarsasapogenin 139	140	141	142	sum
1-PB	0	conj	28.6	0	0	0	0	0
		free	9.1	0	0	0	0	0
1-1B	1	conj	9.6	2.0	1.9	0.3	0	4.2
		free	23.7	0	0	0	0	0
1-3B	3	conj	10.6	12.4	8.8	1.2	0	22.4
		free	22.5	0	0	0	0	0.0
1-4B	5	conj	10.2	11.2	7.8	1.0	0	20.1
		free	21.1	0	0	0	0	0
1-5B	7	conj	11.1	20.7	13.8	1.8	0.4	36.6
		free	28.4	0	0	0	0	0
1-6B	11	conj	12.2	19.1	13.5	1.8	0.3	34.7
		free	26.3	0	0	0	0	0
1-7B	16	conj	25.0	37.3	26.8	3.5	0.7	68.3
		free	19.3	0	0	0	0	0
1-8B	21	conj	10.8	25.0	17.4	2.3	0.5	45.2
		free	27.5	0	0	0	0	0
1-9B	29	conj	12.4	26.1	17.9	2.4	0.5	46.9
		free	21.1	0	0	0	0	0
1-10B	49	conj	3.8	12.8	9.5	1.2	0.2	23.7
		free	16.7	0	0	0	0	0
1-11B	54	conj	5.9	6.1	4.5	0.6	tr	11.1
		free	13.7	0	0	0	0	0
1-12B	62	conj	18.6	8.2	5.5	0.7	tr	14.4
		free	13.0	0	0	0	0	0
1-13B	74	conj	19.6	3.8	2.7	0.3	tr	6.8
		free	12.5	0	0	0	0	0
1-14B	79	conj	5.4	1.5	0.9	tr	0	2.4
		free	20.5	0	0	0	0	0
1-15B	97	conj	3.2	1.2	0.7	0	0	1.9
		free	20.9	0	0	0	0	0

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, chol = cholesterol, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins, sum = m/z 139+m/z 140+m/z 141+m/z 142.

Table 3-17. Levels (mg/kg) of free and conjugated sapogenins and cholesterols in bile samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		chol	episarsasapogenin				
				139	140	141	142	sum
2-PB	0	conj	41.7	0	0	0	0	0
		free	14.3	0	0	0	0	0
2-1B	1	conj	8.0	1.2	0.9	0	0	2.1
		free	22.3	0	0	0	0	0
2-3B	3	conj	21.4	19.2	13.2	1.9	0.4	34.8
		free	22.6	0	0	0	0	0
2-4B	5	conj	23.3	56.7	41.7	6.4	1.5	106
		free	24.5	0	0	0	0	0
2-5B	7	conj	41.2	68.2	46.8	6.7	1.3	123
		free	20.0	0	0	0	0	0
2-6B	11	conj	29.9	46.2	32.2	4.7	0.7	83.7
		free	65.9	0	0	0	0	0
2-7B	16	conj	54.7	29.6	14.9	1.9	tr	46.4
		free	51.1	0	0	0	0	0
2-9B	29	conj	35.6	36.3	21.3	2.9	0.6	61.2
		free	23.4	0	0	0	0	0
2-10B	49	conj	56.4	19.8	13.3	1.6	tr	34.7
		free	5.5	0	0	0	0	0
2-11B	54	conj	67.8	25.1	16.0	2.0	tr	43.1
		free	33.6	0	0	0	0	0
2-12B	62	conj	35.3	17.3	13.4	1.7	tr	32.4
		free	26.6	0	0	0	0	0
2-13B	74	conj	21.8	13.6	12.0	1.6	0.3	27.6
		free	26.1	0	0	0	0	0
2-14B	79	conj	31.4	8.9	7.4	1.0	tr	17.2
		free	20.4	0	0	0	0	0
2-15B	98	conj	51.7	5.0	4.5	0.6	tr	10.0
		free	3.0	0	0	0	0	0

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, chol = cholesterol, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins, sum = m/z 139+m/z 140+m/z 141+m/z 142.

3.5.2.1 Levels of Sapogenins in Rumen, Jejunum and Faeces Samples

Rumen samples

The highest level of free sarsasapogenin (11894 mg/kg) was found in a rumen sample collected 1 hour after dosing. At the same time, a much lower level of conjugated sarsasapogenins (322 mg/kg) was found in the rumen sample (Table 3-13). These results indicate that after an hour most of the dosed sarsasapogenin β-D-glucoside had been hydrolysed to free sarsasapogenin.

Very limited conversion to free episarsasapogenin (1001 mg/kg), presumably via the intermediary of sarsasapogen-3-one (116 mg/kg) was apparent in the rumen after 1 hour. Thereafter free sarsasapogenin levels progressively decreased (8729 → 6615 → 4388 → 4425 → 1233 → 368 mg/kg, after 4, 7, 12, 22, 63 and 80 hours respectively), while conjugated sarsasapogenin levels varied between 368 and 68 mg/kg (Figure 3-10).

No episarsasapogenin conjugates were detected in the rumen samples collected 1 and 4 hours after dosing, however episarsasapogenin conjugates were identified in subsequent samples (Table 3-13).

Free sarsasapogenone was found in all of the rumen extracts at levels in the range 25-194 mg/kg. After 63 hours a downward trend in the level of sarsasapogenone was observed. Low levels of conjugated sarsasapogenone were also detected in 4-12 hour samples (Table 3-13).

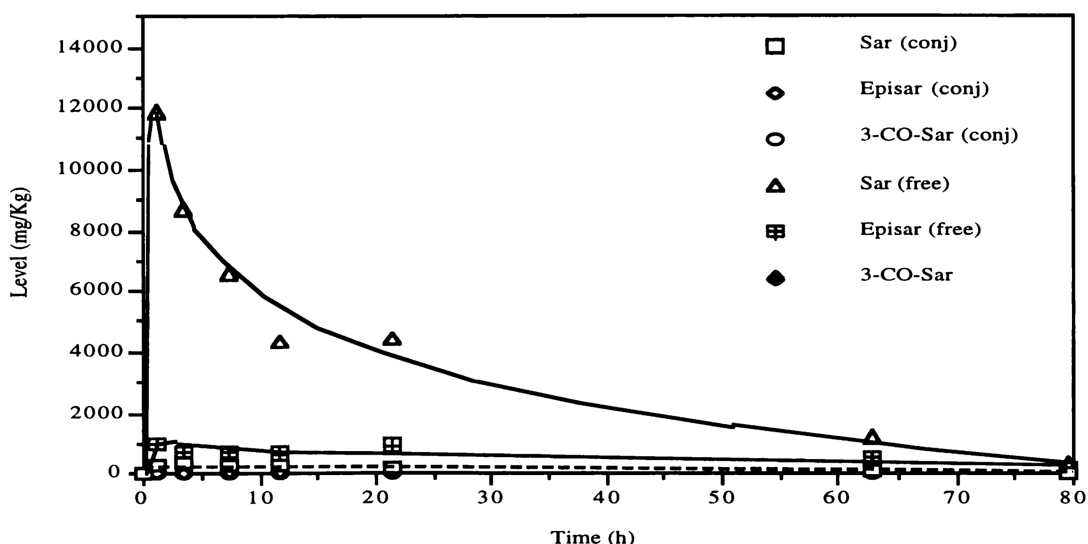


Figure 3-10. Levels of free and conjugated sapogenins in rumen samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

Jejunum samples

As shown in Table 3-14 and Figure 3-11, the level of free sarsasapogenins in jejunum samples rapidly increased during the first three hours (to 22329 mg/kg), and remained at about this level until 8 hours (23365 mg/kg), after which they fell steeply to 1952 mg/kg after 29 hours. Lesser levels were detected in later samples.

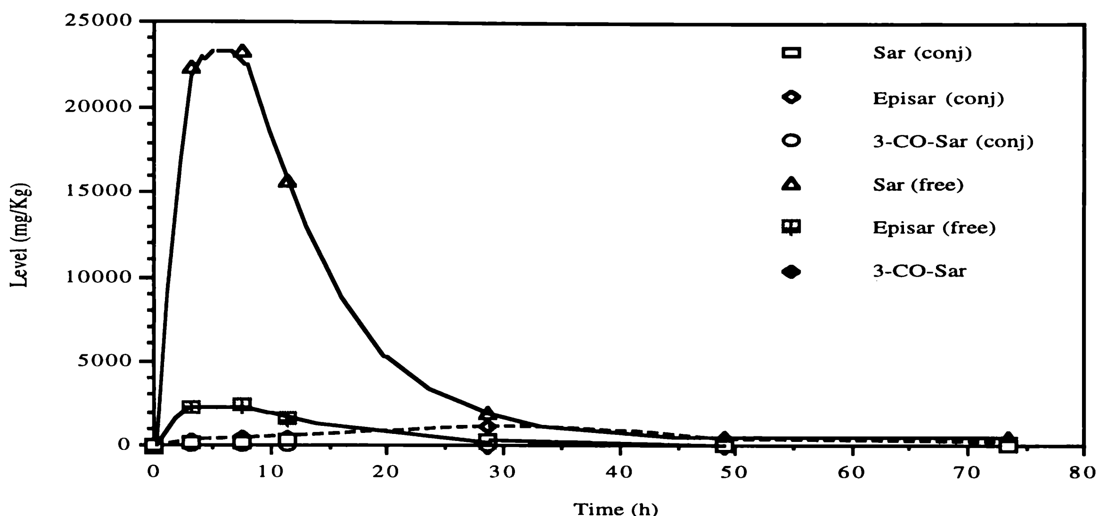


Figure 3-11. Levels of free and conjugated sapogenins in jejunum samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

The level of free episarsasapogenins in the jejunum samples also increased rapidly to 2200-2258 mg/kg after 3-7 hours. The time course of this increase corresponded to that observed for free sarsasapogenins (Figure 3-11). After 12 hours the level of free episarsasapogenins was 1526 mg/kg. It then declined to 179 mg/kg after 29 hours, and remained at about this level until 72 hours.

The level of conjugated episarsasapogenins in the jejunum samples rose from 251 mg/kg after 3 hours to 1094 mg/kg after 29 hours and then decreased to 137 mg/kg after 74 hours (Figure 3-11).

For both free sarsasapogenin and episarsasapogenin, maximum levels occurred in the jejunum after 3-8 hours, whereas conjugated sarsasapogenin and episarsasapogenin levels were at a maximum after 29 hours (Figure 3-11).

Faeces samples

No free or conjugated sapogenins were found in faeces samples 3 hours after dosing commenced. Maximum levels of free sarsasapogenins (18078 mg/kg) and free episarsasapogenins (3310 mg/kg) were detected after 9 hours. Significantly lower levels of

conjugated sarsasapogenins (218 mg/kg) and conjugated episarsasapogenins (208 mg/kg) were also detected in the sample (Table 3-15).

After 9 hours, the level of free sarsasapogenins steadily decreased (Figure 3-12). On the other hand, the level of free episarsasapogenins increased slowly to 4643 mg/kg after 30 hours, and then declined (Figure 3-12). This difference in the time course of free sarsasapogenin and free episarsasapogenins levels corresponded with that noted above for free sarsasapogenins and episarsasapogenins in jejunum samples.

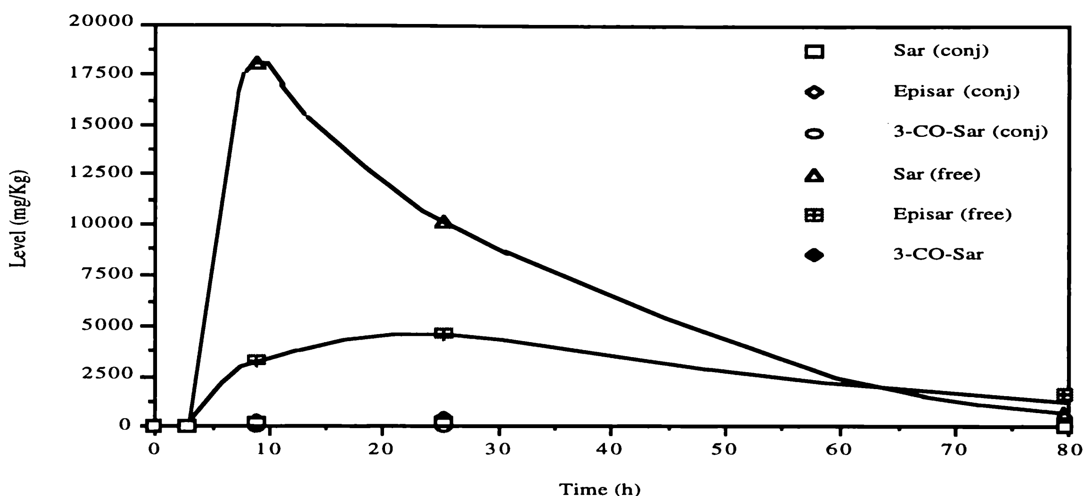


Figure 3-12. Levels of free and conjugated sapogenins in faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

3.5.2.2 Levels of Sapogenins in Bile Samples

The results for bile samples recovered from both sheep 1 and 2 (Tables 3-16 and 3-17) are comparable in so much as episarsasapogenin conjugate levels rose to an initial maximum (68 and 123 mg/kg respectively), and then progressively declined (Figure 3-13). However, sheep 1 exhibited a maximum level 16 hours after dosing, whereas sheep 2 showed a maximum level 7 hours after dosing.

The rate at which episarsasapogenin conjugate levels declined also varied. Sheep 2 showed an essentially linear decay over the period 30-100 hours after dosing, whereas sheep 1 exhibited an exponential-like decay during the same time period. After 5 days, ca 10 mg/kg of episarsasapogenin conjugates were detected in bile samples from sheep 2, compared to ca 2 mg/kg for sheep 1.

No free or conjugated sarsasapogenins, or free episarsasapogenins, were detected in bile samples from sheep 1 or sheep 2.

These results can be compared with those obtained for sheep A (shown in Figure 3-2). That sheep, which was dosed with 2 g of sarsasapogenin β-D-glucuronide (ca half the total level of sapogenin and saponin material dosed to sheep 1 and 2), exhibited a much lower maximum level of episarsasapogenin conjugates (ca 2-3 mg/kg), and the level was maintained for a longer time period (14-30 hours).

It is clear that notwithstanding the common feature of episarsasapogenin conjugate accumulation in the bile, the time course and maximum episarsasapogenin conjugate level can vary substantially from sheep to sheep. Clearly, generalisations based on results from a single sheep (or indeed two sheep as in the present investigation) need to be made with caution.

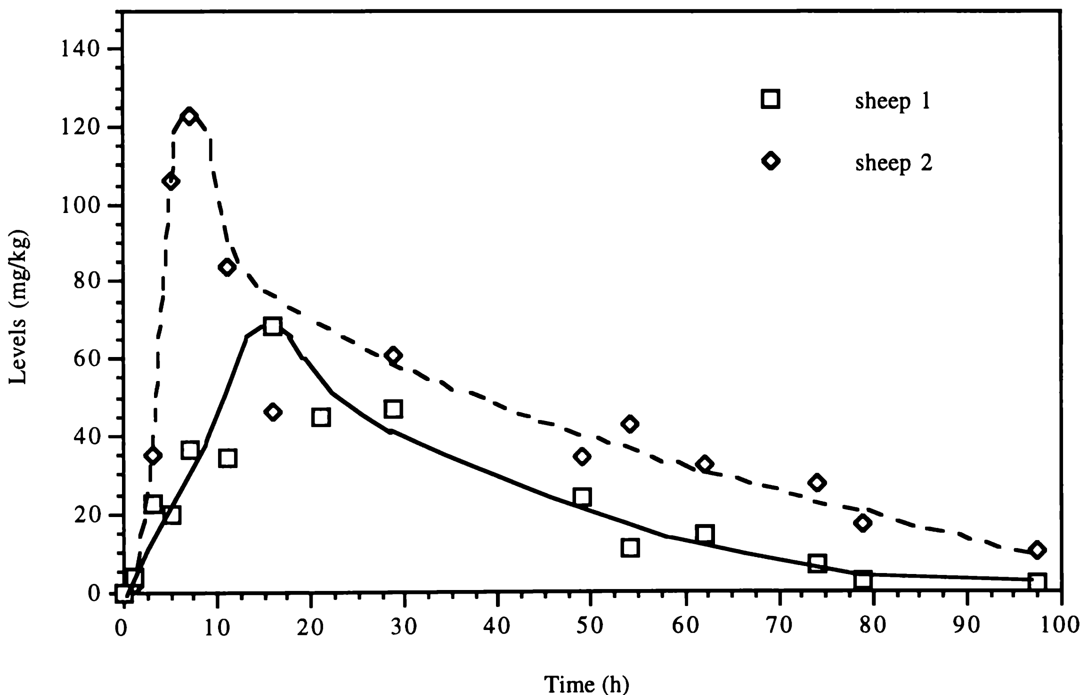


Figure 3-13. Levels of conjugated episarsasapogenins in bile samples from sheep 1 and 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin (data from Tables 3-16 and 3-17).

The biosynthesis of sapogenins, in the plants, has been shown to be related to cholesterol production (Heftmann, 1967; Heftmann, 1968). There is, however, no evidence for the ovine metabolism of cholesterol to sapogenins. No correlation between the levels of

cholesterol and sapogenins were observed in this investigation (see Tables 3-16 and 3-17, and Figure 3-14).

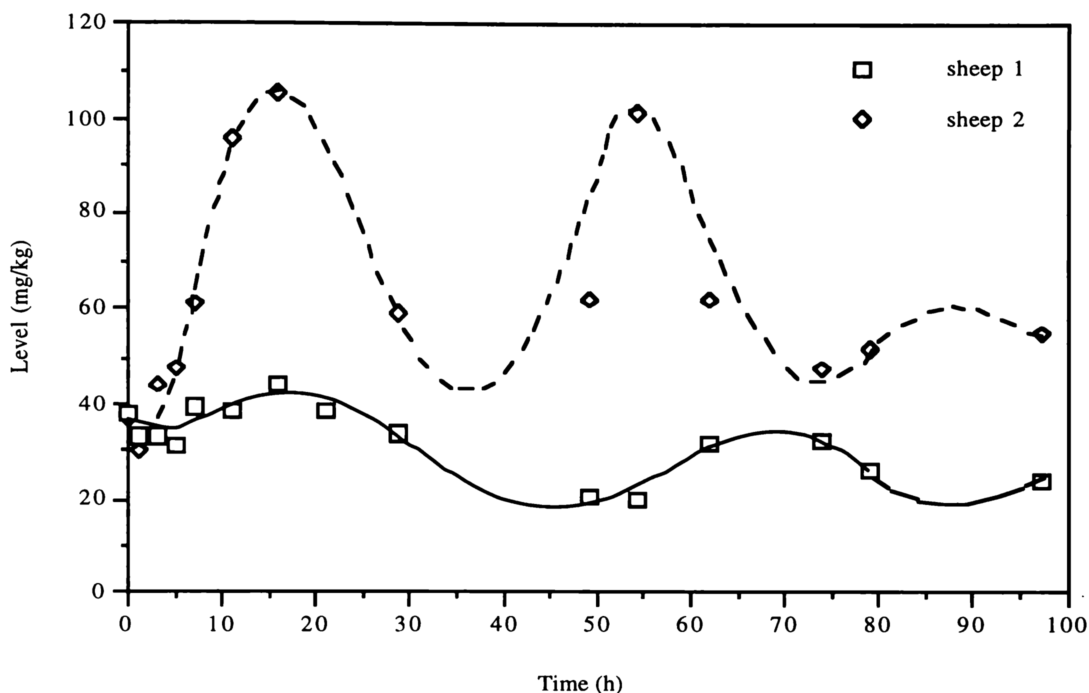


Figure 3-14. Total cholesterol levels in bile samples from sheep 1 and sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

3.5.2.3 Contributions of Deuterated and non-Deuterated Sapogenins to Bile Samples

GC-MS analysis showed that the dosing material, 20,23,23-D_3-sarsasapogenin, contained 1% of non-D (*m/z* 139), 6% of D- (*m/z* 140), 28% of D_2- (*m/z* 141) and 65% of D_3-sarsasapogenin (*m/z* 142), i.e. *m/z* 139, 140, 141 and 142 ion responses in the ratio of 1:6:28:65.

Under the same GC-MS conditions, a standard specimen of sarsasapogenin acetate, and hydrolysed sarsasapogenin glucoside, detected in GC-MS analyses as sarsasapogenin acetate, exhibited *m/z* 139, 140, 141 and 142 responses in the ratio of 87:12:1:0. This pattern can be attributed to the presence, at natural abundance levels, of ^{13}C atoms rather than ^{2}D atoms.

Since a mixture of 2.00 g (0.0035 moles) of sarsasapogenin β-D-glucoside and 2.00 g (0.0048 moles) of 20,23,23-D_3-sarsasapogenin contains 1.46 g (0.0035 moles) of sarsasapogenin and 2.0 g (0.0048 moles) of deuterated sarsasapogenin, the expected ratio of deuterated to non-deuterated sapogenins in ovine samples, assuming no loss of deuterium during metabolism, is 1.46 to 2.0 (or 0.73 to 1.0).

The expected non-D, D-, D_2- and D_3-sarsasapogenin contributions (*m/z* 139, 140, 141 and 142 ion responses, respectively), from a 1.46 to 2.0 by mole mixture of non-deuterated sarsasapogenin (dosed as sarsasapogenin β-D-glucoside) and deuterated sarsasapogenin (dosed as 20,23,23-D_3-sarsasapogenin), would be in the ratios defined by the expressions below:

non-D (*m/z* 139) = 1.46 * 0.87 + 2.00 * 0.01 = 1.29
D_1- (*m/z* 140) = 1.46 * 0.12 + 2.00 * 0.06 = 0.30
D_2- (*m/z* 141) = 1.46 * 0.01 + 2.00 * 0.28 = 0.57
D_3- (*m/z* 142) = 1.46 * 0.00 + 2.00 * 0.65 = 1.30

Since the combined weight of the deuterium-labelled and non-labelled sarsasapogenin was 3.46 g, the percentage contributions of the *m/z* 139, 140, 141 and 142 ions would be expected to be:

non-D (*m/z* 139) = 1.29 / 3.46 = 37%
D- (*m/z* 140) = 0.30 / 3.46 = 9%
D_2- (*m/z* 141) = 0.57 / 3.46 = 16%
D_3- (*m/z* 142) = 1.30 / 3.46 = 38%

Assuming equal rates of metabolism for free and conjugated substrates, and no loss of deuterium by exchange, the expected percentage *m/z* 139, 140, 141, and 142 ion contributions to the rumen, jejunum, faeces and bile sapogenin samples would be 37%, 9%, 16% and 38% respectively (i.e. a ratio of 37:9:16:38) (Figure 3-15 A).

Different percentage contributions were typically observed for episarsasapogenin conjugates recovered from bile samples (Tables 3-18 and 3-19). In general, higher non-labelled and mono-D, and lower D_2- and D_3-episarsasapogenins percentage contributions were observed. For example, the 7 hour bile sample from sheep 1 (sample 1-5B) had 56.5% of non-D, 37.7% of D-, 4.9% of D_2- and 1.0% of D_3-episarsasapogenin (i.e. a ratio of 57:38:5:1) (Figure 3-15 B).

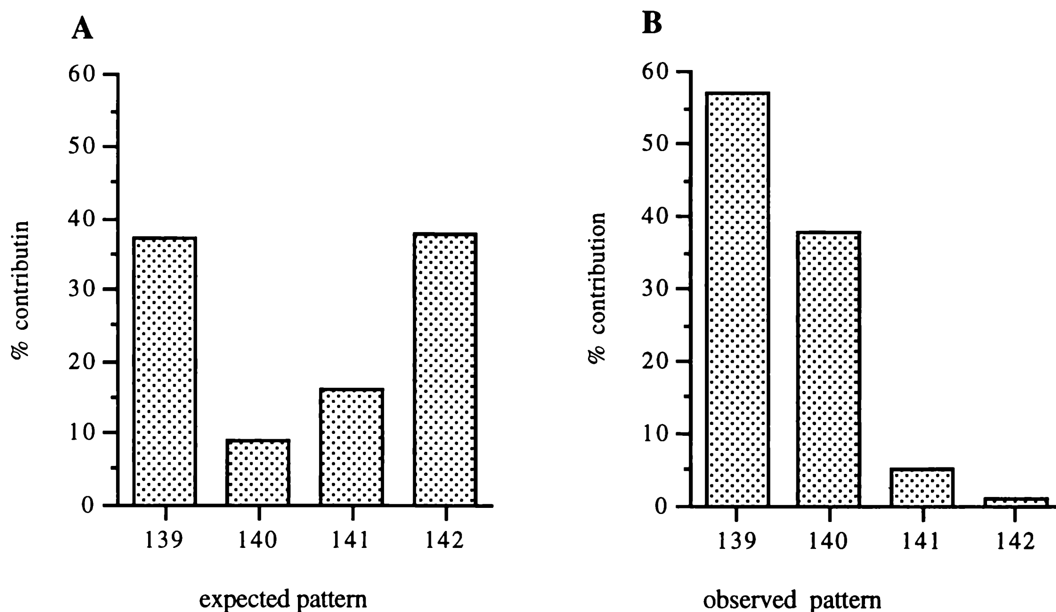


Figure 3-15. Expected percentage contributions (A) and observed percentage contributions (B) for the *m/z* 139, 140, 141, 142 ions of epismilagenins in bile samples recovered from a sheep, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin (sample 1-5B).

These percentage contributions were similar to those determined for episarsasapogenin conjugates present in other samples collected 3-74 hours after dosing, indicating that deuterium exchange was not a significant process in bile samples. This indicates that deuterium loss occurs primarily in other metabolic regions (e.g. the rumen or the liver).

A similar consistency in percentage *m/z* 139, 140, 141 and 142 ion contributions was also observed for sheep 2 bile samples collected 3-74 hours after dosing. The ratio of *m/z* 139-142 ion responses for sheep 2 was typically 55:39:6:1 (sample 2-B) (Table 3-19).

Table 3-18. The percentage contributions (%) of non-D (*m/z* 139), D- (*m/z* 140), D_2- (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) determined in bile samples from sheep 1, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)	episar *m/z* 139	D-episar *m/z* 140	D_2-episar *m/z* 141	D_3-episar *m/z* 142
1-PB	0	0	0	0	0
1-1B	1	48.8	45.1	6.1	0
1-3B	3	55.5	39.3	5.2	0
1-4B	5	55.9	39.1	5.1	0
1-5B	7	56.5	37.7	4.9	1.0
1-6B	11	55.0	38.9	5.1	1.0
1-7B	16	54.6	39.3	5.2	1.0
1-8B	21	55.3	38.5	5.2	1.0
1-9B	29	55.6	38.3	5.1	1.0
1-10B	49	53.7	40.0	5.2	1.0
1-11B	54	54.4	40.4	5.2	0
1-12B	62	57.0	38.0	5.0	0
1-13B	74	55.9	39.1	5.0	0
1-14B	79	63.2	36.8	0	0
1-15B	97	62.4	37.6	0	0

Table 3-19. The percentage contributions (%) of non-D (*m/z* 139), D- (*m/z* 140), D_2- (*m/z* 141) and D_3-episarsasapogenin (*m/z* 142) determined in bile samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)	episar *m/z* 139	D-episar *m/z* 140	D_2-episar *m/z* 141	D_3-episar *m/z* 142
2-PB	0	0	0	0	0
2-1B	1	58.4	41.6	0	0
2-3B	3	55.2	37.9	5.6	1.3
2-4B	5	53.4	39.2	6.0	1.4
2-5B	7	55.4	38.0	5.5	1.1
2-6B	11	55.2	38.4	5.6	0.8
2-7B	16	63.7	32.1	4.1	0
2-9B	29	59.4	34.8	4.7	1.0
2-10B	49	57.0	38.2	4.7	0
2-11B	54	58.3	37.0	4.7	0
2-12B	62	53.3	41.4	5.3	0
2-13B	74	49.4	43.6	5.9	1.0
2-14B	79	51.6	42.9	5.5	0
2-15B	98	49.7	44.4	5.8	0

From Tables 3-18 and 3-19, it is clear that two of the three deuterium atoms present in the dosed 20,23,23-D_3-sarsasapogenin were readily lost during ovine metabolism, and/or the work procedure. Only one of the deuterium atoms was strongly retained (not prone to exchange).

More extensive loss of deuterium (> 99%) was observed in a Norwegian dosing experiment, in which a sheep was dosed with saponin containing plant material and 20,23,23-D_3-sarsasapogenin (initially dissolved in acetic acid and then diluted into water) (Flåøyen and Wilkins, pers commun).

The observation, that the use of acetic acid as a co-solvent for 20,23,23-D_3-sarsasapogenin in a dosing experiment appeared to facilitate deuterium exchange, prompted an investigation of the stability (exchangeability) of D-20, D-23, D-23 atoms in a series of buffer solutions of pH 1, 2, 4, 6 and 8, held at 37°C for 3 days. These pH's are typical of those encountered in the digestive tract of a sheep (Spector, 1956). GC-MS analyses showed that the contributions of D-, D_2-, and D_3-sarsasapogenins remained essentially constant over the 72 hour sampling period (see Section 2, Table 2-10 and Figure 2-12). The results showed that the deuterium atoms were not prone to loss by exchange under the conditions utilised in the stability tests, or encountered during sample work-up and GC-MS analyses.

A standard specimen of sarsasapogenin acetate and a specimen of epismilagenin obtained by hydrolysis of a bile sample recovered from sheep A (trial 1 in which non-deuterated sarsasapogenin was dosed) gave *m/z* 139, 140, 141 and 142 ion responses in the ratio of 87:12:1:0. As indicated above, the *m/z* 140 responses of these compounds can be predominantly attributed to a ^{13}C contributions, rather than to a ^{2}D contributions.

For an experiment in which a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin were dosed, the observed *m/z* 139/140/141/142 isotope pattern of the bile episarsasapogenin conjugates will be derived from a combination of non-deuterated substrate and deuterated substrate contributions.

Dosing of 20,23,23-D_3-sarsasapogenin alone (see Section 3.4) afforded bile episarsasapogenin conjugates which exhibited *m/z* 139, 140, 141 and 142 ion responses in ratios of 18.5:59.8:17.9:3.8 (sheep 2) and 19.3:68.5:11.4:0.9 (sheep 3). The *m/z* 141 and *m/z* 142 ion responses can be considered to arise from the presence of two and three deuterium atoms, respectively.

Mathematical analyses

Two approaches were used to calculate deuterated (20,23,23-D_3-sarsasapogenin) (free sapogenin = X) and non-deuterated (sarsasapogenin β-D-glucoside) (conjugated sapogenin = Y) contributions to bile episarsasapogenin conjugates. All calculations were performed using purpose written Excel spreadsheets. Terms used in these calculations are defined below.

Deuterated, non-deuterated and total sapogenin contributions:

X = deuterated sapogenin contribution (mg/kg) (= free sapogenin contribution)
Y = non-deuterated sapogenin contribution (mg/kg) (= conjugated sapogenin contribution)
X_{139}, X_{140}, X_{141}, X_{142} = non D, D-, D_2- and D_3- contributions from the deuterated sapogenin (mg/kg)
Y_{139}, Y_{140}, Y_{141}, Y_{142} = non D, D-, D_2- and D_3- contributions from the non deuterated sapogenin (mg/kg)
$X_{sum} = X_{139} + X_{140} + X_{141} + X_{142}$
$Y_{sum} = Y_{139} + Y_{140} + Y_{141} + Y_{142}$
T = total sapogenin contribution (mg/kg)
$T_{139} = X_{139} + Y_{139}$
$T_{140} = X_{140} + Y_{140}$
$T_{141} = X_{141} + Y_{141}$
$T_{142} = X_{142} + Y_{142}$
$T_{sum} = T_{139} + T_{140} + T_{141} + T_{142} = X_{sum} + Y_{sum}$

SIM GC-MS determined peak areas were used to determine ion ratios (for example):

$R_{(X)139/140}$ = *m/z* 139 peak area divided by *m/z* 140 peak area for substrate X
$R_{(X)139/141}$ = *m/z* 139 peak area divided by *m/z* 141 peak area for substrate X
$R_{(Y)139/141}$ = *m/z* 139 peak area divided by *m/z* 141 peak area for substrate Y

A mixture of 2.00 g (0.00346 moles) of sarsasapogenin β-D-glucoside and 2.00 g (0.00478 moles) of 20,23,23-D_3-sarsasapogenin (average MWt of ca 418) was administered to each sheep. A conversion factor (CF) is required to convert results to an equimolar basis (ignoring deuterium loss). Assuming 100% of the dosed substrates were

metabolised to bile episarsasapogenin conjugates, 1.46 g (0.00346 moles) of episarsasapogenin and 2.00 g (0.00478 moles) of deuterated episarsasapogenin would be formed (Figure 3-16).

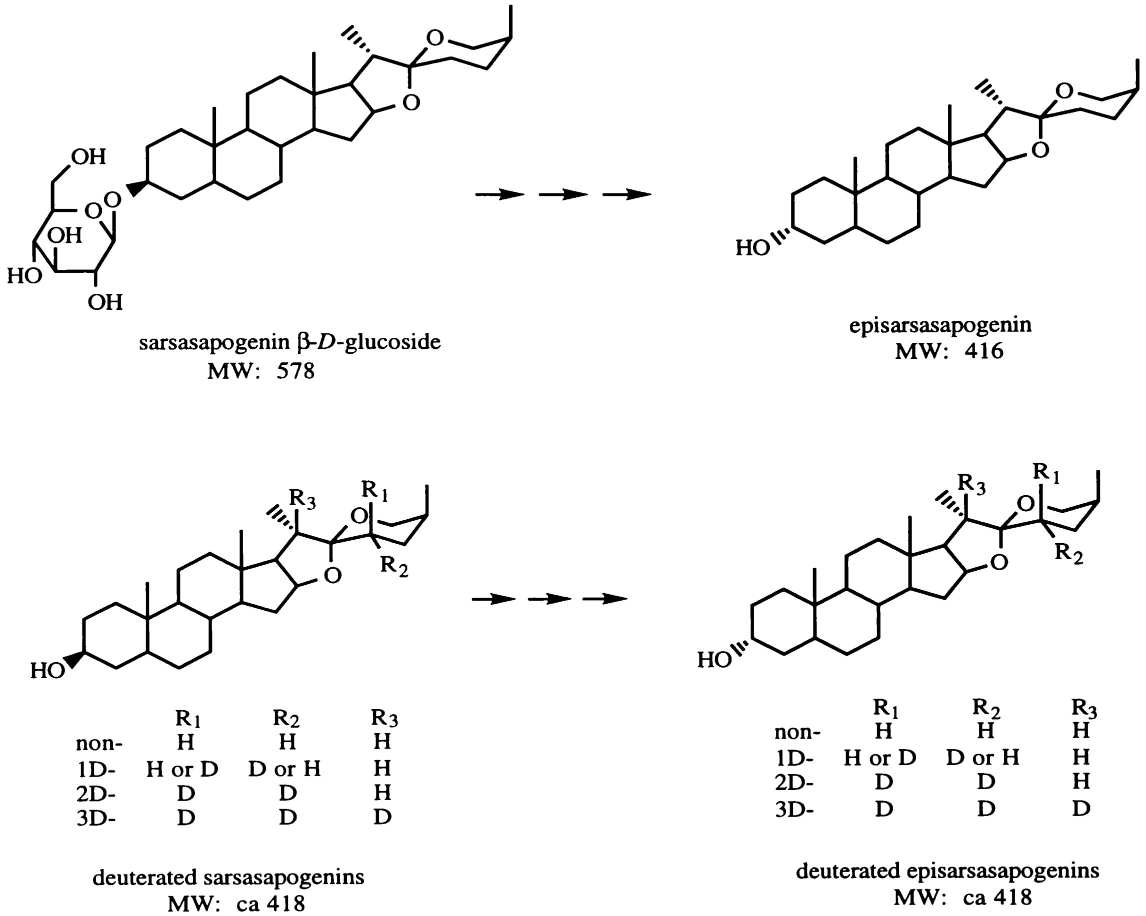


Figure 3-16. Molecular weight changes in the conversion of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin to episarsasapogenins.

Conversion factors (CF) are therefore 1.00 for 20,23,23-D_3-sarsasapogenin (D-sar) and 0.72 for sarsasapogenin β-D-glucoside (sar-glu).

$$CF_{D\text{-}sar} = MWt_{D\text{-}episar} / MWt_{D\text{-}sar} = 418 / 418 = 1.00$$

$$CF_{sar\text{-}glu} = MWt_{episar} / MWt_{sar\text{-}glu} = 416 / 578 = 0.72$$

The X_{sum} and Y_{sum} values can be converted (normalised) to a 1:1 mole basis by dividing by 1.00 and 0.72 respectively, where:

$$N_{D\text{-}sar} = X_{sum} / CF_{D\text{-}sar} = X_{sum} / 1 = X_{sum}$$

$$N_{sar\text{-}glu} = Y_{sum} / CF_{sar\text{-}glu} = Y_{sum} / 0.72$$

Method 1

Since, in SIM GC-MS analyses, the non-deuterated conjugated substrate (Y = sarsasapogenin β-D-glucoside) contributes only *m/z* 139 and 140 ions (ignoring the 1% *m/z* 141 ion contribution and the 0% *m/z* 142 contribution), the *m/z* 141 (free D_2-sapogenin) ion contribution (= X_{141}) to bile episarsasapogenin conjugates can be used to calculate the contributions of the companion non-D (X_{139}), D (X_{140}) and D_3 (X_{142}) to an observed ion ratio pattern.

Figure 3-17 shows the pattern that would be expected for a 1:1 mixture of a non-labelled genin and a metabolised specimen of 20,23,23-D_3-episarsasapogenin, exhibiting *m/z* 139, 140, 141 and 142 ion responses in the ratio 19:69:11:1 (as determined in trial 4 bile samples: see Table 3)

It follows that X_{139} and X_{140} can be calculated from the ratios of *m/z* 141 to 139 ($R_{(X)139/141}$) and *m/z* 141 to 140 ($R_{(X)140/141}$), as determined in an experiment in which only 20,23,23-D_3-sarsasapogenin was dosed to a sheep (see Section 3.5).

$$X_{139} = T_{141} * R_{(X)139/141}$$
$$X_{140} = T_{141} * R_{(X)140/141}$$

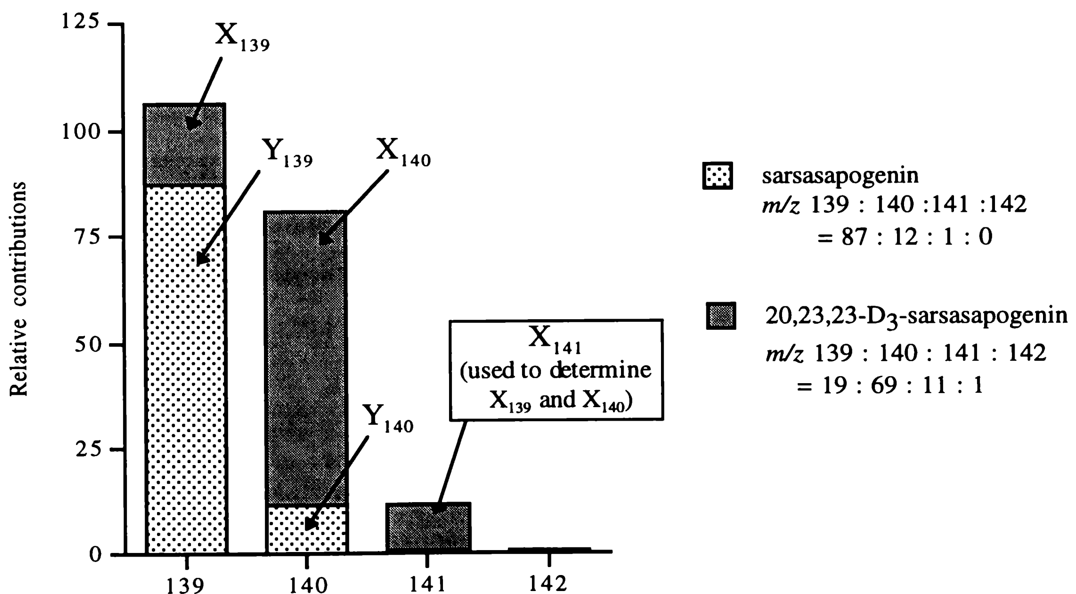


Figure 3-17. Calculated *m/z* 139/140/141/142 ion contributions for a 1:1 mixture of sarsasapogenin and a metabolised specimen of 20,23,23-D_3-sarsasapogenin, exhibiting *m/z* 139, 140, 141 and 142 ion responses in the ratio 19:69:11:1.

These contributions of the free substrates (X_{139}, X_{140}, X_{141} and X_{142}) can then be subtracted from the observed T_{139}, T_{140}, T_{141} and T_{142}, values to give the contributions of the conjugated substrates (Y_{139}, Y_{140}, Y_{141} and Y_{142} values).

$Y_{139} = T_{139} - X_{139}$

$Y_{140} = T_{140} - X_{140}$

$Y_{141} = 0$ (since the 1% Y_{141} contribution can be ignored)

$Y_{142} = 0$ (since a *m/z* 142 response is not found for Y)

In an experiment in which only 20,23,23-D_3-sarsasapogenin was dosed to sheep 2 and sheep 3 (see Section 3.5), SIM GC-MS analysis showed the following average percentage contributions to conjugated bile episapogenins:

sheep 2: 18.5% (*m/z* 139), 59.8% (*m/z* 140), 17.9% (*m/z* 141), 3.8% (*m/z* 142)
sheep 3: 19.3% (*m/z* 139), 68.5% (*m/z* 140), 11.4% (*m/z* 141), 0.9% (*m/z* 142)

The average ratios ($R_{(X)}$) for the *m/z* 139, 140, 141 and 142 ions normalised to the *m/z* 141 ion response were therefore: (see Tables 3-9 and 3-10, Section 3.4.2.2).

sheep 2:
$R_{(X)139/141} = 18.5 / 17.9 = 1.0$
$R_{(X)140/141} = 59.8 / 17.9 = 3.3$
$R_{(X)140/141} = 17.9 / 17.9 = 1.0$
$R_{(X)142/141} = 3.8 / 17.9 = 0.2$

sheep 3
$R_{(X)139/141} = 19.3 / 11.4 = 1.7$
$R_{(X)140/141} = 68.5 / 11.4 = 6.0$
$R_{(X)140/141} = 11.4 / 11.4 = 1.0$
$R_{(X)142/141} = 0.9 / 11.4 = 0.1$

For example, bile sample 2-5B (from sheep 2 dosed with sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin) was found to contain 68.2, 46.8, 6.7 and 1.3 mg/kg of non-D, D-, D_2- and D_3-episarsasapogenin conjugates, respectively (Table 3-17).

i.e. T_{139}, T_{140}, T_{141} and T_{142} = 68.2, 46.8, 6.7 and 1.3 respectively

The levels of episarsasapogenins arising from deuterated sapogenins (X) are: (based on $R_{(x)}$ from sheep 3)

$X_{139} = R_{(x)139/141} * T_{141} = 1.7 * 6.7$ mg/kg = 11.5 mg/kg
$X_{140} = R_{(x)140/141} * T_{141} = 6.0 * 6.7$ mg/kg = 40.4 mg/kg
$X_{141} = T_{141} = 6.7$ mg/kg (contributed from deuterated sapogenins only)
$X_{142} = T_{142} = 1.3$ mg/kg (contributed from deuterated sapogenins only)

Thereafter subtraction of the deuterated sapogenin contributions (X) from the total contributions (T) gives the non-deuterated sapogenin contributions (Y):

$Y_{139} = T_{139} - X_{139} = 68.2 - 11.5 = 56.7$ mg/kg
$Y_{140} = T_{140} - X_{140} = 46.8 - 40.4 = 6.3$ mg/kg
$Y_{141} = 0$ (since X_{141} assumed to equal T_{141})
$Y_{142} = 0$ (since X_{142} assumed to equal T_{142})

The validity of this the calculation methodology, is critically dependent on the *m/z* 139, 140, 141 and 142 ion ratio of bile conjugates derived from the deuterated genin in the mixed dosing experiment being the same as that in another experiment in which only the deuterated genin is dosed. This is a questionable assumption given the likelihood of rumen flora activity variations in the respective trials.

An additional complication in the investigation reported here was that (because of bile flow complications experienced by sheep 1) the mixed dosing experiments were performed using sheep 1 and 2, while the deuterated free sapogenin dosing experiments were subsequently performed using sheep 2 and 3. Clearly, caution has been exercised, when using results from one sheep, to interpret results obtained from another sheep.

The reliability of the calculation methodology can be assessed by comparing the calculated $R_{(Y)140/139} = Y_{140} / Y_{139}$ ratio for the conjugated substrate (sarsasapogenin β-D-glucuronide) with that determined for a specimen of the substrate. As noted above, this ratio should be ca 0.14, based on relative *m/z* 139 and 140 ion contributions of 87% and 12% respectively in the dosed substrate.

$$R_{(Y)140/139} = Y_{140} / Y_{139} = 0.14$$

The X_{139}, X_{140}, X_{141}, X_{142}, X_{sum}, Y_{139}, Y_{140}, Y_{141}, Y_{142}, Y_{sum} and $R_{(Y)140/139}$ values calculated from the four permutations of results determined for sheep 1 and 2 (mixed results), and sheep 2 and 3 (deuterated genin results, see Section 3.5), using method 1, are presented in Table 3-20.

Table 3-20. Contributions (mg/kg) to episarsasapogenins from sarsasapogenin β-D-glucoside (Y) and 20,23,23-D_3-sarsasapogenin (X) and the ratio ($R_{(Y)140/139}$) in bile samples from sheep 1 and 2 determined using *m/z* 141 data from Trial 4 (sheep 2 and 3, dosed with 20,23,23-D_3-sarsasapogenin) (Method 1).

No	Time (h)	X_{139}	X_{140} (Free)	X_{141}	X_{142}	Y_{139}	Y_{140} (Conj)	Y_{141}	Y_{142}	X_{sum}	Y_{sum}	ratio
sheep 1 with sheep 3 D-genin data												
1-PB	0	0	0	0	0	0	0	0	0	0	0	0.00
1-1B	1	0.5	1.8	0.3	0	1.5	0.1	0	0	2.6	1.6	0.07
1-3B	3	2.0	7.2	1.2	0.1	10.4	1.6	0	0	10.5	12.0	0.15
1-4B	5	1.7	6.0	1.0	0.1	9.5	1.8	0	0	8.8	11.3	0.19
1-5B	7	3.1	10.8	1.8	0.1	17.6	3.0	0	0	15.8	20.6	0.17
1-6B	11	3.1	10.8	1.8	0.1	16.0	2.7	0	0	15.8	18.7	0.17
1-7B	16	6.0	21.0	3.5	0.2	31.4	5.8	0	0	30.7	37.2	0.18
1-8B	21	3.9	13.8	2.3	0.2	21.1	3.6	0	0	20.2	24.7	0.17
1-9B	29	4.1	14.4	2.4	0.2	22.0	3.5	0	0	21.0	25.5	0.16
1-10B	49	2.0	7.2	1.2	0.1	10.8	2.3	0	0	10.5	13.1	0.21
1-11B	54	1.0	3.6	0.6	0	5.1	0.9	0	0	5.3	6.0	0.18
1-12B	62	1.2	4.2	0.7	0	7.0	1.3	0	0	6.1	8.3	0.19
1-13B	74	0.5	1.8	0.3	0	3.3	0.9	0	0	2.6	4.2	0.27
1-14B	79	0	0	0	0	1.5	0.9	0	0	0	2.4	0.60
1-15B	97	0	0	0	0	1.2	0.7	0	0	0	1.9	0.58
sheep 2 with sheep 3 D-genin data												
2-PB	0	0	0	0	0	0	0	0	0	0	0	0.00
2-1B	1	0	0	0	0	1.2	0.9	0	0	0	2.1	0.75
2-3B	3	3.2	11.4	1.9	0.1	16.0	1.8	0	0	16.7	17.8	0.11
2-4B	5	10.9	38.4	6.4	0.4	45.8	3.3	0	0	56.1	49.1	0.07
2-5B	7	11.4	40.2	6.7	0.5	56.8	6.6	0	0	58.8	63.4	0.12
2-6B	11	8.0	28.2	4.7	0.3	38.2	4.0	0	0	41.2	42.2	0.10
2-7B	16	3.2	11.4	1.9	0.1	26.4	3.5	0	0	16.7	29.9	0.13
2-9B	29	4.9	17.4	2.9	0.2	31.4	3.9	0	0	25.4	35.3	0.12

2-10B	49	2.7	9.6	1.6	0.1	17.1	3.7	0	0	14.0	20.8	0.22
2-11B	54	3.4	12.0	2.0	0.1	21.7	4.0	0	0	17.5	25.7	0.18
2-12B	62	2.9	10.2	1.7	0.1	14.4	3.2	0	0	14.9	17.6	0.22
2-13B	74	2.7	9.6	1.6	0.1	10.9	2.4	0	0	14.0	13.3	0.22
2-14B	79	1.7	6.0	1.0	0.1	7.2	1.4	0	0	8.8	8.6	0.19
2-15B	98	1.0	3.6	0.6	0	4.0	0.9	0	0	5.3	4.9	0.23
sheep 1 with sheep 2 D-genin data												
1-PB	0	0	0	0	0	0	0	0	0	0	0	0
1-1B	1	0.4	1.3	0.3	0	1.6	0.6	0	0	2.0	2.2	0.38
1-3B	3	1.5	5.2	1.2	0.2	10.9	3.6	0	0	8.0	14.6	0.33
1-4B	5	1.2	4.3	1.0	0.2	10.0	3.5	0	0	6.7	13.5	0.35
1-5B	7	2.2	7.8	1.8	0.3	18.5	6.0	0	0	12.1	24.5	0.32
1-6B	11	2.2	7.8	1.8	0.3	16.9	5.7	0	0	12.1	22.6	0.34
1-7B	16	4.3	15.1	3.5	0.6	33.0	11.7	0	0	23.5	44.7	0.35
1-8B	21	2.8	9.9	2.3	0.4	22.2	7.5	0	0	15.4	29.7	0.34
1-9B	29	3.0	10.3	2.4	0.4	23.1	7.6	0	0	16.1	30.7	0.33
1-10B	49	1.5	5.2	1.2	0.2	11.3	4.3	0	0	8.0	15.7	0.38
1-11B	54	0.7	2.6	0.6	0.1	5.4	1.9	0	0	4.0	7.3	0.35
1-12B	62	0.9	3.0	0.7	0.1	7.3	2.5	0	0	4.7	9.8	0.34
1-13B	74	0.4	1.3	0.3	0	3.4	1.4	0	0	2.0	4.8	0.41
1-14B	79	0	0	0	0	1.5	0.9	0	0	0	2.4	0.60
1-15B	97	0	0	0	0	1.2	0.7	0	0	0	1.9	0.58
sheep 2 with sheep 2 D-genin data												
2-PB	0	0	0	0	0	0	0	0	0	0	0	0.00
2-1B	1	0	0	0	0	1.2	0.9	0	0	0	2.1	0.75
2-3B	3	2.3	8.2	1.9	0.3	16.9	5.0	0	0	12.7	21.9	0.30
2-4B	5	7.9	27.6	6.4	1.0	48.8	14.1	0	0	42.9	62.9	0.29
2-5B	7	8.2	28.9	6.7	1.1	60.0	17.9	0	0	44.9	77.9	0.30
2-6B	11	5.8	20.3	4.7	0.8	40.4	11.9	0	0	31.5	52.4	0.29
2-7B	16	2.3	8.2	1.9	0.3	27.3	6.7	0	0	12.7	34.0	0.25
2-9B	29	3.6	12.5	2.9	0.5	32.7	8.8	0	0	19.4	41.5	0.27
2-10B	49	2.0	6.9	1.6	0.3	17.8	6.4	0	0	10.7	24.2	0.36
2-11B	54	2.5	8.6	2.0	0.3	22.6	7.4	0	0	13.4	30.0	0.33
2-12B	62	2.1	7.3	1.7	0.3	15.2	6.1	0	0	11.4	21.3	0.40
2-13B	74	2.0	6.9	1.6	0.3	11.6	5.1	0	0	10.7	16.7	0.44
2-14B	79	1.2	4.3	1.0	0.2	7.7	3.1	0	0	6.7	10.8	0.40
2-15B	98	0.7	2.6	0.6	0.1	4.3	1.9	0	0	4.0	6.2	0.44

Surprisingly the combinations of results which yielded acceptable $R_{(Y)140/139}$ ratios (to within ±30%) were sheep 1 and 2 data (mixed dosing experiment) with sheep 3 data (deuterated genin dosing experiment).

Whilst this combination was fortuitous, it also shows that even for the same sheep, inter-experiment metabolism variations (possibly arising from variations in rumen microflora activities) must be considered when analysing data from a series of experiments.

A higher $R_{(Y)140/139}$ ratio (e.g. 0.30) overestimates the conjugated sapogenin contribution, and underestimates the free deuterated genin contribution.

The calculated free and conjugated genin contributions to bile episarsasapogenins recovered from sheep 1 and 2, using sheep 3 free deuterated genin ion ratio data, are depicted in Figure 3-18 A and B.

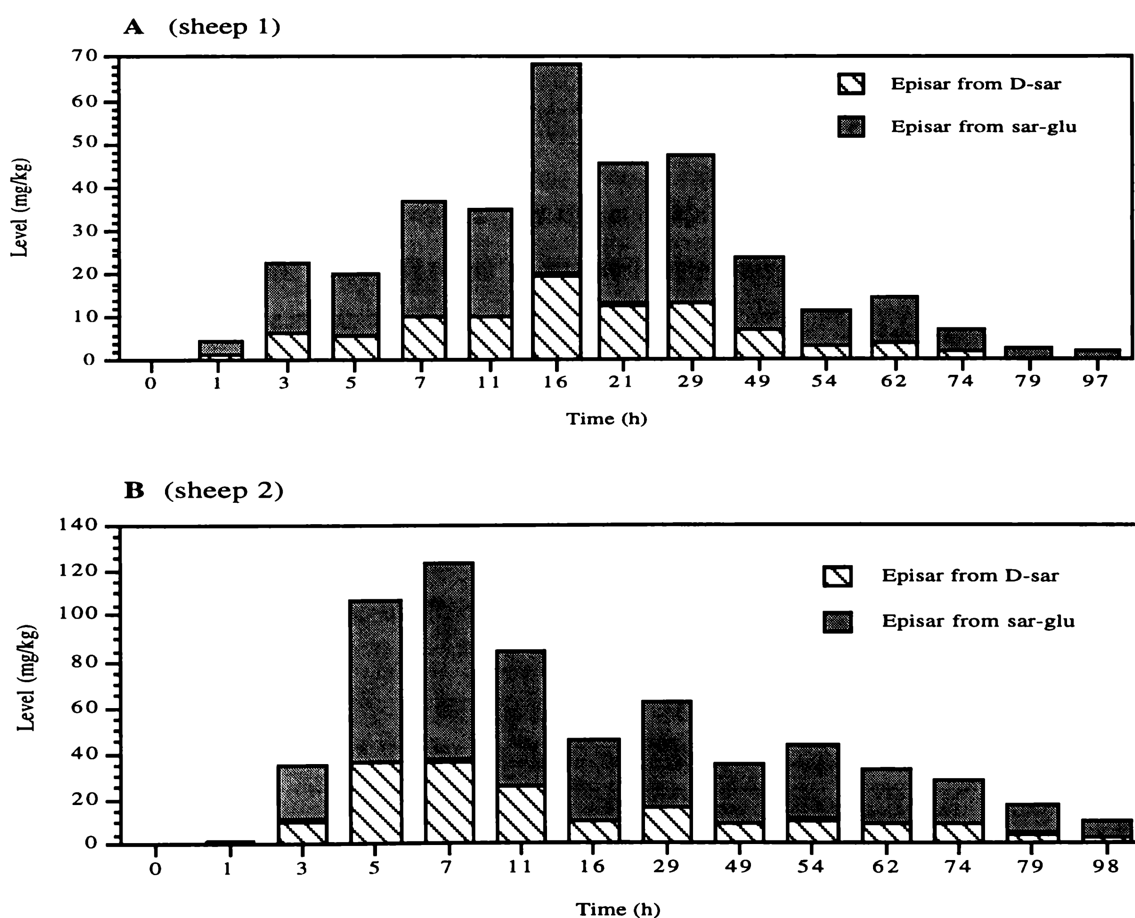


Figure 3-18. Contributions to episarsasapogenins from sarsasapogenin β-D-glucoside (sar-glu) and 20,23,23-D_3-sarsasapogenin (D-sar) in the bile samples from sheep 1 (**A**) and sheep 2 (**B**).

Normalised 20,23,23-D_3-sarsasapogenin (D-sar) and sarsasapogenin β-D-glucoside (sar-glu) contributions ($N_{D\text{-}sar}$ and $N_{sar\text{-}glu}$ respectively) to bile episapogenin conjugate samples, were calculated using conversion factors (CF) of 1.00 for 20,23,23-D_3-sarsasapogenin (D-sar) and 0.72 for sarsasapogenin β-D-glucoside (sar-glu), are presented in Table 3-21.

For example, the conjugated episapogenin content of bile sample 1-7B was calculated to be comprised of 37.2 mg/kg (Y_{sum}) of episarsasapogenin derived from sarsasapogenin β-D-glucoside and 30.7 mg/kg (X_{sum}) of episarsasapogenins from 20,23,23-D_3-sarsasapogenin (Tables 3-14 and 3-15), hence the calculated normalised incorporations ($N_{D\text{-}sar}$ and $N_{sar\text{-}glu}$) are

$$N_{sar\text{-}glu} = Y_{sum} / CF_{sar\text{-}glu} = 37.2 / 0.72 = 51.7$$
$$N_{D\text{-}sar} = X_{sum} / CF_{D\text{-}sar} = 30.7 / 1 = 30.7$$

therefore

$$N_{sar\text{-}glu} / N_{D\text{-}sar} = 51.7 / 30.7 = 1.7$$

These calculations reveal that bio-availability of sarsasapogenin β-D-glucoside, in the form that it was dosed to the sheep (i.e. as an ethanol-water suspension) to be ca 1.7 times greater than that of free sarsasapogenin.

It can be seen that the average bio-availability of sarsasapogenin β-D-glucoside is ca 1.6-1.7 times greater than that of free sarsasapogenin (Table 3-20). This observation, while derived from results obtained using an ethanol-water suspension, is consistent with the belief that for ingested plant material, conjugated sapogenins (saponins) are more readily metabolised (bio-available) than is the case for free sapogenins.

Table 3-21. Normalised contributions (Method 1) of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin to episarsasapogenin conjugates identified in bile samples from sheep 1 and 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)	X_{sum}	Y_{sum}	$N_{D\text{-}sar}$	$N_{sar\text{-}glu}$	$N_{sar\text{-}glu}/N_{D\text{-}sar}$
sheep 1						
1-PB	0	0	0	0	0	0
1-1B	1	2.6	1.6	2.6	2.2	0.9
1-3B	3	10.5	12.0	10.5	16.7	1.6
1-4B	5	8.8	11.3	8.8	15.7	1.8
1-5B	7	15.8	20.6	15.8	28.6	1.8
1-6B	11	15.8	18.7	15.8	26.0	1.6
1-7B	16	30.7	37.2	30.7	51.7	1.7
1-8B	21	20.2	24.7	20.2	34.3	1.7
1-9B	29	21.0	25.5	21.0	35.4	1.7
1-10B	49	10.5	13.1	10.5	18.2	1.7
1-11B	54	5.3	6.0	5.3	8.3	1.6
1-12B	62	6.1	8.3	6.1	11.5	1.9
1-13B	74	2.6	4.2	2.6	5.8	2.2
1-14B	79	0	2.4	0	3.3	0
1-15B	97	0	1.9	0	2.6	0
sheep 2						
2-PB	0	0	0	0	0	0
2-1B	1	0	2.1	0	2.9	0
2-3B	3	16.7	17.8	16.7	24.7	1.5
2-4B	5	56.1	49.1	56.1	68.2	1.2
2-5B	7	58.8	63.4	58.8	88.1	1.5
2-6B	11	41.2	42.2	41.2	58.6	1.4
2-7B	16	16.7	29.9	16.7	41.5	2.5
2-9B	29	25.4	35.3	25.4	49.0	1.9
2-10B	49	14.0	20.8	14.0	28.9	2.1
2-11B	54	17.5	25.7	17.5	35.7	2.0
2-12B	62	14.9	17.6	14.9	24.4	1.6
2-13B	74	14.0	13.3	14.0	18.5	1.3
2-14B	79	8.8	8.6	8.8	11.9	1.4
2-15B	98	5.3	4.9	5.3	6.8	1.3

Method 2

Close inspection of the % *m/z* 139-142 ion contributions reported in Tables 3-9 and 3-10 (Section 3.4) revealed that while the *m/z* 141 ion (D_2-sapogenin) levels varied significantly for sheep 2 and 3, the ratio of the *m/z* 139/140 contributions was essentially identical (0.30 and 0.28 respectively).

Normalised to the *m/z* 140 ion contributions, the ratios of *m/z* 140 to 139, 140, 141 and 142 were 0.30:1.0:0.30:0.06 and 0.28:1.0:0.17:0.01 for sheep 2 and 3 respectively. When this information (i.e. that $X_{139}/X_{140} = 0.29$) is combined with the requirement that $Y_{140}/Y_{139} = 0.14$ (for sarsasapogenin acetate, see preceding discussion) and the experimentally determined T_{139} and T_{140} values, four equations involving the X_{139}, X_{140}, Y_{139} and Y_{140} terms can be generated, and solved for unique values of these terms.

$$R_{(Y)140/139} = Y_{140} / Y_{139} = 0.14$$
$$R_{(X)139/140} = X_{139} / X_{140} = 0.29$$
$$T_{139} = X_{139} + Y_{139}$$
$$T_{140} = X_{140} + Y_{140}$$

therefore

$$Y_{139} = (T_{139} - R_{(X)139/140} * T_{140}) / (1 - R_{(Y)140/139} * R_{(X)139/140})$$
$$= (T_{139} - 0.29 * T_{140}) / 0.96$$

Substitution of the Y_{139} value into the preceding four equations allow the X_{139}, X_{140} and Y_{140} terms to be calculated.

For instance, bile sample 2-5B (from sheep 2 dosed with sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin) was found to contain 68.2, 46.8, 6.7 and 1.3 mg/kg of non-D, D-, D_2- and D_3-episarsasapogenin conjugates, respectively (Table 3-16) (i.e. T_{139}, T_{140}, T_{141} and T_{142} = 68.2, 46.8, 6.7 and 1.3 respectively).

The calculated levels of episarsasapogenins arising from deuterated sapogenins (X) and from non-deuterated saponins (Y) are: (mg/kg)

$$Y_{139} = (T_{139} - 0.29 * T_{140}) / 0.96 = (68.2 - 0.29 * 46.8) / 0.96 = 57.4$$
$$Y_{140} = 0.14 * Y_{139} = 0.14 * 57.4 = 8.0$$
$$X_{139} = T_{139} - Y_{139} = 68.2 - 57.4 = 10.8$$
$$X_{140} = X_{139} / 0.29 = 10.8 / 0.29 = 38.6$$

The contributions of sarsasapogenin β-D-glucoside (non-deuterated) and 20,23,23-D_3-sarsasapogenin (deuterated) to bile episarsasapogenins calculated by this procedure are given in Table 3-22.

Normalised 20,23,23-D_3-sarsasapogenin (D-sar) and sarsasapogenin β-D-glucoside (sar-glu) contributions ($N_{D\text{-}sar}$ and $N_{sar\text{-}glu}$ respectively) to bile episapogenin conjugate samples, according to the conversion factors (CF) of 1.00 for 20,23,23-D_3-sarsasapogenin (D-sar) and 0.72 for sarsasapogenin β-D-glucoside (sar-glu), are presented in Table 3-23.

For example, the conjugated episapogenin content of bile sample 2-5B was calculated to be comprised of 65.4 mg/kg (Y_{sum}) of episarsasapogenin derived from sarsasapogenin β-D-glucoside and 57.4 mg/kg (X_{sum}) of episarsasapogenins from 20,23,23-D_3-sarsasapogenin (see Tables 3-21), hence the calculated normalised incorporations are

$$N_{sar\text{-}glu} = Y_{sum} / CF_{sar\text{-}glu} = 65.4 / 0.72 = 90.9$$
$$N_{D\text{-}sar} = X_{sum} / CF_{D\text{-}sar} = 57.4 / 1 = 57.4$$

therefore

$$N_{sar\text{-}glu} / N_{D\text{-}sar} = 90.9 / 57.4 = 1.6$$

These calculations reveal that bio-availability of sarsasapogenin β-D-glucoside, in the form that it was dosed to the sheep, to be ca 1.6 times greater than that of free sarsasapogenin.

Table 3-22. Contributions (mg/kg) to bile episarsasapogenins from sarsasapogenin β-D-glucoside (Y) and 20,23,23-D_3-sarsasapogenin (X) in bile samples from sheep 1 and 2 determined using *m/z* 139/140 ion ratio data from Trial 4 (sheep 2 and 3) (Method 2).

No	Time (h)	X_{139}	X_{140}	X_{141}	X_{142}	Y_{139}	Y_{140}	Y_{141}	Y_{142}	X_{sum}	Y_{sum}
1-PB	0	0	0	0	0	0	0	0	0	0	0
1-1B	1	0.5	1.7	0.3	0	1.5	0.2	0	0	2.5	1.7
1-3B	3	2.1	7.3	1.2	0	10.4	1.4	0	0	10.6	11.8
1-4B	5	1.8	6.5	1.0	0	9.4	1.3	0	0	9.3	10.7
1-5B	7	3.2	11.3	1.8	0.4	17.5	2.5	0	0	16.7	20.0
1-6B	11	3.1	11.2	1.8	0.3	16.0	2.2	0	0	16.5	18.2
1-7B	16	6.3	22.4	3.5	0.7	31.0	4.3	0	0	32.8	35.4
1-8B	21	4.0	14.4	2.3	0.5	21.0	2.9	0	0	21.2	23.9
1-9B	29	4.1	14.8	2.4	0.5	22.0	3.1	0	0	21.8	25.0
1-10B	49	2.2	8.0	1.2	0.2	10.6	1.5	0	0	11.6	12.0
1-11B	54	1.1	3.8	0.6	tr	5.0	0.7	0	0	5.4	5.7
1-12B	62	1.3	4.5	0.7	tr	6.9	1.0	0	0	6.5	7.9
1-13B	74	0.6	2.2	0.3	tr	3.2	0.4	0	0	3.2	3.6
1-14B	79	0.2	0.7	tr	0	1.3	0.2	0	0	0.9	1.5
1-15B	97	0.2	0.6	0	0	1.0	0.1	0	0	0.7	1.2
2-PB	0	0	0	0	0	0	0	0	0	0	0
2-1B	1	0.2	0.8	0	0	1.0	0.1	0	0	1.0	1.1
2-3B	3	3.1	10.9	1.9	0.4	16.2	2.3	0	0	16.2	18.4
2-4B	5	9.8	35.0	6.4	1.5	46.9	6.6	0	0	52.7	53.5
2-5B	7	10.8	38.6	6.7	1.3	57.4	8.0	0	0	57.4	65.4
2-6B	11	7.5	26.7	4.7	0.7	38.7	5.4	0	0	39.5	44.2
2-7B	16	3.1	11.1	1.9	tr	26.5	3.7	0	0	16.1	30.2
2-9B	29	4.7	16.8	2.9	0.6	31.6	4.4	0	0	25.0	36.0
2-10B	49	3.1	10.9	1.6	tr	16.7	2.3	0	0	15.6	19.1
2-11B	54	3.6	12.9	2.0	tr	21.5	3.0	0	0	18.6	24.5
2-12B	62	3.2	11.4	1.7	tr	14.1	2.0	0	0	16.3	16.1
2-13B	74	2.9	10.5	1.6	0.3	10.7	1.5	0	0	15.3	12.2
2-14B	79	1.8	6.4	1.0	tr	7.1	1.0	0	0	9.2	8.1
2-15B	98	1.1	3.9	0.6	tr	3.9	0.5	0	0	5.6	4.4

Table 3-23. Normalised contributions (Method 2) of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin to episarsasapogenin conjugates identified in bile samples from sheep 1 and 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)	X_{sum} (free)	Y_{sum} (conj)	$N_{D\text{-}sar}$ (free)	$N_{sar\text{-}glu}$ (conj)	$N_{sar\text{-}glu}/N_{D\text{-}sar}$
sheep 1						
1-PB	0	0	0	0.0	0.0	0.0
1-1B	1	2.5	1.7	2.5	2.4	1.0
1-3B	3	10.6	11.8	10.6	16.4	1.6
1-4B	5	9.3	10.7	9.3	14.9	1.6
1-5B	7	16.7	20.0	16.7	27.8	1.7
1-6B	11	16.5	18.2	16.5	25.3	1.5
1-7B	16	32.8	35.4	32.8	49.1	1.5
1-8B	21	21.2	23.9	21.2	33.2	1.6
1-9B	29	21.8	25.0	21.8	34.8	1.6
1-10B	49	11.6	12.0	11.6	16.7	1.4
1-11B	54	5.4	5.7	5.4	8.0	1.5
1-12B	62	6.5	7.9	6.5	11.0	1.7
1-13B	74	3.2	3.6	3.2	5.0	1.6
1-14B	79	0.9	1.5	0.9	2.1	2.3
1-15B	97	0.7	1.2	0.7	1.7	2.3
sheep 2						
2-PB	0	0.0	0.0	0.0	0.0	0.0
2-1B	1	1.0	1.1	1.0	1.6	1.6
2-3B	3	16.2	18.4	16.2	25.6	1.6
2-4B	5	52.7	53.5	52.7	74.3	1.4
2-5B	7	57.4	65.4	57.4	90.9	1.6
2-6B	11	39.5	44.2	39.5	61.3	1.6
2-7B	16	16.1	30.2	16.1	41.9	2.6
2-9B	29	25.0	36.0	25.0	50.0	2.0
2-10B	49	15.6	19.1	15.6	26.5	1.7
2-11B	54	18.6	24.5	18.6	34.0	1.8
2-12B	62	16.3	16.1	16.3	22.3	1.4
2-13B	74	15.3	12.2	15.3	16.9	1.1
2-14B	79	9.2	8.1	9.2	11.3	1.2
2-15B	98	5.6	4.4	5.6	6.2	1.1

Table 3-24. Percentages (%) of free and conjugated sapogenins in rumen samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		sarsasapogenin 139	140	141	142	episarsasapogenin 139	140	141	142	3-CO-sarsasapogenin 139	140	141	142
2-1R	1	conj	60.6	30.4	9.0	0	0	0	0	0	0	0	0	0
		free	14.6	22.5	32.0	30.9	17.9	36.9	34.5	10.7	36.6	14.7	21.1	27.5
2-3R	4	conj	60.4	30.0	7.9	1.7	0	0	0	0	0	0	0	0
		free	15.9	26.2	32.2	25.8	23.8	40.3	29.6	6.3	34.1	28.3	23.5	14.1
2-5R	7	conj	65.4	26.9	5.9	1.8	64.6	29.3	6.1	tr	tr	tr	tr	0
		free	16.4	25.4	32.4	25.8	45.3	28.3	20.9	5.5	34.5	26.9	23.8	14.8
2-6R	12	conj	65.3	25.5	7.1	2.1	65.1	29.7	5.2	tr	tr	tr	tr	0
		free	16.3	21.2	33.1	29.4	50.4	20.1	18.0	11.5	39.1	21.1	22.3	17.5
2-8R	22	conj	67.8	26.6	4.4	1.3	63.6	32.2	4.2	tr	tr	tr	0	0
		free	19.1	24.5	29.7	26.8	54.8	20.8	8.6	15.8	42.8	22.4	17.3	17.5
2-12R	63	conj	49.2	36.7	14.1	tr	61.5	38.5	tr	tr	tr	tr	0	0
		free	20.0	19.0	30.1	30.8	44.1	19.4	21.8	14.8	39.7	17.4	20.7	22.2
2-14R	80	conj	54.7	45.3	tr	tr	62.3	37.7	tr	tr	tr	0	0	0
		free	23.4	19.1	28.3	29.2	37.5	20.1	22.2	20.2	31.8	21.8	24.5	21.9

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins.

Table 3-25. Percentages (%) of free and conjugated sapogenins in jejunum samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		sarsasapogenin				episarsasapogenin				3-CO-sarsasapogenin			
			139	140	141	142	139	140	141	142	139	140	141	142
2-3J	3	conj	57.8	30.9	11.3	0	51.0	40.2	8.8	0	0	0	0	0
		free	12.1	32.5	35.1	20.2	15.2	43.7	31.6	9.5	16.8	39.3	28.6	15.3
2-5J	8	conj	62.1	26.3	8.7	2.9	54.8	37.6	6.2	1.3	tr	tr	tr	0
		free	11.4	24.2	34.5	29.9	16.9	37.4	34.1	11.6	25.5	26.8	25.3	22.5
2-6J	12	conj	69.7	20.9	6.7	2.7	58.2	34.7	5.8	1.4	tr	tr	0	0
		free	11.7	28.3	34.9	25.1	17.4	38.2	33.2	11.2	17.1	31.8	32.1	19.0
2-9J	29	conj	40.9	47.5	11.5	0	59.0	35.3	4.4	1.3	0	0	0	0
		free	14.6	42.6	30.7	12.1	36.4	36.9	26.8	tr	0	0	0	0
2-10J	49	conj	46.8	53.2	tr	tr	54.0	40.8	5.3	tr	0	0	0	0
		free	22.6	40.1	25.8	11.6	44.5	34.5	21.0	tr	0	0	0	0
2-13J	74	conj	60.7	39.3	tr	tr	48.1	43.9	8.0	tr	0	0	0	0
		free	20.1	32.6	30.7	16.6	38.2	29.2	24.4	8.2	55.3	44.7	tr	0

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, 139 = non-D sapogenins, 140 = D-sapogenins, 141 = D_2-sapogenins, 142 = D_3-sapogenins.

Table 3-26. Percentages (%) of free and conjugated sapogenins in faeces samples from sheep 2, dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin.

No	Time (h)		sarsasapogenin				episarsasapogenin				3-CO-sarsasapogenin			
			139	140	141	142	139	140	141	142	139	140	141	142
2-3F	3	conj	0	0	0	0	0	0	0	0	0	0	0	0
		free	0	0	0	0	0	0	0	0	0	0	0	0
2-5F	9	conj	51.7	32.0	11.2	5.1	53.4	38.6	8.1	0	0	0	0	0
		free	11.6	30.3	35.5	22.7	41.5	27.0	20.3	11.2	19.0	34.6	36.2	10.2
2-9F	25	conj	54.7	31.4	9.7	4.2	56.7	33.0	7.6	2.8	0	0	0	0
		free	14.2	32.1	34.2	19.5	38.0	32.1	21.8	8.0	35.5	36.8	27.7	tr
2-14F	80	conj	54.3	45.7	tr	tr	54.2	36.8	9.0	0	0	0	0	0
		free	18.7	32.7	30.7	17.9	41.1	25.2	20.5	13.3	0	0	0	0

Time = interval time for collecting samples, conj = conjugated extract, free = free extract, tr = trace, 139 = non-D sapogenins, 140 = D-sapogenins,

141 = D_2-sapogenins, 142 = D_3-sapogenins.

A rumen sample collected 1 hour after dosing of a mixture of 2 g of sarsasapogenin β-D-glucoside and 2 g of 20,23,23-D_3-sarsasapogenin, was found to be comprised dominantly of deuterated free sarsasapogenins (32 and 30% of D_2- and D_2-sarsasapogenin respectively), and non-deuterated conjugated sarsasapogenins (61% of non-D sarsasapogenin).

Thereafter the percentage contribution of conjugated non-deuterated sarsasapogenin (*m/z* 139) rose slightly to 65-68% after 7-22 hours, and then fell to 49-55% after 63-80 hours. Over the same time period the percentage contribution of free D_2- and D_3-sarsasapogenin remained relatively consistent (may be with a slight downward trend) in the ranges 32-28% and 31-25%, respectively.

It can be inferred from these results that, in rumen, the conjugate and free sarsasapogenins behave as two separate pools, and that these pools largely retain their integrity. In particular, it was apparent (Figure 3-19) that labelling pattern of rumen episarsasapogenin conjugates corresponded to that of the dosed sarsasapogenin conjugate (rather than that of dosed 20,23,23-D_3-sarsasapogenin). This can be interpreted as indicating that once dosed, non-labelled sarsasapogenin β-D-glucoside is adsorbed onto an active site ('bound site'), hydrolysis to free sarsasapogenin, oxidation to sarsasapogenone, reduction to episarsasapogenin, and re-conjugation (pathway A, Figure 3-19) occur with only limited release of free episarsasapogenin (pathway B, Figure 3-19). Thus the labelling pattern of ruminal episarsasapogenin conjugates correspond to that of dosed sarsasapogenin β-D-glucoside.

On the other hand, free episarsasapogenin, at least after 7 hours, appears to be derived in part from oxidation and reduction of free 20,23,23-D_3-sarsasapogenin (pathway D, Figure 3-19) and in part from non-labelled free episarsasapogenin released from the bound route (pathway B, Figure 3-19). The comparatively high D- and D_2- level observed for free episarsasapogenin after 7 hours indicates that more free episarsasapogenin originates from pathway B than from pathway D, i.e. free episarsasapogenin is initially produced more quickly (faster) via pathway B than is the case for pathway D.

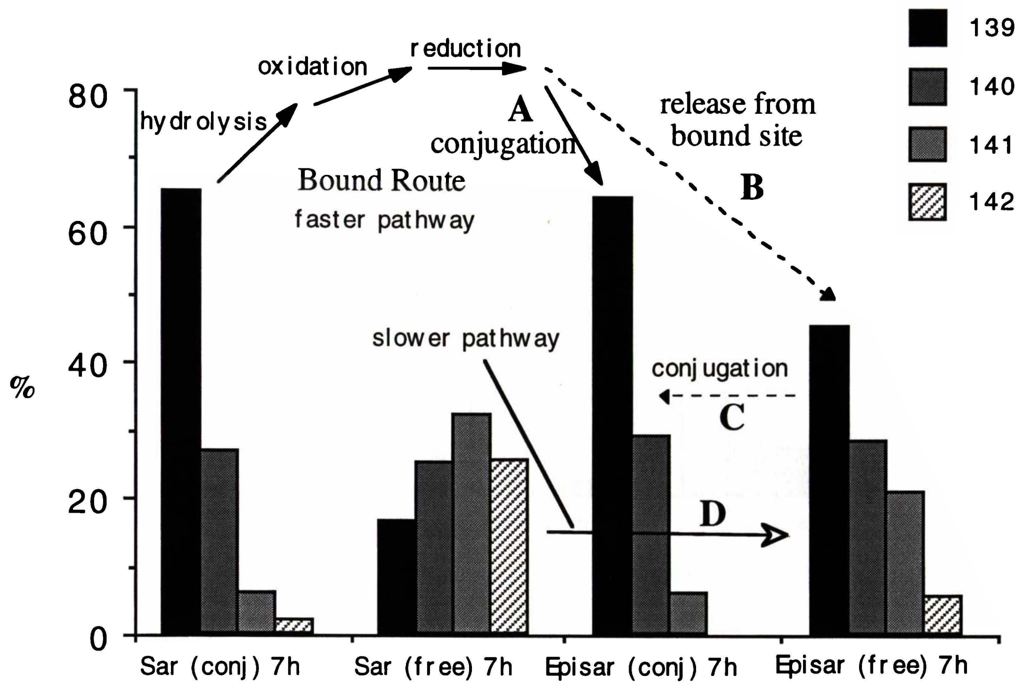


Figure 3-19. Diagrammatic representation of *m/z* 139-142 ion ratios, and proposed metabolic pathways, for free and conjugated sapogenins and saponin during ovine ruminal metabolism.

These observations support the earlier hypothesis, that conjugated sapogenins are more effectively "taken up" and metabolised in an ovine environment than is the case for free sapogenins.

After 7 hours the labelling pattern of bile episarsasapogenin conjugates corresponded with that observed for 7-63 hour rumen samples. There was also a close correspondence in the labelling patterns of the free and conjugated sarsasapogenin and episarsasapogenins found in the 49-74 hour jejunum and 9-80 hour faeces samples (Tables 3-25 and 3-26, Figure 3-20).

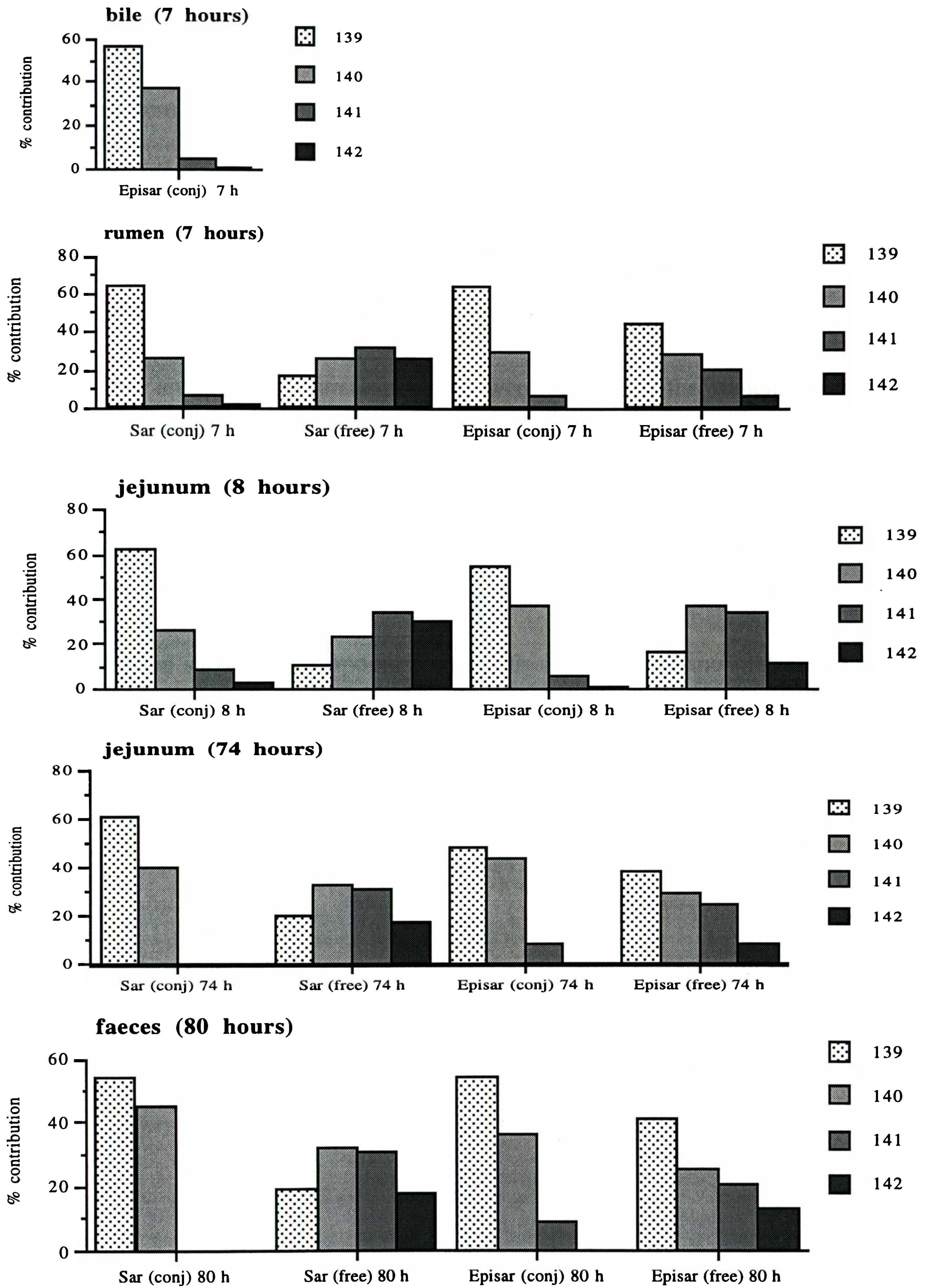


Figure 3-20. Non-D, D-, D_2- and D_3-sapogenin (free form) and saponin (conjugated form) contributions to selected rumen, jejunum, faeces and bile samples.

3.5.3 Summary and Conclusions

It is apparent from the results presented above that:

i) maximum levels of conjugated episarsasapogenin appeared in bile samples 12-24 hours after dosing commenced. Thereafter levels decreased rapidly, and then declined slowly. Low levels of episarsasapogenin conjugates were detectable in the bile after 4 days.

ii) elevated levels of free sarsasapogenins were present in the rumen, jejunum and faeces samples. Low levels of conjugated sarsasapogenins, free episarsasapogenins and conjugated episarsasapogenins were also identified in the samples.

iii) considerable loss of deuterium occurred when 20,23,23-D_3-sarsasapogenin was dosed.

iv) conjugated sapogenins (saponins), when dosed as an ethanol-water suspension, were more readily metabolised than free sapogenins. Typically conjugated sapogenins were 1.7 times more bio-available.

3.6 Trial 3 - Dosing with 3β-D-Epismilagenin β-D-Glucoside

3.6.1 Introduction

Miles et al. (1992b, 1993, 1994b) and Wilkins et al. (1994) have proposed that 3-keto-sapogenins are intermediates in the ovine metabolism of free and conjugated sapogenins to episapogenins. The detection of 3-keto-sarsasapogenin in rumen and other animal samples during previous dosing experiments (see Sections 3.3, 3.4 and 3.5) is consistent with the proposal of Miles et al. (1992b, 1993, 1994b) and Wilkins et al. (1994) that ruminal metabolism of sarsasapogenin to episarsasapogenin proceeds via sarsasapogenone.

An as yet undefined aspect of the conversion of sapogenins to episapogenins is the reversibility, or otherwise, of the oxidation and reduction processes involved in this conversion. This aspect of ovine metabolism was investigated by orally dosing the C-3 deuterium labelled saponin, 3β-D-epismilagenin β-D-glucoside (prepared as described in Section 5.2.2) to two sheep. Samples were collected from the bile, rumen, jejunum and faeces of the sheep, and analysed using selected ion mode (SIM) GC-MS protocols.

Specific objectives of the dosing experiment were to determine:

i) the reversibility or otherwise of C-3 oxidation and reduction processes;

ii) the time course of deuterium loss at C-3;

iii) the extent of C-3 deuterium retention in free and conjugated sapogenins;

iv) the stereoselectivity of the reduction process.

3.6.2 Results and Discussion

Quantitative analyses of the bile, rumen, jejunum and faeces samples from the sheep dosed with 3β-D-epismilagenin β-D-glucoside were performed by the method of Wilkins et al. (1994) using a SIM GC-MS *m/z* 139 profile for sapogenins and a *m/z* 368 profile for cholesterol. The peaks arising from sapogenin acetates and smilagenone were identified by comparison of the retention times of standard specimens of these compounds. The presence

of a C-3 deuterium atom was established using ion ratio data derived from SIM GC-MS *m/z* 284, 285, 344 and 345 ion profiles.

The mass spectra of sapogenin acetates, such as epismilagenin acetate, exhibit a base peak at *m/z* 139 and characteristic fragment ions at *m/z* 284 and *m/z* 344 (Figure 3-21). When the hydrogen at C-3 is replaced by deuterium, the foregoing sapogenin acetate *m/z* 284 and *m/z* 344 fragment ions are replaced by *m/z* 285 and *m/z* 345 fragment ions (Figure 3-21).

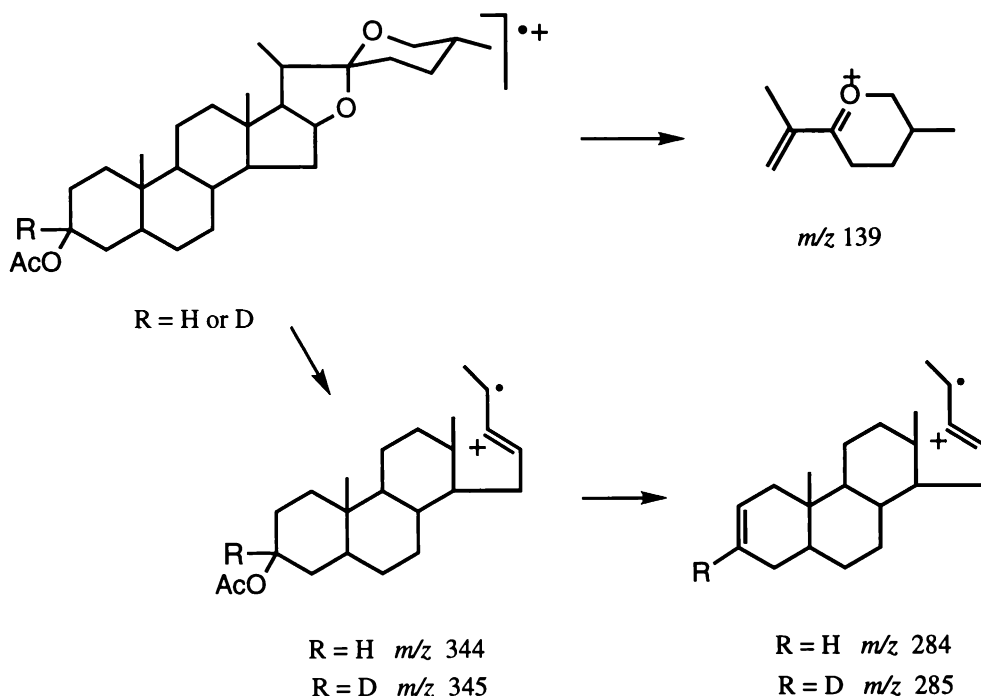


Figure 3-21. Proposed structures of *m/z* 139, 284, 285, 344 and 345 fragment ions appearing in the mass spectra of smilagenin acetate and its 3-deuterated analogue.

The extent of C-3 deuteration in metabolised sapogenins was established by the ratio of the integrated *m/z* 285 to 284 and *m/z* 345 to 344 ion responses. For example, a standard (non-deuterated) specimen of epismilagenin acetate exhibited *m/z* 285/284 and *m/z* 345/344 ion ratios of 0.27 and 0.31 respectively, while 3β-D-epismilagenin acetate exhibited *m/z* 285/284 and *m/z* 345/344 ion ratios of 29.7 and 31.1 respectively (Tables 3-28, 3-30 and 3-31).

3.6.2.1 Levels of Sapogenins in Rumen, Jejunum and Faeces Samples

The rumen, jejunum and faeces samples from sheep 4 were freeze-dried for 24 hours. The percentage dry matter content of the samples is given in Table 3-27. The levels of free and conjugated sapogenins and *m/z* 285/284 ion ratios in rumen, jejunum and faeces samples from sheep 4 are presented in Table 3-28.

Table 3-27. Wet weight, dried weight and % dry matter contents of rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

No	Time (h)	wet weight (g)	dried weight (g)	% dry matter
rumen				
4-1R	1	24.3	1.7	7.1
4-2R	2	24.6	1.5	6.1
4-3R	4	24.8	1.5	6.2
4-4R	5	21.9	1.5	7.0
4-5R	7	20.7	1.9	9.3
4-6R	12	23.2	2.3	10.1
4-7R	17	18.5	2.3	12.6
4-8R	22	17.2	1.8	10.7
4-10R	50	24.0	1.4	6.0
4-12R	63	19.6	2.4	12.1
4-15R	99	27.7	3.3	12.0
jejunum				
4-3J	3	24.5	0.6	2.5
4-5J	7	28.7	0.5	1.9
4-7J	16	27.0	0.8	2.9
4-8J	21	18.9	0.5	2.8
4-10J	49	24.3	0.7	2.9
4-15J	98	27.0	0.8	2.9
faeces				
4-3F	4	6.7	2.6	39.1
4-7F	16	1.7	0.7	38.2
4-9F	25	7.4	3.0	40.3
4-12F	59	6.9	3.2	46.1
4-15F	99	6.2	2.8	44.9

Table 3-28. Levels (mg/kg, dry matter) of smilagenins, epismilagenins and smilagenones and *m/z* 285/284 ion ratios of epismilagenins in rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

No	Time		Levels (mg/kg)			Ratio (epismil)
	(h)		smilagenin	epismilagenin	smilagenone	*m/z* 285/284
epismilagenin						0.27
3β-D-epismilagenin						29.7
rumen						
4-1R	1	conj	95.7	589	5.4	29.4
		free	545	4670	41.9	24.9
4-2R	2	conj	103	672	10.1	27.2
		free	584	5613	69.8	22.4
4-3R	4	conj	77.8	431	6.6	22.2
		free	604	5755	60.6	21.7
4-4R	5	conj	74.1	361	9.0	21.2
		free	376	4292	79.6	19.2
4-5R	7	conj	60.6	262	6.3	16.6
		free	430	4449	47.4	17.1
4-6R	12	conj	63.4	242	8.3	10.2
		free	372	4154	59.8	14.0
4-7R	17	conj	45.9	195	7.2	6.9
		free	369	3795	49.6	11.6
4-8R	22	conj	51.8	198	7.5	5.2
		free	292	3178	49.9	9.5
4-10R	50	conj	51.1	180	11.7	2.6
		free	214	2193	43.5	4.6
4-12R	63	conj	24.8	123	5.2	2.7
		free	60.2	728	20.9	3.9
4-15R	99	conj	34.3	161	10.0	1.4
		free	166	734	32.7	1.8
jejunum						
4-3J	3	conj	25.9	1017	11.9	8.6
		free	71.1	855	17.4	23.2
4-5J	7	conj	45.9	1002	22.4	7.6
		free	207	3144	22.9	19.6
4-7J	16	conj	NQ	795	12.8	4.5
		free	280	3179	32.5	13.3
4-8J	21	conj	34.1	433	19.4	2.9
		free	247	2813	27.3	11.9
4-10J	49	conj	11.9	306	6.4	2.6
		free	83.0	1582	12.2	6.3
4-15J	98	conj	NQ	323	3.5	1.2
		free	19.9	112	5.6	2.1

faeces						
4-3F	4	conj	0	0	0	0
		free	2.0	67.7	0	6.4
4-7F	16	conj	41.9	565	14.0	5.3
		free	179	2592	48.3	9.4
4-9F	25	conj	40.2	485	13.0	6.3
		free	84.8	1708	23.5	6.4
4-12F	59	conj	52.6	466	13.4	3.6
		free	NQ	1345	11.3	4.7
4-15F	99	conj	49.6	327	9.8	2.3
		free	19.6	556	6.2	3.6

NQ = not quantifiable

A rapid increase in free epismilagenin concentrations (to 4670-5555 mg/kg) occurred in rumen samples 2-4 hours after dosing. Thereafter levels declined steadily to 733 mg/kg at 99 hours. The levels of conjugated epismilagenins were significantly lower (with a maximum level of 671 mg/kg 2 hours after dosing commenced) than those of free epismilagenin. After 99 hours, this level fell to 120 mg/kg (Figure 3-22). Moderate levels of free and conjugated smilagenone (3-CO-smilagenin) and smilagenin were also detected in the rumen samples (Table 3-28).

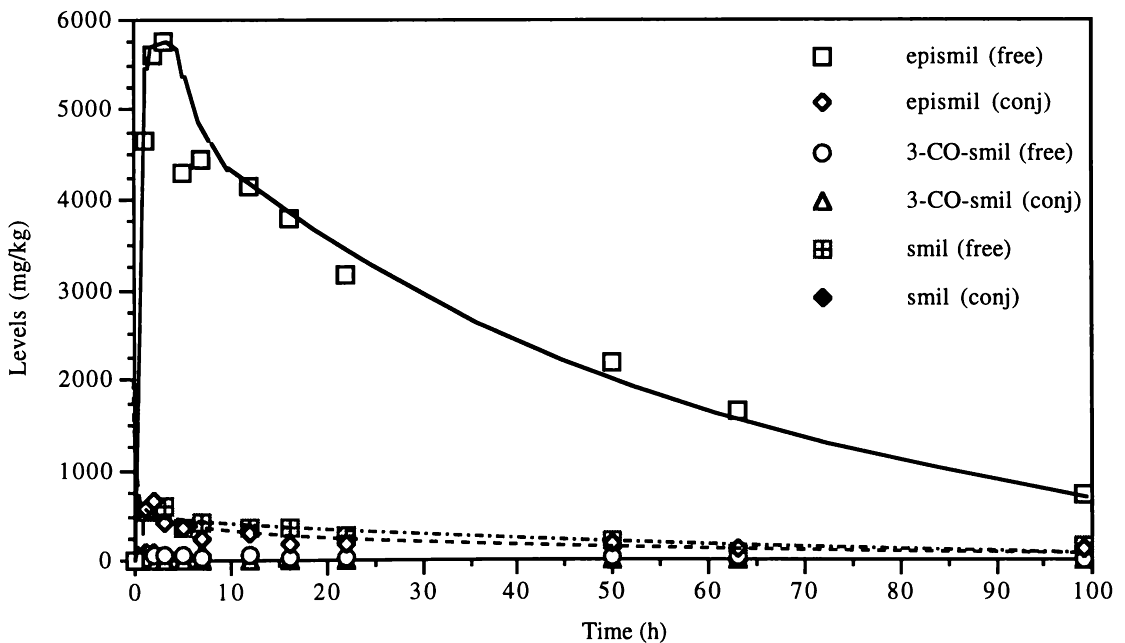


Figure 3-22. Levels of free and conjugated sapogenins in rumen samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

The variations in levels of free and conjugated epismilagenins, smilagenins and smilagenones (3-CO-smilagenins) in jejunum samples (Figure 3-23) paralleled those observed for rumen samples (Figure 3-22). Epismilagenin levels rose rapidly 7 hours after dosing, to 3200 mg/kg for free epismilagenin, and 1000 mg/kg for conjugated epismilagenins, after which levels fell progressively. Free and conjugated smilagenins and smilagenones were minor jejunum constituents.

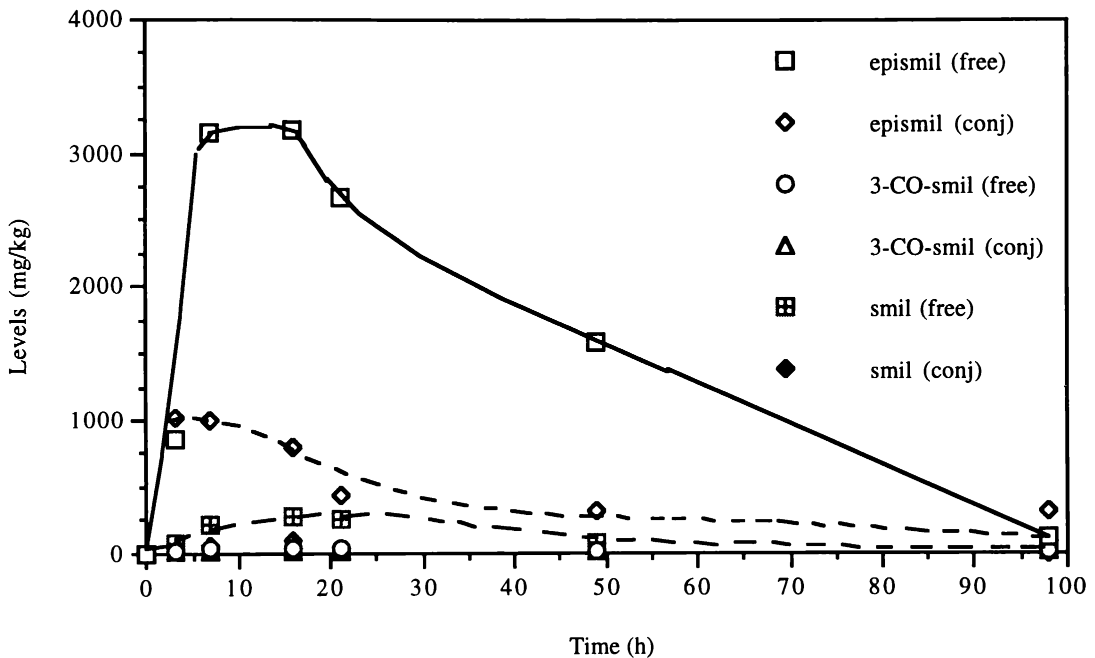


Figure 3-23. Levels of free and conjugated sapogenins in jejunum samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

Maximum levels of free and conjugated epismilagenins occurred in faeces samples 16 hours after dosing (Figure 3-24), slightly longer than was the case for jejunum samples (at ca 12 hours). Much lower levels of free and conjugated forms of smilagenones (3-CO-smilagenins) and smilagenins were also detected in the faeces samples (Table 3-28).

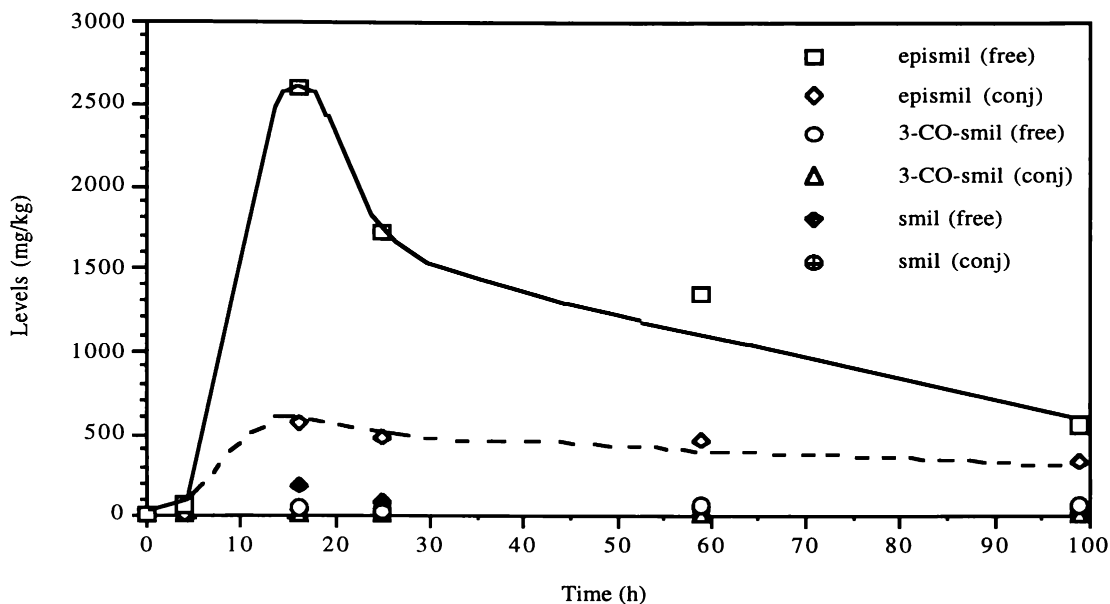


Figure 3-24. Levels of free and conjugated sapogenins in faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

3.6.2.2 Stereoselectivity of Reduction at C-3

The results, presented in Tables 3-28 and 3-29, indicate that 3β-D-epismilagenin β-D-glucoside is rapidly hydrolysed to free 3β-D-epismilagenin, reversibly oxidised to smilagenone and reduced to afford smilagenin and epismilagenin (as evidenced by the progressively declining *m/z* 285/284 ion ratios) (Table 3-28), which can be reconjugated to form conjugated smilagenins and epismilagenins (Figure 2-25).

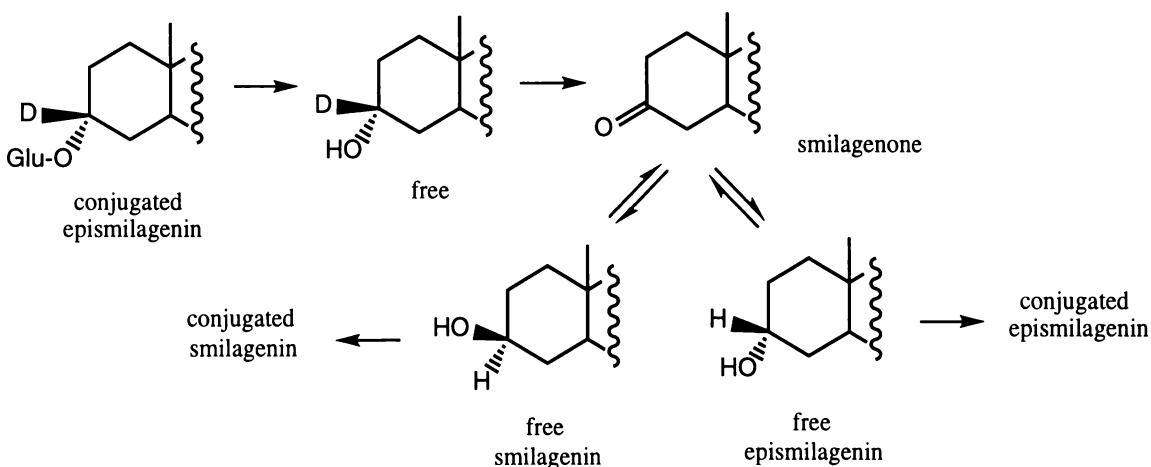


Figure 3-25. Conversion of conjugated 3β-D-epismilagenin to epismilagenin and smilagenin and their conjugates.

Table 3-29. Percentage contributions (%) of free and conjugated smilagenins and epismilagenins identified in rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

No	Time (h)		Ratio (%) smilagenin	epismilagenin
epismilagenin			7	93
3β-D-epismilagenin			4	96
rumen				
4-1R	1	conj	14	86
		free	10	90
4-2R	2	conj	13	87
		free	9	91
4-3R	4	conj	15	85
		free	10	90
4-4R	5	conj	17	83
		free	8	92
4-5R	7	conj	19	81
		free	9	91
4-6R	12	conj	21	79
		free	8	92
4-7R	17	conj	19	81
		free	9	91
4-8R	22	conj	21	79
		free	8	92
4-10R	50	conj	22	78
		free	9	91
4-12R	63	conj	17	83
		free	4	96
4-15R	99	conj	21	79
		free	18	82
jejunum				
4-3J	3	conj	2	98

		free	8	92
4-5J	7	conj	4	96
		free	6	94
4-7J	16	conj	NQ	NQ
		free	8	92
4-8J	21	conj	7	93
		free	6	94
4-10J	49	conj	4	96
		free	5	95
4-15J	98	conj	NQ	NQ
		free	15	85
faeces				
4-3F	4	conj	0	0
		free	3	97
4-7F	16	conj	7	93
		free	6	94
4-9F	25	conj	8	92
		free	5	95
4-12F	59	conj	10	90
		free	NQ	NQ
4-15F	99	conj	13	87
		free	3	97

NQ = not quantifiable

The data presented in Table 3-29 shows that the reversible oxidation of epismilagenin to smilagenone, followed by reduction to smilagenin and epismilagenin proceeds to afford predominantly episapogenin. This dominance was apparent for all of the rumen (ca 80% for conjugates and 90% for free), jejunum (92-99%) and faeces (93-94%) samples (Figure 3-26). The lesser contribution observed for rumen samples may be a consequence of the presence of ca 4% of smilagenin in the dosed material.

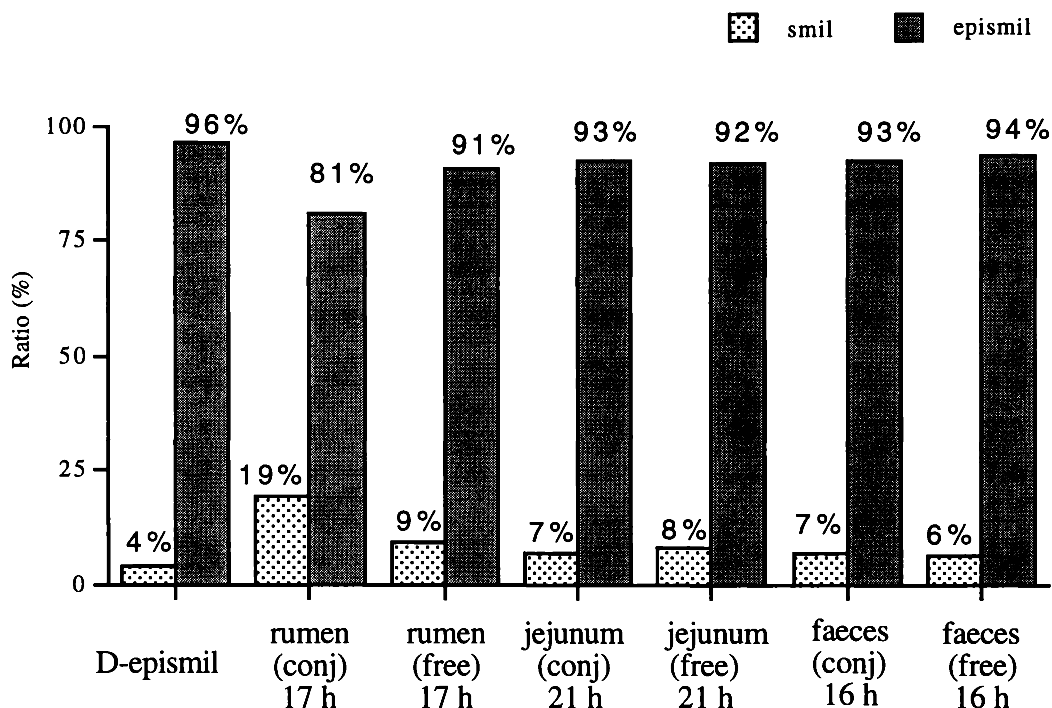


Figure 3-26. Percentage contributions (%) of free and conjugated smilagenins and epismilagenins in rumen, jejunum and faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

3.6.2.3 Levels of Conjugated Epismilagenins in Bile Samples

The levels of conjugated epismilagenins and *m/z* 285/284 and *m/z* 345/344 ion ratios of the epismilagenins in bile samples from sheep 3 and 4 dosed with 3β-D-epismilagenin β-D-glucoside are presented in Tables 3-30 and 3-31. No free sapogenins were detected in the bile samples.

Table 3-30. Levels (mg/kg) of free and conjugated epismilagenins and *m/z* 285/284 and *m/z* 345/344 ion ratios of conjugated epismilagenin in bile samples from sheep 3, dosed with 3β-D-epismilagenin β-D-glucoside.

No	Time (h)		Level epismilagenin	Ratio *m/z* 285/284	*m/z* 345/344
epismilagenin				0.27	0.31
3-D-epismilagenin				29.7	31.1
3-PB	0	conj	0	0	0
3-1B	1	conj	13.1	14.1	0
3-3B	3	conj	38.3	11.3	12.8
3-4B	5	conj	96.4	11.1	12.3
3-5B	7	conj	80.1	7.5	7.6
3-6B	11	conj	65.8	5.5	5.5
3-7B	16	conj	59.6	4.8	4.6
3-8B	21	conj	43.5	3.3	3.2
3-10B	49	conj	9.0	1.7	1.5
3-11B	54	conj	11.3	1.4	1.2
3-12B	62	conj	5.3	1.2	1.1
3-13B	74	conj	5.3	1.8	1.2
3-14B	79	conj	3.8	1.9	0
3-15B	97	conj	1.3	1.4	0

Table 3-31. Levels (mg/kg) of free and conjugated epismilagenins and *m/z* 285/284 and *m/z* 345/344 ion ratios of conjugated epismilagenin in bile samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

No	Time (h)		Level epismilagenin	Ratio (*m/z*) 285/284	(*m/z*) 345/344
epismilagenin				0.27	0.31
3-D-epismilagenin				29.7	31.1
4-PB	0	conj	0	0	0
4-1B	1	conj	10.2	10.9	0
4-3B	3	conj	42.0	9.3	10.0
4-4B	6	conj	87.8	6.2	6.4
4-5B	8	conj	58.6	4.6	4.9
4-6B	12	conj	61.2	3.6	3.6
4-7B	16	conj	51.4	2.9	2.8
4-9B	29	conj	27.3	2.3	1.9
4-10B	49	conj	29.4	1.8	1.7
4-11B	54	conj	31.5	1.7	1.7
4-12B	62	conj	22.3	1.8	1.7
4-13B	74	conj	14.8	1.7	1.5
4-14B	79	conj	17.9	1.5	1.4
4-15B	98	conj	10.8	1.2	1.1

Conjugated epismilagenins appeared in the bile samples 1 hour after dosing commenced (Figure 3-27). Levels of conjugated epismilagenins in the bile samples increased quickly, reaching their peak levels of 96 mg/kg (sheep 3) and 87 mg/kg (sheep 4) after 6 hours. Thereafter levels decreased quickly until 11-12 hour after dosing, and then continued to decrease more slowly. After 50 and 97 hours the levels of conjugated epismilagenins were below 10 and 1.3 mg/kg respectively for sheep 3, and 30 and 11 mg/kg respectively for sheep 4.

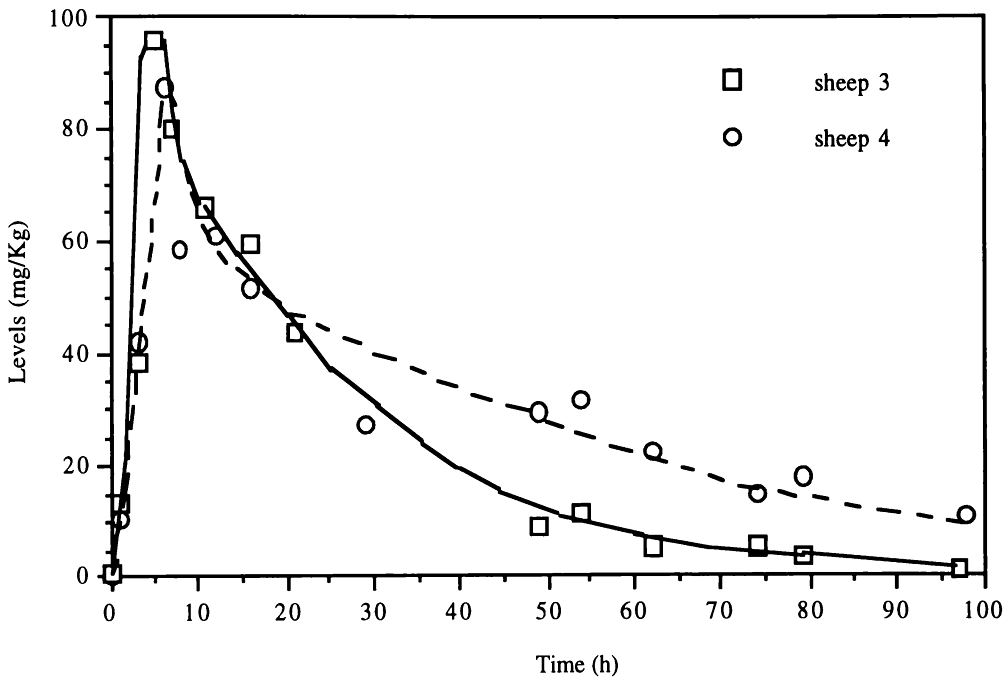


Figure 3-27. Levels of conjugated epismilagenins in bile samples from sheep 3 and 4, dosed with 3β-D-epismilagenin β-D-glucoside.

It is of note that the *m/z* 285/284 ion ratios of epismilagenins in the bile samples consistently decreased during the dosing period (Figure 3-28). This is consistent with the continuing loss of the C-3 deuterium by reversible oxidation/reduction during the ovine metabolism.

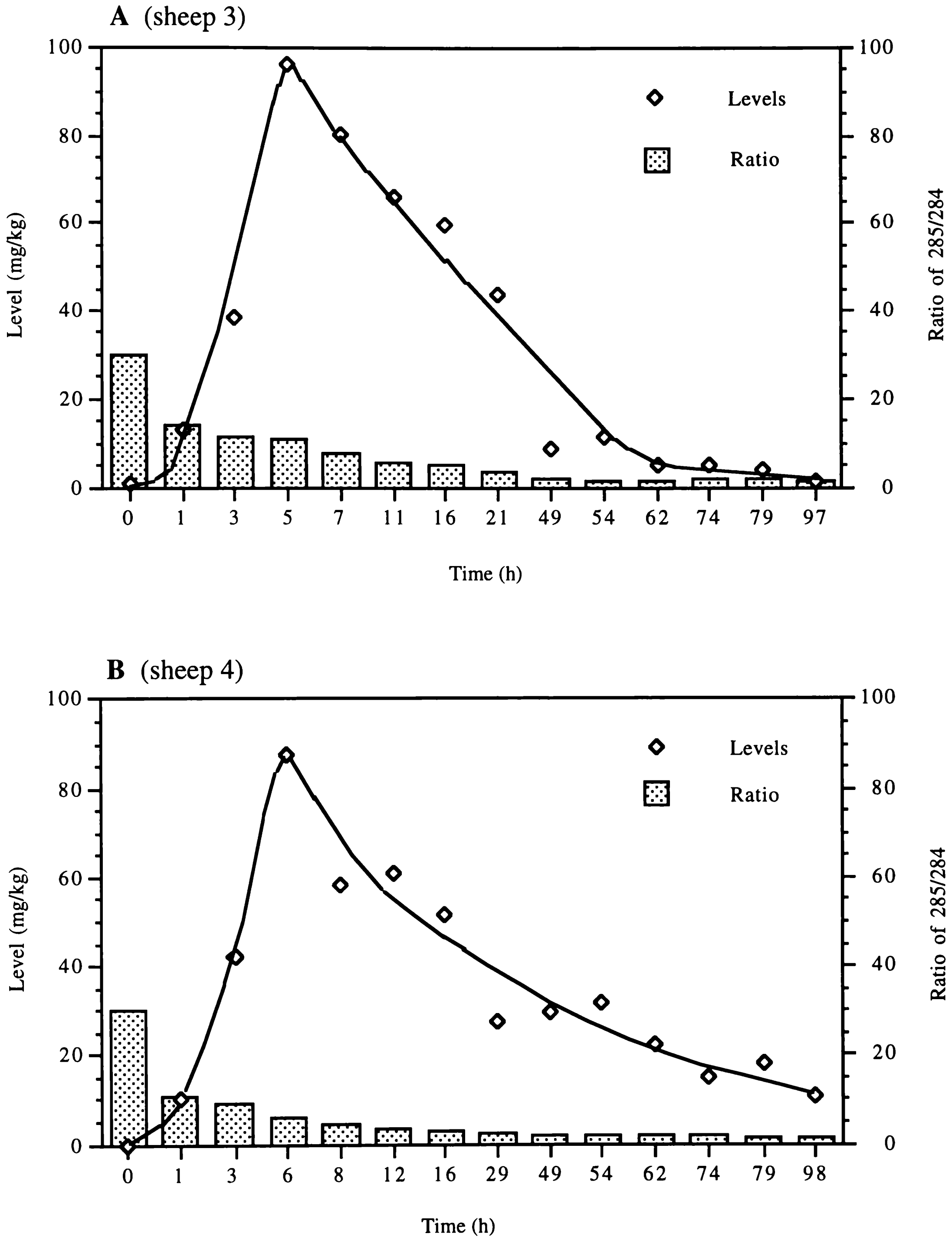


Figure 3-28. Levels and *m/z* 285/284 ion ratios of epismilagenin conjugates identified in bile samples from sheep 3 (A) and sheep 4 (B), dosed with 3β-D-epismilagenin β-D-glucoside.

3.6.2.4 Bile Cholesterol Levels

The sum of free and conjugated cholesterols in bile samples increased after dosing commenced. Free cholesterol levels did not vary obviously (Table 3-32), however the levels of conjugated cholesterols increased significantly after dosing commenced and reached their peaks after 7 hours for sheep 3 (rising from 0 to 22 mg/kg) and after 12 hours for sheep 4 (rising from 51 to 130 mg/kg) (Figure 3-29). Conjugated epismilagenin levels increased and reached the peak value at the same time.

The increase in conjugated cholesterol levels appears to correspond with the rise in conjugated epismilagenin levels in the bile of sheep 4. This is suggestive of a relationship between cholesterol and episapogenin levels, especially for the conjugated forms of these compounds.

Table 3-32. Levels (mg/kg) of free and conjugated cholesterols in bile samples from sheep 3 and 4, dosed with 3β-D-epismilagenin β-D-glucoside.

No	Time (h)	cholesterol conjugated	free	sum
sheep 3				
3-PB	0	0.3	5.0	5.3
3-1B	1	1.8	13.1	14.9
3-3B	3	14.7	17.7	32.4
3-4B	5	14.0	11.7	25.7
3-5B	7	22.0	20.6	42.6
3-6B	11	16.7	15.8	32.5
3-7B	16	3.9	21.7	25.6
3-8B	21	8.5	17.9	26.4
3-10B	49	0.8	32.3	33.1
3-11B	54	0.6	18.5	19.1
3-12B	62	0.4	20.5	20.9
3-13B	74	5.5	35.4	40.9
3-14B	79	1.6	30.5	32.1
3-15B	97	1.7	45.0	46.7
sheep 4				
4-PB	0	50.5	26.8	77.3
4-1B	1	60.8	29.7	90.5
4-3B	3	72.0	10.8	82.8
4-4B	6	86.0	37.9	124

4-5B	8	118	15.2	133
4-6B	12	130	11.4	141
4-7B	16	82.7	75.5	158
4-9B	29	106	26.3	132
4-10B	49	108	13.4	121
4-12B	62	119	7.1	127
4-13B	74	83.4	8.8	92.2
4-14B	79	76.1	22.7	98.8
4-15B	98	79.1	15.1	94.2

sum = conjugated cholesterol + free cholesterol

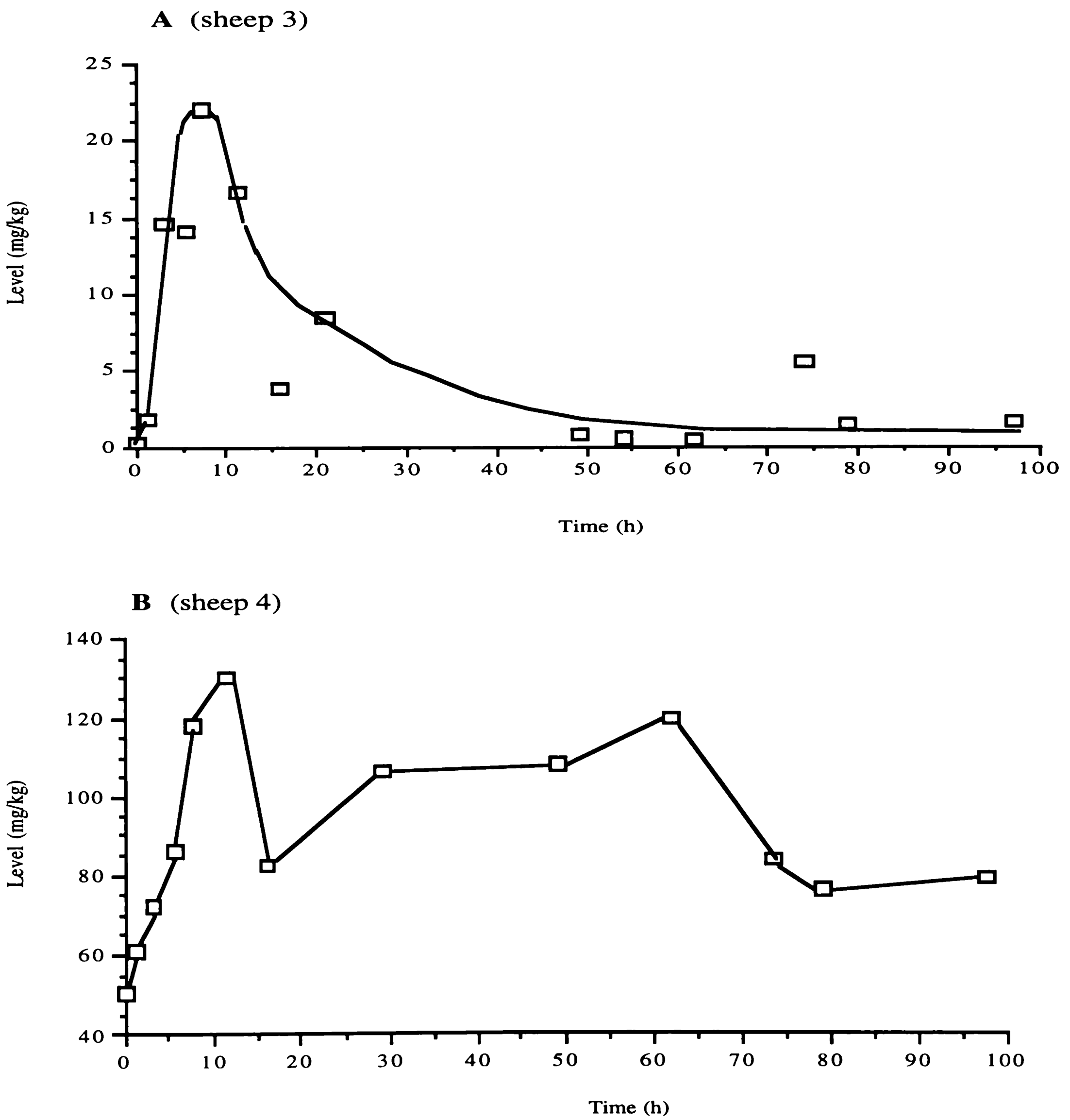


Figure 3-29. Levels of conjugated cholesterols in the bile samples from sheep 3 (**A**) and sheep 4 (**B**), dosed with 3β-D-epismilagenin β-D-glucoside.

3.6.2.5 Ion Ratio Analyses

A comparison of the *m/z* 285/284 ion ratios determined for free and conjugated episapogenins in rumen samples (see Table 3-25 and Figure 3-30) shows the C-3 deuterium atom is lost from the conjugated epismilagenin faster than is the case for free epismilagenin. For example, the *m/z* 285/284 ion ratio decreased from 30 to 10 after 12 hours for conjugated epismilagenin, whereas 21 hours was required for the equivalent change in ratio for free epismilagenin.

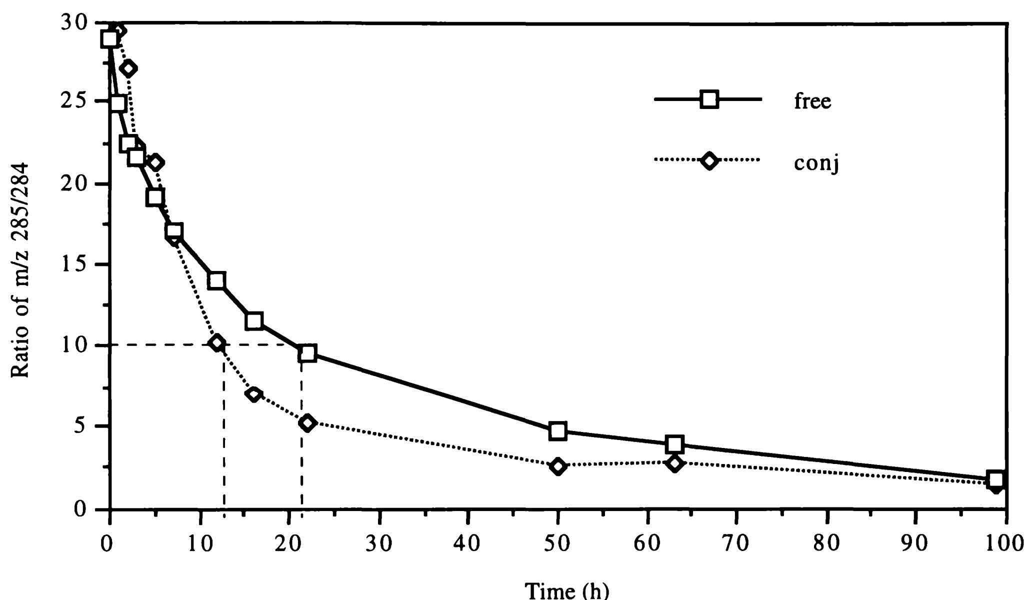


Figure 3-30. Ratios of *m/z* 285/284 ions in the free and conjugated epismilagenins in rumen samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

This observation indicates that free and conjugated epismilagenins behave as two discrete pools of substrates (Figure 3-31). In the case of the conjugate pool, the faster loss of label can be interpreted as indicating that after initial absorption onto an active enzyme site, hydrolysis, oxidation, reduction (predominantly to epismilagenin rather than smilagenin) and re-conjugation, proceeds faster than is the case for the oxidation and reduction of free epismilagenin - i.e. free epismilagenin is less bio-available (at least with respect to oxidation/reduction) than is the case for conjugated epismilagenin.

This finding can be compared with observation that in the dosing experiment in which free and conjugated sapogenins were dosed, the conjugated substrate was bio-utilised ca 1.7 faster than was the case for the free substrate (see Section 3.5).

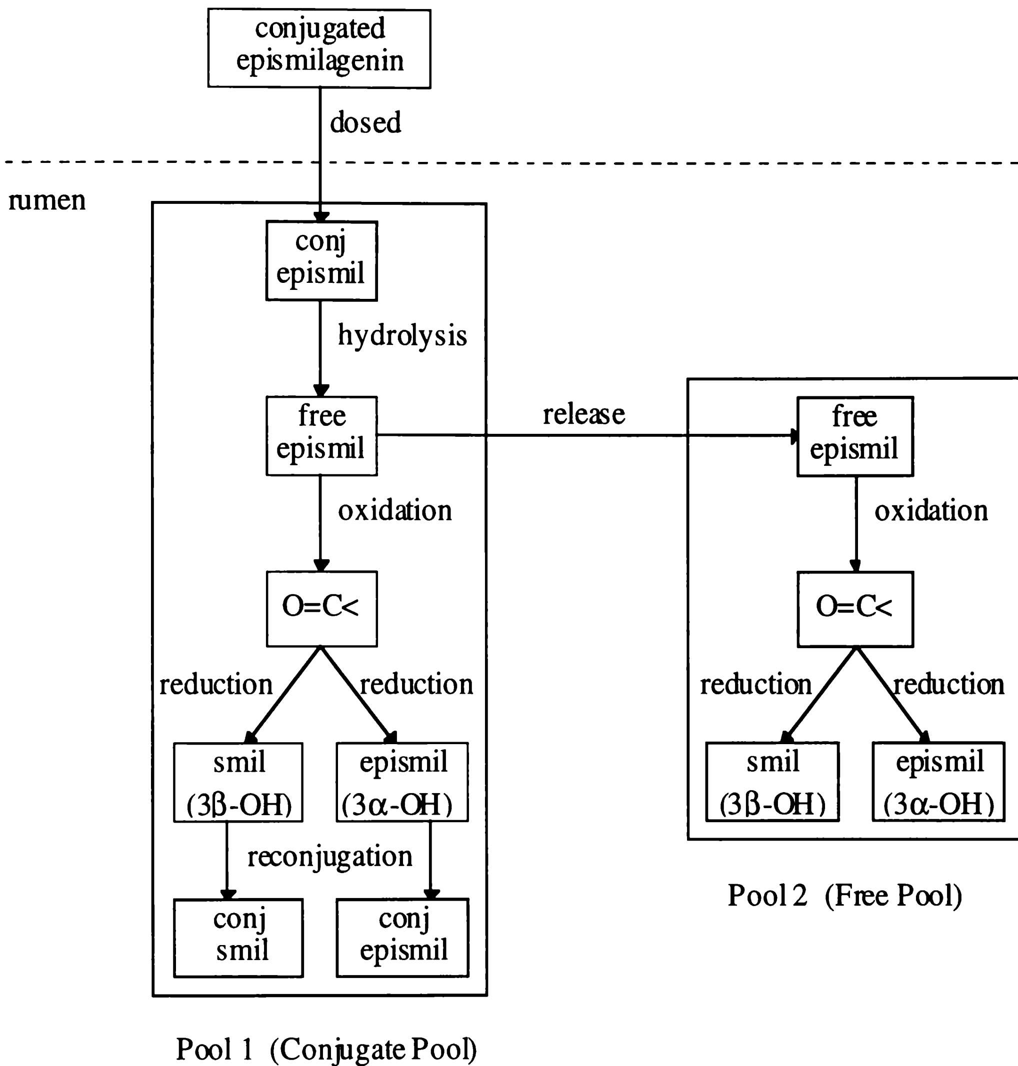


Figure 3-31. Proposed metabolic pools (Conjugated Pool and Free Pool) in the rumen of sheep.

Variations were also observed in the *m/z* 285/284 ion ratios of free and conjugated epismilagenins in the jejunum and faeces samples (Figures 3-32 and 3-33). The *m/z* 285/284 ion ratios of conjugated epismilagenins decreased more quickly than those of free epismilagenins - that is deuterium loss at C-3 occurs more quickly for conjugated epismilagenins than is the case for free epismilagenin.

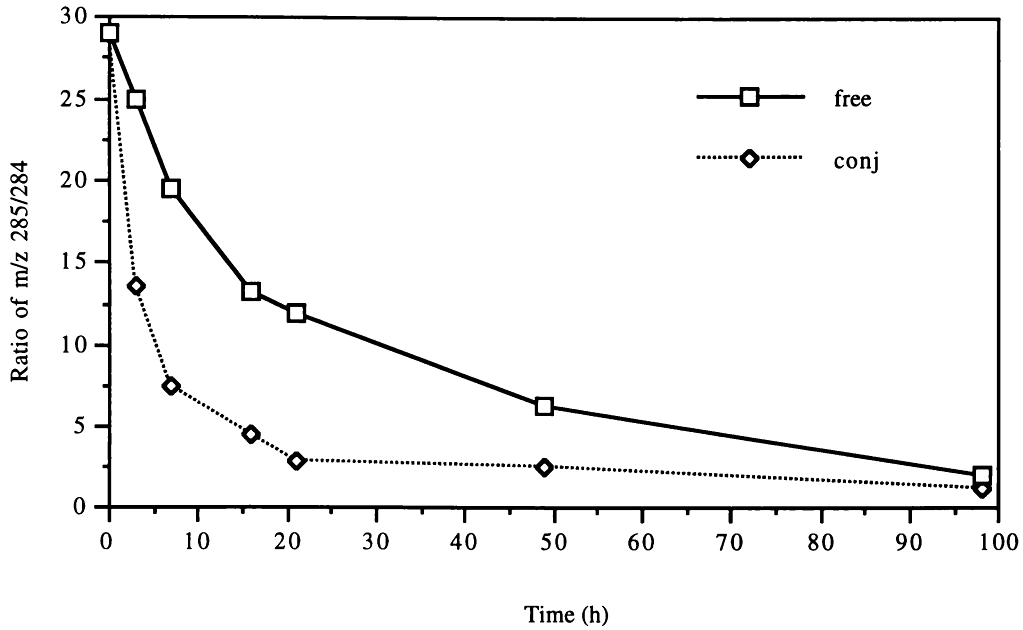


Figure 3-32. Time variation of *m/z* 285/284 ion ratios for free and conjugated epismilagenins in jejunum samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

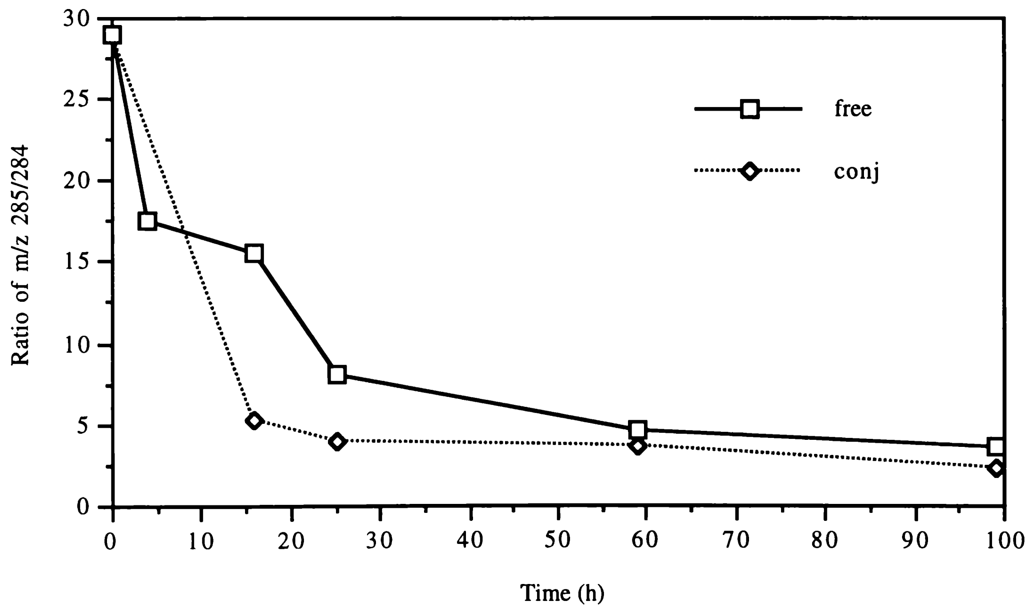


Figure 3-33. Time variation of *m/z* 285/284 ion ratios for free and conjugated epismilagenins in faeces samples from sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

M/z 285/284 ion ratio results for free and conjugated epismilagenins in the rumen, jejunum and bile samples are combined in Figure 3-34. This figure shows that there are marked differences in the rates at which the 3-deuterium is lost from free and conjugated substrates in the different metabolic regions (the rumen, jejunum and bile).

The slowest rates of deuterium loss (and therefore oxidation at C-3) were displayed by free epismilagenin in rumen and jejunum samples, whereas the fastest rates of deuterium loss were shown by conjugated epismilagenins in bile and jejunum samples.

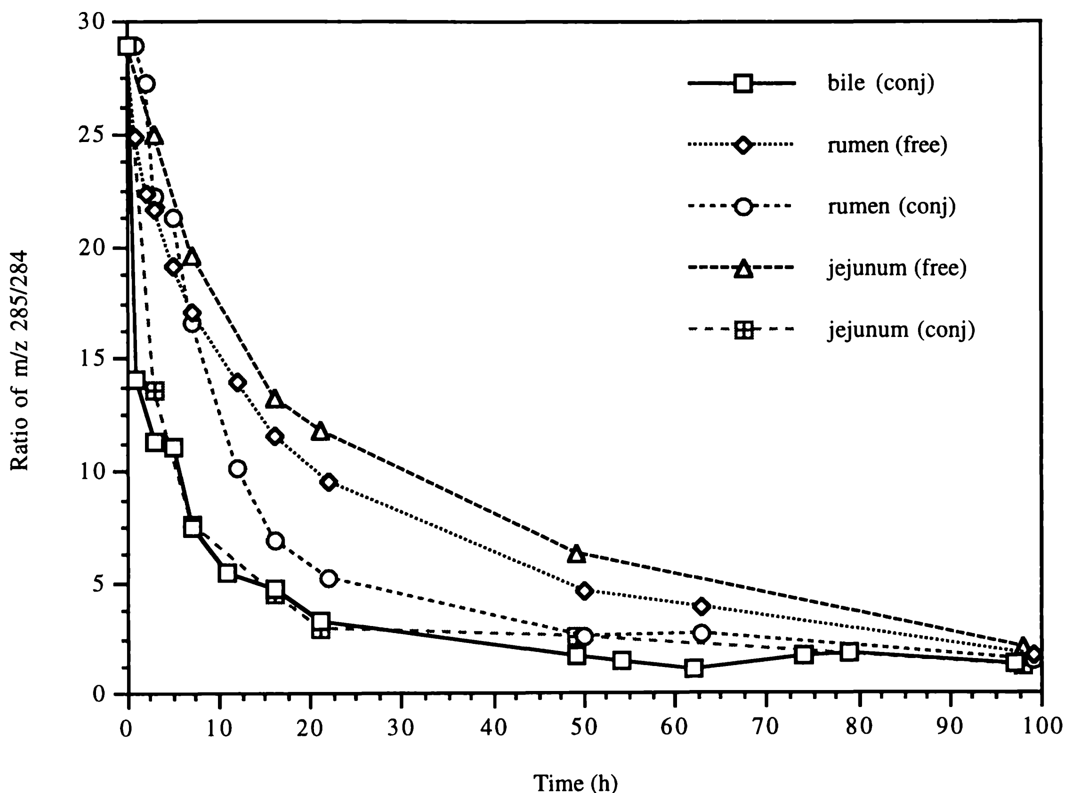


Figure 3-34. Ratios of *m/z* 285/284 ions in free and conjugated epismilagenins identified in extracts from bile, rumen and jejunum samples of sheep 4, dosed with 3β-D-epismilagenin β-D-glucoside.

Rate analyses

The time required for the *m/z* 285/284 ion ratios of bile, jejunum and rumen epismilagenin conjugates to decrease from 30 to 10 is ca 5, 5 and 12 hours respectively. These differences are consistent with the proposal of Flåøyen and Wilkins (1997) that the rumen and jejunum

are distinct metabolic regions, each of which is characterised by different metabolic activities.

Curve fitting of the *m/z* 285/284 ion ratio profiles determined for bile, rumen and jejunum samples from sheep 4 recovered 0-96 hours after the commencement of dosing showed good fits for exponential and logarithmic decay functions (r^2 values in the range 0.93-0.99).

The rate equation for a first order process takes the form $F_t = F_0 e^{-kt}$ where k = the rate constant for the process and F_t and F_0 are the values of function F at time = t and time = 0 respectively (Barrow, 1961).

This equation can be applied to the deuterium exchange process in the form $y = a * e^{-kx}$ where y = *m/z* 285/284 ion ratio, x = time, and k = rate constant for deuterium loss.

	$y = a * e^{-kx}$	
hence	$\ln y = \ln a - kx$	
or	$\log y = \log a - kx / 2.303$	(conversion to $\log_{10}$)
i.e.	$y = a * 10^{-kx/2.303}$	
or	$y = a * 10^{-bx}$	where $b = k / 2.303$

Therefore, provided deuterium loss is a first order process (i.e. a process dependent only on the concentration of the deuterated substrate), plots of the *m/z* 285/284 ion ratio profiles determined for the rumen, jejunum and bile samples on semi-log scale (log y against time x) should afford straight line profiles of slope b.

As shown in Figure 3-35, when plotted on semi-log scale (log ratio vs time) for 0-96 hours period, good linearities were observed for the period 1-24 hours after dosing, while significant deviations from the initial linearity were observed for the 48-96 hour results (as the level of deuterium approached its limiting value). Care must be exercised in interpreting the initial 1 hour results, especially for regions other than the rumen, since at time zero the maximum level of epismilagenin is present in the rumen (delivered as the dosed substrate), whereas generally only lower levels of recently transported free and conjugated genins are present in jejunum and bile samples. It is doubtful that for shorter times (1-3 hours after dosing) material recently transported from the rumen to the jejunum, and thence to the liver (bile material) will have been present in the jejunum or liver for a period of time sufficient for it to exhibit the deuteration loss profile of a particular metabolic region, as opposed to one it has recently arrived from.

Notwithstanding these and other reservations in respect of the possible significance of the results, a first order rate analyses was applied to the period 1-24 hours after dosing, assuming the initial loss of deuterium arose from a single process, rather than two competing first order process (e.g. a fast initial process and a subsequent slower process).

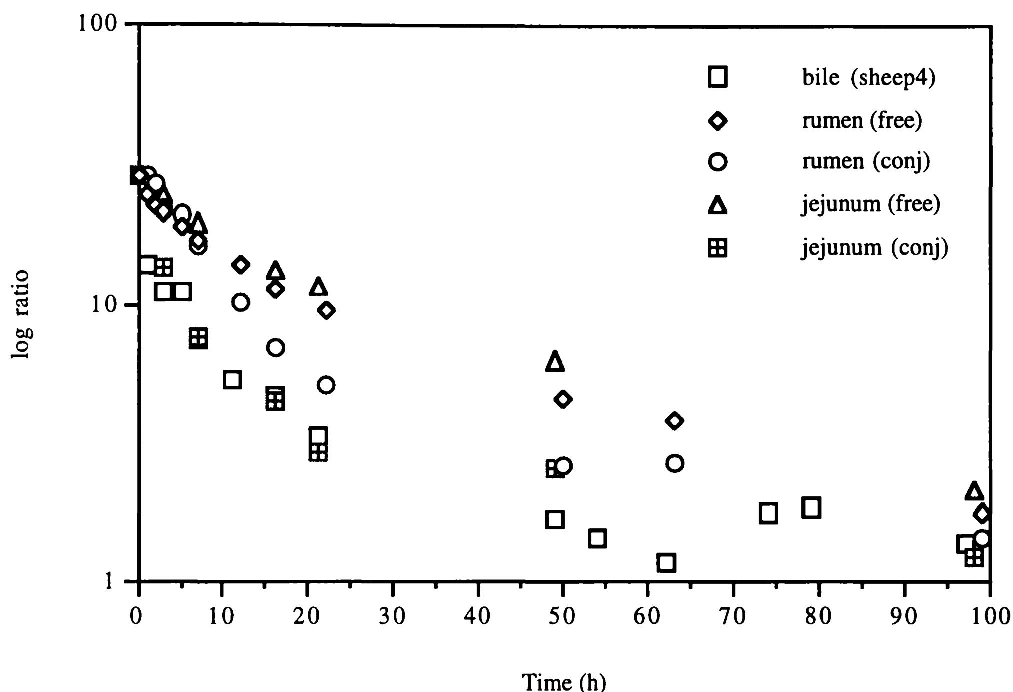


Figure 3-35. Plots of log *m/z* 285/284 ion ratio versus time (0-96 hours) determined for rumen, jejunum and bile samples.

The regression equations, obtained when exponential decay curves of the form $y = a * 10^{-bx}$ were fitted to the *m/z* 285/284 ion ratios determined during the 1-24 hours period after dosing (Figure 3-36), are given below:

A (bile conjugates)	$y = 14.075 * 10^{-0.031x}$	(r = 0.979)
B (rumen free)	$y = 24.541 * 10^{-0.021x}$	(r = 0.994)
C (rumen conjugates)	$y = 30.584 * 10^{-0.037x}$	(r = 0.992)
D (jejunum free)	$y = 27.113 * 10^{-0.018x}$	(r = 0.990)
E (jejunum conjugates)	$y = 15.453 * 10^{-0.035x}$	(r = 0.985)

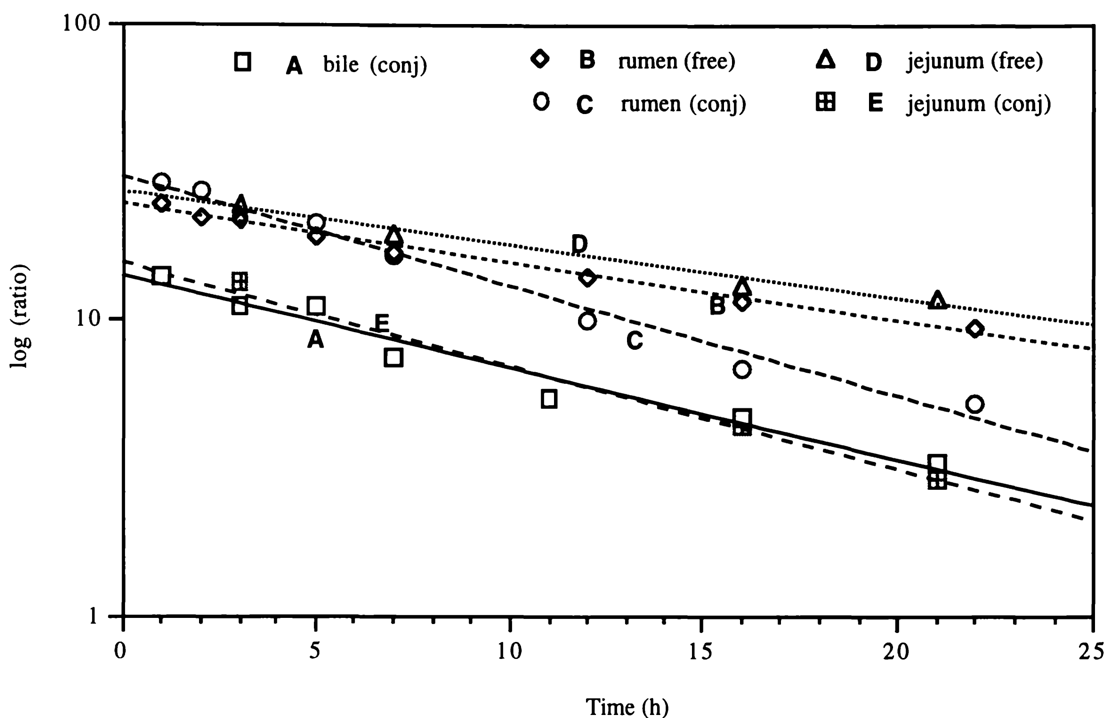


Figure 3-36. Plots of log *m/z* 285/284 ion ratio versus time (1-24 hours) determined for rumen, jejunum and bile samples, with exponential curve fitting of the form $y = a * 10^{-bx}$.

The straight line profiles, shown in Figure 3-36, are consistent with the existence of a single first order process for deuterium loss in the rumen and jejunum during the initial 24 hour period after dosing. It is apparent from the greater negative slopes of the conjugate bile, rumen and jejunum profiles (b terms of -0.031, -0.037 and -0.035) (curve A, C and E, Figure 3-34), compared to those of the free rumen and jejunum profiles (b values of -0.020 and -0.018 respectively) (curve B and D), that during this period, loss of labelled deuterium from conjugated epismilagenin occurs ca 1.8 times faster than is the case from free epismilagenin.

The 'a' and 'b' values for bile conjugates and for jejunum conjugates (a = 14.075 and 15.453 respectively; b = -0.031 and -0.035 respectively) were also similar (equations A and E, Figure 3-36).

Possible significance of ion ratio data

The similarity in bile and jejunum conjugate ion ratio profiles (Figure 3-34) can be rationalised in several ways.

Flåøyen and Frøslie (1997) have proposed that, in the jejunum, free sapogenins are absorbed and transported via the portal vein to the liver where further metabolism takes place (i.e. oxidation and reduction of sapogenins to episapogenins). Thereafter episapogenins are conjugated with D-glucuronic acid and excreted into the bile. This is a commonly accepted pathway for the absorption of xenobiotica (see Figure 3-37), and their removal from ovine digestive tracts. The diminished *m/z* 285/284 ion ratio of bile conjugates (essentially identical to that of jejunum conjugates: see Figure 3-34) can be attributed to loss of deuterium during oxidation and reduction in the liver. Return of bile conjugates to the small intestine (see Figure 3-37) may also (at least in part) account for the similar bile and jejunum conjugate ion ratio values.

Alternatively, bile epismilagenin conjugates may be derived by absorption of epismilagenin conjugates from the jejunum, or other metabolic regions such as the ileum, as is the case for bile acid conjugates (Davenport, 1978) (see Figure 3-38), and transported to the liver where de-conjugation and re-conjugation with D-glucuronic acid may occur without loss of the 3-deuterium atom. If this were the case, similar ion ratio profiles (Figure 3-34) would be expected for jejunum and bile epismilagenin conjugates.

The available experimental evidence does not distinguish these two possibilities. The identification, in future work, of the conjugated forms of sapogenins present in rumen, duodenum, jejunum, ileum, large intestine, portal vein samples, etc., may contribute to an improved understanding of absorption and transportation pathways. In the case of bile samples, deposited crystalloid material has been shown to be comprised predominantly of the Ca salts of β-D-glucuronides of epismilagenin and episarsasapogenin (Miles et al., 1993; Miles et al., 1994b), however the form of the extractable bile conjugates has not been established (although they are presumably also glucuronides).

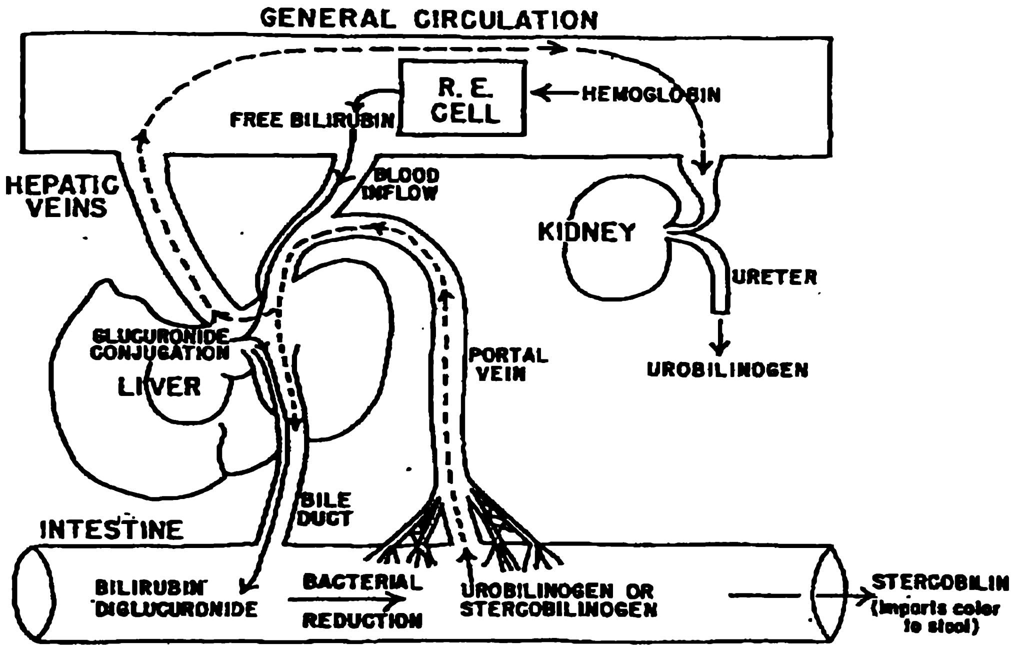


Figure 3-37. Enterohepatic circulation in sheep as related to bilirubin excretion (from Kaneko and Cornelius, 1970).

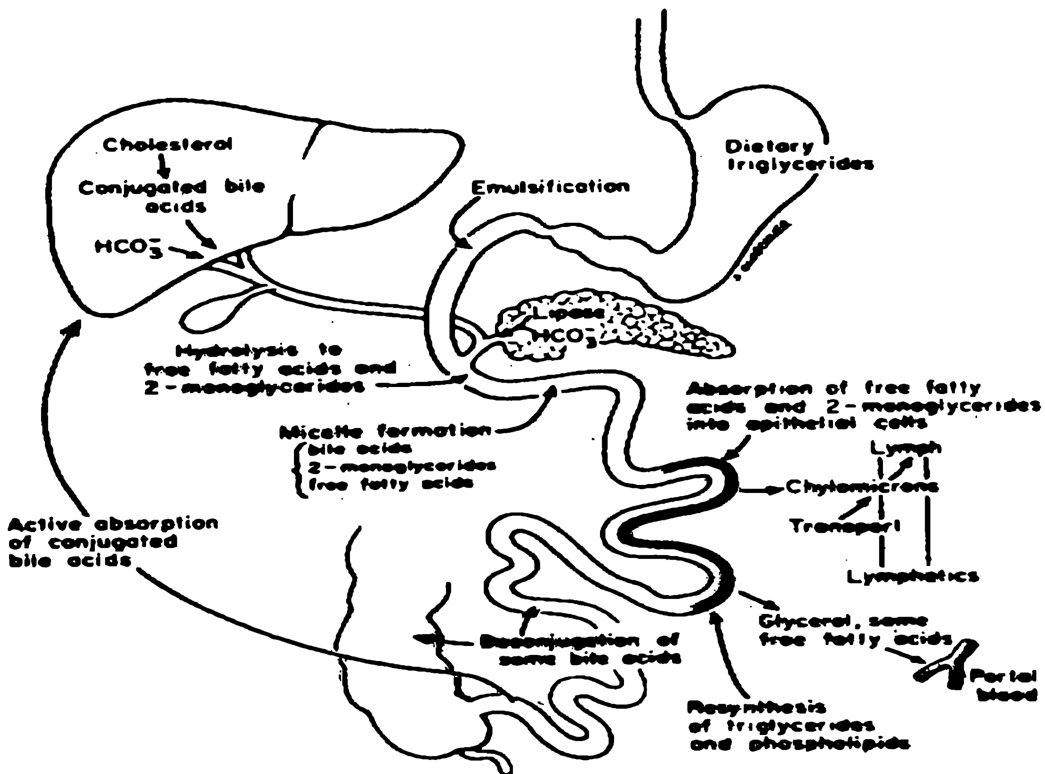


Figure 3-38. The process of active absorption of conjugated bile acids (from Davenport, 1978).

3.6.3 Summary and Conclusions

i) Oxidation/reduction at C-3 is a reversible process, as evidenced by the progressive loss of the 3β-D atom of 3β-D-epismilagenin β-D-glucoside.

ii) Epismilagenin, rather than smilagenin is the favoured reduction product of smilagen-3-one.

iii) Deuterium loss by reversible oxidation/reduction occurs at faster rates for conjugated epismilagenins than is the case for free epismilagenins. Conjugated epismilagenins are more bioavailable than free epismilagenins.

iv) Deuterium loss occurs at different rates in different metabolic regions (e.g. the rumen and the jejunum).

v) The *m/z* 285 / *m/z* 284 ion ratio profile of jejunum conjugates was similar to that of bile conjugates.

Chapter Four

SAPONIN CONSTITUENTS OF SELECTED PLANTS

4.1 Introduction

The identification of the crystalloid material deposited in the bile ducts of sheep suffering from hepatogenous photosensitization diseases as primarily insoluble calcium salts of epismilagenin (**4-1**) and/or episarsasapogenin (**4-2**) β-D-glucuronides (Miles et al., 1992b; Miles et al., 1994b; Wilkins et al., 1994) demonstrates that saponins play a significant role in the development of photosensitization diseases. It is now accepted that plant saponins are the source of the episapogenins characterised in biliary crystals, however it is not known if the biliary crystalloid material is the primary cause of diseases, or if the deposition of crystalline material is a secondary response to other causative factors (Smith and Miles, 1993).

Previous studies have revealed the widespread occurrence of sapogenins and saponins in the plants which are associated with occasional outbreaks of hepatogenous photosensitizations of ruminants. Sarsasapogenin (**4-3**), smilagenin (**4-4**) and their glycosides occur in *N. ossifragum* (Ceh and Hauge, 1981; Miles et al., 1993; Wilkins, pers commun); smilagenin (**4-4**) occurs in *A. lecheguilla* (Wall et al., 1962; Camp et al., 1988); diosgenin (**4-5**) and yamogenin (**4-6**) saponins are present in *B. decumbens* (Wilkins et al., 1994; Meagher et al., 1996); a diosgenin saponin has been identified in *P. dichotomiflorum* (Miles et al., 1991; Miles et al., 1992b; Munday et al., 1993b); diosgenin and yamogenin saponins have been detected in *P. coloratum* (Patamalai et al., 1990; Wilkins, pers commun); diosgenin (**4-5**) has been identified as the sapogenin component of saponins in *P. schinzii* (Miles et al., 1992b); and a variety of sapogenins including diosgenin (**4-5**), yamogenin (**4-6**), epismilagenin (**4-1**), tigogenin (**4-7**), neotigogenin (**4-8**), gitogenin (**4-9**), neogitogenin (**4-10**), ruscogenin (**4-11**) and neoruscogenin (**4-12**) have been identified as genin components of saponins from *T. terrestris* (Wilkins et al., 1996; Wilkins et al., 1994; Miles et al., 1993; Miles et al., 1994b). The structures of some *T. terrestris* saponins (e.g. tribulosin, doscin and a β-glucosidase treated ruscogenin saponin) have been reported (Mahato et al., 1982; Wilkins et al.; 1996).

In this study, the saponin contents of some collections of *N. ossifragum* from Norway, *P. virgatum* from the USA, *P. miliaceum* from New Zealand, *N. texana* from the USA, *B. decumbens* from Papua New Guinea (PNG) and *T. terrestris* from Iran, are reported.

4.2 *Narthecium ossifragum*

4.2.1 Introduction

Grazing of *N. ossifragum* has been reported to cause outbreaks of photosensitization diseases amongst ruminant animals (Ender, 1955; Ford, 1964; Flåøyen, 1993; Flåøyen et al., 1994).

Stabursvik (1959) concluded that a sarsasapogenin glycoside containing glucose, galactose, xylose and arabinose was present in *N. ossifragum.* Subsequently Ceh and Hauge (1981) reported the occurrence in *N. ossifragum* extracts of two sapogenin trisaccharides (saponins), narthecin and xylosin, and an open ring F trisaccharide, narthecioside (Figure 4-1), which on mild acid hydrolysis afforded sarsasapogenin (**4-3**), glucose, galactose and arabinose or glucose, galactose and xylose, respectively. However the positions of the glycoside linkages were not determined.

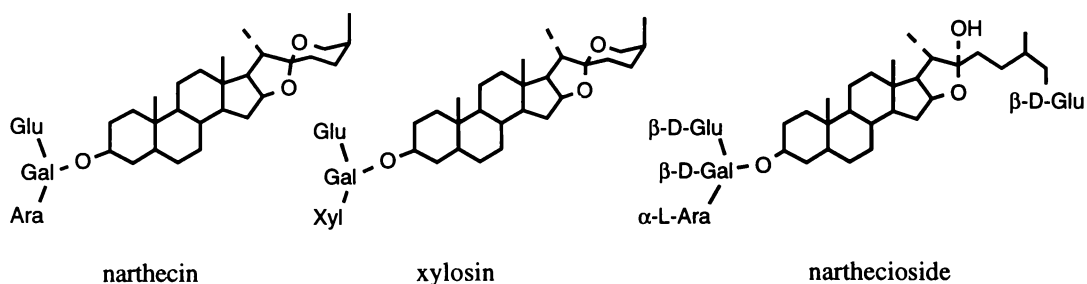


Figure 4-1. Structures of narthecin, xylosin and narthecioside, as reported by Ceh and Hauge (1981).

Recently, *N. ossifragum* saponins have been shown to contain sarsasapogenin (**4-3**) and smilagenin (**4-4**) in a ratio of ca 10:1 (Miles et al., 1993).

Kobayashi et al. (1993) and Inoue et al. (1995) have reported the isolation and structure elucidation of five saponins from *N. asiaticum*, a species occurring in Japan. The five saponins were identified as **4-13**, **4-14**, **4-15**, **4-16** and **4-17** (Figure 4-2). Inoue et al. (1995) suggested that saponins **4-13** and **4-14** might correspond to narthecin and xylosin respectively.

Subsequently Berndt (1997) showed that the two major *N. ossifragum* saponins, which presumably corresponded to the specimens of narthecin (**4-13**) and xylosin (**4-14**) isolated by Ceh and Hauge (1981), exhibited ^{1}H and ^{13}C NMR data which identified them as saponins (**4-13**) and (**4-14**), respectively. A third (minor) saponin exhibited ^{1}H and ^{13}C NMR data which identified it as the disaccharide (**4-15**) (Figure 4-2) (Berndt, 1997).

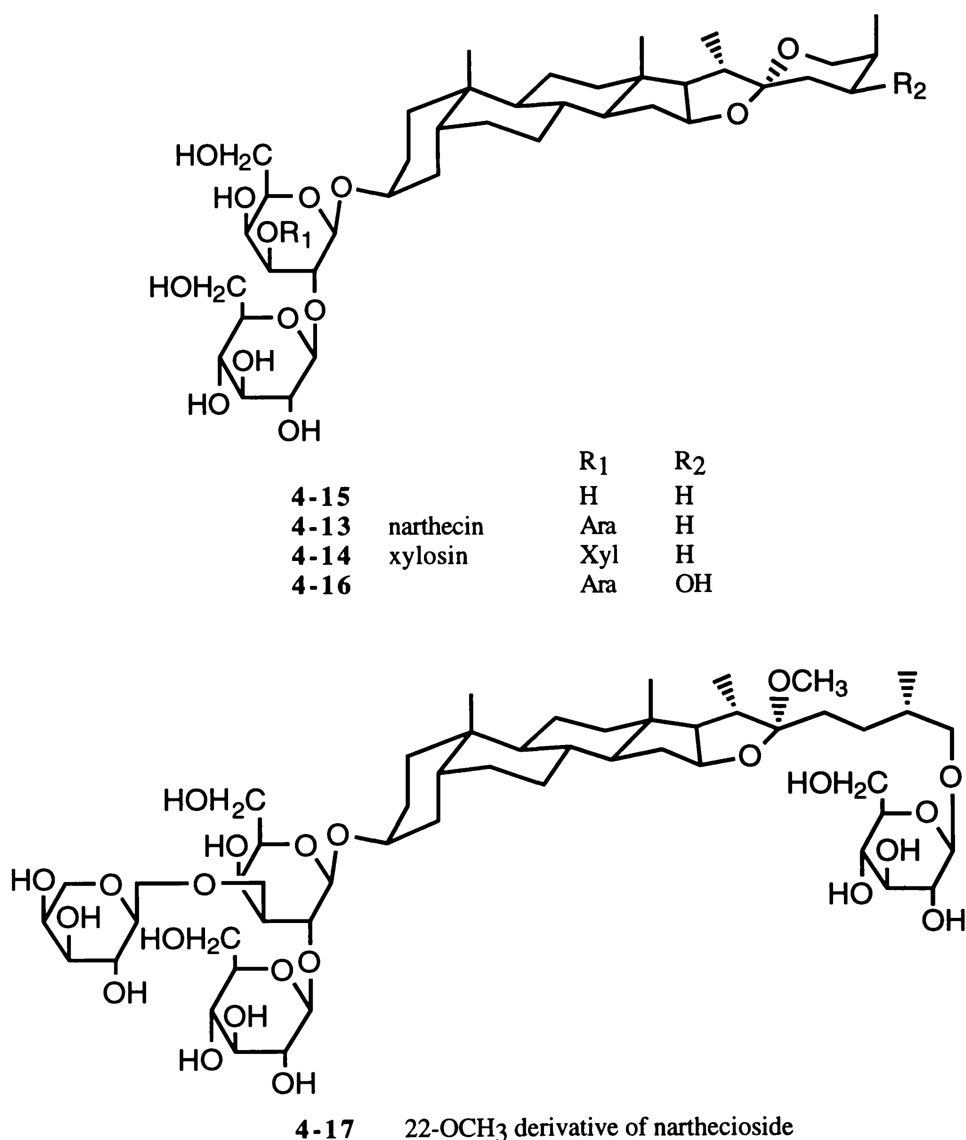


Figure 4-2. Structures of saponins isolated from *N. asiaticum* by Kobayashi et al. (1993) and Inoue et al. (1995).

In this work, the isolation and structure elucidation of saponin constituents of *N. ossifragum* samples collected from pastures in the Faroe Island, and in the vicinity of Hemne Selbu, Sør-Trøndelag County, Norway, are described. The structures of these saponins were elucidated using one- and two-dimensional NMR and electrospray mass

spectroscopy (ES-MS) spectral data. Saponin levels in the *N. ossifragum* specimen were determined by GC-MS analysis of the genins derived by acid hydrolysis of the parent saponins.

4.2.2 Results and Discussion

Quantitative analysis

Quantitative analysis of *N. ossifragum* samples, collected from pastures in the Faroe Island, Norway in 1995, was performed as reported by Wilkins et al. (1994) using the selected ion mode (SIM) GC-MS *m/z* 139, 368, 382, 394 and 396 ion profiles for sapogenins, cholesterol (**4-18**), campesterol (**4-19**), stigmasterol (**4-20**) and β-sitosterol (**4-21**) respectively (see Section 5.1.2).

The results, presented in Table 4-1, showed that high levels of sarsasapogenin (**4-3**) and smilagenin (**4-4**) saponins, in a ratio of ca 4:1, were present in the Faroe Island samples. For example, sample 3 exhibited conjugated sarsasapogenin (**4-3**) and smilagenin (**4-4**) levels of 2121 mg/kg and 557 mg/kg respectively. No free sapogenins were found in the samples.

Table 4-1. Levels (mg/kg) of free and conjugated sapogenins and sterols identified in Norwegian *N. ossifragum* samples.

samples		chol	camp	stig	sito	smil	sar
1	conj	4.9	8.5	83.4	32.8	366	1317
	free	0	0	0	0	0	0
2	conj	4.3	4.6	61.8	26.0	243	1406
	free	0	0	0	0	0	0
3	conj	9.1	14.3	196	75.4	557	2121
	free	2.7	4.7	55.5	20.8	0	0

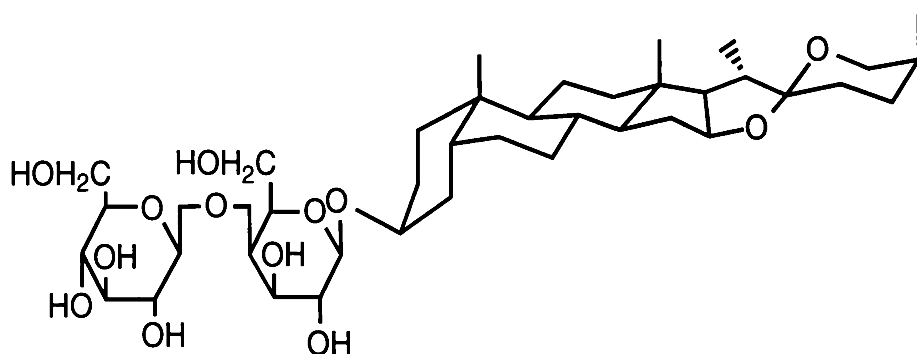
conj = conjugated extract, free = free extract, chol = cholesterol, camp = campesterol, stig = stigmasterol, sito = β-sitosterol, smil = smilagenin, sar = sarsasapogenin

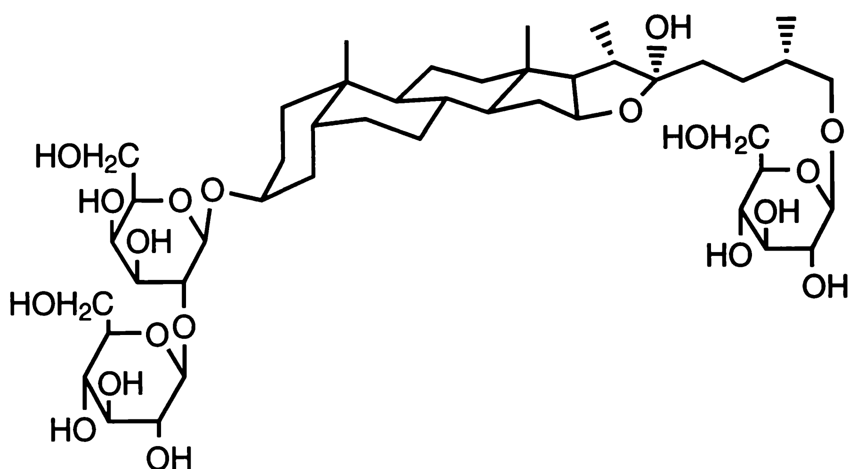
Identification of saponins

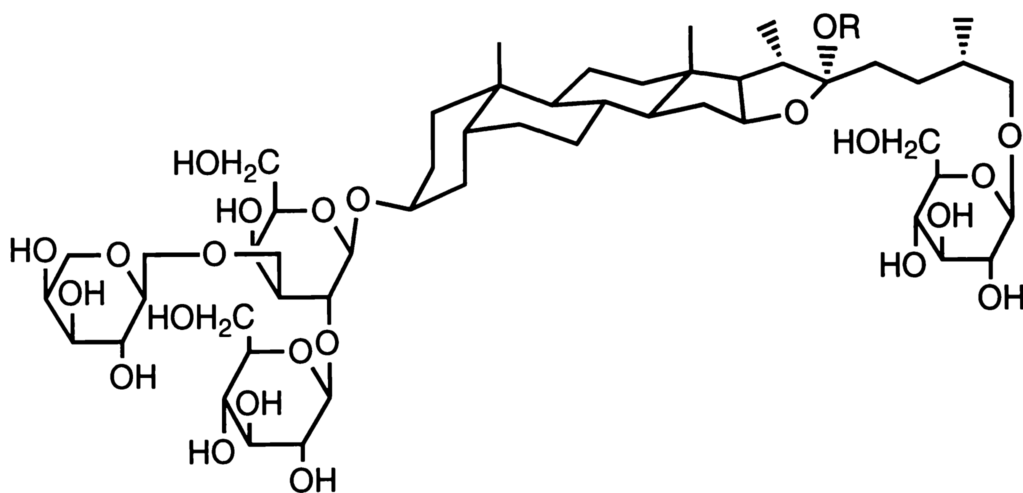
Separation of the crude saponin material, recovered from the ethanol-water extract of freeze-dried Faroe Island plant samples (which had previously been extracted with dichloromethane), by radial chromatography on silica gel using mixtures of chloroform and aqueous methanol as eluant, afforded saponins which were identified as (25*R/S*)-5β-spirostan-3β-ol 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside] (**4-22**), (25*R/S*)-5β-spirostan-3β-ol 3-*O*-[*O*-β-D-glucopyranosyl-(1→2)-β-D-galactopyranoside] (**4-15**), (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-[*O*-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl]-26-*O*-β-D-glucopyranoside (**4-23**), (25*R/S*)-5β-spirostan-3β-ol 3-*O*-{*O*-β-D-glucopyranosyl)-(1→2)-*O*-[α-L-arabinopyranosyl)-(1→3)]-β-D-galactopyranoside} (**4-13**) (narthecin), (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**), and (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-22-*O*-methyl-26-*O*-β-D-glucopyranoside (**4-17**) (Figure 4-3).

The trivial name faroecin is proposed for saponin **4-22** since it was first isolated from Faroe island *N. ossifragum*. Since saponin **4-23** is the 26-glucosyl-furostanol analogue of saponin **4-15**, isolated by Inoue et al. (1995) from *N. asiaticum*, the trivial designations asiaticin and asiaticioside are proposed for saponins **4-15** and **4-23** respectively. Saponin **4-24** appears to correspond to the furostanol saponin narthecioside detected by Ceh and Hauge (1981).

The 22-*O*-methyl-furostanol saponin **4-17** is believed to be an artefact derived from methylation of saponin **4-24** during the chromatographic separation of the extracts.







4-24 R = H
4-17 R = CH_3

Figure 4-3. Structures of faroecin (**4-22**), asiaticioside (**4-23**), narthecioside (**4-24**) and 22-*O*-methyl-narthecioside (**4-17**) isolated from Norwegian *N. ossifragum*.

Structure elucidation of saponin 4-15

The ^{1}H NMR spectrum of **4-15** included signals attributable to the presence of two axially oriented β-anomeric glycosyl protons (4.92 ppm, d, J = 7.7 Hz and 5.29 ppm, d, J = 7.7 Hz). The ^{13}C NMR resonances of **4-15** corresponded closely to those reported by Inoue et al. (1995) for a specimen of **4-15** (Table 4-2 and 4-3).

Analyses of one- and two-dimensional COSY, TOCSY, ROESY, HMBC and HSQC spectral data revealed that the assignments of Inoue et al. (1995) for the C-3' and C-2'' resonances of **4-15** should be reversed. In particular, the HSQC spectrum of **4-15** demonstrated that H-3' (4.26 ppm) and H-2'' (4.07 ppm) correlated with carbon resonances which occurred at 75.5 ppm (C-3') and 76.9 ppm (C-2''), respectively.

Correlations observed in the COSY and TOCSY spectra of **4-15** established that the proton signals which occurred at 4.26 and 4.07 ppm were associated with the galactosyl and glucosyl residues, respectively. Correlations observed in the ROESY spectrum of **4-15** between H-1' (4.92 ppm) and H-3 (4.32 ppm), and between H-1'' (5.29 ppm) and H-2' (4.63 ppm) verified the presence of 3-*O*-galactopyranosyl and glucopyranosyl-(1→2)-galactopyranosyl linkages, respectively.

Berndt (1997) has also concluded that the C-3' and C-2'' resonances of **4-15** reported by Inoue et al. (1995) should be reversed.

The negative ion ES-MS of **4-15**, determined at -110 V, using acetonitrile-water (1:1) as solvent, included a pseudomolecular ion at *m/z* 739.6 [$(M-H)^-$].

Table 4-2. ^{13}C NMR assignments (ppm in C_5D_5N) for the aglycone atoms of faroecin (**4-22**), asiaticin (**4-15**), asiaticioside (**4-23**), narthecin (**4-13**), narthecioside (**4-24**) and 22-*O*-methyl-narthecioside (**4-17**).

	4-17*	4-13*	4-15*	4-24	4-17	4-13	4-15	4-23	4-22
1	30.7	30.7	30.9	30.7	30.7	30.7	31.0	31.0	30.9
2	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0	27.0
3	75.0	75.1	75.2	75.1	75.0	75.1	75.2	75.4	74.7
4	30.0	30.7	30.9	30.7	30.7	30.7	30.9	31.0	30.7
5	36.6	36.5	36.9	36.6	36.6	36.6	37.0	37.0	37.0
6	26.9	26.8	26.8	26.9	26.9	27.0	26.8	26.8	26.8
7	26.8	26.9	26.8	27.0	26.8	26.8	26.8	26.8	27.0
8	35.6	35.6	35.5	35.6	35.6	35.6	35.5	35.5	35.6
9	40.3	40.3	40.2	40.3	40.3	40.3	40.2	40.3	40.3
10	35.3	35.3	35.2	35.3	35.3	35.3	35.3	35.3	35.2
11	21.1	21.1	21.2	21.1	21.1	21.2	21.1	21.2	21.2
12	40.2	40.3	40.3	40.4	40.2	40.3	40.3	40.4	40.3
13	41.3	40.9	40.9	41.3	41.3	40.9	40.9	41.3	40.9
14	56.4	56.4	56.4	56.5	56.4	56.5	56.4	56.5	56.5
15	32.2	32.2	32.2	32.5	32.2	32.2	32.2	32.4	32.2
16	81.5	81.4	81.3	81.3	81.5	81.4	81.3	81.3	81.4
17	64.5	63.0	63.0	64.1	64.5	63.0	63.0	64.1	63.0
18	16.5	16.6	16.6	16.7	16.5	16.6	16.6	16.7	16.6
19	24.0	24.0	24.0	24.0	23.9	24.0	24.0	24.0	23.9
20	40.5	42.5	42.5	40.7	40.5	42.5	42.5	40.7	42.5
21	16.4	14.9	14.9	16.5	16.4	16.9	14.9	16.5	14.9
22	112.7	109.7	109.7	110.7	112.7	109.7	109.7	110.6	109.7
23	30.7	26.2	26.2	30.7	30.7	26.3	26.2	30.0	26.2
24	28.2	26.5	26.4	28.4	28.2	26.5	26.4	28.4	26.5
25	34.5	27.6	27.5	34.5	34.5	27.6	27.6	34.4	27.6
26	75.2	65.1	65.1	75.4	75.0	65.1	65.1	75.2	65.1
27	17.6	16.3	16.3	17.5	17.6	16.3	16.3	17.5	16.3
OMe	47.4				47.4				

*assignments reported by Kobayashi et al. (1993) and Inoue et al. (1995).

Table 4-3. ^{1}H and ^{13}C NMR assignments (ppm in C_5D_5N) for the glycoside atoms of faroecin (**4-22**), asiaticioside (**4-23**), narthecioside (**4-24**), 22-*O*-methyl narthecioside (**4-17**), narthecin (**4-13**) and asiaticin (**4-15**).

	4-17*	**4-13***		**4-15***		**4-24**		**4-17**		**4-13**		**4-15**		**4-23**		**4-22**	
	^{13}C	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Gal																	
1'	102.0	102.0	4.91	102.2	4.92	102.0	4.89	102.0	4.89	102.1	4.89	102.6	4.88	102.6	4.92	103.7	4.75
2'	76.5	77.6	4.81	81.9	4.66	77.8	4.80	77.8	4.80	77.8	4.80	81.9	4.63	81.9	4.66	73.5	4.36
3'	84.3	84.3	4.30	76.9	4.26	84.3	4.28	84.3	4.28	84.3	4.28	75.5	4.23	75.5	4.26	75.3	4.18
4'	69.8	69.8	4.83	69.8	4.56	69.8	4.79	69.8	4.79	69.9	4.79	69.9	4.53	69.9	4.56	80.1	4.67
5'	77.6	76.5	4.05	76.6	4.02	76.5	4.02	76.5	4.02	76.5	4.02	76.6	4.00	76.6	4.02	75.4	4.00
6'	63.5	62.2	4.39	62.2	4.41	62.2	4.40	62.2	4.40	62.3	4.39	62.2	4.39	62.2	4.41	61.0	4.57
																	4.17
Glu																	
1"	106.4	104.4	5.55	106.1	5.29	104.4	5.53	104.4	5.53	104.4	5.53	106.2	5.26	106.1	5.29	107.2	5.24
2"	76.4	76.4	4.03	75.5	4.07	76.5	4.02	76.5	4.02	76.5	4.01	76.9	4.06	76.9	4.07	76.0	4.09
3"	78.6	78.6	4.17	78.4	4.20	78.6	4.14	78.6	4.14	78.6	4.14	78.0	4.16	78.4	4.20	78.8	4.19
4"	72.8	72.8	4.19	71.7	4.32	72.8	4.16	72.8	4.16	72.8	4.16	71.8	4.28	71.7	4.32	72.4	4.03
5"	77.8	77.8	3.72	78.0	3.86	77.6	3.68	77.6	3.68	77.6	3.68	78.4	3.38	78.0	3.86	78.5	3.98
6"	62.2	63.5	4.44	62.8	4.47	63.6	4.43	63.6	4.43	63.6	4.42	62.8	4.44	62.9	4.47	63.2	4.63
			4.33				4.33		4.33		4.32						4.19

* assignments reported by Kobayashi et al. (1993) and Inoue et al. (1995).

continued ....

Table 4-3. (Continued)

	4-17*	4-13*		4-15*		4-24		4-17		4-13		4-15		4-23		4-22	
	^{13}C	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
Ara																	
1'''	104.4	106.4	5.19			106.4	5.17	106.4	5.17	106.4	5.17						
2'''	72.8	72.8	4.45			72.8	4.45	72.8	4.45	72.8	4.44						
3'''	74.7	74.7	4.09			74.7	4.06	74.7	4.06	74.7	4.06						
4'''	69.9	69.8	4.19			69.8	4.22	69.8	4.22	69.9	4.22						
5'''	67.5	67.5	4.22			67.5	4.18	67.5	4.18	67.5	4.18						
			3.69				3.69		3.69		3.70						
26-Glu																	
1''''	105.1					105.1	4.81	105.1	4.81					105.1	4.79		
2''''	75.2					75.2	4.01	75.2	4.01					75.2	3.99		
3''''	78.7					78.7	4.22	78.7	4.22					78.6	4.20		
4''''	71.8					71.8	4.22	71.8	4.22					71.8	4.20		
5''''	78.5					78.5	3.93	78.5	3.93					78.5	3.90		
6''''	62.9					62.9	4.53	62.9	4.53					62.9	4.52		
							4.37		4.37						4.35		

* assignments reported by Kobayashi et al. (1993) and Inoue et al. (1995).

Structure elucidation of saponin 4-13

The ^{1}H NMR spectrum of **4-13** included three lowfield methine signals at 4.89, 5.17 and 5.53 ppm, the coupling constants of which (J = 7.7, 7.6 and 7.8 Hz, respectively) indicated the presence of three axially oriented anomeric glycosyl protons. The ^{13}C NMR resonances of **4-13**, presented in Table 4-2 and 4-3, corresponded closely to those reported by Inoue et al. (1995) for a specimen of **4-13** isolated from a Japanese collection of *N. asiaticum.*

Correlations observed in the two-dimensional HMQC, HMBC, COSY, TOCSY and ROESY spectra of **4-13** substantiated the assignments presented in Tables 4-2 and 4-3, and were consistent with the identification of this saponin as (25*R/S*)-5β-spirostan-3β-ol 3-*O*-{*O*-β-D-glucopyranosyl)-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranoside} (narthecin).

The negative ion ES-MS of **4-13**, determined at -110 V, using acetonitrile-water (1:1) as solvent , included a pseudomolecular ion at *m/z* 871.6 [$(M-H)^-$].

Structure elucidation of saponin 4-22

A minor, early eluting fraction, was identified as (25*R/S*)-5β-spirostan-3β-ol 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside] (**4-22**).

The ^{1}H NMR spectrum of **4-22** showed the presence of two axially (β-) oriented anomeric glycosyl protons at 4.75 ppm (d, J = 7.7 Hz) and 5.24 ppm (d, J = 7.8 Hz), and four spirostanol-type methyl group signals at 0.82 (s), 0.83 (s), 1.07 (d, J = 7.0 Hz) and 1.16 ppm (d, J = 6.9 Hz).

The occurrence of a quaternary ^{13}C NMR signal at 109.7 ppm showed that **4-22** possessed a closed ring F spirostanol-type structure (Agrawal et al., 1985). Two anomeric glycoside carbon resonances were observed at 103.7 ppm (C-1') and 107.2 ppm (C-1") in the ^{13}C NMR spectrum. These ^{13}C NMR signals indicated **4-22** to be a spirostanol disaccharide.

The resonances of protons associated with each of the glycosyl residues were identified from correlations observed in COSY and HOHAHA (TOCSY) spectra (starting from the anomeric H-1' and H-1'' protons (4.75 and 5.24 ppm respectively). Glycosidic carbon and proton resonances were correlated in a HSQC experiment.

A combination of ^{1}H, ROESY, COSY and HSQC NMR data led to the conclusion that galactosyl and glucosyl sugar units were present in saponin **4-22**. ^{1}H NMR coupling constant data demonstrated that the anomeric glycosyl H-1' and H-1" protons (4.75 ppm, *J* = 7.8 Hz and 5.24 ppm, *J* = 7.7 Hz respectively) of **4-22** were axially oriented and coupled to axially oriented H-2' and H-2" protons, respectively, while the ROESY correlations showed that the respective glycosyl H-3', H-5', H-3", H-5" protons were also axially oriented. In the COSY spectrum, weak correlations between H-3' and H-4' and between H-4' and H-5' revealed that H-4' was equatorially oriented, while strong correlations between H-3" and H-4" and between H-4" and H-5" showed that H-4" was axially oriented (Agrawal, 1992). Further analyses of coupling constant data, and ^{1}H and ^{13}C NMR data, also led to the conclusion that H-4' (4.67 ppm) was equatorially oriented, whereas H-4" (4.03 ppm) was axially oriented. It was therefore concluded that the glycosyl residues were galactosyl and glucosyl residues, respectively.

Correlation peaks were observed in the ROESY spectrum of **4-22**, between H-1' and H-3, and between H-1" and H-4' (Table 4-4 and Figure 4-4). These observations showed that the inner galactosyl residue was attached to the genin at C-3, while the outer glucosyl residue was attached to C-4' of the galactosyl residue. Consistent with this conclusion, correlations were also observed in the HMBC spectrum of **4-22** between H-1' and C-3, and between H-1" and C-4' (Table 4-5).

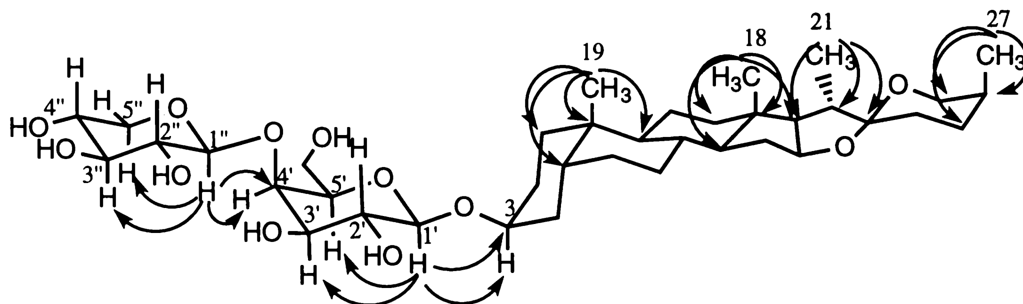


Figure 4-4. Selected ROESY (^{1}H-^{1}H) and HMBC (^{1}H-^{13}C) correlations observed for faroecin (**4-22**).

Table 4-4. Selected ROESY correlations observed for faroecin (**4-22**) (ppm in C_5D_5N).

irradiated signal	correlated signals
4.75 (H-1')	4.28 (H-3), 4.18 (H-3'), 4.00 (H-5')
5.24 (H-1")	4.47 (H-4'), 4.19(H-3"), 4.00 (H-5")

Table 4-5. Selected HMBC correlations observed for faroecin (**4-22**) (ppm in C_5D_5N).

^{1}H signal	correlated ^{13}C signal(s)
5.24 (H-1")	80.1 (C-4')
4.75 (H-1')	74.7 (C-3)
1.16 (H-21)	109.7 (C-22), 63.0 (C-17), 42.5 (C-20)
1.08 (H-27)	65.1 (C-26), 27.6 (C-25), 26.5 (C-24)
0.83 (H-19)	40.3 (C-9), 37.0 (C-5), 35.2 (C-10), 30.9 (C-1)
0.82 (H-18)	63.0 (C-17), 56.5 (C-14), 40.9 (C-13), 40.3 (C-12)

The C-2' and C-4' resonances of **4-22** (73.5 and 80.1 ppm respectively) differed appreciably from those of the C-2' and C-4' signals of **4-15** (81.9 and 69.7 ppm respectively). When a hydroxy group in a sugar unit is glycosylated (linked to another sugar unit), the resonance of the glucosylated carbon atom is typically shifted to lower field by 7-10 ppm (Agrawal, 1992). The foregoing differences in chemical shifts are consistent with the outer glucosyl residue of **4-22** being linked to C-4' of the inner galactosyl residue, rather than to C-2', as is the case for **4-15**.

The ^{13}C NMR assignments, presented in Tables 4-2 and 4-3 for the glucosyl and galactosyl residues of **4-22**, can be compared to those reported for the corresponding *O*-[*O*-β-D-glucopyranosyl-(1"→4')-β-D-galactopyranosyl-(1'→3)]-diosgenin saponin (Agrawal et al., 1985).

The negative ion ES-MS of **4-22**, determined at -110 V, using acetonitrile-water (1:1) as solvent, included a pseudomolecular ion at *m/z* 739.5 [$(M-H)^-$].

The ES-MS and one- and two-dimensional NMR data determined for **4-22** showed it to be (25*R/S*)-5β-spirostan-3β-ol 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside].

Structure elucidation of saponin 4-24

The ^{1}H NMR spectrum of saponin **4-24** included signals attributable to the presence of four axially β-oriented anomeric glycosyl protons at 4.81 (d, J = 7.7 Hz), 4.89 (d, J = 7.7 Hz), 5.17 (d, J = 7.6 Hz) and 5.53 ppm (d, J = 7.8 Hz), and 18-Me, 19-Me, 21-Me, and 27-Me protons at 0.88 (s), 0.97 (s), 1.03 (d, J = 6.6 Hz) and 1.32 ppm (d, J = 6.8 Hz), respectively.

The ^{13}C NMR resonances of **4-24** (presented in Tables 4-2 and 4-3) corresponded closely to those reported by Kobayashi et al. (1993) for the 22-methoxy furostanol saponin (**4-17**) isolated from *N. asiaticum*. However, the ^{1}H and ^{13}C NMR spectra of **4-24** did not include signals at ca 3.3 ppm and 47 ppm respectively, attributable to the presence of a methoxy group.

The occurrence of the C-22 resonance of **4-24** at 110.7 ppm (rather than 112.7 ppm as in **4-17**) was consistent with presence of a 22-hydroxy group, rather than a 22-methoxy group (Agrawal et al., 1985).

Analyses of correlation peaks observed in the COSY spectrum of **4-24** lead to the conclusion that the ^{1}H NMR resonances of a pair of glucosyl methine protons were almost identical. While cross peaks identified the H-2'' (4.02 ppm) (via a correlation with H-1'') and H-4'' (4.16 ppm) (via a correlation with H-5'') resonances respectively, they did not establish the resonance of H-3'' (essentially co-incident with either H-2'' or H-4''). The ^{1}H-^{13}C correlated HSQC spectrum of **4-24** revealed two methine proton resonances occurred in the region 4.14-4.16 ppm, while only one methine resonance occurred in the vicinity of 4.02 ppm. Hence H-3'' was found to occur at 4.14 ppm (rather than 4.02 ppm). The resonances (4.22 ppm) of another pair of coincident 26-glucosyl protons (H-3'''' and H-4'''') were established from similar analyses of COSY and HSQC NMR spectral data.

Detailed analyses of one- and two-dimensional COSY, TOCSY, ROESY, HMBC and HSQC spectral data confirmed that saponin **4-24** was the 26-*O*-glucosyl-furostanol analogue of saponin **4-13**.

Correlations, observed in the ROESY spectrum of **4-24**, between H-1' and H-3, H-1'' and H-2', H-1''' and H-3', and H-1'''' and H-26 (Table 4-6 and Figure 4-5), demonstrated that the presence of 1'-galactosyl to 3-*O*-genin, 1''-glucosyl to 2'-*O*-galactosyl, 1'''-arabinosyl to 3'-*O*-galactosyl and 1''''-glucosyl to 26-*O*-genin linkages, respectively.

Multiple bond ^{1}H-^{13}C correlations in the HMBC spectrum of **4-24** also verified the linkages of 1'-galactosyl to 3-*O*-genin, 1''-glucosyl to 2'-*O*-galactosyl, 1'''-arabinosyl to 3'-*O*-galactosyl and 1'''-glucosyl to 26-*O*-genin. Structurally significant HMBC correlations observed for **4-24** are given in Table 4-7, and depicted in Figure 4-5.

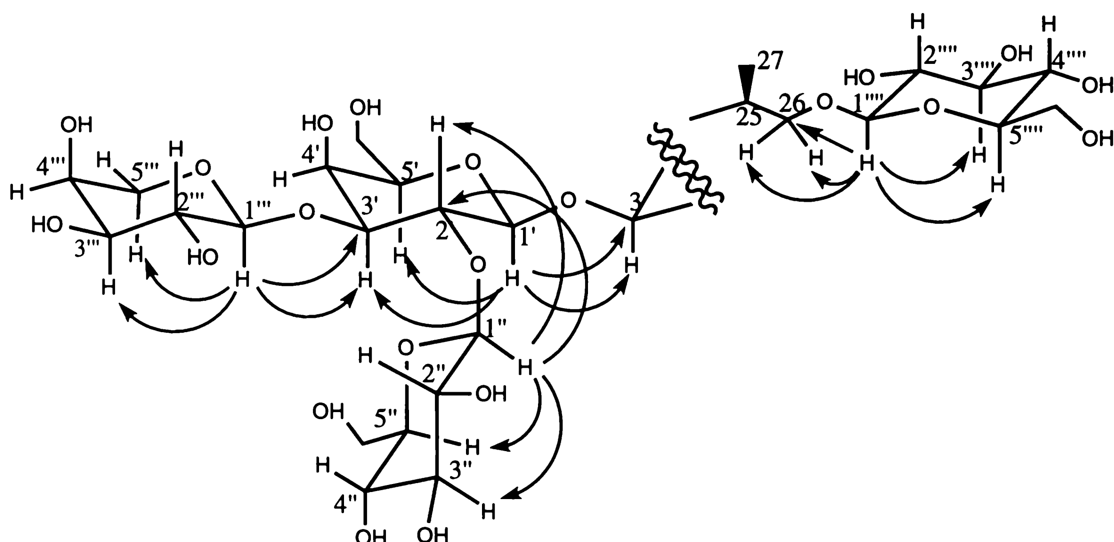


Figure 4-5. Selected ROESY (^1H-^1H) and HMBC (^1H-^{13}C) correlations observed for narthecioside (**4-24**).

Table 4-6. Selected ROESY correlations observed for narthecioside (**4-24**) (ppm in C_5D_5N).

irradiated signal	correlated signals
4.89 (H-1')	4.34 (H-3), 4.28 (H-3'), 4.02 (H-5')
5.53 (H-1")	4.80 (H-2'), 4.15 (H-3"), 3.68 (H-5")
5.17 (H-1"')	4.28 (H-3'), 4.06 (H-3"'), 3.69 (H-5"')
4.81 (H-1"")	3.49 (H-26a), 4.22 (H-3""), 3.93 (H-5"")

Table 4-7. Selected HMBC correlations observed for narthecioside (**4-24**) (ppm in C_5D_5N).

^1H signal	correlated ^{13}C signal(s)
4.89 (H-1')	75.1 (C-3)
5.53 (H-1")	77.8 (C-2')
5.17 (H-1"')	84.3 (C-3')
4.81 (H-1"")	75.4 (C-26)
1.32 (H-21)	110.7 (C-22), 64.1 (C-17), 40.7 (C-20)
1.03 (H-27)	75.4 (C-26), 34.5 (C-25), 28.4 (C-24)
0.97 (H-19)	40.3 (C-9), 36.6 (C-5), 35.3 (C-10), 30.7 (C-1)
0.88 (H-18)	64.1 (C-17), 56.5 (C-14), 41.3 (C-13), 40.4 (C-12)

The homonuclear Hartmann-Hahn (HOHAHA) spectrum of **4-24**, optimised for the detection of long range couplings, showed that the anomeric protons of each of the glycosyl units exhibited correlations at the resonance frequencies of their respective H-2, H-3, H-4, H-5, and H-6 signals (Table 4-8).

Table 4-8. Selected HOHAHA correlations observed for narthecioside (**4-24**) (ppm in C_5D_5N).

irradiated signal	correlated signals
5.53 (H-1")	4.43 (H-6"b), 4.33 (H-6"a), 4.15 (H-3" and H-4"), 4.02 (H-2"), 3.68 (H-5")
5.17 (H-1"')	4.45 (H-2"'), 4.22 (H-4"'), 4.18 (H-5"'b), 4.06 (H-3"'), 3.70 (H-5"'a)
4.89 (H-1')	4.80 (H-2' and H-4'), 4.40 (H-6'), 4.28 (H-3'), 4.02 (H-5')
4.81 (H-1"")	4.53 (H-6""b), 4.37 (H-6""a), 4.22 (H-3"" and H-4""), 4.01 (H-2""), 3.93 (H-5"")

The negative ion ES-MS of **4-24** included pseudomolecular ions at *m/z* 1051.8 [$(M-H)^-$] and 1087.9 [$(M+Cl)^-$].

The ES-MS and one- and two-dimensional NMR data determined for **4-24** showed it to be (25*S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside. Hitherto Ceh and Hauge (1981) have proposed the trivial name, narthecioside for this saponin.

Structure elucidation of saponin 4-17

The negative ion ES-MS [*m/z* 1065.8 $(M-H)^-$] and ^1H and ^{13}C NMR data (Tables 4-2 and 4-3) identified saponin **4-17** as the 22-*O*-methyl (22-methoxy) analogue of saponin **4-24**. The ^{13}C NMR assignments presented in Tables 4-2 and 4-3 for saponin **4-17** are comparable to those reported by Kobayashi et al. (1993) for this saponin.

Since **4-17** was only detected in fractions after separation of the extracts, it may be concluded that **4-17** is an artefact derived from the methylation of **4-24** during radial chromatography using $MeOH-CHCl_3-H_2O$ mixtures as the developing solvent. Kobayashi et al. (1993) have also suggested **4-17** to be an artefact.

Structure elucidation of saponin 4-23

The ^{1}H NMR spectrum of **4-23** included signals attributable to the presence of three axially β-oriented anomeric glycosyl protons at 4.79 (d, J = 7.8 Hz), 4.92 (d, J = 7.6 Hz) and 5.29 ppm (d, J = 7.6 Hz), and four methyl signals at 0.88 (s), 0.94 (s), 1.01 (d, J = 6.9 Hz) and 1.30 ppm (d, J = 7.0 Hz).

The chromatographic behaviour of saponin **4-23** indicated it to be more polar than the known disaccharide saponin **4-15** (asiaticin), but less polar than the trisaccharide saponin **4-13** (narthecin).

The ^{13}C NMR signals of **4-23** corresponded closely to those reported for the ring A and B genin carbon signals of **4-15**, and the ring F carbon signals and 26-glucosyl residue carbon signals of the 26-furostanol-glucoside **4-13** (Tables 4-2 and 4-3). The remaining glycosyl residue carbon signals of saponin **4-23** corresponded closely to those reported for saponin **4-15**.

ROESY (Table 4-9) and HMBC (Table 4-10) NMR spectral data verified the presence in **4-23** of 1'-galactosyl to 3-*O*-genin, 1''-glucosyl to 2'-*O*-galactosyl and 1'''-glucosyl to 26-*O*-genin linkages (Figure 4-6).

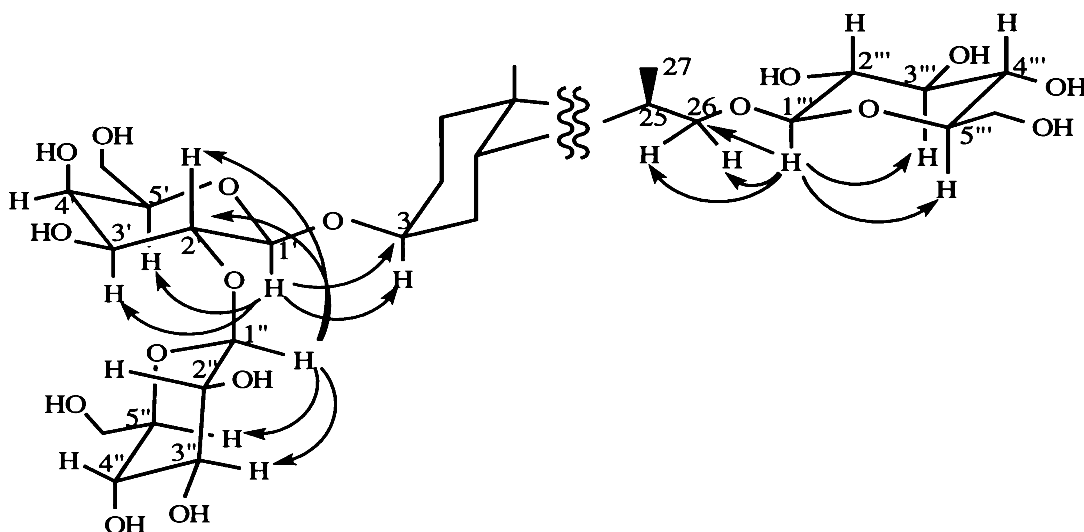


Figure 4-6. Selected ROESY (^{1}H-^{1}H) and HMBC (^{1}H-^{13}C) correlations observed for asiaticioside (**4-23**).

Table 4-9. Selected ROESY correlations observed for asiaticioside (**4-23**) (ppm in C_5D_5N).

irradiated signal	correlated signals
5.29 (H-1")	4.66 (H-2'), 4.20 (H-3"), 3.86 (H-5")
4.92 (H-1')	4.29 (H-3), 4.26 (H-3'), 4.02 (H-5')
4.79 (H-1"')	4.00 (H-26b), 3.46 (H-26a), 4.20 (H-3"'), 3.90 (H-5"')

Table 4-10. Selected HMBC correlations observed for asiaticioside (**4-23**) (ppm in C_5D_5N).

^{1}H signal	correlated ^{13}C signal(s)
5.29 (H-1")	81.9 (C-2')
4.92 (H-1')	75.4 (C-3)
4.79 (H-1"')	75.2 (C-26)
4.66 (H-2')	106.1 (C-1"), 102.6 (C-1'), 75.5 (C-3')
1.30 (H-21)	110.6 (C-22), 64.1 (C-17), 40.7 (C-20)
1.01 (H-27)	75.2 (C-26), 34.4 (C-25), 28.4 (C-24)
0.97 (H-19)	40.3 (C-9), 37.0 (C-5), 35.3 (C-10), 31.0 (C-1)
0.86 (H-18)	64.1 (C-17), 56.5 (C-14), 41.3 (C-13), 40.4 (C-12)

The HOHAHA spectrum of **4-23** included correlations between the anomeric glycosidic protons (H-1) and the H-2, H-3, H-4, H-5 and H-6 resonances within each of the sugar ring systems (Table 4-11).

Table 4-11. Selected HOHAHA correlations observed for asiaticioside (**4-23**) (ppm in C_5D_5N).

irradiated signal	correlated signals
4.92 (H-1')	4.66 (H-2'), 4.56 (H-4'), 4.41 (H-6'), 4.26 (H-3'), 4.02 (H-5')
5.29 (H-1")	4.47 (H-6"), 4.32 (H-4"), 4.20 (H-3"), 4.07 (H-2"), 3.86 (H-5")
4.79 (H-1"')	4.52 (H-6"'b), 4.35 (H-6"'a), 4.20 (H-3"' and H-4"'),
	3.99 (H-2"'), 3.90 (H-5"')

The negative ion ES-MS of **4-23** included a pseudomolecular ion at *m/z* 919.8 [$(M-H)^-$].

The ES-MS and one- and two-dimensional NMR data determined for **4-23** showed it to be (25*S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside.

While NMR data (chemical shifts, coupling constants and ROESY correlations) readily identifies the glycosyl units it does not distinguish D- or L-forms. The configurations of the sugar residues presented here for **4-13** and **4-15**, and implied for **4-22, 4-23, 4-24** and **4-17**, are based on the findings of Stabursvik (1959), Ceh and Hauge (1981), Kobayashi et al. (1993) and Inoue et al. (1995) that D- for glucosyl and galactosyl units and L- for arabinosyl unit (rather than D-analogues) occur in *N. asiaticum* and *N. ossifragum.*

The conclusion that saponins **4-13, 4-15, 4-22, 4-23, 4-24** and **4-17** are constituents of *N. ossifragum* is consistent with the earlier investigations of Stabursvik (1954, 1959) and Ceh and Hauge (1981). The results presented here, and those of Berndt (1997), confirm the proposal of Inoue et al. (1995) that narthecin (**4-13**) occurs in *N. ossifragum* as well as in *N. asiaticum*, and show that asiaticin (**4-15**) is also a common constituent of the 1-butanol extracts of these plants.

4.3 *Panicum virgatum*

4.3.1 Introduction

P. virgatum (swichgrass) has been reported to be implicated in photosensitization of sheep in the United States (Puoli et al., 1992). In a preliminary investigation, Berndt (1997) concluded that diosgenin and yamogenin saponins (ca 10:1) were present in *P. virgatum*, and that the molecular weight of these saponins was 1194 daltons. Berndt (1997) also noted that this molecular weight corresponded to those of the *25R*- and 25*S*-furostanol saponins previously identified as constituents of *P. dichotomiflorum* (Munday et al., 1993b) and *P. coloratum* (Wilkins, pers commun) respectively.

Diosgenin (**4-5**) and yamogenin (**4-6**) have been found to be the principle sapogenin constituents of saponins from some other *Panicum* species, including *P. dichotomiflorum* (Miles et al., 1991; Miles et al., 1992b; Munday et al., 1993b), *P. coloratum* (Patamalai et al., 1990; Wilkins, pers commun), and *P. miliaceum* (Miles et al., 1993). Munday et al. (1993) have reported the isolation and structure elucidation of a diosgenin-derived

furostenol saponin, dichotomin, from *P. dichotomiflorum* (Figure 4-7). A 25*S*- analogue of dichotomin, for which the trivial designation coloratin has been proposed, has been isolated from an Australian collection of *P. coloratum* (Wilkins, pers commun) (Figure 4-7).

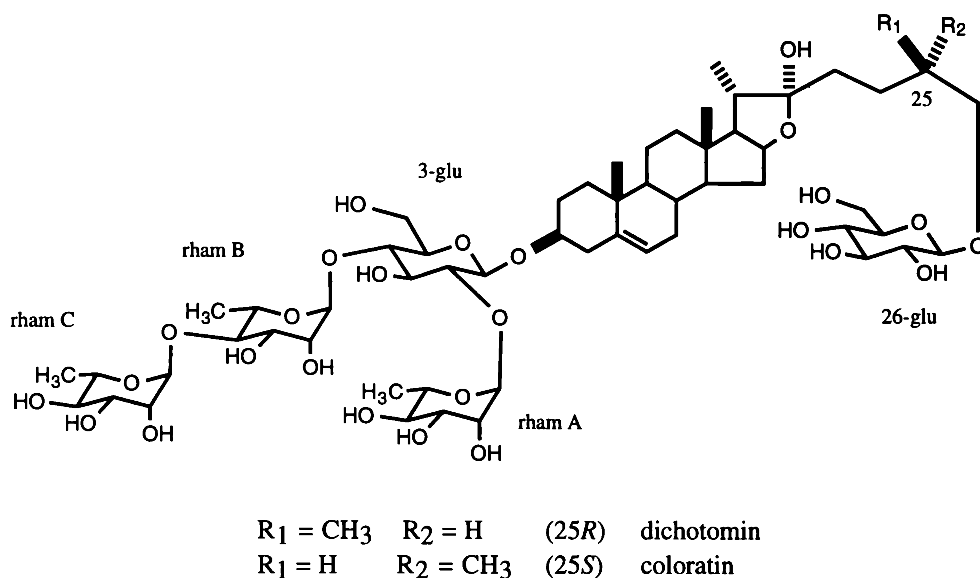


Figure 4-7. Structures of dichotomin (Munday et al., 1993b) and coloratin (Wilkins, pers commun).

In this section, levels of free and conjugated sapogenins in 13 USA collections of *P. virgatum,* and the identification of dichotomin as the principal furostenol saponin of the *P. virgatum* samples are reported.

4.3.2 Results and Discussion

Quantitative analysis

Analyses of the sapogenin and sterol constituents of the hexane and hydrolysed ethanol-water extracts of *P. virgatum,* collected by Dr R. L. Reid from sites in the vicinity of Morgantown, WV, USA in 1993, were performed using the total ion chromatograms (TIC) and selected ion mode (SIM) GC-MS (Wilkins et al., 1994) (see Section 5.1.2).

GC-MS analyses revealed the presence in the *P. virgatum* samples of diosgenin (**4-5**) and yamogenin (**4-6**) in a ratio of ca 10:1. Dehydrated forms of diosgenin and yamogenin were also detected in all of the acid hydrolysates. Dehydration of diosgenin-derived saponins during acid hydrolysis, to produce spirosta-3,5-diene, has been reported

previously (de Kock and Enslin, 1958). Peak identifications were confirmed using *m/z* 139, 282 and 396 ion ratio data (Wilkins et al., 1994).

The levels of free and conjugated forms of sterols, diosgenin (**4-5**) and yamogenin (**4-6**), including dehydrated diosgenin and yamogenin, identified in the *P. virgatum* samples are presented in Table 4-12.

Table 4-12. Levels (mg/kg) of sapogenins and sterols in the free extracts and the conjugated extracts from USA *P. virgatum* samples.

date	sample (code)	extract	dio	yam	chol	stig	sito
31-5-91	*P. virgatum* (93/7)	free	-	-	284	247	419
		conj	175	15	260	275	452
27-6-91	*P. virgatum* (93/5)	free	-	-	135	197	292
		conj	25	-	50	77	126
2-7-91	*P. virgatum* (93/1)	free	-	-	116	146	234
		conj	177	31	32	37	77
16-6-92	*P. virgatum* + clover (93/10)	free	-	-	177	210	228
		conj	198	21	74	100	150
16-6-92	*P. virgatum* + fert (93/8)	free	-	-	176	239	251
		conj	85	4.9	77	128	173
16-6-92	cool season grass (93/11)	free	-	-	177	210	228
		conj	-	-	7.6	36	162
25-6-92	*P. virgatum* + clover (93/4)	free	-	-	167	211	206
		conj	218	21	94	157	235
25-6-92	*P. virgatum* + fert (93/13)	free	-	-	132	162	161
		conj	115	19	49	51	74
25-6-92	cool season grass (93/3)	free	-	-	13	81	293
		conj	-	-	20	63	298
30-7-92	*P. virgatum* + clover (93/9)	free	-	-	143	197	195
		conj	113	9.8	61	96	124
30-7-92	*P. virgatum* + fert. (93/2)	free	-	-	142	207	195
		conj	102	16	63	108	132
10-8-92	*P. virgatum* + clover (93/12)	free	-	-	126	157	162
		conj	288	42	65	96	139
10-8-92	*P. virgatum* + fert. (93/6)	free	-	-	160	21	204
		conj	43	4.2	106	176	205

conj = conjugated extract, free = free extract, chol = cholesterol, stig = stigmasterol, site = β-sitosterol, dio = diosgenin, yam = yamogenin

In all cases only conjugated sapogenins (saponins) were detected in the plant samples (Table 4-12). Variable levels of saponins were detected in the 13 samples. The highest levels of saponins (329 mg/kg in sample 93/12 and 238 mg/kg in sample 93/4) were present in samples collected at times which corresponded with photosensitization outbreaks.

Identification of saponins

Separation of the crude saponin extract, recovered after 1-butanol-water partition of the ethanol-water extract of dried *P. virgatum*, on a silica gel column using mixtures of chloroform and aqueous methanol as eluent, afforded a specimen of a 25*R/S*-saponin which was identified as (25*R/S*)-furost-5-ene-3β,22α,26-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranoside (**4-25**) [25*R*-isomer, dichotomin, (Munday et al., 1993b) and 25*S*-isomer, coloratin, (Wilkins, pers commun)].

The ^{1}H NMR spectrum of **4-25** included three anomeric proton signals at 6.32 (br s), 6.21 (br s) and 5.77 ppm (br s), five doublet methyl proton signals at 1.73 (d, J = 6.2 Hz), 1.56 (d, J = 5.8 Hz), 1.54 (d, J = 5.7 Hz), 1.30 (d, J = 6.8 Hz) and 0.96 ppm (d, J = 6.7 Hz), and two singlet methyl proton signals at 1.03 ppm (s) and 0.87 ppm (s). The doublet signals at 1.73, 1.56 and 1.54 ppm were due to the methyl groups of 6-deoxyhexoses. The COSY and HOHAHA spectra revealed five sugar units, amongst which were two anomeric protons at 4.92 (d, J = 7.7 Hz) and 4.78 ppm (d, J = 7.9 Hz) concealed by peak overlap in the ^{1}H NMR spectrum.

The (25*R/S*)-furost-5-ene skeleton of **4-25** was revealed by the ^{13}C resonances at 110.6 ppm (C-22) (open ring F structure), 140.7 ppm (C-5) and 121.7 ppm (C-6) [5(6)-double bond] (Agrawal et al., 1985), and by the ^{1}H signals at 3.94 ppm (H-26a) and 3.63 ppm (H-26b) (25*R*-isomer) and at 4.07 ppm (H-26a) and 3.38 ppm (H-26b) (25*S*-isomer).

A complete assignment of the ^{1}H and ^{13}C NMR signals of dichotomin was achieved in a manner similar to that described by Munday et al. (1993).

The ^{13}C NMR resonances of the dominant 25*R*-isomer of **4-25** corresponded closely to those reported by Munday et al. (1993) for a specimen of dichotomin, isolated from *P. dichotomiflorum* (Table 4-13 and 4-14). The much lower level peaks attributable to a 25*S*-isomer in the sample could be seen in the ^{1}H, COSY and HSQC spectra.

Table 4-13. ^{13}C NMR chemical shifts (ppm in C_5D_5N) for the aglycone atoms of saponin **4-25** isolated from *P. virgatum.*

No	dichotomin[a] (25*R*, open ring F)	coloratin[b] (25*S*, open ring F)	(*2*)[a] (25*R*, closed ring F)	**4-25**
1	37.5	37.5	37.3	37.3
2	30.2	30.1	30.0	30.0
3	78.2	78.0	78.0	78.2
4	38.9	38.9	38.8	38.8
5	140.8	140.8	140.6	140.7
6	121.9	121.8	121.6	121.7
7	32.4	32.4	32.1	32.3
8	31.6	31.7	31.5	31.5
9	50.3	50.3	50.1	50.2
10	37.3	37.1	37.0	37.4
11	21.1	21.1	20.9	21.0
12	39.9	39.9	39.7	39.8
13	40.8	40.8	40.0	40.7
14	56.6	56.6	56.5	56.4
15	32.1	32.3	32.0	32.2
16	81.1	81.1	80.9	81.0
17	63.6	63.7	62.7	63.6
18	16.4	16.4	16.2	16.3
19	19.4	19.4	19.2	19.2
20	40.6	40.7	41.8	40.5
21	16.4	16.4	14.9	16.2
22	110.8	110.7	109.1	110.6
23	37.1	37.1	31.7	36.9
24	28.3	28.3	29.1	28.2
25	34.2	34.4	30.4	34.1
26	75.3	75.3	66.7	75.1
27	17.4	17.5	17.2	17.3

[a] reported by Munday et al. (1993b)

[b] reported by Wilkins (pers commun)

Table 4-14. ^{13}C and ^{1}H chemical shifts (ppm in C_5D_5N) for the glycoside atoms of saponin **4-25** isolated from *P. virgatum*.

	dichotomin[a] (25*R*, open ring F)		coloratin[b] (25*S*, open ring F)		*(2)*[a] (25*R*, closed ring F)		**4-25**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
3-glu								
1'	100.3	4.91	100.3	4.93	100.1	4.95	100.2	4.92
2'	78.2	4.15	78.5	4.20	77.8	4.21	77.7	4.17
3'	77.6	4.15	77.7	4.20	77.5	4.22	77.5	4.17
4'	78.0	4.32	78.1	4.38	77.6	4.40	77.8	4.34
5'	76.9	3.60	77.0	3.56	76.8	3.62	76.7	3.60
6'	61.2	4.04	61.2	4.04	61.0	4.05	61.1	4.03
		4.16		4.20		4.20		4.19
rham A								
1''	102.2	6.27	102.2	6.37	102.0	6.39	102.0	6.32
2''	72.4	4.80	72.5	4.83	72.3	4.85	72.3	4.81
3''	72.8	4.59	72.8	4.62	72.7	4.63	72.6	4.60
4''	74.0	4.33	73.9	4.34	73.9	4.37	73.9	4.33
5''	69.7	4.86	69.5	4.94	69.4	4.94	69.3	4.91
6''	18.6	1.72	18.6	1.72	18.5	1.78	18.4	1.73
rham B								
1'''	102.2	5.72	102.2	5.78	102.2	5.83	102.1	5.77
2'''	72.8	4.52	72.8	4.52	72.7	4.56	72.6	4.54
3'''	73.1	4.48	73.2	4.52	73.2	4.56	73.0	4.50
4'''	80.3	4.36	80.3	4.42	80.2	4.44	80.2	4.39
5'''	68.4	4.78	68.3	4.91	68.2	4.92	68.1	4.84
6'''	18.8	1.52	18.8	1.52	18.7	1.59	18.7	1.54
rham C								
1''''	103.2	6.18	103.2	6.25	103.1	6.28	103.1	6.21
2''''	72.5	4.85	72.6	4.89	72.4	4.90	72.4	4.86
3''''	72.8	4.44	72.8	4.49	72.7	4.52	72.6	4.47
4''''	73.8	4.27	73.9	4.30	73.8	4.31	73.7	4.27
5''''	70.4	4.25	70.4	4.33	70.2	4.36	70.2	4.29
6''''	18.4	1.54	18.4	1.54	18.3	1.60	18.2	1.56
26-glu								
1'''''	104.8	4.77	105.1	4.76			104.8	4.78
2'''''	75.1	3.99	75.2	3.98			75.0	3.99
3'''''	78.4	4.24	78.4	4.19			78.3	4.20
4'''''	71.6	4.16	71.7	4.19			71.5	4.20
5'''''	78.3	3.92	77.9	3.91			78.2	3.92
6'''''	62.7	4.31	62.8	4.35			62.6	4.35
		4.50		4.56				4.50

[a] reported by Munday et al. (1993b)

[b] reported by Wilkins (pers commun)

In the HMBC spectrum of **4-25** (Table 4-15 and Figure 4-8), correlations between the anomeric proton signals and the carbon signals at 6.32 ppm (H-1'', rham A) to 77.7 ppm (C-2', 3-glu), 6.21 ppm (H-1'''', rham C) to 80.2 ppm (C-4''', rham B), 5.77 ppm (H-1''', rham B) to 77.8 ppm (C-4', 3-glu), 4.92 ppm (H-1', 3-glu) to 78.2 ppm (C-3, genin), and 4.78 ppm (H-1''''', 26-glu) to 75.1 ppm (C-26, genin) revealed the linkages of the sugar units and genin (Figure 4-8). The ROESY spectrum of **4-25** also verified the linkages, including the correlations of H-1' to H-3, H-1'' to H-2', H-1''' to H-4', H-1'''' to H-4''' and H-1''''' to H-26 (see Table 4-16 and Figure 4-8).

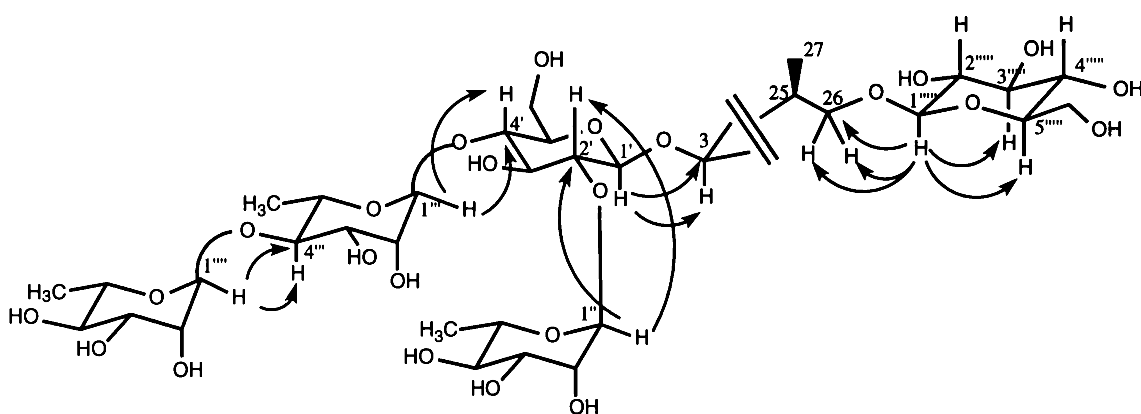


Figure 4-8. Selected ROESY (^{1}H-^{1}H) and HMBC (^{1}H-^{13}C) correlations observed for saponin **4-25**.

Table 4-15. Selected HMBC correlations observed for saponin **4-25** (ppm in C_5D_5N).

^{1}H signal	correlated ^{13}C signal(s)
6.32 (H-1'')	77.7 (C-2'), 72.6 (C-3''), 72.3 (C-2''), 69.3 (C-5'')
6.21 (H-1'''')	80.2 (C-4'''), 72.5 (C-3'''' and C-2''''), 70.2 (C-5'''')
5.77 (H-1''')	77.8 (C-4'), 73.0 (C-3''' and C-2'''), 68.1 (C-5''')
4.92 (H-1')	78.2 (C-3)
4.78 (H-1''''')	75.1 (C-26)
1.73 (H-6'')	69.3 (C-5''), 73.9 (C-4'')
1.56 (H-6'''')	70.2 (C-5''''), 73.7 (C-4'''')
1.54 (H-6''')	68.1 (C-5'''), 80.2 (C-4''')
1.30 (H-21)	110.6 (C-22), 63.6 (C-17), 40.5 (C-20)
1.03 (H-19)	140.7 (C-5), 50.2 (C-9), 37.4 (C-10), 37.3 (C-1)
0.96 (H-27)	75.1 (C-26), 34.1 (C-25), 28.2 (C-24)
0.87 (H-18)	63.6 (C-17), 56.4 (C-14), 40.7 (C-13), 39.8 (C-12)

Table 4-16. Selected ROESY correlations observed for **4-25** (ppm in C_5D_5N).

irradiated signal	correlated signals
4.92 (H-1')	4.17 (H-3'), 3.86 (H-3), 3.60 (H-5')
6.32 (H-1")	4.17 (H-2')
5.77 (H-1"')	4.34 (H-4')
6.21 (H-1"")	4.39 (H-4"')
4.78 (H-1""')	4.20 (H-3""'), 3.92 (H-5""' and H-26a), 3.62 (H-26b)

The negative ion ES-MS of **4-25** included pseudomolecular ions at *m/z* 1229 [(M+Cl)$^-$], 1193 [(M-H)$^-$], and fragment ions at *m/z* 1047 (loss of one rhamnose unit from a compound of molecular weight 1194 daltons), 753 (loss of three rhamnose unit from a compound of molecular weight 1194). The positive ion ES-MS of **4-25** showed a pseudomolecular ion at *m/z* 1217 (addition of sodium ion to a compound of molecular weight 1194 daltons).

Because of some differences in the ^{1}H NMR chemical shifts presented in Tables 4-13 and 4-14 for dichotomin, compared to those reported by Munday et al. (1993), possibly attributable to variations in the water content of the NMR solvent and/or the determination of the published NMR spectral data at a unspecified temperature (40 or 50°C?), a series of ^{1}H NMR experiments were performed to determine if the chemical shifts were sensitive to temperature.

The temperature dependency of the chemical shifts of water, anomeric protons, rhamnose methyl protons and each of the angular genin methyl protons of **4-25** is presented in Figure 4-9.

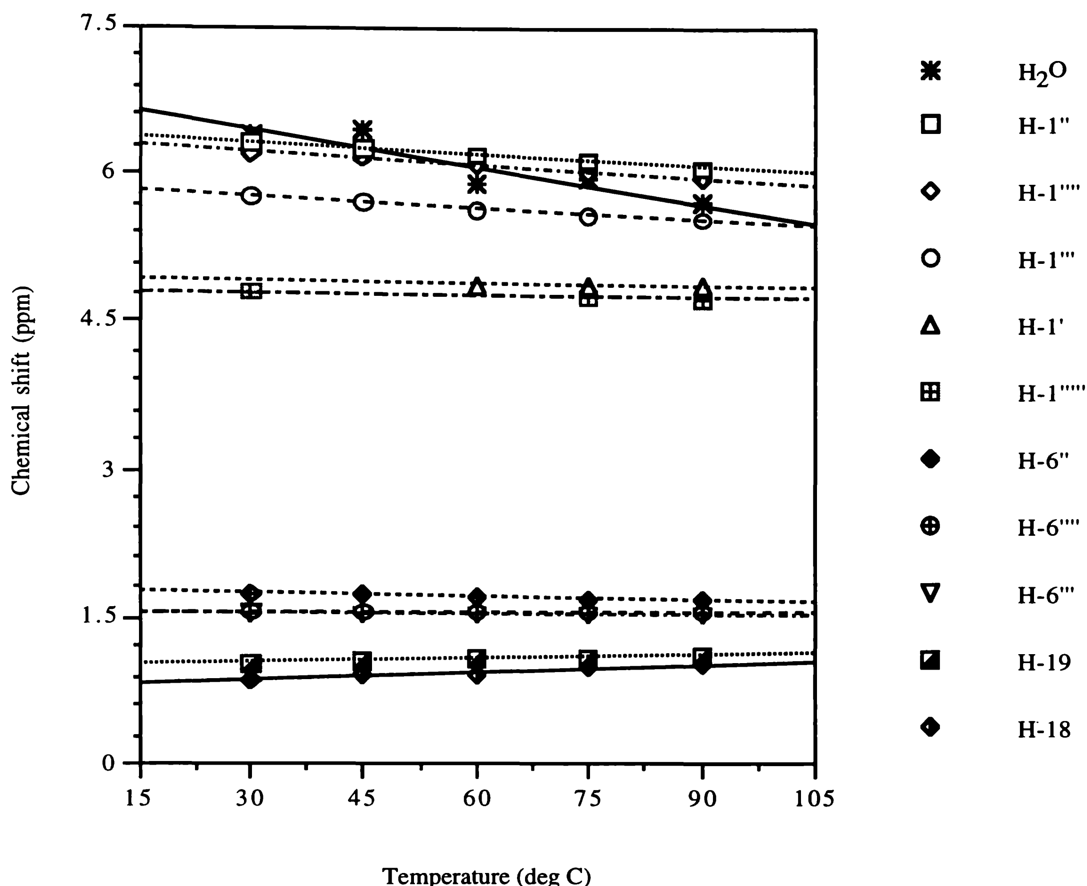


Figure 4-9. Temperature dependency of the chemical shifts of selected ^{1}H NMR signals of **4-25**.

As the temperature increased low field glycosyl protons generally exhibited upfield shifts, while high field genin signals generally exhibited downfield shifts. For example, the resonance of anomeric proton H-1'' shifted from 6.33 ppm at 30°C to 6.07 ppm at 90°, whereas the resonance of the angular methyl protons (H-18) increased from 0.87 ppm at 30°C to 1.01 ppm at 90°C.

The chemical shift of the water peak, which occurred at 6.3-6.5 ppm in the 30°C ^{1}H NMR spectrum, decreased more quickly than saponin proton chemical shifts as the probe temperature was raised. The water signal was shifted to 5.71 ppm at 90°C. The variable temperature analyses showed that no glycosyl proton resonances were concealed under the broad water peak in the standard 30°C ^{1}H NMR spectrum.

4.4 *Panicum miliaceum*

4.4.1 Introduction

P. miliaceum is one of the plants which associated with occasional outbreaks of hepatogenous photosensitization of ruminants (Clare, 1952; Holland et al., 1991; Miles et al., 1991; Miles et al., 1992a, Miles et al., 1992b). Hydrolysed extract of this plant has been reported to contain two sapogenins, diosgenin (**4-5**) and yamogenin (**4-6**) in a 4:1 ratio (Miles et al., 1993). No reports of the saponin constituents in this plant have appeared in the literature.

In this work, quantitative analyses of the free and conjugated sapogenin components in a *P. miliaceum* sample (stem and seed) collected from a domestic garden, Hamilton, New Zealand in 1994, were performed. Two saponins were isolated from the sample and identified by electrospray mass spectral (ES-MS) and one- and two-dimensional NMR spectral data.

4.4.2 Results and Discussion

Quantitative analysis

GC-MS analyses of hexane extracts and ethanol-water extracts were performed by the method of Wilkins et al. (1994), using selected ion mode (SIM) GC-MS detection (see Section 5.1.2). The results are presented in Table 4-17.

High levels of diosgenin (**4-5**) and yamogenin (**4-6**) (127 and 33 mg/kg respectively) were found in the hydrolysed conjugated stem extract (Table 4-17). The ca 4:1 ratio of **4-5** to **4-6** is close to that reported by Miles et al. (1993) for another *P. miliaceum* sample. Lower levels of **4-5** and **4-6** (69 and 5 mg/kg respectively) appeared in the hydrolysed conjugated seed extract. The detection of **4-5** and **4-6** in the hydrolysed conjugated extracts is consistent with presence of either, or both, of 25*R/S*-spirostenol (diosgenin and yamogenin) and 25*R/S*-furostenol saponins in the conjugated extracts.

The sapogenins in the samples were mainly present in conjugated forms, rather than as free sapogenins, e.g. 127 mg/kg of conjugated **4-5** compared to 12 mg/kg of free **4-5** were found in the stem sample, and no free sapogenins were detected in the seed sample.

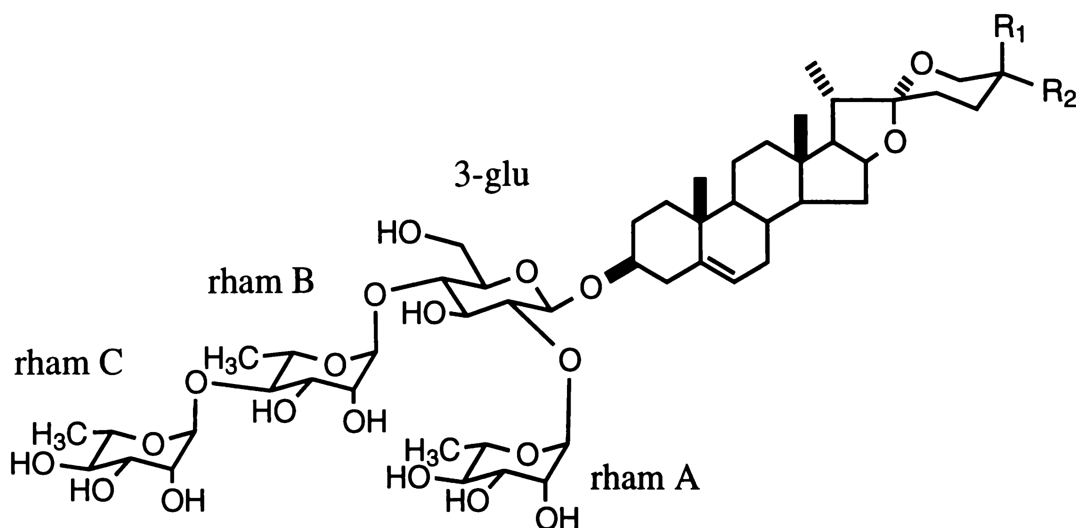
Table 4-17. Levels (mg/kg) of sapogenins and sterols in the free and conjugated extracts from New Zealand *P. miliaceum* samples.

		chol	camp	stig	sito	dio	yam
seeds	conj	54.7	34.3	86.5	191	69.0	5.2
	free	51.5	25.4	58.5	128	0	0
stems	conj	39.2	16.9	57.6	94.7	127	33.2
	free	68.7	29.0	79.0	138	11.7	3.8

conj = conjugated extract, free = free extract, chol = cholesterol, camp = campesterol, stig = stigmasterol, site = β-sitosterol, dio = diosgenin, yam = yamogenin

Identification of saponins

Fractionation of the ethanol-water extract, obtained from the dried stems of *P. miliaceum* by column chromatography on silica gel, using mixtures of chloroform and aqueous methanol as eluent, afforded two saponins which were identified as (25*R/S*)-furost-5-ene-3β,22α,26-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranoside (**4-25**) [25*R*-isomer, dichotomin, (Munday et al., 1993b) and 25*S*-isomer, coloratin, (Wilkins, pers commun)] and (25*R/S*)-spirost-5-en-3β-ol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside} (**4-26**) (Figure 4-10).



4-26 (25*R*: R_1 = H, R_2 = CH_3 , 25*S*: R_1 = CH_3, R_2 = H)

Figure 4-10. Structure of saponin **4-26** isolated from *P. miliaceum*.

Structure elucidation of saponin 4-25

Compound **4-25** was obtained as a yellowish amorphous powder. The positive ion ES-MS of **4-25** at 100 V using CH_3CN-H_2O (1:1) as solvent showed a pseudomolecular ion at *m/z* 1233 (addition of potassium to a compound of molecular weight 1194 daltons).

The ^{1}H NMR spectrum of **4-25** showed four anomeric proton signals at 6.37 (br s, H-1''), 6.26 (br s, H-1''''), 5.81 (br s, H-1'''), and 4.81 ppm (d, *J* = 7.8 Hz, H-1'''''), three CH_3-CH proton signals at 1.76 (d, *J* = 6.1 Hz, H-6''), 1.59 (d, *J* = 6.6 Hz, H-6''''), 1.58 (d, *J* = 5.9 Hz, H-6'''), and four angular methyl protons at 0.90 ppm (s, H-18), 0.99 ppm (d, *J* = 6.7 Hz, H-27, 25*R*) / 1.03 ppm (d, *J* = 6.8 Hz, H-27, minor 25*S*), 1.06 (s, H-19) and 1.33 (d, *J* = 6.5 Hz, H-21). The H-1' glucosyl signal was not observed in the ^{1}H NMR spectrum due to overlap with other signals. However, HMBC, HMQC and COSY spectra showed that a doublet methine proton signal occurred at 4.95 ppm.

The ^{13}C NMR spectrum of **4-25** (Table 4-18 and 4-19) contained 7 methyl, 12 methylene, 34 methine and 4 quaternary carbons. The resonance of C-22 (110.7 ppm) indicated **4-25** to be a open ring F sapogenin (Agrawal et al., 1985). The ^{13}C NMR data showed **4-25** to be a furost-5-en-3-ol possessing two glucopyranosyl and three rhamnopyranosyl units. The ^{13}C NMR signals of **4-25** corresponded closely to those reported by Munday et al. (1993) for the 25*R*-furost-5-ene tetrasaccharide, dichotomin isolated from *P. dichotomiflorum*, and those determined by Wilkins (pers commun) for coloratin (the 25*S*-isomer of dichotomin).

Detailed analyses of the one- and two-dimensional COSY, HSQC, HMBC and ROESY spectral data confirmed saponin **4-25** to be a 4:1 mixture of (25*R/S*)-furost-5-ene-3β,22α,26-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranoside, a specimen of which was also isolated from *P. virgatum* (see Section 4.3).

A complete assignment of the proton and carbon resonances of the dominant 25*R* component of saponin **4-25** is given in Table 4-18, and can be compared with those determined for a specimen of saponin **4-25** isolated from *P. virgatum*.

Table 4-18. ^{13}C NMR chemical shifts (ppm in C_5D_5N) for the aglycone atoms of saponins **4-25** and **4-26** isolated from *P. miliaceum*.

No	dichotomin[a]	coloratin[b]	(2)[a]	**4-25**	**4-26**
1	37.5	37.5	37.3	37.5	37.5
2	30.2	30.1	30.0	30.2	30.1
3	78.2	78.0	78.0	78.1	78.1
4	38.9	38.9	38.8	40.0	38.9
5	140.8	140.8	140.6	140.8	140.8
6	121.9	121.8	121.6	121.8	121.8
7	32.4	32.4	32.1	32.5	32.3
8	31.6	31.7	31.5	31.7	31.7
9	50.3	50.3	50.1	50.4	50.3
10	37.3	37.1	37.0	37.1	37.1
11	21.1	21.1	20.9	21.1	21.1
12	39.9	39.9	39.7	39.9	39.8
13	40.8	40.8	40.0	40.8	40.4
14	56.6	56.6	56.5	56.6	56.6
15	32.1	32.3	32.0	32.3	32.2
16	81.1	81.1	80.9	81.1	81.1
17	63.6	63.7	62.7	63.8	62.9
18	16.4	16.4	16.2	16.5	16.3
19	19.4	19.4	19.2	19.4	19.4
20	40.6	40.7	41.8	40.7	42.0
21	16.4	16.4	14.9	16.4	15.0
22	110.8	110.7	109.1	110.7	109.3
23	37.1	37.1	31.7	37.1	31.8
24	28.3	28.3	29.1	28.3	29.3
25	34.2	34.4	30.4	34.3	30.5
26	75.3	75.3	66.7	75.2	66.9
27	17.4	17.5	17.2	17.5	17.3

[a] reported by Munday et al. (1993b)

[b] reported by Wilkins (pers commun)

Table 4-19. ^{13}C and ^{1}H chemical shifts (ppm in C_5D_5N) for the glycoside atoms of saponins **4-25** and **4-26** isolated from *P. miliaceum*.

	dichotomin[a]		coloratin[b]		(*2*)[a]		**4-25**		**4-26**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
3-glu										
1'	100.3	4.91	100.3	4.93	100.1	4.95	100.4	4.95	100.3	4.96
2'	78.2	4.15	78.5	4.20	77.8	4.21	78.0	4.21	78.0	4.21
3'	77.6	4.15	77.7	4.20	77.5	4.22	77.7	4.22	77.7	4.21
4'	78.0	4.32	78.1	4.38	77.6	4.40	77.9	4.38	77.7	4.39
5'	76.9	3.60	77.0	3.56	76.8	3.62	77.0	3.62	77.0	3.62
6'	61.2	4.04	61.2	4.04	61.0	4.05	61.3	4.04	61.2	4.05
		4.16		4.20		4.20		4.16		4.20
rham A										
1''	102.2	6.27	102.2	6.37	102.0	6.39	102.1	6.37	102.1	6.37
2''	72.4	4.80	72.5	4.83	72.3	4.85	72.5	4.84	72.5	4.84
3''	72.8	4.59	72.8	4.62	72.7	4.63	72.8	4.63	72.8	4.63
4''	74.0	4.33	73.9	4.34	73.9	4.37	74.1	4.35	74.1	4.35
5''	69.7	4.86	69.5	4.94	69.4	4.94	69.5	4.94	69.5	4.95
6''	18.6	1.72	18.6	1.72	18.5	1.78	18.6	1.76	18.6	1.76
rham B										
1'''	102.2	5.72	102.2	5.78	102.2	5.83	102.2	5.81	102.2	5.82
2'''	72.8	4.52	72.8	4.52	72.7	4.56	72.8	4.55	72.8	4.55
3'''	73.1	4.48	73.2	4.52	73.2	4.56	73.2	4.55	73.3	4.55
4'''	80.3	4.36	80.3	4.42	80.2	4.44	80.4	4.42	80.4	4.43
5'''	68.4	4.78	68.3	4.91	68.2	4.92	68.3	4.89	68.3	4.91
6'''	18.8	1.52	18.8	1.52	18.7	1.59	18.8	1.58	18.9	1.58
rham C										
1''''	103.2	6.18	103.2	6.25	103.1	6.28	103.2	6.26	103.3	6.26
2''''	72.5	4.85	72.6	4.89	72.4	4.90	72.6	4.88	72.6	4.89
3''''	72.8	4.44	72.8	4.49	72.7	4.52	72.8	4.49	72.8	4.50
4''''	73.8	4.27	73.9	4.30	73.8	4.31	74.0	4.28	74.0	4.29
5''''	70.4	4.25	70.4	4.33	70.2	4.36	70.4	4.34	70.4	4.35
6''''	18.4	1.54	18.4	1.54	18.3	1.60	18.4	1.59	18.4	1.59
26-glu										
1'''''	104.8	4.77	105.1	4.76			104.9	4.81		
2'''''	75.1	3.99	75.2	3.98			75.2	4.01		
3'''''	78.4	4.24	78.4	4.19			78.6	4.22		
4'''''	71.6	4.16	71.7	4.19			71.7	4.22		
5'''''	78.3	3.92	77.9	3.91			78.4	3.93		
6'''''	62.7	4.31	62.8	4.35			62.8	4.38		
		4.50		4.56				4.53		

[a] reported by Munday et al. (1993b)

[b] reported by Wilkins (pers commun)

Structure elucidation of saponin 4-26

The ^{1}H NMR spectrum of compound **4-26** included signals attributable to the presence of three equatorally α-oriented rhamnosyl anomeric protons at 6.37 (br s), 6.26 (br s) and 5.82 ppm (br s), and an axially β-oriented glucosyl anomeric protons at 4.96 ppm (d, J = 7.8 Hz).

The ^{13}C NMR spectrum of **4-26** consisted of 7 methyl, 11 methylene, 29 methine and 4 quaternary carbon signals, and the total of 51 carbon signals. These data corresponded closely to those reported by Munday et al. (1993) for a specimen of the saponin tetrasaccharide (*2*) obtained by enzymatic hydrolysis of **4-25**.

The ^{13}C NMR spectrum of **4-26** included a signal at 109.3 ppm (C-22), indicative of a diosgenin-type saponin (closed ring F) rather than a furost-5-ene saponin (open ring F) characterised by a signal at 110.2 ppm (Agrawal et al., 1985; Agrawal et al., 1995).

^{1}H and ^{13}C NMR spectral data indicated the 25*R*/25*S*-isomer in a ratio of approximately 4:1. COSY experiments also showed a major 25*R*-isomer pattern (3.52 ppm ↔ 3.59 ppm correlation between H-26a and H-26b) and a minor 25*S*-isomer pattern (3.38 ppm ↔ 4.07 ppm correlation between H-26a and H-26b).

A combination of the one- and two-dimensional COSY, HSQC, HMBC and ROESY spectral data led to the assignment of the ^{1}H and ^{13}C signals of **4-26** and the confirmation of the linkages of 1'-glucosyl to 3-*O*-genin, 1''-rhamnosyl to 2'-*O*-glucosyl, 1'''-rhamnosyl to 4'-*O*-rhamnosyl and 1''''-rhamnosyl to 4'''-*O*-rhamnosyl.

The negative ion ES-MS of **4-26** at -110 V using CH_3CN-H_2O (1:1) as solvent showed a pseudomolecular ion at *m/z* 1013 (loss of a proton from a compound of molecular weight 1014 daltons).

The ES-MS and one- and two-dimensional NMR data determined for **4-26** showed it to be (25*R/S*)-spirost-5-en-3β-ol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamno-pyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside}.

4.5 *Nolina texana*

4.5.1 Introduction

N. texana is a perennial desert shrub (family Liliaceae) growing over a wide area of the southwestern United States and northern Mexico. The buds, blossoms and seeds of this plant have been reported to be highly poisonous to sheep and goats (Tunnicliff, 1929; Mathews, 1937; Mathews, 1940; Hershey, 1945; Rankins et al., 1993).

Thus far no accounts of the saponin, or sapogenin, constituents of *N. texana* have appeared in the literature. However saponins have been reported as constituents of a related species, namely *N. recurvata.*

N. recurvata, a species found in Mexico, has been reported to contain a series of five hydroxylated spirosta-5,25(27)-diene saponins (**4-27** to **4-31**) (Figure 4-11) (Takaashi et al., 1995). All of these saponins are characterised by the presence of 3β- and 23-hydroxy groups and by 1β-glycosyl residues. Additionally, hydroxy and/or glycosyl residues (attached to C-24 and/or C-21) were identified in saponins **4-30** and **4-31** isolated by Takaashi et al. (1995) (Figure 4-11).

Subsequently, Mimaki et al. (1996) reported the characterisation of a series of eight ruscogenin and spirosta-5,25(27)-diene type-saponins (**4-32** to **4-39**), possessing closed or open (furostenol-type) ring F structures (Figure 4-11).

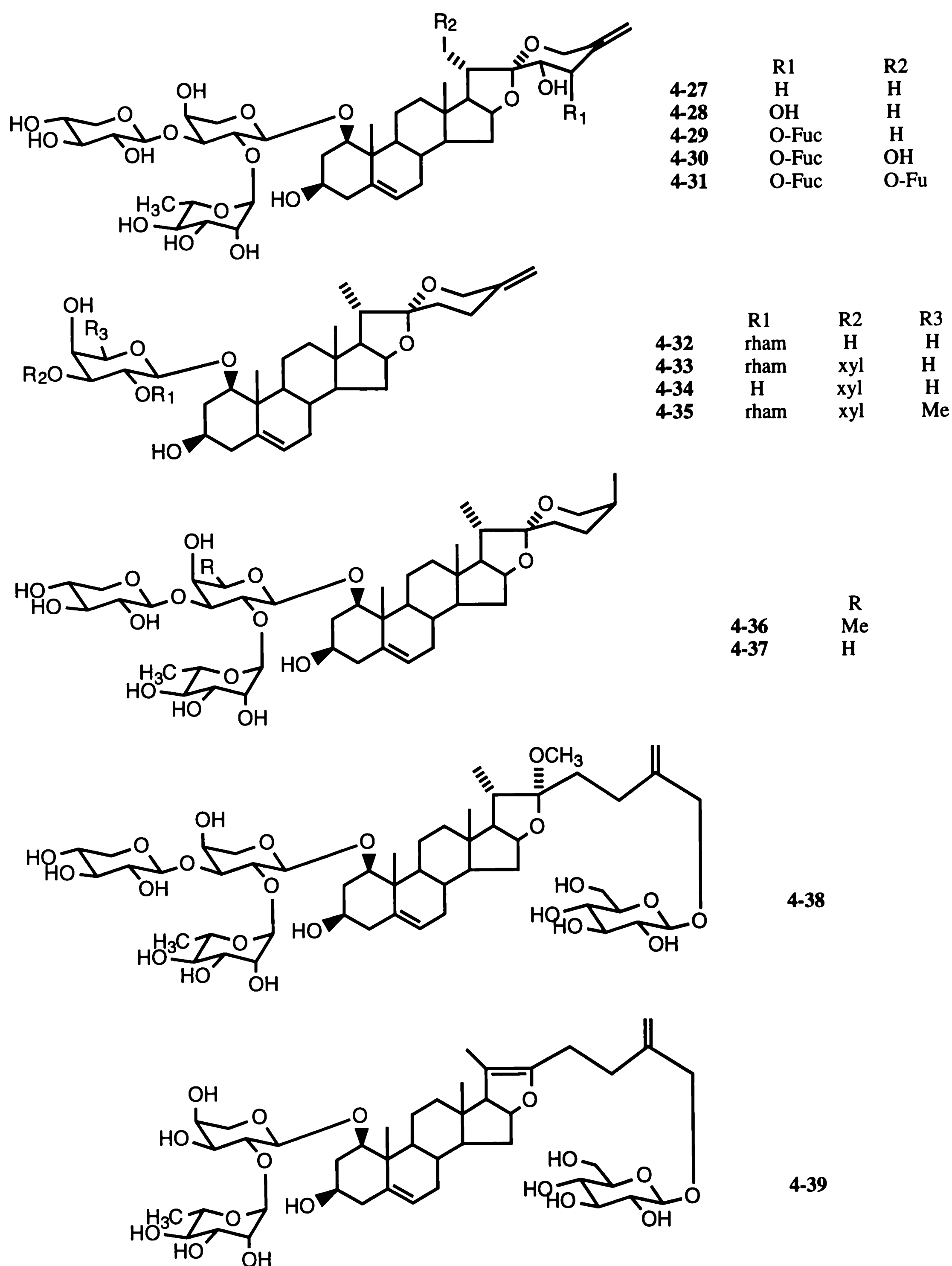


Figure 4-11. Structures of saponins isolated from *N. recurvata* by Takaashi et al. (1995) and Mimaki et al. (1996).

Some confusion exists in the literature about the trivial designation of sapogenins (and saponins) possessing sapogenins **4-12** and **4-40** (Figure 4-12). Some authors [e.g. Wilkins et al. (1994), Agrawal et al. (1985)] refer to **4-12** as neoruscogenin, whereas others [e.g. Takaashi et al. (1995), Mimaki et al. (1996)] refer to **4-40** as neoruscogenin. The former approach is analogous to the use of the neo- prefix to differentiate the 25*R*- and 25*S*- forms of tigogenin (**4-7**, **4-8**) and gitogenin (**4-9**, **4-10**), respectively (see figure 4-12), whereas the latter approach applies the neo- prefix to the 25(27)-ene (**4-40**), and **4-11** and **4-12** are consequentially trivially designated as ruscogenin and 25*S*-ruscogenin respectively.

In this document the approach, which designates **4-11**, **4-12** and **4-40** as ruscogenin, neoruscogenin and $\Delta^{25(27)}$-ruscogenin analogues respectively, is applied to all ruscogenin-type sapogenins and saponins, irrespective of the designations used in the original publications.

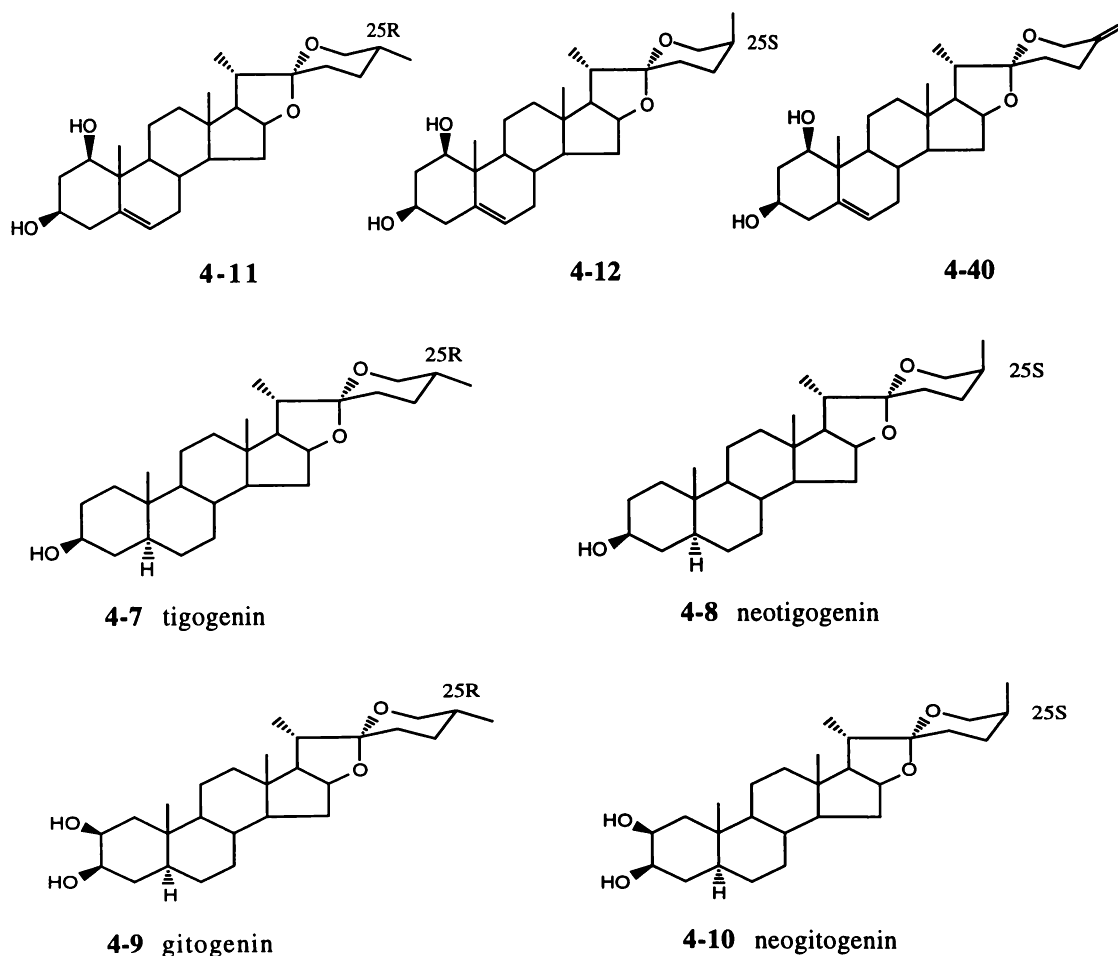


Figure 4-12. Structures of ruscogenin (**4-11**), neoruscogenin (**4-12**), $\Delta^{25(27)}$-ruscogenin (**4-40**), tigogenin (**4-7**), neotigogenin (**4-8**), gitogenin (**4-9**) and neogitogenin (**4-10**).

Little information is available about the saponin constituents of *N. mirocarpa*, other than that they constituted 4.6% of the material dosed in sheep trials (Rankins et al., 1993). Cheeke and Shull (1985) have stated that this level of saponins may be sufficient to cause heptatoxicoses.

In this work, quantitative analyses of free and conjugated sapogenins in a USA collection of *N. texana*, supplied by Professor Murl Bailey, College of Veterinary Medicine, Texas A&M University, and the characterisation of three sapogenins and three saponins from the sample using GC-MS (free sapogenins only), ES-MS and one- and two-dimensional NMR spectral data are described.

4.5.2 Results and Discussion

4.5.2.1 Characterisation of Sapogenin Constituents

Sequential extraction of the plant material with hexane and ethanol-water (4:1) afforded extracts containing free and conjugated sterols and sapogenins, respectively. Hydrolysis of the concentrated conjugated extracts for 2 hours at 85°C with 1 M HCl yielded the parent sterols and sapogenins. The identity and levels of sterols and sapogenins in the extracts was elucidated by TIC and SIM GC-MS analyses of the acetylated free and hydrolysed conjugated extracts (Section 5.1.2).

In addition to peaks attributable to the presence in the extracts of the common plant sterols, cholesterol (**4-18**), campesterol (**4-19**), stigmasterol (**4-20**) and β-sitosterol (**4-21**), three later eluting peaks (peak A, B, C) exhibited intense *m/z* 139 or 137 ions, suggestive of the presence of sapogenins, or ring F dehydro-sapogenins, respectively. The area ratio of these three peaks was ca 1:1.2:2.5.

The mass spectra of peaks A and B exhibited strong *m/z* 139, 280, 394 and 118 fragment ions, and were similar to those of specimens of ruscogenin and neoruscogenin which were available at the University of Waikato (Meagher, 1996). The mass spectrum of peak C (the later eluting peak) showed strong fragment ions at *m/z* 137, 280, 392 and 118.

The *m/z* 139 fragment ion (peaks A and B) can be attributed to the characteristic ring F fragment of sapogenins (see Figure 2-9), while the *m/z* 137 fragment ion (peak C) suggested the presence of a double bond or a hydroxy group in ring F.

The *m/z* 280 and 394 fragment ions observed for peaks A and B suggested these compounds contained one more double-bond or hydroxy group than was the case for diosgenin, which showed *m/z* 282 and 396 fragments ions. The *m/z* 392 fragment ion observed for peak C suggested the presence of two more double-bonds or hydroxy groups (including one in ring F) than was the case for diosgenin. Each of peaks A, B and C, exhibited a significant *m/z* 118 fragment ion suggestive of a ruscogenin-type 1β,3β-dihydroxy-spirost-5-ene structure (Meagher, 1996).

Since peaks A and B had almost identical mass spectra, it appeared that they might be a pair of 25*R*- and 25*S*-isomers, (25*R*-isomers typically elute before 25*S*-isomers on a HP-1 column), while the mass spectrum of peak C was consistent with it being a ring F double bond analogue of peaks A and B.

Separation of the hydrolysed conjugated extract by radial chromatography using mixtures of hexane-ethyl acetate as the developing solvents, afforded a sapogenin fraction.

The ^{1}H NMR spectrum (in $CDCl_3$) of the sapogenin fraction showed the presence of three sapogenins in a ratio of ca 1:1.2:2.5. The ^{13}C and DEPT NMR spectra of the fraction also included three signals in the region 109.3-109.8 ppm in a ratio of 1:1.2:2.5. Three carbon signals, in the same ratios, were observed in the regions 80.8-81.1, 62.2-62.4 and 65.0-66.9 ppm.

The ^{1}H NMR spectrum of the mixture exhibited three singlet methyl group signals in the region 1.04-1.03 ppm. A further three methyl group signal occurred in the region 0.78-0.80 ppm. The ^{1}H NMR also included a major doublet signal at 0.96 ppm (J = 7.0 Hz), and four less intense doublet signals at 1.07 ppm (J = 7.1 Hz), 0.99 ppm (J = 6.8 Hz), 0.98 ppm (J = 6.0 Hz) and 0.77 ppm (J = 5.7 Hz).

The HMBC spectrum of the sapogenin mixture (in $CDCl_3$) identified the three 19-CH_3 signals (overlapped in the region 1.04-1.03 ppm) by correlations with C-5 (138.3 ppm), C-1 (77.9 ppm), C-9 (50.4 ppm) and C-10 (42.9 ppm). The chemical shift of C-1 indicated that this carbon was hydroxylated, as required for ruscogenin and its analogues.

The HMBC spectrum also confirmed the occurrence of three overlapping singlet signals at 0.78-80 ppm, and that three overlapping doublet signals at ca 0.95 ppm arose from the 18-CH_3 and 21-CH_3 methyl groups respectively. The minor doublet signal at 1.07 ppm was identified as the 27-CH_3 (25*S*) signal, while the doublet signal at 0.77 ppm was identified

at the 27-CH_3 (25*R*) signal, by comparison with the 27-CH_3 correlation patterns observed in the HMBC spectra of sarsasapogenin (25*S*) and diosgenin (25*R*) respectively.

The foregoing ^1H, ^{13}C and HMBC NMR spectral features are consistent with the sapogenin sample being comprised of a 1:1.2:2.5 mixture of ruscogenin (25*R*) (**4-11**), neoruscogenin (25*S*) (**4-12**) and $\Delta^{25(27)}$-ruscogenin (**4-40**) respectively.

A notable feature of the ^1H NMR spectrum of the sapogenin mixture was the absence of a doublet-like methyl group signal in the region 0.7-1.1 ppm, attributable to the presence of a 27-CH_3 group in the major compound. This is consistent with the presence of a double-bond between C-25 and C-27 in the structure of **4-40**. The ^{13}C NMR spectrum of the mixture included signals at 108.6 ppm (=CH_2) and 143.8 ppm (>C=) assignable to the C-27 and C-25 resonances of **4-40** respectively.

The HMBC spectrum of the mixture revealed that H-27a of **4-40** (4.76 ppm) correlated with C-26 (65.0 ppm), H-27b (4.72 ppm) correlated with C-24 (28.6 ppm), H-26β (4.29 ppm) correlated with C-25 (143.8 ppm) and C-27 (108.6 ppm), and H-26α (3.84 ppm) correlated with C-25 (143.8 ppm), C-27 (108.6 ppm), C-22 (109.5 ppm) and C-24 (28.6 ppm). These correlations verified the presence of 25(27)-double in ring F of **4-40** (Figure 4-13 and Table 4-20).

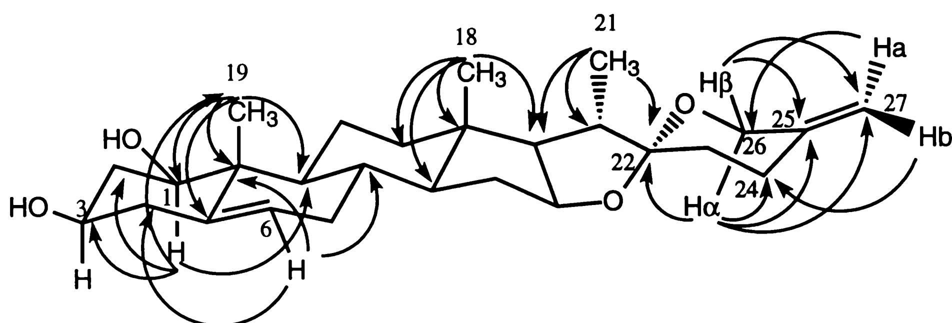


Figure 4-13. Selected HMBC (^1H-^{13}C) correlations observed for $\Delta^{25(27)}$-ruscogenin (**4-40**).

Table 4-20. Selected HMBC correlations observed for $\Delta^{25(27)}$-ruscogenin (**4-40**) (ppm in $CDCl_3$).

^{1}H signal	correlated ^{13}C signal(s)
5.25 (H-6)	42.9 (C-10), 42.0 (C-4), 32.5 (C-8)
4.76 (H-27a)	65.0 (C-26)
4.72 (H-27b)	28.6 (C-24)
4.29 (H-26β)	143.8 (C-25), 108.6 (C-27)
3.84 (H-26α)	143.8 (C-25), 108.6 (C-27), 109.5 (C-22), 28.6 (C-24)
3.41 (H-1)	68.1 (C-3), 50.4 (C-9), 42.9 (C-10), 42.5 (C-2), 13.1 (C-19)
2.23 (H-4)	138.3 (C-5), 125.4 (C-6), 68.1 (C-3), 42.9 (C-10), 42.5 (C-2)
2.00 (H-7a)	138.3 (C-5), 125.4 (C-6), 56.5 (C-14), 50.4 (C-9), 32.5 (C-8)
1.04 (H-19)	138.3 (C-5), 77.9 (C-1), 51.4 (C-9), 43.6 (C-10)
0.95 (H-21)	109.5 (C-22), 62.4 (C-17), 41.6 (C-20)
0.79 (H-18)	62.4 (C-17), 56.4 (C-14), 40.0 (C-12/C-13)

The HSQC spectrum of the mixture showed that H-26β and H-26α signals of **4-40** (the major component of the mixture) occurred at 4.28 and 3.84 ppm, while those of **4-12** and **4-11** occurred at 3.93 and 3.28 ppm, and at 3.44 and 3.38 ppm, respectively. These H-26β and H-26α signals for **4-12** and **4-11** corresponded closely to those observed for sarsasapogenin (25*S*) and diosgenin (25*R*) respectively.

A complete assignment of the ^{1}H and ^{13}C NMR spectra of the sapogenin mixture was derived by careful analyses of signals due to common, and differing, portions of the three sapogenins. For example, the ^{1}H NMR spectrum in $CDCl_3$ showed superimposed signals at 5.25 ppm (m, H-6), 3.53 ppm (m, H-3) and 3.41 ppm (d, J = 6.5 Hz, H-1), confirming the presence of 1β- and 3β-hydroxy groups in each of the sapogenins. The ^{13}C NMR spectrum exhibited the same chemical shifts for C-1 to C-15 (rings A, B and C carbon atoms) and for C-18 and C-19 (the angular methyl group carbons attached to these rings).

The ^{13}C NMR spectrum of the mixture for **4-11**, **4-12** and **4-40** was also determined in C_5D_5N (Table 4-21). The chemical shifts determined in C_5D_5N differed slightly from those determined in $CDCl_3$. There was a close similarity between the chemical shifts of **4-11**, **4-12** and **4-40** determined in C_5D_5N in this investigation and those of ruscogenin, neoruscogenin and $\Delta^{25(27)}$-ruscogenin reported by Agrawal et al. (1985) and Mimaki et al. (1996).

Table 4-21. ^{13}C NMR spectral data of ruscogenin (**4-11**), neoruscogenin (**4-12**) and $\Delta^{25(27)}$-ruscogenin (**4-40**) determined in C_5D_5N and $CDCl_3$ (ppm).

	4-11			**4-12**			**4-40**		
	C_5D_5N*	C_5D_5N	$CDCl_3$	C_5D_5N*	C_5D_5N	$CDCl_3$	C_5D_5N**	C_5D_5N	$CDCl_3$
1	78.2	78.1	77.9	78.2	78.1	77.9	78.1	78.1	77.9
2	44.0	44.0	42.5	44.0	44.0	42.5	44.0	44.0	42.5
3	68.3	68.1	68.1	68.2	68.1	68.1	68.1	68.1	68.1
4	43.7	43.6	42.0	43.6	43.6	42.0	43.6	43.6	42.0
5	140.5	140.4	138.3	140.3	140.4	138.3	140.4	140.4	138.3
6	124.3	124.4	125.4	124.3	124.4	125.4	124.7	124.4	125.4
7	33.2	32.3	32.0	33.1	32.3	32.0	32.3	32.3	32.0
8	32.5	33.0	32.5	32.4	33.0	32.5	33.0	33.0	32.5
9	51.6	51.4	50.4	51.4	51.4	50.4	51.4	51.4	50.4
10	43.7	43.6	42.9	43.6	43.6	42.9	43.6	43.6	42.9
11	24.4	24.2	23.8	24.3	24.2	23.8	24.2	24.2	23.8
12	40.8	40.6	40.2	40.7	40.6	40.2	40.6	40.5	40.1
13	40.4	40.3	39.9	40.3	40.3	39.9	40.3	40.3	40.0
14	57.2	57.0	56.5	57.0	57.0	56.5	57.0	57.0	56.5
15	32.6	32.4	32.0	42.5	32.4	32.0	32.4	32.4	32.0
16	81.2	81.1	80.8	81.2	81.2	80.9	81.4	81.5	81.1
17	63.4	63.2	62.3	63.1	63.0	62.2	63.2	63.2	62.4
18	16.7	16.7	16.6	16.7	16.7	16.6	16.6	16.7	16.6
19	14.0	13.9	13.1	14.0	13.9	13.1	15.0	13.9	13.1
20	42.2	42.0	41.7	42.6	42.5	42.2	41.9	41.9	41.6
21	15.1	15.0	14.9	15.0	14.9	14.4	13.9	15.0	14.9
22	109.3	109.3	109.3	109.8	109.8	109.8	109.5	109.5	109.5
23	32.1	31.9	31.5	26.5	26.4	26.0	33.3	33.3	32.9
24	29.5	29.3	28.9	26.3	26.2	25.8	29.0	29.0	28.6
25	30.7	30.6	30.3	27.6	27.6	27.1	144.5	144.5	143.8
26	66.1	66.9	66.9	65.2	65.1	65.2	65.0	65.0	65.0
27	17.4	17.4	17.2	16.4	16.3	16.1	108.6	108.7	108.6

* reported by Agrawal et al. (1985)

** reported by Mimaki et al. (1996)

4.5.2.2 Quantitative Analyses

Quantitative analyses of the hexane and ethanol-water extracts of freeze-dried leaf, stem and blossom samples of *N. texana* were performed by the GC-MS method of Wilkins et al. (1994) (see Section 5.1.2). The levels of the respective sapogenins (**4-11, 4-12, 4-40**) were determined assuming a unit response factor for their *m/z* 139 or 137 ion responses, relative to the *m/z* 139 ion responses of sarsasapogenin propionate, while the levels of cholesterol (**4-18**), stigmasterol (**4-20**) and β-sitosterol (**4-21**) were determined relative to the *m/z* 368, 394 and 396 ion responses, respectively, of authentic specimens of these compounds. Campesterol (**4-19**) was quantified relative to its *m/z* 382 ion response, assuming its response factor to be the average of that determined for **4-18** (*m/z* 368) and **4-21** (*m/z* 396) ion responses (see Section 5.1.2)

The levels of free and conjugated sapogenins and sterols identified in the specimens of *N. texana* are presented in Table 4-22. No diosgenin, yamogenin, sarsasapogenin, smilagenin or other monohydroxy sapogenins were detected in the free and conjugated extracts from the *N. texana* samples.

Table 4-22. Levels (mg/kg) of free and conjugated sapogenins and sterols in the leaves, stems, blossoms of USA *N. texana*.

	RT	Leaves		Stems		Blossoms	
	(min)	conj	free	conj	free	conj	free
cholesterol	13.2	19.3	27.8	25.1	69.4	32.6	44.2
campesterol	14.9	3.8	6.3	9.1	21.0	10.4	28.3
stigmasterol	15.3	17.9	27.6	19.6	40.9	33.1	43.8
β-sitosterol	16.4	38.8	61.1	54.9	125	108	120
sarsasapogenin	14.9	0	0	0	0	0	0
diosgenin	15.5	0	0	0	0	0	0
4-11	20.5	0	0	23.2	0	49.1	31.9
4-12	20.9	0	0	14.2	0	57.2	39.3
4-40	21.1	0	0	6.1	0	110	87.2

RT = retention time, conj = conjugated fraction, free = free fraction

The results in Table 4-22 exhibited that blossoms had the highest levels of free and conjugated sapogenins, and that leaves did not contain free or conjugated sapogenins. Stems showed no detectable free sapogenins and only low levels of conjugated sapogenins.

Mathews (1937, 1940) and Rankins et al. (1993) have suggested that saponins present in flower stalks and seed pods of sacahuiste plants (*N. microcarpa, N. texana*) might contribute to toxicosis when ingested by livestock. A possible point of significance in respect of Mathews' (1940) early observation is that no reports of toxicity have appeared for cattle which mainly graze leaves of *Nolina* species, whereas toxicity outbreaks were observed amongst sheep grazing both leaves and blossoms.

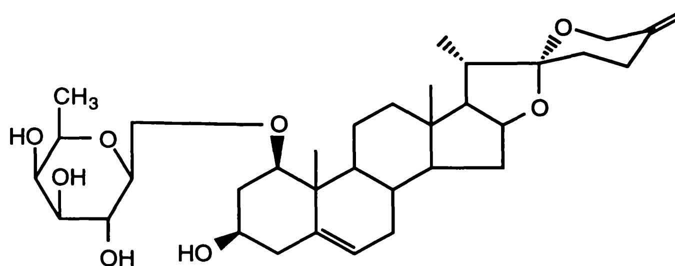
Evidence to date indicates that dihydroxy saponins (e.g. tigogenin and neotigogenin, both of which are 5α-H-sapogenins) are not transported to the liver and released into the bile, whereas epismilagenin and episarsasapogenin (5β-H-sapogenins), derived from metabolism of diosgenin and yamogenin, are transported to the liver and released into the bile (Miles et al., 1993; Miles et al., 1994b; Wilkins et al., 1994). It is not known if 5β-H dihydroxy sapogenins derive from ruminal hydrogenation of ruscogenin and its analogues are transported to the liver and released into the bile.

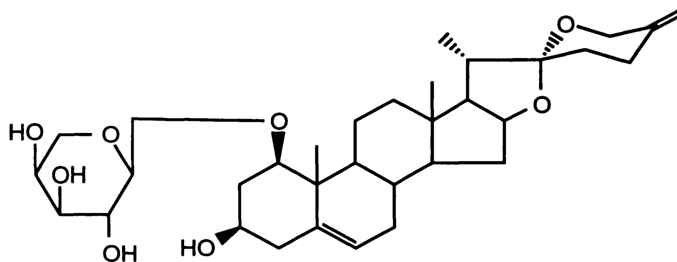
Alternatively, it is possible that the photosensitization outbreaks observed by Mathews (1940) were a consequence of elevated levels of diosgenin and yamogenin (the monohydroxylated biosynthetic precursors of ruscogenin and neoruscogenin respectively) being present in the plant at the time it was ingested by affected sheep.

4.5.2.3 Characterisation of Saponin Constituents

Radial chromatography of the ethanol-water extract, obtained from freeze-dried blossoms of *N. texana* which had previously been extracted with hexane, using mixtures of chloroform and aqueous as development solvents, afforded five saponin fractions (fraction I, II, III, IV and V).

Fraction I, the least polar fraction, when separated by HPLC using an ODS silica gel column and methanol-water (4:1) as eluent, gave three fractions (fraction Ia, Ib and Ic), which were identified by TLC, GC-MS and NMR data as mixtures of saponin monoglycosides. Careful analyses of spectral data of these fractions, including the addition and subtraction of appropriate one and two-dimensional NMR data sets, led to the structural elucidation of the predominant saponin constituents of these fractions. The saponins were identified as spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-fucopyranoside (**4-41**), spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-α-L-arabinopyranoside (**4-42**) and spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-galactopyranoside (**4-43**) (Figure 4-14).





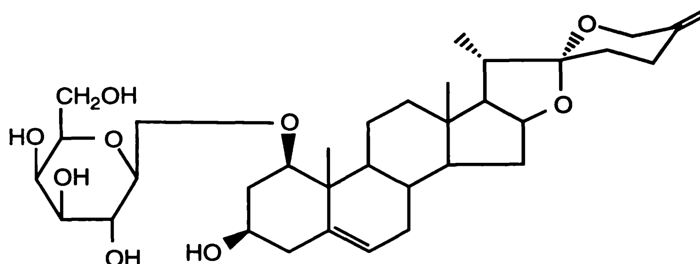


Figure 4-14. Structures of saponin (**4-41**), saponin (**4-42**) and saponin (**4-43**) characterised from *N. texana*.

ES-MS of other more polar fractions (fractions II, III, IV and V) showed that these fractions might contain more complex glycosidic systems. For instance, fraction II exhibited a *m/z* 757.7 ion (-100 V), fraction III gave *m/z* 791.0 and 644.9 ions (-100 V) and a *m/z* 731 ion (+100 V), fraction IV showed *m/z* 936.9 and 919.2 ions (-100 V) and a *m/z* 885.2 ion (+100 V), while fraction V showed a *m/z* 835.6 ion (-100 V) and a *m/z* 1015.9 ion (+100 V). Further work is required to elucidate the structures of the principal components of these fractions.

Structure elucidation of saponin 4-41

The ^{1}H NMR spectrum of fraction Ic, determined in C_5D_5N, included methyl proton signals at 0.85 (s), 0.90 (s), 1.04 (d, J = 7.0 Hz), 1.22 (s), 1.33 (s) and 1.55 ppm (d, J = 6.4 Hz), and lowfield signals at 4.29 (1H, t, J = 7.8 and 7.8 Hz), 4.71 (1H, d, J = 7.7 Hz), and 4.77 ppm (2H, d, J = 13.4 Hz). The chemical shifts and ratios of the methyl group signals, observed for fraction Ic, suggested the possible presence of a sapogenin and a saponin containing a deoxy-sugar.

The ^{13}C NMR spectrum (in C_5D_5N) showed that pairs of peaks occurred in regions where typically only a single peak is observed for a sapogenin. For example, two peaks appeared at 144.3 and 139.2 ppm (C-25 signals), 140.2 and 139.4 ppm (C-5 signals), 124.5 and 124.1 ppm (C-6 signals), 81.3 and 81.2 ppm (C-16 signals), 63.0 and 62.9 ppm (C-17 signals), two 56.9 and 56.7 ppm (C-14 signals), and at 24.0 and 23.6 ppm (C-11 signals). These observations are consistent with the presence of two closely related sapogenins in the mixture.

The HMBC spectrum confirmed that two compounds were present in fraction Ic. In particular the 19-CH_3 protons (1.33 ppm) of the minor component exhibited correlations with carbon resonances at 140.2 (C-5), 78.0 (C-1), 51.2 (C-9) and 43.4 ppm (C-10), while the 19-CH_3 protons (1.22 ppm) of the major component exhibited correlations with carbon resonances at 139.5 (C-5), 83.8 (C-1), 50.3 (C-9) and 43.4 ppm (C-10).

The marked difference in the C-1 signals (78.0 and 83.8 ppm for the minor and major constituents respectively) is consistent with the presence of a glycosyl group at C-1 in the major component, and a free hydroxy group in the minor component. A correlation was also observed between a doublet methine proton (4.71 ppm, J = 7.7 Hz), assignable to an anomeric glycosyl proton, and the C-1 resonance which occurred at 83.8 ppm.

Careful analyses of the ^{13}C NMR spectral data, determined for fraction Ic and those reported by Agrawal et al. (1985) and Mimaki et al. (1996) for $\Delta^{25(27)}$-ruscogenin (**4-40**), revealed fraction Ic to be a mixture of free $\Delta^{25(27)}$-ruscogenin (**4-40**) and a glycosylated $\Delta^{25(27)}$-ruscogenin (**4-41**).

The ^{13}C NMR spectrum of fraction Ic exhibited a total of 60 peaks. After subtraction of the 27 peaks arising from free $\Delta^{25(27)}$-ruscogenin (**4-40**), 33 peaks were found, of which 27 peaks would belong to the genin component, and the remaining 6 peaks to a glycoside residue.

The ^{1}H NMR spectrum of fraction Ic (determined at 27°C) included two doublet signals at 4.77 ppm (2H, J = 13.4 Hz), 4.71 ppm (1H, J = 7.7 Hz) and a very large water peak at 4.87 ppm. In order to ascertain if other glycoside signals were concealed under the large water peak, the ^{1}H NMR spectrum was also determined at 45°C, according to the preceding described in Section 4.3.2.

The ^{1}H NMR spectrum determined at 45°C showed that the water peak was shifted upfield from 4.87 (27°C) to 4.67 ppm (45°C), while glycoside signals were not significantly shifted (Figure 4-15). No signals were found to be concealed under the water peak.

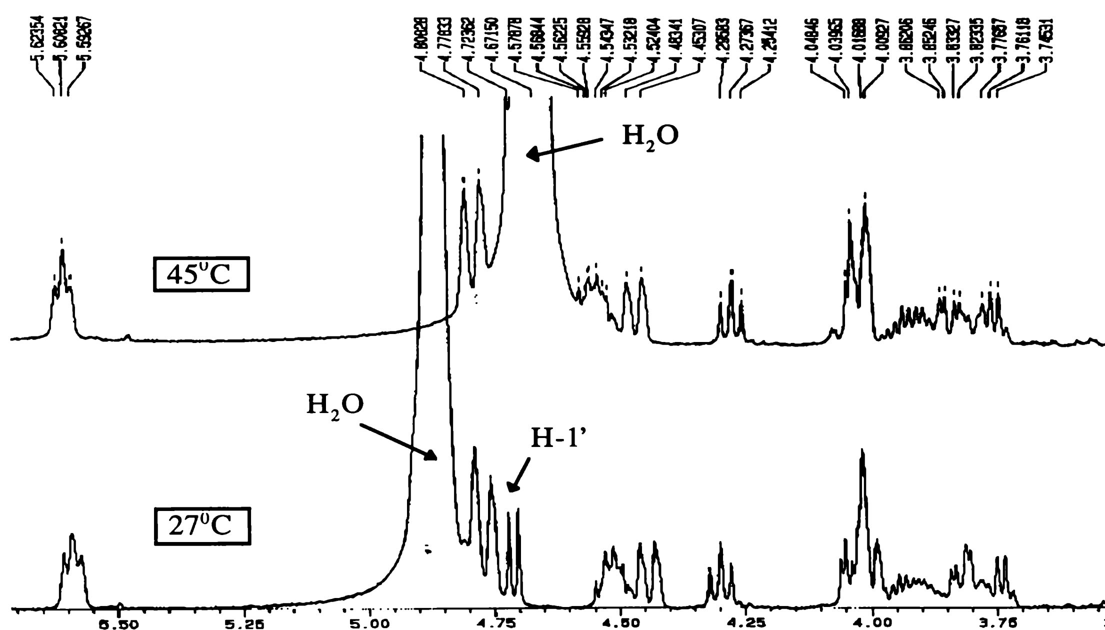


Figure 4-15. ^{1}H NMR spectra of fraction Ic determined at 27°C and 45°C.

The coupling constant of H-1' (J = 7.7 Hz) in the ^{1}H NMR spectrum indicated that H-1' and H-2' were both axially oriented (Agrawal, 1992), and was consistent with the presence of a β-glycoside linkage. Correlations observed for H-1' ↔ H-3' ↔ H-5' in the ROESY spectrum of fraction Ic also verified that H-1', H-3' and H-5' were axially oriented (Figure 4-16).

In COSY spectrum of fraction Ic correlations were observed for H-1' (4.71 ppm) ↔ H-2' (4.29 ppm) ↔ H-3' (4.06 ppm), and H-6' (1.55 ppm) ↔ H-5' (3.74 ppm), but not H-3' ↔ H-4' or H-4' ↔ H-5'. A HOHAHA experiment, optimised for the detection of long range couplings, revealed weak but definable correlations between H-3' (4.06 ppm) and

H-4' (4.04 ppm), and between H-4' and H-5' (3.74 ppm) as well as correlations between H-1', H-2' and H-3' and between H-6' and H-5'. These observations revealed H-4' to be equatorially oriented.

The foregoing observations identified the glycosyl residue as a fucosyl residue (Figure 4-16).

The point of attachment between the sugar unit and the genin (1'↔1) was established from the correlation between H-1' (4.71 ppm) and C-1 (83.8 ppm) observed in the HMBC spectrum (Table 4-23 and Figure 4-16) and from the correlation between H-1' (4.71 ppm) and H-1 (3.94 ppm) observed in the ROESY spectrum of fraction Ic (Figure 4-16).

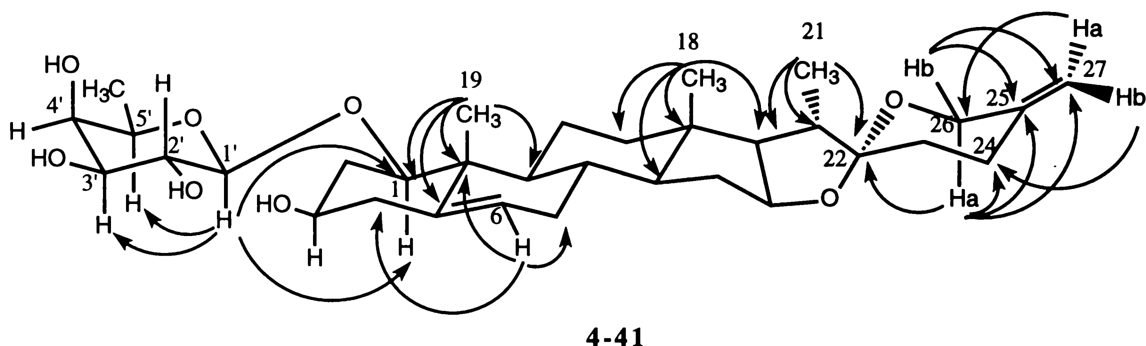


Figure 4-16. Correlations of HMBC (^{1}H-^{13}C) and ROESY (^{1}H-^{1}H) observed for spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-fucopyranoside (**4-41**).

Table 4-23. Selected HMBC correlations observed for saponin **4-41** (ppm in C_5D_5N).

^{1}H signal	correlated ^{13}C signal(s)
5.59 (H-6)	43.7 (C-4), 43.4 (C-10), 31.7 (C-7)
4.78 (H-27a)	64.8 (C-26)
4.76 (H-27b)	28.8 (C-24)
4.71 (H-1')	83.8 (C-1)
4.44 (H-26b)	144.3 (C-25), 108.4 (C-27)
4.29 (H-2')	102.4 (C-1'), 75.2 (C-3')
4.00 (H-26a)	144.3 (C-25), 109.2 (C-22), 108.4 (C-27), 28.8 (C-24)
3.74 (H-5')	102.4 (C-1'), 72.3 (C-4'), 17.2 (C-6')
2.60 (H-4)	139.5 (C-5), 124.5 (C-6), 68.0 (C-3), 43.4 (C-10), 38.0 (C-2)
1.55 (H-6')	71.1 (C-5')
1.22 (H-19)	139.5 (C-5), 83.8 (C-1), 50.3 (C-9), 43.4 (C-10)
1.04 (H-21)	109.2 (C-22), 62.9 (C-17), 40.1 (C-13)
0.85 (H-18)	62.9 (C-17), 56.8 (C-14), 40.4 (C-12), 40.1 (C-13)

The HSQC spectrum of fraction Ic revealed the ^{13}C resonances of the glycoside 1', 2', 3', 4', 5' and 6' carbons via ^{13}C- ^{1}H correlations observed for the corresponding protons (Table 4-24).

The negative ion ES-MS of fraction Ic showed a pseudomolecular ion at *m/z* 573.5 which corresponded to a $(M-H)^-$ ion (loss of a proton from a compound of molecular weight 574 daltons), while the positive ES-MS revealed a pseudomolecular ion at *m/z* 597.5 which corresponded to a $(M+Na)^+$ ion (the addition of sodium ion to a compound of molecular weight 574 daltons).

The foregoing analyses of ES-MS and one- and two-dimensional NMR spectral data showed the dominant saponin component of fraction Ic to be spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-fucopyranoside (**4-41**).

Close inspection of the ^{1}H, ^{13}C and COSY, HSQC spectra of fraction Ic, revealed the presence of very small signals of which could also be attributed to the possible presence of low levels of the corresponding 25*R*- (ruscogenin) and 25*S*- (neoruscogenin) analogues of the fucopyranosyl saponins.

Table 4-24. ^{1}H and ^{13}C NMR assignments (ppm in C_5D_5N) for spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-fucopyranoside (**4-41**), spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-α-L-arabinopyranoside (**4-42**) and spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-galactopyranoside (**4-43**).

No	4-41		4-42		4-43	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
1	83.8	3.94	83.1	3.83	83.2	4.43
2	38.0	2.81, 2.12	37.7	2.79, 2.14	37.5	2.74, 2.08
3	68.0	3.99	68.0	3.86	68.0	4.43
4	43.7	2.60	43.6	2.66	43.8	2.59
5	139.5	-	139.5	-	-	-
6	124.5	5.59	124.5	5.60	124.5	5.56
7	31.7	1.95, 1.59	32.0	1.93, 1.58	31.9	1.88, 1.53
8	32.9	1.78	33.0	1.78	32.9	1.73
9	50.3	1.51	50.1	1.50	50.3	1.44
10	43.4	-	43.6	-	-	-
11	24.1	2.95, 1.52	23.7	2.95, 1.55	24.0	2.87, 1.50
12	40.4	1.66, 1.35	40.2	1.62, 1.32	40.1	1.54, 1.23
13	40.1	-	40.1	-	-	-
14	56.8	1.18	57.6	1.15	56.8	1.08
15	32.2	2.05, 1.48	32.3	2.04, 1.45	32.1	1.97, 1.41
16	81.3	4.52	81.4	4.52	81.2	4.49
17	62.9	1.83	62.9	1.80	62.9	1.74
18	16.6	0.85	16.5	0.84	16.7	0.82
19	14.8	1.22	14.8	1.23	14.8	1.21
20	41.7	1.97	41.8	1.95	41.9	1.90
21	14.8	1.04	14.9	1.03	14.9	1.02
22	109.2	-	109.2	-	-	-
23	33.0	1.63	33.0	1.59	32.9	1.55
24	28.8	2.73, 2.26	28.8	2.74, 2.26	28.8	2.67, 2.20
25	144.3	-	144.0	-	-	-
26	64.8	4.44, 4.00	64.9	4.45, 4.01	64.9	4.42, 3.98
27	108.4	4.77	108.5	4.79	108.4	4.76
	Fuc		Arab		Gal	
1'	102.4	4.71	102.2	4.74	101.5	4.91
2'	72.0	4.29	72.6	4.39	75.2	4.00
3'	75.2	4.06	74.8	4.11	78.4	4.18
4'	72.3	4.04	69.6	4.26	72.1	4.09
5'	71.1	3.74	67.5	4.32, 3.74	78.0	4.89
6'	17.2	1.55			63.5	4.52, 4.32

Structure elucidation of saponin 4-42

One- and two-dimensional NMR spectra showed fraction Ib to be a mixture of two saponins, one of which was **4-41** (the dominant saponin component of fraction Ic). Difference NMR spectra, generated by subtracting spectral data determined for fraction Ic from those determined for fraction Ib (one- and two-dimensional data sets), afforded difference HMBC, HOHAHA, ROESY spectra which identified the second saponin component of fraction Ib as spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-α-L-arabinopyranoside (**4-42**).

The HOHAHA spectrum of **4-42** showed weak correlations between H-3' and H-4' and between H-4' and H-5'. The ROESY spectrum exhibited strong mutual correlations between H-1', H-3' and H-5', which suggested that H-1', H-3' and H-5' were axially oriented and H-4' was equatorially oriented as those of **4-41**.

The HMQC spectrum of **4-42** identified 5 (rather than 6) glycoside carbon resonances, and that two protons (3.74 and 4.32 ppm) were attached to C-5' (67.5 ppm). The chemical shifts of the glycoside protons and carbons corresponded to those reported by Kobayashi et al. (1993) for an α-L-arabinopyranosyl residue.

Correlation between H-1' (4.74 ppm) and C-1 (83.1 ppm) in the HMBC spectrum (Table 4-25) and correlation between H-1' (4.74 ppm) and H-1 (3.83 ppm) in the ROESY spectrum verified a (1' ↔ 1) linkage between the glycoside unit and the genin.

Table 4-25. Selected HMBC correlations observed for saponin **4-42** (ppm in C_5D_5N).

^{1}H signal	correlated ^{13}C signal(s)
5.60 (H-6)	43.6 (C-4, C-10), 32.0 (C-7)
4.74 (H-1')	83.1 (C-1)
4.45 (H-26a)	144.0 (C-25), 108.5 (C-27)
4.01 (H-26b)	144.0 (C-25), 109.2 (C-22), 108.5 (C-27), 28.8 (C-24)
1.23 (H-19)	139.5 (C-5), 83.1 (C-1), 50.1 (C-9), 43.6 (C-10)
1.03 (H-21)	109.2 (C-22), 62.9 (C-17), 40.1 (C-13)
0.84 (H-18)	62.9 (C-17), 57.6 (C-14), 40.2 (C-12), 40.1 (C-13)

The negative ion ES-MS of fraction Ib included pseudomolecular ions [$(M-H)^-$] at *m/z* 559.4 (**4-42**) and at *m/z* 573.4 (**4-41**), and positive ES-MS showed pseudomolecular ions [$(M+Na)^+$] at *m/z* 583.4 (**4-42**) at *m/z* 597.5 (**4-41**).

The foregoing analyses of NMR and ES-MS spectral data showed the main saponin component of fraction Ib to be spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-α-L-arabinopyranoside (**4-42**).

Structure elucidation of saponin 4-43

The NMR spectra of fraction Ia showed it to be a ca 2:3 mixture of **4-42** and **4-43**. Detailed analyses of one- and two-dimensional NMR data determined for **4-43**, including ^{1}H, COSY, HMBC and HSQC data, showed that **4-43** differed from **4-41** and **4-42** only in respect of its glycoside residue.

Weak correlations were observed in the COSY and HOHAHA spectra between the glycoside H-3' and H-4' signals and between the H-4' and H-5' signals. This suggested that the C-3', C-4' and C-5' stereochemistry of **4-43** was similar to that of **4-41** and **4-42**.

No glycosyl methyl signals appeared in the ^{1}H NMR spectrum in the region 1.6-1.5 ppm where the CH_3 signals of deoxy sugars is normally found. The HSQC spectrum revealed the presence of 6 (rather than 5) glycosyl resonances in **4-43**, and showed that two protons (4.32 and 4.52 ppm) correlated with the glycosyl methylene carbon resonance (63.5 ppm). These spectral features were consistent with the identification of the glycosyl residue of **4-43** as a galactosyl residue (Figure 4-14).

The anomeric H-1' signal of **4-43** was not observed in the ^{1}H NMR spectrum, due to it being concealed under a large water peak, centred at 4.8 ppm. However, the COSY spectrum of **4-43** identified the chemical of H-1' (4.91 ppm), by an off-diagonal cross peak with H-2' (4.00 ppm). When the ^{1}H NMR spectrum of **4-43** was determined at 45°, the large water peak was shifted upfield, and a well defined doublet peak was observed at 4.91 ppm (1H, J = 7.8 Hz). The coupling constant showed that H-1' was axially α-oriented and the galactosyl linkage was equatorially β-oriented. ^{1}H and ^{13}C NMR assignments for **4-43** are presented in Table 4-23.

The negative ion ES-MS of fraction Ia included pseudomolecular ions [(M-H)$^-$] at *m/z* 559.6 (**4-42**) and *m/z* 589.4 (**4-43**). The positive ion ES-MS showed pseudomolecular ions [(M+Na)$^+$] at *m/z* 583.4 (**4-42**) and *m/z* 613.3 (**4-43**).

The foregoing of ES-MS and one- and two-dimensional NMR spectral data identified **4-43** as spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-D-galactopyranoside.

While NMR data (chemical shifts, coupling constants and ROESY correlations) readily identifies the glycosyl units it does not distinguish D- or L-forms. The configuration of the sugar residues presented here for **4-41**, **4-42** and **4-43** are tentatively assigned as being the same as those established by Takaashi et al. (1995) and Mimaki et al. (1996).

4.6 *Tribulus terrestris*

4.6.1 Introduction

T. terrestris has been reported to cause photosensitization disease in South African sheep, known as geeldikkop (Theiler, 1918; Coetzer et al., 1983; Miles et al., 1993; Miles et al., 1994a; Miles et al., 1994b; Wilkins et al., 1994; Wilkins et al., 1996). Photosensitization outbreaks have also been reported amongst sheep grazing *T. terrestris* in Australia (Glastonbury et al., 1984), the United States (Camp et al., 1988), Argentina (Tapia et al., 1994) and Iran (Amjadi et al., 1977).

Collections of Australian and South African *T. terrestris* have been found to contain a variety steroidal sapogenins, including diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin, neogitogenin, and ruscogenin (Miles et al., 1994b; Wilkins et al., 1994; Wilkins et al., 1996). Hitherto, a number of saponins, including tribulosin and dioscin, have been isolated from *T. terrestris* (Mahato et al., 1982). Wilkins et al. (1996) have also reported the structure elucidation of a ruscogenin-derived saponin (saponin C) isolated from the β-glucosidase-treated extracts of a South African collection of *T. terrestris*.

In this work, 4 samples of *T. terrestris*, collected by Prof A Rezakhani, from sites in Northern Iran in 1994, were analysed using the SIM GC-MS method of Wilkins et al. (1994). The samples were from an area where photosensitization outbreaks have been periodically occurred.

4.6.2 Results and Discussion

The air-dried and ground samples were extracted with hexane in a Soxhlet apparatus for 16 hours to give free sapogenin extracts, and then with ethanol-water for 20 hours to give conjugated sapogenin extracts which were hydrolysed using 1 M hydrochloric acid to afford the corresponding free sapogenins.

GC-MS analyses of the hexane and hydrolysed ethanol-water extracts of the samples were performed by the method of Wilkins et al. (1994) using selected ion mode (SIM) GC-MS ion profiles *m/z* 139 for sapogenins, *m/z* 368 for cholesterol, *m/z* 382 for campesterol, *m/z* 394 for stigmasterol, and *m/z* 396 for β-sitosterol (see Section 5.1.2). The levels of free and conjugated sapogenins and sterols identified in the four Iranian *T. terrestris* samples are presented in Table 4-26.

Confirmation of the identification of the smilagenin, sarsasapogenin, diosgenin, yamogenin, tigogenin, neotigogenin, hecogenin and neohecogenin peaks was carried out by comparison of the retention times of authentic standards and from ion ratio data (*m/z* 139, 284, 344, 282 and 396 ions) (Wilkins et al., 1994). TIC GC-MS analyses of the samples also verified the identification the sapogenin peaks.

The retention times of yamogenin and tigogenin on a HP-1 column are essentially identical (Wilkins et al., 1994). These sapogenins can however be distinguished by ion ratio analysis (Wilkins et al., 1994). Comparison of the *m/z* 282 and 344 ion profiles (for yamogenin and tigogenin respectively) showed that the overlapping yamogenin and tigogenin peak arose mainly from tigogenin, and that only a trace of yamogenin was present in the conjugated extract (Figure 4-17).

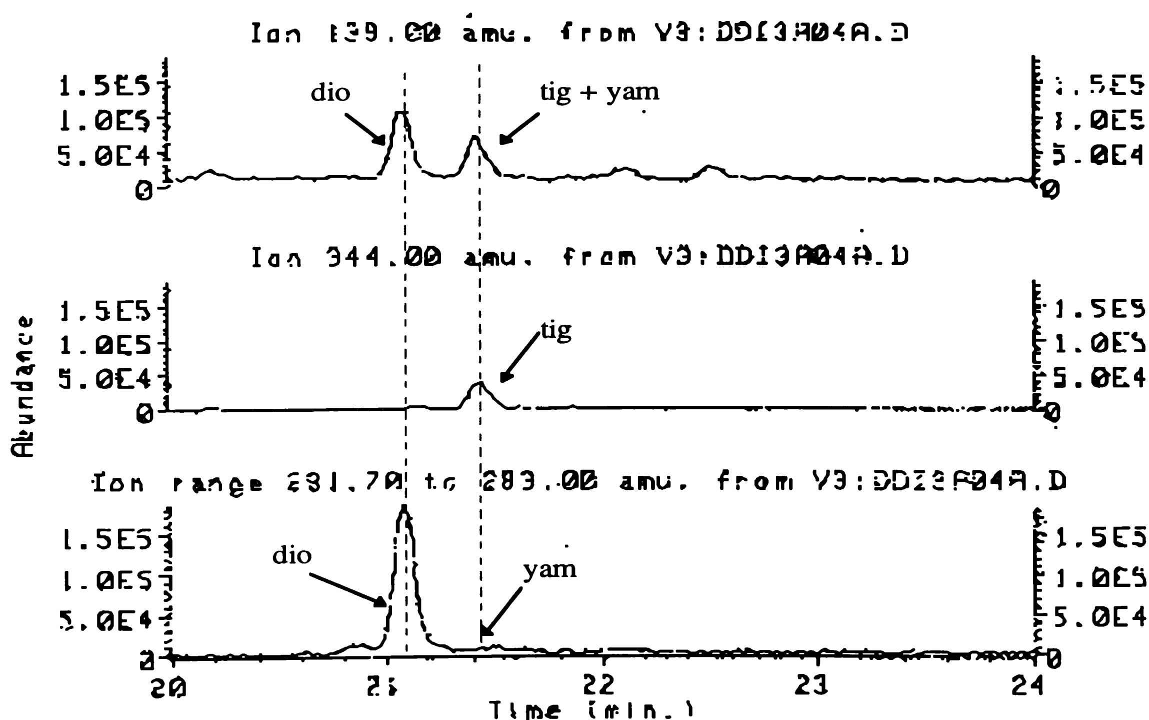


Figure 4-17. GC-MS *m/z* 139, 344 and 282 ion profiles of determined for sapogenins in the hydrolysed conjugated extract of an Iranian collection of *T. terrestris* (sample 4).

Table 4-26. Levels (mg/kg) of sapogenins and sterols in the free and conjugated extracts from Iranian *T. terrestris* samples.

samples		chol	camp	stig	sito	smil	sar	dio	tig	neotig	heco	neoheco
Iran94/1	conj	4.7	2.4	10.6	26.4	9.0	5.9	79.8	161	45.8	66.4	56.4
	free	26.9	6.8	36.8	65.2	4.0	1.4	0	0	0	0	0
Iran94/2	conj	5.9	0	3.0	11.6	48.2	21.9	11.5	11.3	0	12.5	0
	free	22.2	4.9	30.9	72.6	20.5	6.8	0	0	0	0	0
Iran94/3	conj	117	33.4	224	404	0	0	54.4	40.2	0	33.9	0
	free	16.5	3.2	25.7	44.0	6.1	2.5	0	0	0	0	0
Iran94/4	conj	40.1	11.5	82.9	131	0	0	183	104	0	55.3	0
	free	15.9	3.3	25.6	38.6	6.5	2.9	0	0	0	0	0

conj = conjugated extract, free = free extract, chol = cholesterol, camp = campesterol, stig = stigmasterol, sito = β-sitosterol,

smil = smilagenin, sar = sarsasapogenin, dio = diosgenin, tig = tigogenin, neotig = neotigogenin, heco = hecogenin, neoheco = neohecogenin

Several chemotypes of *T. terrestris* have been identified. For example, one South African chemotype is characterised by the presence of variable levels of diosgenin, yamogenin, tigogenin, neotigogenin, gitogenin and neogitogenin saponins (Miles et al., 1994a), while another South African chemotype is characterised by the presence of elevated levels of ruscogenin and neoruscogenin saponins (Wilkins et al., 1996). Meagher (1996) has noted that Queensland specimens of *T. terrestris* differ from New South Wales specimens, in that they do not contain detectable levels of diosgenin and yamogenin sapogenins or saponins. Chinese collections of *T. terrestris* are characterised by the presence of diosgenin, tigogenin and hecogenin (a 12-keto-sapogenin) (Wang and Lu, 1991). Isozyme analysis has also identified genic variations in *T. terrestris* populations (Morrison and Scott, 1996).

The Iranian collections of *T. terrestris* examined in this investigation exhibited a genin chemistry different to those of the chemotypes described above. Saturated (5α-H: tigogenin and neotigogenin and 5β-H: smilagenin and sarsasapogenin) and unsaturated (diosgenin and yamogenin) monohydroxy saponins, and/or sapogenins, were found in the samples. Hecogenin and neohecogenin conjugates were also detected in most of the samples (Table 4-26).

When the level of diosgenin glycosides was low (e.g. 11.5 mg/kg, sample 2), higher levels of smilagenin, sarsasapogenin and/or tigogenin (5β- and 5α-sapogenins) glycosides (48.2, 21.9 and 11.3 mg/kg respectively for sample 2) were found (Table 4-26). On the other hand, when the level of diosgenin glycosides was high (e.g. 183 mg/kg, sample 4), elevated levels of tigogenin (a 5α-sapogenin glycoside) (104 mg/kg, sample 4), but not smilagenin and sarsasapogenin glycosides (5β-sapogenin glycosides) were detected.

Miles et al. (1994a) have suggested that only collections of *T. terrestris* possessing elevated of diosgenin saponins are likely to be lithogenic. The level of diosgenin detected in at least one of Iranian samples (sample 4) is such that toxicity outbreaks might be anticipated. It can be concluded that reports of periodic toxicity outbreaks amongst Iranian sheep grazing *T. terrestris* are consistent with Miles et al's proposal that elevated levels of diosgenin and/or yamogenin saponins are likely to be implicated in photosensitization outbreaks.

4.7 *Brachiaria decumbens*

4.7.1 Introduction

B. decumbens has been reported to cause photosensitization in sheep, goats and cattle in Australia, Malaysia, Indonesia, Nigeria, Brazil and Papua New Guinea (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). Diosgenin (**4-5**) and yamogenin (**4-6**) have been identified as genin components of saponins present in hydrolysed *B. decumbens* extracts (Smith and Miles, 1993; Wilkins et al., 1994). Higher levels of **4-5** and **4-6** may be present in young plants (Wilkins et al., 1994).

Analyses of grass samples collected from a South American pasture during a photosensitization outbreak revealed that elevated levels of diosgenin and yamogenin saponins were present in plant samples (Meagher et al., 1996).

Berndt (1997) has shown that the dominant *B. decumbens* saponins are the furostenol saponins, protodioscin (**4-44**) and protoyamoscin (**4-45**) (the 25*S*-analogue of **4-44**) (see Figure 1-7), and that hydrolysis of these saponins affords diosgenin (**4-5**) and yamogenin (**4-6**) respectively.

In this work, 82 samples of *B. decumbens*, harvested by Mrs S. Low from research plots of *B. decumbens* growing at Papua New Guinea University of Technology, Lae, Papua New Guinea, over the period June 1994 to 1997 were analysed. Grass samples were dried and ground to fine powders in PNG, and forwarded to New Zealand for extraction and GC-MS analyses.

The results reported here, together with those obtained by Berndt (1997) for other samples, represent a contribution to a research programme in progress in Papua New Guinea, directed towards an improved understanding of factors which may contribute to seasonal fluctuations in saponin levels, and to occasional toxicity outbreaks.

4.7.2 Results and Discussion

The air-dried and ground samples were extracted with hexane in a Soxhlet apparatus for 16 hours to give free sapogenin extracts, and then with ethanol-water for 20 hours to give conjugated sapogenin extracts which were hydrolysed using 1 M hydrochloric acid to afford the corresponding free sapogenins.

GC-MS analyses of the hexane and hydrolysed ethanol-water extracts of the samples were performed by the method of Wilkins et al. (1994) using selected ion mode (SIM) GC-MS ion profiles *m/z* 139 for sapogenins, *m/z* 368 for cholesterol, *m/z* 382 for campesterol, *m/z* 394 for stigmasterol, and *m/z* 396 for β-sitosterol (see Section 5.1.2). The identity of the diosgenin and yamogenin peaks was confirmed by comparison of retention time and ion ratio data (*m/z* 282 and 396 ions) for authentic specimens.

The levels of free and conjugated sapogenins and sterols identified in the PNG samples are presented in Table 4-27. In general diosgenin (**4-5**) and yamogenin (**4-6**) were accompanied by peaks arising from the corresponding, dehydrated, spirosta-3,5-dienes. Since these compounds are considered to be extraction and/or derivatization artefacts (Meagher, 1996), the total levels of diosgenin (**4-5**) and yamogenin (**4-6**) sapogenins were calculated as the sum of the levels of diosgenin and dehydrated diosgenin, and yamogenin and dehydrated yamogenin, respectively.

All of the *B. decumbens* samples contained diosgenin (**4-5**) and yamogenin (**4-6**) in a ratio of ca 1:1 (Table 4-27), irrespective of the time (season) samples were collected. Berndt (1997) has showed that the dominant *B. decumbens* saponins are the furostenol saponins **4-44** and **4-45**, and that acid hydrolysis of these saponins afforded diosgenin (**4-5**) and yamogenin (**4-6**) respectively.

Conjugated diosgenin (**4-5**) and yamogenin (**4-6**) were identified as the predominant constituents of samples from research plots 1 to 5. Only in one case (plot 3, June 95) was an appreciable level of free diosgenin (**4-5**) and yamogenin (**4-6**) detected. Appreciable levels of free sapogenins were however detected in many of the plot 6 to 8 samples, and some of the 1997 field samples (e.g. Marrumbung and Theo's-M.L, see Table 4-27). Previous experience has shown that the detection of free sapogenins does not necessarily imply their presence in plant material at the time of harvesting. For example, Meagher et al. (1996) have noted that enzymatic or chemically induced hydrolysis of saponins may occur during the drying and storage samples.

The levels of free and conjugated sapogenins detected in the supplied samples fluctuated greatly. In some cases (e.g. plot 1 samples) levels of conjugated sapogenins were ca 10 times higher in the September, October-November, 1995 and February, 1996 samples (spring-summer samples) than was the case for June-August, 1995 samples (winter samples) (see Figure 4-19). In other cases, however, (e.g. plots 7 and 8) elevated levels of saponins (ca 10 times higher than summer levels) were observed for autumn-winter (May or July 1996) samples.

Whilst there was a tendency for elevated saponin levels to be present in summer (December) and rainy season (May) samples, no consistent pattern in saponin levels was apparent. The extent to which saponin and sapogenin levels in grasses are influenced by climatic (e.g. rainfall levels, temperature variations, etc), environmental (soil types, etc) and management practices (e.g. fertiliser applications, etc) is not known.

The levels of free and conjugated sterols in the PNG *B. decumbens* samples also fluctuated (see Table 4-27 and Figure 4-20). Comparison of Figure 4-20 with Figure 4-19 suggests that the levels of conjugated sterols may increase when elevated levels of conjugated sapogenins are present in the samples. For example, in plot 3 samples, the levels of conjugated sapogenins (diosgenin and yamogenin) rose from 19 mg/kg (October-November, 1995) to 228 mg/kg (December, 1995), while the levels of the sum of conjugated sterols (cholesterol, campesterol, stigmasterol and β-sitosterol) were 49 mg/kg and 153 mg/kg respectively at the same times.

However, conjugated sterols levels were higher at the times when lower levels of conjugated sapogenins were present in the experiment plots, especially so for June (winter) samples. For instance, conjugated sterol and conjugated sapogenin levels of 133 mg/kg and 97 mg/kg respectively were identified in the June 1994, plot 3 sample.

While it is well known that cholesterol is the biosynthetic precursor of sapogenins (Heftmann, 1967; Heftmann, 1968), the relationship between levels of sapogenins and saponins and the levels of sterols in plants is not understood.

Table 4-27. Levels (mg/kg) of sapogenins and sterols in the free extracts and conjugated extracts from PNG *B. decumbens* samples.

samples			chol	camp	stig	sito	dio	yam
plot 1	June/94	Conj	8.5	5.2	18.2	31.8	4.1	4.5
		Free	0	0	0	0	0	0
plot 1	June/95	Conj	2.5	1.8	5.9	10.4	6.3	6.9
		Free	1.9	1.2	5.0	8.9	0	0
plot 1	Aug/95	Conj	2.7	1.8	5.3	10.1	5.9	6.6
		Free	0	0	0	2.1	0	0
plot 1	Sept/95	Conj	7.6	6.0	16.7	30.1	45.8	48.0
		Free	0	0	0	3.2	0	0
plot 1	Oct-Nov/95	Conj	5.0	4.4	13.9	25.7	54.8	64.6
		Free	3.9	2.5	8.6	15.0	0	0
plot 1	Dec/95	Conj	3.5	2.3	7.2	13.4	5.7	6.0
		Free	0	0	2.1	6.3	0	0
plot 1	May/96	Conj	4.3	3.3	10.8	16.6	7.3	8.2
		Free	0	0	2.1	3.0	0	0
plot 1	Feb/96	Conj	5.8	6.1	18.0	29.2	45.9	58.6
		Free	0	0	0	1.0	0	0
plot 1	Sept/96	Conj	2.8	2.7	8.7	14.7	13.0	15.2
		Free	0	0	0	0	0	0
plot 2	Apr/94	Conj	11.4	6.4	21.8	35.9	21.8	23.1
		Free	0	0	0.8	1.8	0	0
plot 2	July/94	Conj	4.2	2.8	9.2	16.2	17.2	18.8
		Free	1.2	0.9	2.5	5.6	0	0
plot 2	Sept/94	Conj	7.1	6.6	18.4	33.4	42.6	48.3
		Free	0	0	0	0	0	0
plot 2	June/95	Conj	11.3	9.3	32.2	46.3	31.5	33.6
		Free	3.5	2.7	11.2	14.0	0	0
plot 2	Aug/95	Conj	1.2	1.0	3.3	5.9	13.5	14.9
		Free	0	0	0	2.1	0	0
plot 2	Sept/95	Conj	4.0	2.6	8.2	15.2	18.1	18.7
		Free	7.1	6.0	19.8	32.1	0	0
plot 2	Oct-Nov/95	Conj	3.3	2.0	6.2	12.0	8.6	9.3
		Free	1.1	0.9	2.4	5.8	0	0

continued....

Table 4-27. (Continued)

Samples			chol	camp	stig	sito	dio	yam
plot 2	Dec/95	Conj	1.2	0	2.6	5.4	11.2	12.6
		Free	0	0	1.4	4.0	0	0
plot 2	Feb/96	Conj	5.9	5.2	15.7	25.0	4.2	5.1
		Free	1.9	1.5	5.0	7.6	0	0
plot 3	July/94	Conj	19.9	12.9	43.7	72.2	39.5	39.3
		Free	0	0	0	0	0	0
plot 3	Sept/94	Conj	11.8	10.1	32.4	52.2	41.4	46.2
		Free	0	0	0	0	0	0
plot 3	June/95	Conj	21.0	11.8	42.4	58.0	47.9	48.6
		Free	11.3	8.8	34.6	41.6	21.9	20.6
plot 3	Aug/95	Conj	11.4	7.4	23.9	44.4	34.6	36.2
		Free	0.8	0	2.3	3.6	0	0
plot 3	Sept/95	Conj	5.4	3.3	10.5	22.6	8.6	9.4
		Free	2.3	1.8	6.9	12.6	0	0
plot 3	Oct-Nov/95	Conj	8.3	4.9	13.9	29.7	9.2	9.8
		Free	1.3	0.9	2.6	5.3	0	0
plot 3	Dec/95	Conj	18.9	14.4	36.9	83.0	215	228
		Free	0	0	0	1.8	0	0
plot 3	Feb/96	Conj	8.3	7.8	23.6	40.6	138	166
		Free	0	0	0	0	0	0
plot 3	May/96	Conj	6.0	4.1	14.5	25.0	56.5	68.4
		Free	0	0	0	1.1	0	0
plot 4	July/94	Conj	17.5	13.0	38.2	68.7	26.0	26.0
		Free	3.4	2.5	8.2	12.8	2.4	1.9
plot 4	June/95	Conj	23.3	18.0	50.8	89.9	96.2	93.4
		Free	3.3	2.7	9.8	15.1	6.4	6.0
plot 4	Aug/95	Conj	11.5	9.8	29.8	56.3	95.3	102
		Free	4.4	3.3	11.4	20.4	0	0
plot 4	Sept/95	Conj	8.0	4.9	16.0	28.2	13.2	13.4
		Free	2.0	1.3	4.3	7.3	0	0
plot 4	Oct-Nov/95	Conj	11.2	7.7	21.6	40.8	11.2	11.3
		Free	1.4	1.0	2.9	5.2	0	0

continued....

Table 4-27. (Continued)

Samples			chol	camp	stig	sito	dio	yam
plot 4	Dec/95	Conj	15.1	12.2	32.6	68.1	121	133
		Free	3.8	2.7	7.9	15.0	0	0
plot 4	Feb/96	Conj	10.8	10.9	31.4	51.4	112	134
		Free	8.6	7.5	24.0	31.5	15.5	15.3
plot 4	Apr/96	Conj	7.3	4.8	14.1	27.3	31.4	32.7
		Free	3.8	2.8	8.4	14.6	1.9	1.8
plot 4	May/96	Conj	6.3	4.3	14.9	22.6	10.2	10.3
		Free	5.5	4.1	14.6	19.3	3.8	3.5
plot 5	Aug/95	Conj	5.6	3.2	11.1	21.7	24.1	24.6
		Free	0	0	2.8	3.3	0	0
plot 5	Sept/95	Conj	4.2	2.8	9.7	20.6	23.3	26.5
		Free	0.5	0.6	2.4	39.4	0	0
plot 5	Oct-Nov/95	Conj	4.7	2.6	8.8	19.2	12.5	13.8
		Free	0	0	2.3	3.8	0	0
plot 5	Dec/95	Conj	6.3	3.7	12.3	27.7	8.9	10.3
		Free	0.9	0	2.7	5.6	0	0
plot 5	Apr/96	Conj	4.2	1.7	5.6	14.2	7.4	7.6
		Free	1.2	1.3	4.4	8.4	0	0
plot 5	May/96	Conj	2.7	2.6	9.0	16.1	23.2	24.4
		Free	4.1	3.1	12.9	17.8	3.7	3.8
plot 6	Sept/94	Conj	3.1	2.4	7.9	18.9	21.7	24.1
		Free	6.3	5.3	21.8	39.5	5.0	5.2
plot 6	Dec/94	Conj	4.2	3.9	15.8	27.2	10.9	11.6
		Free	5.0	5.4	26.3	31.5	14.2	15.8
plot 6	June/95	Conj	6.2	5.4	24.5	32.0	25.1	26.8
		Free	3.9	3.3	15.1	19.0	26.1	28.7
plot 6	Aug/95	Conj	1.6	1.1	5.0	9.9	44.0	47.0
		Free	5.6	4.6	15.5	26.8	7.6	7.9
plot 6	Sept/95	Conj	1.6	0	2.3	5.1	15.2	17.0
		Free	9.8	7.2	25.8	43.7	7.5	8.0
plot 6	Feb/96	Conj	6.6	1.0	5.7	8.2	41.7	53.3
		Free	8.9	8.5	29.6	42.4	19.1	20.9

continued....

Table 4-27. (Continued)

Samples			chol	camp	stig	sito	dio	yam
plot 6	Apr/96	Conj	1.5	0.5	5.5	6.1	18.6	22.4
		Free	6.2	4.0	13.2	24.8	3.6	4.4
plot 6	May/96	Conj	1.8	0.6	4.3	6.1	18.5	23.1
		Free	6.7	4.9	18.8	26.7	7.6	8.7
plot 7	Feb/95	Conj	2.0	0.6	4.2	5.5	8.1	9.0
		Free	5.5	3.9	13.6	24.9	2.0	2.1
plot 7	June/95	Conj	1.9	1.2	6.7	7.9	24.3	24.3
		Free	7.6	6.4	27.3	33.6	26.9	27.0
plot 7	Aug/95	Conj	1.3	0.7	2.3	5.7	9.7	10.6
		Free	6.4	5.0	18.3	33.4	5.9	5.8
plot 7	Sept/95	Conj	2.2	0.6	2.6	6.1	22.2	21.8
		Free	10.8	7.5	28.8	49.6	2.1	2.4
plot 7	Dec/95	Conj	2.4	0	2.1	5.2	68.1	75.9
		Free	11.1	7.8	25.5	49.9	7.6	7.6
plot 7	Feb/96	Conj	3.2	1.2	4.9	8.3	67.2	81.8
		Free	10.1	9.5	35.6	48.2	25.0	25.7
plot 7	May/96	Conj	1.7	1.4	5.6	11.0	268	323
		Free	7.2	5.2	19.7	31.2	8.2	8.3
plot 8	Sept/94	Conj	1.0	0.7	2.7	8.0	115	139
		Free	7.0	5.6	22.0	40.0	5.7	5.9
plot 8	June/95	Conj	2.7	2.6	12.9	11.5	46.1	59.1
		Free	11.6	10.9	55.0	53.3	95.7	97.9
plot 8	Aug/95	Conj	1.0	0.6	2.8	6.5	55.7	64.5
		Free	5.4	4.8	17.7	32.5	6.7	6.9
plot 8	Sept/95	Conj	1.1	0	3.0	9.8	26.3	31.4
		Free	7.0	5.5	20.6	35.9	3.3	3.5
plot 8	Dec/95	Conj	4.4	3.9	12.4	30.3	85.0	98.6
		Free	0	0	0.7	1.6	0	0
plot 8	Feb/96	Conj	2.2	2.4	8.7	14.9	49.9	64.7
		Free	0	0	0	0.9	0	0
plot 8	Apr/96	Conj	1.3	1.0	3.2	8.4	6.0	6.9
		Free	5.5	4.2	13.5	26.7	0	0

continued....

Table 4-27. (Continued)

Samples			chol	camp	stig	sito	dio	yam
plot 8	May/96	Conj	7.2	7.2	27.8	50.7	218	262
		Free	5.0	3.6	14.5	20.4	3.9	3.8
plot 8	July/96	Conj	6.7	5.9	19.5	47.6	184	202
		Free	8.0	5.2	18.3	36.6	0	0
plot 4	97	Conj	11.3	11.3	34.7	71.4	27.1	25.8
		Free	17.6	16.0	49.4	82.6	8.7	10.0
plot 5	97	Conj	12.9	13.6	43.0	74.5	120	126
		Free	8.8	8.1	26.2	41.6	21.9	21.2
plot 11	97	Conj	17.3	26.4	66.5	111	27.9	26.2
		Free	8.2	8.3	22.1	33.3	9.2	7.8
plot 14	97	Conj	12.6	12.9	46.1	71.4	39.0	36.2
		Free	24.3	21.2	75.6	101	39.6	35.2
plot 15	97	Conj	14.4	13.9	47.4	77.2	118	111
		Free	19.7	15.0	56.5	76.3	51.2	53.1
Base	97	Conj	0.8	3.1	4.9	17.2	0	0
		Free	1.9	6.5	8.4	27.7	0	0
Paragrass	97	Conj	1.7	7.3	14.5	20.5	14.1	17.5
		Free	22.6	92.7	167	212	3.5	3.8
Top Stud-Trid	97	Conj	21.3	17.2	59.5	145	77.0	80.2
		Free	8.8	7.2	25.2	54.0	0	0
Marrumbung	97	Conj	14.3	15.3	58.0	96.4	211	232
		Free	15.1	3.9	60.8	85.3	29.1	28.6
Tank PDK	97	Conj	5.2	7.2	32.2	20.1	162	24.9
		Free	29.0	29.8	140	73.2	10.1	2.2
/9	97	Conj	15.9	15.5	57.8	104	212	239
		Free	19.6	20.8	72.9	106	23.3	25.2
Theo's-M.L.	97	Conj	9.4	9.2	35.0	61.8	147	165
		Free	20.0	20.7	75.5	113	20.3	22.3
Morgon (96)	97	Conj	0	1.7	2.5	8.9	0	0
		Free	17.0	89.9	129	307	0	0
Bottom Hand	97	Conj	1.0	0	3.5	8.8	54.1	59.7
		Free	61.7	64.3	188	284	0	0

Conj = conjugated extract, Free = free extract, chol = cholesterol, camp = campesterol, stig = stigmasterol, sito = β-sitosterol, dio = diosgenin, yam = yamogenin

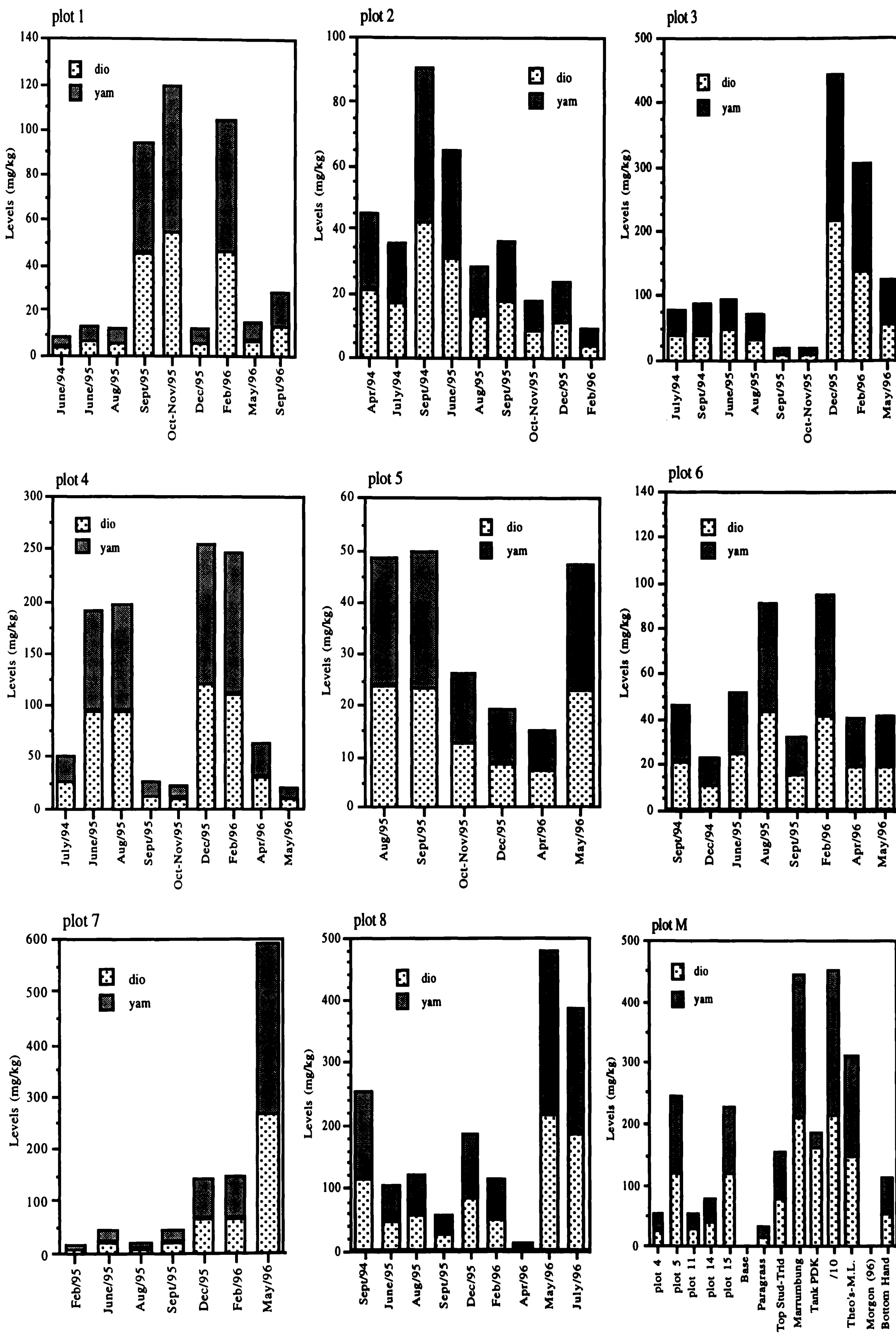


Figure 4-18. Levels of conjugated sapogenins in *B. decumbens* samples from the research plots in PNG.

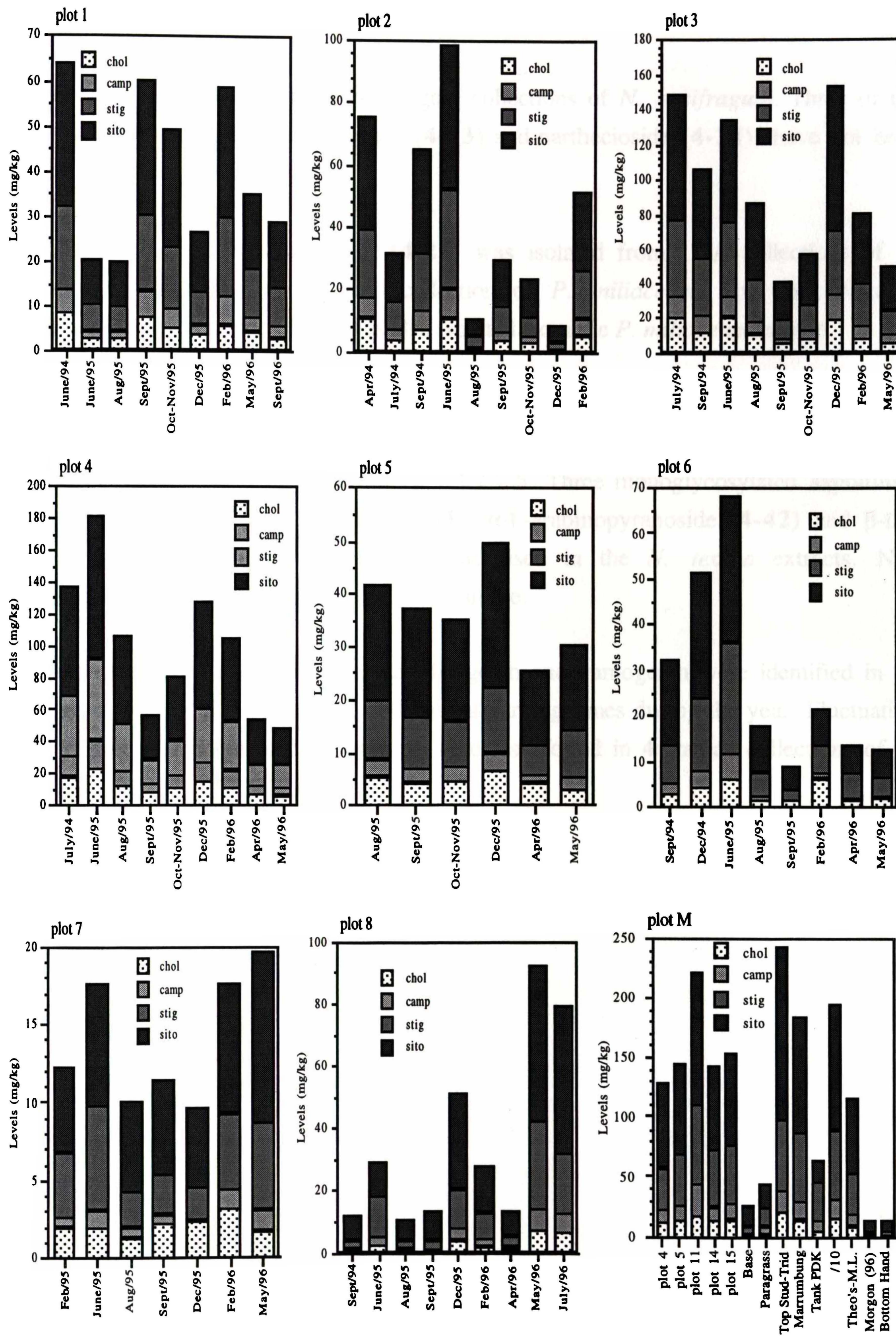


Figure 4-19. Levels of conjugated sterols in *B. decumbens* samples from the research plots in PNG.

4.8 Summary and Conclusions

Six saponins were isolated from Norwegian collections of *N. ossifragum*. Three of the saponins, faroecin (**4-22**), asiaticioside (**4-23**) and narthecioside (**4-24**), have not been previously reported in the literature.

The 25*R/S*-furostenol tetrasaccharide (**4-25**) was isolated from USA collections of *P. virgatum* and from a New Zealand collection of *P. miliaceum*. The corresponding spirostenol tetrasaccharide (**4-26**) was also isolated from the *P. miliaceum* sample.

Three unsaturated dihydroxy sapogenins, ruscogenin (**4-11**), neoruscogenin (**4-12**) and $\Delta^{25(27)}$-ruscogenin (**4-40**), were identified in an USA collection of *N. texana*. These sapogenins were present in a ratio of ca 1:1.2:2.5. Three monoglycosylated saponins, $\Delta^{25(27)}$-ruscogenin β-D-fucopyranoside (**4-41**), α-L-arabinopyranoside (**4-42**) and β-D-galactopyranoside (**4-43**), were also characterised in the *N. texana* extracts. No monohydroxy sapogenins were found in the sample.

Fluctuating levels of free and conjugated diosgenin and yamogenin were identified in 82 PNG specimens of *B. decumbens*, collected at varying times during the year. Fluctuating levels of free and conjugated sapogenins were also found in 4 Iranian collections of *T. terrestris*.

Chapter Five

EXPERIMENTAL

5.1 General

5.1.1 Nuclear Magnetic Resonance (NMR) Experiments

One- and two-dimensional ^{1}H and ^{13}C NMR spectra were obtained from $CDCl_3$, CD_3SOCD_3 or C_5D_5N solutions using either a Bruker AC-300 instrument fitted with a 5 mm dual probehead operating at 300.13 (^{1}H) and 75.47 (^{13}C) MHz, or a Bruker DRX-400 instrument fitted 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).

^{13}C NMR signal multiplicities (C, CH, CH_2 or CH_3: s, d, t, or q respectively) were determined using the DEPT sequence. NOE-difference experiments were performed with sequential irradiation of each line in the multiplets using the method of Kinns and Sanders (1984). Two-dimensional COSY, long-range COSY and inverse mode heteronuclear multiple-bond correlation (HMBC) spectra were determined in absolute value mode. NOESY, ROESY, HOHAHA, and ^{13}C-^{1}H correlated inverse mode HMQC or HSQC spectra were determined in phase-sensitive mode.

^{1}H NMR spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW (sweep width) = 5593 Hz, 90° pulse, SI = 32 K, TD = 32 K, Acq = 2.9 sec, D1 (repetition delay) = 0.1 sec, LB (line broadening factor) = 0.

^{13}C NMR spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW = 24691 Hz, 70° pulse, SI = 32 K, TD = 32 K, Acq = 0.664 sec, D1 = 1 sec, LB = 2.

DEPT135 NMR spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW = 24154 Hz, SI = 32 K, TD = 32 K, Acq = 0.664 sec, D1 = 1 sec, D2 = $1/2J$ = 3.8 msec, LB = 2.

COSY spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW1 = SW2 = 2688 Hz, SI1 = SI2 = 1 K, TD1 = 400, TD2 = 1 K, Acq = 0.19 sec, D1 = 0.25 sec, MC2 = QF, SSB1 = SSB2 = 0, WDW1 = WDW2 = SINE.

ROESY spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW1 = SW2 = 2874 Hz, SI1 = SI2 = 1 K, TD1 = 400, TD2 = 1 K, Acq = 0.178 sec, D1 = 1 sec, P15 (spinlock time, with PL1 = 3 db) = 250 msec, MC2 = TPPI, SSB1 = SSB2 = 2, WDW1 = WDW2 = QSINE.

HOHAHA (= TOCSY) spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW1 = SW2 = 2723 Hz, SI1 = SI2 = 1 K, TD1 = 320, TD2 = 1 K, Acq = 0.188 sec, D1 = 1 sec, mixing time = 200 msec, MC2 = TPPI, SSB1 = SSB2 = 2, WDW1 = WDW2 = QSINE.

HMQC and HSQC spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW1 = 15092 Hz, SW2 = 2723 Hz, SI1 = SI2 = 1 K, TD1 = 400, TD2 = 1 K, Acq = 0.188 sec, D1 = 0.4 sec, D2 = $1/2J$ = 3.45 msec, MC2 = TPPI, SSB1 = SSB2 = 2, WDW1 = WDW2 = QSINE.

HMBC spectra were typically acquired using the DRX-400 spectrometer and the following conditions: SW1 = 19118 Hz, SW2 = 2723 Hz, SI1 = SI2 = 1 K, TD1 = 160, TD2 = 1 K, Acq = 0.188 sec, D1 = 0.7 sec, D2 = $1/2J$ = 3.45 msec, MC2 = QF, SSB1 = SSB2 = 0, WDW1 = WDW2 = SINE.

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

5.1.2.1 Sample Preparation

An accurately weighed sample (2 g) of air-dried, or freeze-dried, ground plant or animal material was placed in a Whatman cellulose extraction thimble and sequentially extracted in a Soxhlet apparatus with *n*-hexane (100 mL) for 20 h to afford a free extract, and then with ethanol-water (4:1) (100 mL) for 24 h to give a conjugated extract. The respective extracts, in 250 mL round bottom flasks, were evaporated to dryness on a rotary evaporator at 40°C.

An accurately determined amount of internal standard, either sarsasapogenin propionate (0.208 mg/mL in chloroform, for samples containing mainly saturated sapogenins) or diosgenin propionate (0.214 mg/mL in chloroform, for samples containing mainly unsaturated and dihydroxylated sapogenins), was added to the evaporated free (*n*-hexane) extract, which was further diluted with chloroform and transferred to a GC vial. Pyridine (0.5 mL) and acetic anhydride (0.5 mL) were added, and the mixture was allowed to stand for 2 h prior to GC-MS analysis.

The conjugated (ethanol-water) extract was taken up in 1 M hydrochloric acid (70 mL) and heated to 80-85°C for 1.5-2 h. After cooling, the aqueous solution was extracted with chloroform (3 × 20 mL). The chloroform extracts were combined, dried over anhydrous sodium sulfate, filtered and the solvent was removed by rotary evaporation. An accurately determined amount of internal standard, sarsasapogenin propionate (0.208 mg/mL in chloroform, for samples containing mainly saturated sapogenins) or diosgenin propionate (0.214 mg/mL in chloroform, for samples containing mainly unsaturated and dihydroxylated sapogenins), was added to the evaporated chloroform extract, which was further diluted with chloroform and transferred to a GC vial. Pyridine (0.5 mL) and acetic anhydride (0.5 mL) were added, and the mixture was allowed to stand for 2 h prior to GC-MS analysis.

5.1.2.2 Primary Standard Solutions

- **Standard solution of sarsasapogenin propionate**: 0.0104 g of sarsasapogenin propionate was weighed into a 15 mL glass vial, dissolved in chloroform (ca 5 mL), and transferred with repeated washing to a 50 mL volumetric flask and made up to the mark with chloroform, to give a standard solution of 0.208 mg/mL of sarsasapogenin propionate.

- **Standard solution of diosgenin propionate**: 0.0107 g of diosgenin propionate was weighed into a 15 mL glass vial, dissolved in chloroform (ca 5 mL), and transferred with repeated washing to a 50 mL volumetric flask and made up to the mark with chloroform to give a standard solution of 0.214 mg/mL of diosgenin propionate.

- **Standard solution of sapogenins and sterols**:
  The following quantities of sapogenins and sterols:

0.0019 g of cholesterol acetate
0.0022 g of stigmasterol acetate
0.0019 g of β-sitosterol acetate
0.0020 g of sarsasapogenin acetate
0.0025 g of sarsasapogenin propionate
0.0021 g of diosgenin acetate
0.0021 g of diosgenin propionate

were weighed into a 20 mL volumetric flask and made up to mark with chloroform to give the standard solution.

- **Standard solution of fatty acids**:

The following quantities of fatty acids:

0.0020 g of palmitic acid
0.0020 g of stearic acid
0.0026 g of oleic acid
0.0021 g of sarsasapogenin propionate

were weighed into a 20 mL volumetric flask and made up to mark with chloroform to give the standard solution.

5.1.2.3 GC-MS

Sapogenin and steroid components

GC-MS analysis of sapogenin acetates was performed using a 0.22 mm id (internal diameter) × 25 m HP-1 (Hewlett Packard) methylsilicone capillary column installed in a HP-5980 gas chromatography 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 programmed from 200°C to 250°C at 35°C/min, then to 295°C at 3°C/min with a 18 minutes hold. Column head pressure was typically set at 15 kPa.

Identification of sapogenin and steroid 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* 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 following selected ion mode (SIM) chromatograms:

m/z 139 for sapogenins	(base peak *ex* ring F)
m/z 368 for cholesterol	(M^+-HOAc fragment ion)
m/z 382 for campesterol	(M^+-HOAc fragment ion)
m/z 394 for stigmasterol	(M^+-HOAc fragment ion)
m/z 396 for β-sitosterol	(M^+-HOAc fragment ion)

Concentrations of these components were determined relative to an internal standard (sarsasapogenin propionate or diosgenin propionate) using appropriate response factors (R_F).

Response factors (R_F) were determined by comparison of the integrated peak areas and weights of the internal standards (sarsasapogenin propionate or diosgenin propionate) and authentic specimens of sarsasapogenin acetate, diosgenin acetate, cholesterol acetate, stigmasterol acetate, and β-sitosterol acetate.

Fatty acids

Fatty acid analyses were performed as described for sterols and sapogenins, other than that the GC oven temperature was programmed from 100°C to 295°C at 8°C/min with a final 10 minutes hold. Column head pressure was typically set at 15 kPa.

Identification of fatty acid methyl esters was carried out by comparison of the retention times of a series of fatty acid standards, and by examination of the TIC and SIM profiles (*m/z* 74 and 79 ions).

Quantitation of fatty acids was performed relative to sarsasapogenin propionate as internal standard, by integration of SIM *m/z* 74 (saturated fatty acids) and 79 (unsaturated fatty acids) ion profiles respectively. Response factors (R_F) were determined by comparison of the integrated areas and weights of sarsasapogenin propionate (internal standard) and palmitic acid methyl ester, stearic acid methyl ester, and oleic acid methyl ester, present in a reference solution.

5.1.2.4 Calculations

Response factor (R_F):

$$R_F(x) = \frac{A(sx)/W(sx)}{A(std)/W(std)}$$

where A(sx) is the integrated peak area of a known weight of the standard compound x;
A(std) is the integrated peak area of internal standard;
W(sx) is the weight of standard compound x;
W(std) is the weight of internal standard.

Levels (mg/kg):

$$Level(x) = \frac{A(x)/W(sm)}{A(istd)/W(istd)} * \frac{1}{R_F(x)} * R_M(x) * 1000$$

where A(x) is the integrated peak area of compound x;
A(istd) is the integrated peak area of internal standard added to the sample;
W(sm) is the weight of plant or animal sample;
W(istd) is the weight of internal standard added in the sample;
$R_M(x)$ is the ratio of the molecular weight 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).

5.1.3 Electrospray Mass Spectroscopy (ES-MS)

ES-MS was performed in negative ion mode, or positive ion mode, using a VG Platform II instrument, with a cone voltage of -40 V to -110 V (negative ion MS), or 40 V to 110 V (positive ion MS) and acetonitrile-water (1:1) as the solvent. Ethanolic saponin extracts were diluted with acetonitrile-water and injected directly into the ES-MS. Solid samples

were dissolved in acetonitrile-water or pyridine and diluted with acetonitrile-water prior to ES-MS analysis.

The addition of Na^+, K^+ and/or Cl^- to sample solutions was often used to assist in identification of the molecular ion.

5.1.4 Chromatography

Thin-layer chromatography (TLC)
TLC was performed on silica gel plates (E. Merck 5554) using the following solvent systems: **A**: chloroform-methanol-water (40:19:1) for saponins; **B**: hexane-ethyl acetate (2:1) for sapogenins. Plates were visualised by spraying with a saturated dodecamolybdophosphoric acid ethanol solution, and heating at ca 90°C.

Radial chromatography (RC)
Radial chromatography was typically performed using a Chromatotron 7924T and a 22.5 cm diameter × 2 mm silica gel (60 G, Merck 7731) plate. Radial chromatography plates were developed with the mixtures of chloroform and 5% aqueous methanol.

Column chromatography (CC)
Column chromatography was carried out using 50 × 2 cm silica gel (60-120 mesh, BDH 30061) columns. Columns were developed with the mixtures of chloroform and 5% aqueous methanol.

High performance liquid chromatography (HPLC)
High performance liquid chromatography (HPLC) was performed using a Waters Radial Pak RP18 column (8 mm id × 100 mm) equipped with a Waters 515 HPLC Pump, a Waters 410 Differential Refractometer and a Hewlett Packard 3394A Integrator. Methanol-water (4:1; 0.5 mL/min) was used as column eluent.

5.1.5 Chemicals

Authentic specimens of diosgenin, cholesterol acetate, stigmasterol acetate, β-sitosterol acetate were purchased from Sigma Chemical Co., MO, USA. Sarsasapogenin was purchased from The Upjohn Co., MI, USA. Specimens of sarsasapogenin propionate and diosgenin propionate were available from the work of Berndt (1997).

5.2 Experimental, Chapter Two

5.2.1 Synthesis of Sarsasapogenin β-D-Glucoside (2-2)

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

1,2,3,4,6-Penta-*O*-acetyl-β-D-glucopyranose (**2-3**) (30.0 g, 0.077 moles), glacial acetic acid (35 mL) and 45% hydrobromic acid in acetic acid (75 mL, 0.6 moles) were mixed and held at 5°C for 24 h until TLC showed complete reaction. The solution was poured into a mixture of chloroform (500 mL) and ice (200 g) and separated. The aqueous layer was extracted with chloroform (2 × 150 mL). The combined chloroform extracts were washed with 2% sodium hydroxide solution (3 × 200 mL) and water (2 × 200 mL), and dried with anhydrous sodium sulfate for 16 h. Removal of solvent using a rotary evaporator gave an oil. Recrystallisation from diethyl ether afforded **2-4** as white needles (30.2 g, 95.7%), m.p. 87-88°C [lit. 87-88°C (Redemann and Niemann, 1955)]; ^{1}H NMR (δ ppm, $CDCl_3$): 1.98 (3H, s, OAc), 1.99 (3H, s, OAc), 2.04 (3H, s, OAc), 2.05 (3H, s, OAc), 4.23 (1H, br d, H-6b), 4.25 (1H, m, H-5), 4.29 (1H, m, H-6a), 4.78 (1H, dd, J = 6.0 and 4.0 Hz, H-2), 5.10 (1H, t, J = 9.7 and 9.7 Hz, H-4), 5.49 (1H, t, J = 10.1 and 9.8 Hz, H-3), 6.55 (1H, d, J = 3.98 Hz, H-1); ^{13}C NMR (δ ppm, $CDCl_3$): 20.5-20.6 (4 × OAc), 61.0 (C-6), 67.2 (C-4), 70.1 (C-3), 70.6 (C-2), 72.2 (C-5), 86.6 (C-1), 169.4 (OAc), 169.7 (OAc), 169.8 (OAc), 170.4 (OAc); COSY correlations: 6.55 (H-1) ↔ 4.78 (H-2) ↔ 5.49 (H-3) ↔ 5.10 (H-4) ↔ 4.25 (H-5, H-6b) ↔ 4.29 (H-6a).

Sarsasapogenin β-D-glucoside tetraacetate (2-5)

A mixture of sarsasapogenin (**2-1**) (5.0 g, 0.0125 moles), cadmium carbonate (5.0 g, 0.025 moles) and toluene (200 mL) was refluxed in a 500 mL round bottom flask fitted with a Dean and Stark trap for 0.5 h. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**2-4**) (11.0 g, 0.025 moles) in toluene (100 mL) was added to the reaction mixture dropwise over 1 h. The mixture was then refluxed for 24 h, until TLC showed complete reaction. The reaction mixture was filtered while still hot. After cooling, removal of solvent using a rotary evaporator gave an oil. The oil was recrystallised from diethyl ether to give sarsasapogenin β-D-glucoside tetraacetate (**2-5**) (3.8 g, 40.7%), m.p. 204-212°C; ES-MS (+40V, CH_3CN-H_2O): *m/z* 746.2 $(M+NH_4)^+$ and *m/z* 769.4 $(M+Na)^+$; ^{1}H NMR (δ ppm, $CDCl_3$): 0.75 (3H, s, H-18), 0.92 (3H, s, H-19), 0.98 (3H, d, J = 6.6 Hz, H-27), 1.07 (3H, d, J = 7.1 Hz, H-21), 2.00 (3H, s, OAc), 2.01 (3H, s, OAc), 2.02 (3H, s, OAc), 2.06 (3H, s, OAc), 3.29 (1H, dd, H-26a), 3.66 (1H, m, H-5'), 3.94 (1H, dd, H-26b), 3.99 (1H, b s, H-3), 4.10 (1H, dd,

H-6'a), 4.25 (1H, dd, H-6'b), 4.39 (1H, q, H-16), 4.54 (1H, d, $J = 7.98$ Hz, H-1'), 4.99 (1H, q, H-2'), 5.07 (1H, t, H-4'), 5.20 (1H, t, H-3'); ^{13}C NMR (δ ppm, $CDCl_3$): 14.4 (C-21), 16.0 (C-27), 16.5 (C-18), 20.6 (OAc), 20.65 (OAc), 20.7 (OAc), 20.8 (OAc), 20.9 (C-11), 24.0 (C-19), 25.8 (C-24), 26.0 (C-23), 26.4 (C-7), 26.6 (C-6), 26.7 (C-2), 27.1 (C-25), 29.7 (C-1), 30.2 (C-4), 31.8 (C-15), 35.0 (C-10), 35.3 (C-8), 36.8 (C-5), 40.1 (C-9), 40.3 (C-12), 40.7 (C-13), 42.2 (C-20), 56.5 (C-14), 62.2 (C-17, C-6'), 65.7 (C-26), 68.7 (C-3), 71.5 (C-5'), 71.7 (C-2'), 73.0 (C-4'), 74.4 (C-3'), 81.0 (C-16), 98.6 (C-1'), 109.8 (C-22), 169.1 (OAc), 169.4 (OAc), 170.4 (OAc), 170.7 (OAc); COSY correlations: 4.54 (H-1') ↔ 4.99 (H-2') ↔ 5.21 (H-3') ↔ 5.07 (H-4') ↔ 3.66 (H-5') ↔ 4.10 (H-6'a) ↔ 4.25 (H-6'b), 4.39 (H-16) ↔ 1.99 (H-15a) ↔ 1.75 (H-17) ↔ 1.18 (H-15b), 3.94 (H-26b) ↔ 3.29 (H-26a).

Sarsasapogenin β-D-glucoside (2-2)

(A) A 2.5 M solution of sodium hydroxide in methanol (8 mL, 0.02 moles) was added to a suspension of sarsasapogenin β-D-glucoside tetraacetate (**2-5**) (3.6 g, 0.0048 moles) in methanol (120 mL) and the mixture was stirred at 50-60°C for 1.5 h. After cooling, the mixture was poured into 700 mL of water and extracted with 1-butanol (3 × 150 mL). The 1-butanol solution was washed with water, dried with anhydrous sodium sulfate and evaporated *in vacuo*. The residue was recrystallised from 70% aqueous acetone to give sarsasapogenin β-D-glucoside (**2-2**) (2.5 g, 89.3%), m.p. 223-229°C; ES-MS (-100V, CH_3CN-H_2O): *m/z* 577.5 $(M-H)^-$; ^{1}H NMR (δ ppm, DMSO-D_6): 0.81 (3H, s, H-18), 1.00 (3H, s, H-19), 1.02 (3H, d, $J = 7.0$ Hz, H-21), 1.10 (3H, d, $J = 6.9$ Hz, H-27), 3.02 (1H, t, H-2'), 3.14 (2H, m, H-4', H-5'), 3.22 (1H, t, H-3'), 3.31 (1H, d, H-26a), 3.51 (1H, br d, H-6'a), 3.73 (1H, br d, H-6'b), 3.88 (1H, dd, H-26b), 4.01 (1H, s, H-3), 4.23 (1H, d, $J = 7.75$, H-1'), 4.38 (1H, m, H-16), 4.50 (1H, br s, H-OH), 4.78 (1H, br s, H-OH), 5.00 (1H, br s, H-OH); ^{13}C NMR (δ ppm, DMSO-D_6): 14.4 (C-21), 15.9 (C-27), 16.1 (C-18), 20.4 (C-11), 23.5 (C-19), 25.3 (C-24), 25.4 (C-23), 25.9 (C-7), 26.1 (C-6), 26.2 (C-2), 26.3 (C-25), 29.4 (C-1), 30.1 (C-4), 31.3 (C-15), 34.5 (C-10), 34.8 (C-8), 35.8 (C-5), 39.2 (C-9), 39.5 (C-12), 39.7 (C-13), 41.5 (C-20), 55.6 (C-14), 61.0 (C-6'), 61.7 (C-17), 64.2 (C-26), 70.1 (C-3), 72.5 (C-5'), 73.5 (C-2'), 76.6 (C-4'), 76.8 (C-3'), 80.3 (C-16), 100.9 (C-1'), 108.8 (C-22); COSY correlations: 4.23 (H-1') ↔ 3.02 (H-2') ↔ 3.22 (H-3') ↔ 3.14 (H-4', H-5') ↔ 3.73 (H-6'b) ↔ 3.51 (H-6'a), 4.38 (H-16) ↔ 1.99 (H-15a) ↔ 1.76 (H-17) ↔ 1.25 (H-15b), 4.01 (H-3) ↔ 2.0 (H-2) ↔ 1.5 (H-4), 3.88 (H-26b) ↔ 3.31 (H-26a).

(B) A mixture of sarsasapogenin (**2-1**) (15.0 g, 0.036 moles), cadmium carbonate (15.0 g, 0.072 moles) and toluene (400 mL) was refluxed in a 1000 mL round bottom flask fitted with a Dean-Stark trap for 0.5 h. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-

glucopyranosyl bromide (**2-4**) (16.50 g, 0.072 moles) in toluene (200 mL) was added to the reaction mixture dropwise over 1.5 h. The mixture was then refluxed for 24 h, until TLC showed complete reaction. The reaction mixture was filtered while still hot. After cooling, removal of solvent using a rotary evaporator gave an oil which was suspended in a solution of methanol (350 mL) and 2.5 M methanolic sodium hydroxide (24 mL), and stirred at 50-60°C for 2 h. After cooling, the mixture was poured into water (1500 mL) and extracted with 1-butanol (3 × 300 mL). The 1-butanol solution was washed with water, dried with anhydrous sodium sulfate and evaporated *in vacuo*. The residue was recrystallised from 70% aqueous acetone to give sarsasapogenin β-D-glucoside (**2-2**) (16.72 g, 83.6%), m.p. 222-230°C; ^{1}H and ^{13}C NMR data were the same as those determined for a specimen prepared by method (A).

5.2.2 Synthesis of 3β-D-Epismilagenin β-D-Glucoside (2-8)

Smilagenin (2-9)

A mixture of sarsasapogenin (**2-1**) (20.0 g), 95% aqueous ethanol (1000 mL) and concentrated hydrochloric acid (200 mL) was refluxed for 120 h, then poured into water (3000 mL). The precipitate in the mixture was collected and washed with water. Recrystallisation from acetone (× 3) afforded white needles of **2-9**, m.p. 176-8°C [lit 183°C (Wall et al., 1955a)], yield 47% (9.4 g); ^{1}H NMR (δ ppm, $CDCl_3$): 0.75 (3H, s, H-18), 0.77 (3H, d, J = 7.0 Hz, H-21), 0.95 (3H, d, J = 7.0 Hz, H-27), 0.99 (3H, s, H-19), 3.36 (1H, t, H-26a), 3.46 (1H, m, H-26b), 4.09 (1H, br s, H-3), 4.37 (2H, m, H-16); ^{13}C NMR (δ ppm, $CDCl_3$): 14.5 (C-21), 16.5 (C-18), 17.1 (C-27), 20.9 (C-11), 23.9 (C-19), 26.6 (C-6, C-7), 27.9 (C-2), 28.8 (C-24), 30.0 (C-1), 30.3 (C-25), 31.4 (C-23), 31.8 (C-15), 33.6 (C-4), 35.3 (C-8, C-10), 36.6 (C-5), 39.9 (C-9), 40.3 (C-12), 40.7 (C-13), 41.6 (C-20), 56.5 (C-14), 62.3 (C-17), 66.9 (C-26), 67.1 (C-3), 81.0 (C-16), 109.7 (C-22).

Smilagenone (2-10)

A solution of chromium trioxide (Djerassi et al., 1956) (15 mL) was added to a solution of smilagenin (**2-9**) (8.3 g, 0.02 moles) in acetone (800 mL) and stirred at room temperature for 2 h. The reaction solution was then poured into water (2000 mL) and the precipitate was collected, washed with water and dried. Recrystallisation from acetone gave smilagenone (**2-10**) (7.7 g, 93%), m.p. 186-8°C [lit. 188°C (Wall et al., 1955)]; ^{1}H NMR (δ ppm, $CDCl_3$): 0.78 (3H, d, J = 7.1 Hz, H-21), 0.79 (3H, s, H-18), 0.96 (3H, d, J = 7.0 Hz, H-27), 1.03 (3H, s, H-19), 3.36 (1H, t, H-26a), 3.46 (1H, m, H-26b), 4.40 (2H, m, H-16); ^{13}C NMR (δ ppm, $CDCl_3$): 14.5 (C-21), 16.5 (C-18), 17.2

(C-27), 21.1 (C-11), 22.7 (C-19), 26.5 (C-6), 26.6 (C-7), 28.8 (C-24), 30.3 (C-25), 31.4 (C-23), 31.8 (C-15), 35.1 (C-10), 35.2 (C-8), 37.0 (C-2), 37.2 (C-2), 40.2 (C-12), 40.7 (C-13), 40.9 (C-9), 41.7 (C-20), 42.4 (C-4), 44.3 (C-6), 56.3 (C-14), 62.3 (C-17), 66.9 (C-26), 80.8 (C-16), 109.3 (C-22), 213.2 (C-3).

3β-D-Epismilagenin (2-11)

A solution of smilagenone (**2-10**) (16.32 g, 0.04 moles) in ether (200 mL) was added during 1 h to a vigorously stirred solution of lithium aluminium deuteride (2.42 g, 0.057 moles) in diethyl ether (150 mL). The mixture was stirred at room temperature for 2 h and the excess of lithium aluminium deuteride was destroyed with ice-cold 0.5 M sulfuric acid. The ethereal layer was separated, and the aqueous layer was extracted with diethyl ether (2 × 50 mL). The diethyl ether extracts were combined, washed with water, sodium hydrogen carbonate solution, and again with water, dried with anhydrous sodium sulfate, evaporated to give 3β-D-epismilagenin (**2-11**) (16.21 g, 98.6%), m.p. 180-3°C; ^{1}H NMR (δ ppm, $CDCl_3$): 0.74 (3H, s, H-18), 0.78 (3H, d, J = 6.9 Hz, H-21), 0.92 (3H, s, H-19), 0.95 (3H, d, J = 7.0 Hz, H-27), 3.36 (1H, t, H-26a), 3.46 (1H, m, H-26b), 4.09 (1H, br s, H-3), 4.38 (2H, m, H-16); ^{13}C NMR (δ ppm, $CDCl_3$): 14.5 (C-21), 16.5 (C-18), 17.2 (C-27), 20.7 (C-11), 23.4 (C-19), 26.7 (C-6), 27.1 (C-7), 28.8 (C-24), 30.3 (C-25), 30.4 (C-2), 31.4 (C-23), 31.8 (C-15), 34.7 (C-10), 35.4 (C-1), 35.5 (C-8), 36.4 (C-4), 40.3 (C-12), 40.5 (C-9), 41.4 (C-13), 41.7 (C-20), 42.1 (C-5), 56.4 (C-14), 62.3 (C-17), 66.9 (C-26), 80.9 (C-16), 109.3 (C-22).

3β-D-Epismilagenin β-D-glucoside tetraacetate (2-12)

A mixture of 3β-D-epismilagenin (**2-11**) (3.80 g, 0.009 moles), cadmium carbonate (4.47 g, 0.026 moles) and toluene (150 mL) was refluxed in a 500 mL round bottom flask fitted with a Dean-Stark trap for 1 h. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide (**2-4**) (10.91 g, 0.026 moles) in toluene (100 mL) was added dropwise to the mixture during 1 h. The mixture was refluxed for 24 h, until TLC showed complete reaction, and then filtered while still hot. The filtrate was evaporated *in vacuo* and recrystallised from methanol to give 3β-D-epismilagenin β-D-glucoside tetraacetate (**2-12**) (2.84 g, 42.2%), m.p. 159-162°C; ES-MS (+100 V): *m/z* 748.2 $(M+H)^+$, *m/z* 765.5 $(M+NH_4)^+$, *m/z* 770.4 $(M+Na)^+$; ^{1}H NMR (δ ppm, $CDCl_3$): 0.73 (3H, s, H-18), 0.77 (3H, d, J = 6.1 Hz, H-27), 0.91 (3H, s, H-19), 0.94 (3H, d, J = 6.8 Hz, H-21), 1.99 (3H, s, OAc), 2.01 (3H, s, OAc), 2.02 (3H, s, OAc), 2.06 (3H, s, OAc), 3.36 (1H, t, J = 10.6 and 10.9 Hz, H-26a), 3.46 (1H, m, H-26b), 3.67 (1H, m, H-5'), 4.09 (1H, br d, H-6'a), 4.24 (1H, dd, H-6'b), 4.39 (1H, m, H-16), 4.57 (1H, d, J = 7.7 Hz, H-1'), 4.92 (1H, t, J = 8.0 and 7.9 Hz, H-2'), 5.05 (1H, t, J = 9.7 and 9.4 Hz, H-4'), 5.18 (1H, t, J = 7.8 and 7.8 Hz, H-3'); ^{13}C NMR (δ ppm, $CDCl_3$):

14.5 (C-21), 16.5 (C-18), 17.1 (C-27), 20.6 (OAc), 20.7 (OAc), 20.8 (OAc), 20.8 (OAc), 20.9 (C-11), 23.4 (C-19), 28.8 (C-24), 26.4 (C-7), 26.6 (C-6), 29.7 (C-2), 30.2 (C-25), 31.4 (C-23), 31.8 (C-15), 34.1 (C-1), 35.0 (C-10), 35.2 (C-4), 35.5 (C-8), 40.1 (C-9), 40.3 (C-12), 40.7 (C-13), 41.6 (C-20), 42.2 (C-5), 56.2 (C-14), 62.2 (C-6'), 62.3 (C-17), 66.9 (C-26), 68.7 (C-4'), 71.7 (C-2', C-5'), 72.9 (C-3'), 81.0 (C-16), 99.9 (C-1'), 109.2 (C-22), 169.1 (OAc), 169.4 (OAc), 170.4 (OAc), 170.7 (OAc); COSY correlations: 4.57 (H-1') ↔ 4.92 (H-2') ↔ 5.18 (H-3') ↔ 5.05 (H-4') ↔ 3.67 (H-5') ↔ 4.24 (H-6'b) ↔ 4.09 (H-6'a), 4.39 (H-16) ↔ 1.97 (H-15a) ↔ 1.75 (H-17) ↔ 1.19 (H-15b).

3β-D-Epismilagenin β-D-glucoside (2-8)

A 2.5 M solution of sodium hydroxide in methanol (5 mL, 0.013 moles) was added to a suspension of 3β-D-epismilagenin β-D-glucoside tetraacetate (**2-12**) (2.84 g, 0.0038 moles) in methanol (80 mL). The mixture was stirred at 50-60°C for 1.5 h, poured into water (300 mL) and extracted with 1-butanol (4 × 70 mL). The combined 1-butanol solution was washed with water (3 × 40 mL), dried with sodium sulfate for 16 h (overnight) and evaporated *in vacuo*. The residue was recrystallised from acetone-water (3:1) to give 3β-D-epismilagenin β-D-glucoside (**2-8**) (2.04 g, 92.7%), m.p. 195-210°C; ES-MS (-100 V): *m/z* 578.2 $(M-H)^-$; ^1H NMR (δ ppm, DMSO-D_6): 0.78 (3H, s, H-18), 0.81 (3H, d, J = 6.2 Hz, H-21), 0.97 (3H, s, H-19), 0.98 (3H, d, J = 6.3 Hz, H-27), 2.96 (1H, t, H-2'), 3.12 (2H, m, H-4', H-5'), 3.20 (1H, t, H-3'), 3.29 (1H, d, J = 10.8 Hz, H-26a), 3.71 (1H, br d, J = 11.0 Hz, H-6'b), 4.29 (1H, d, J = 8.07, H-1'), 4.47 (1H, br s, HO-6'), 4.84 (1H, br s, HO-2'), 4.89 (1H, br s, HO-4'), 4.92 (1H, br s, HO-3'); ^{13}C NMR (δ ppm, DMSO-D_6): 18.6 (C-21), 20.1 (C-18), 21.0 (C-27), 24.1 (C-11), 27.1 (C-19), 30.1 (C-7), 30.3 (C-6), 30.6 (C-2), 32.4 (C-24), 33.8 (C-25), 34.9 (C-23), 35.4 (C-15), 37.6 (C-1), 37.8 (C-4), 38.3 (C-10), 38.9 (C-8), 43.6 (C-12), 43.7 (C-9), 44.0 (C-13), 45.0 (C-20), 45.4 (C-5), 59.6 (C-14), 65.1 (C-6'), 65.8 (C-17), 69.8 (C-26), 74.1 (C-4'), 77.5 (C-2'), 80.6 (C-5'), 80.7 (C-3'), 84.2 (C-16), 104.3 (C-1'), 112.3 (C-22); COSY correlations: 4.29 (H-1') ↔ 2.96 (H-2') ↔ 3.20 (H-3') ↔ 3.12 (H-4', H-5') ↔ 3.48 (H-6'a) ↔ 3.71 (H-6'b), 4.38 (H-16) ↔ 1.99 (H-15a) ↔ 1.77 (H-17) ↔ 1.24 (H-15b), 3.49 (H-26b) ↔ 3.29 (H-26a).

5.2.3 Preparation of 20,23,23-D_3-Sarsasapogenin

Preparation of deuterioacetic acid (AcOD)

A mixture of acetic anhydride (52 mL, 0.56 moles) and deuterium oxide (10 mL, 0.56 moles) was stirred at room temperature for 16 h (overnight) to give deuterioacetic acid (CH_3COOD).

Preliminary deuteration reactions

A mixture of sarsasapogenin (1 g) and deuterioacetic acid (30 mL) was refluxed, using an oil-bath as the heat source. Sub-samples recovered from the reaction mixture after 15, 30 and 45 minutes, and 1, 2, 3, 4, 5, 6, 10, 12, 24, 48, 72, 96, 120, 148 and 168 h, were acetylated using acetic anhydride and pyridine (1:1), and analysed using selected ion mode (SIM) GC-MS (*m/z* 139, 140, 141, 142 ion profiles, to determine the percentage contributions non-D, D-, D_2- and D_3-sarsasapogenins. Results are presented in Table 2-9, Section 2.4.2)

Preparation of 20,23,23-D_3-sarsasapogenin

(A) A solution of sarsasapogenin (5.0 g) in deuterioacetic acid (150 mL) was refluxed for 24 h, after which the solvent was removed *in vacuo*. Recrystallisation from acetone gave 20,23,23-D_3-sarsasapogenin (3.5 g) (containing non-D-sarsasapogenin: 1%, D-sarsasapogenin: 6%, D_2-sarsasapogenin: 28% and D_3-sarsasapogenin: 65%, determined by SIM GC-MS); ^{1}H NMR (δ ppm, $CDCl_3$): 0.76 (3H, s, H-18), 0.975 (3H, s, H-19), 0.98 (3H, s, CH_3CD-, H-20) .1.07 (3H, d, J = 7.0 Hz, H-27), 3.29 (1H, m, H-26a), 3.94 (1H, dd, H-26b), 4.10 (1H, br s, H-3), 4.40 (1H, m, H-16); ^{13}C NMR (δ ppm, $CDCl_3$): 14.2 (C-21), 16.1 (C-27), 16.5 (C-18), 21.0 (C-11), 24.0 (C-19), 25.6 (C-24), 26.6 (C-6, C-7), 27.1 (C-25), 27.9 (C-2), 30.0 (C-1), 31.8 (C-15), 33.6 (C-4), 35.4 (C-8, C-10), 36.6 (C-5), 39.9 (C-9), 40.4 (C-12), 40.7 (C-13), 56.6 (C-14), 62 1 (C-17), 65.2 (C-26), 67.2 (C-3), 81.1 (C-16), 109.8 (C-22), (C-20 and C-23 signals were suppressed due to deuteration).

(B) A solution of sarsasapogenin acetate (5.0 g) in deuterioacetic acid (150 mL) was refluxed for 24 h, after which the solvent was removed *in vacuo*. The residue was dissolved in methanol (100 mL), a 2.5 M solution of sodium hydroxide in methanol (4 mL) was added, and the reaction mixture was stirred at 60-70°C for 1 h. The mixture was then poured into water (200 mL), extracted with chloroform (3 × 50 mL) and the chloroform extracts were combined and concentrated using a rotary evaporator. Recrystallisation from acetone gave 20,23,23-D_3-sarsasapogenin (2.8 g) (containing

non-D-sarsasapogenin: 2%, D-sarsasapogenin: 8%, D_2-sarsasapogenin: 28% and D_3-sarsasapogenin: 62%, as shown by GC-MS). ^{1}H and ^{13}C NMR data were identical to those determined for a specimen prepared by method (A).

5.2.4 20,23,23-D_3-Sarsasapogenin Stability Tests

The following buffer solutions were prepared (CRC Handbook, 59th Ed):

pH 1: 25 mL of 0.2 M KCl + 67 mL of 0.2 M HCl
pH 2: 25 mL of 0.2 M KCl + 6.5 mL of 0.2 M HCl
pH 4: 50 mL of 0.1 M potassium hydrogen phthalate + 0.1 mL of 0.1 M HCl
pH 6: 50 mL of 0.1 M potassium dihydrogen phosphate + 5.6 mL of 0.1 M NaOH
pH 8: 50 mL of 0.025 M borax + 20.5 mL of 0.1 M HCl

20,23,23-D_3-Sarsasapogenin (0.1 g) was suspended in the buffer solutions (pH 1, 2, 4, 6 and 8), and in acetic acid (7 mL each), respectively. The resulting suspensions were kept in a water-bath at 37°C. A solution of 20,23,23-D_3-sarsasapogenin (0.1 g) in acetic acid (7 mL) was kept at room temperature (ca 17°C). Sub-samples were collected after 0.25, 0.5, 1, 2, 3, 4, 6, 12, 24, 48 and 72 h, extracted with chloroform (1 mL), acetylated with acetic anhydride and pyridine (1:1), and analysed using SIM GC-MS (*m/z* 139, 140, 141, 142 ion profiles to determine the percentage contributions of non-D, D-, D_2- and D_3-sarsasapogenins respectively). Results are presented in Table 2-10.

5.3 Experimental, Chapter Three

5.3.1 Trial 1 – Dosing Trial with Sarsasapogenin β-D-Glucoside

A 12 week old 20 kg Romney lamb (sheep A) was given sedative and local anaesthetic and operated on to insert a bile cannula in the bile duct (by Dr Barry Smith, Veterinary Scientist, AgResearch, Ruakura, Hamilton, New Zealand). The sheep was allowed to recover for a week before the commencement of dosing.

Sarsasapogenin β-D-glucoside (2 g), prepared as described in Section 5.2.1, was suspended in 20% ethanol (100 mL) and dosed to sheep A by intraruminal intubation. A series of bile samples were recovered from this sheep after 0, 0.5, 1, 2, 3, 5, 6, 12, 14, 24, 27 and 30 h respectively. A rumen sample was collected after 30 h.

Rumen sample

An accurately weighed freeze-dried rumen material (2 g) was extracted in a Soxhlet apparatus with *n*-hexane (100 mL) for 20 h to afford a free sapogenin extract, and then with ethanol-water (4:1) (100 mL) for 24 h to give a conjugated extract which was then hydrolysed in a 1 M hydrochloric acid solution at 80-85°C for 2 h and extracted with chloroform (3 × 20 mL). The free and conjugated extracts analysed using SIM GC-MS methodology as described in Section 5.1.2, with the addition of 1,000 μL of standard sarsasapogenin propionate solution (as internal standard) for the free extract and 1,500 μL of standard sarsasapogenin propionate solution for the conjugated extract.

Bile samples

Protocol A: An accurately weighed bile sample (5 g) was extracted with *n*-hexane (4 × 5 mL) to afford a free sapogenin extract. Thereafter a 1 M hydrochloric acid solution (5 mL) was added and warmed to 75-80°C for 1.5 h, to hydrolyse conjugated sapogenins to free sapogenins. The mixture was then cooled and extracted with chloroform (5 × 5 mL) to give a hydrolysed conjugated extract (i.e. material considered to be originally present in the bile in a conjugated form). The free and conjugated extracts were analysed by GC-MS methodology as described in Section 5.1.2, with the addition of 50 μL of standard sarsasapogenin propionate solution (as internal standard) for the free extract and 100 μL of standard sarsasapogenin propionate solution for the conjugated extract.

Protocol B: An accurately weighed bile sample (5 g) was extracted with chloroform (4 × 5 mL) to afford a free sapogenin extract. Thereafter a 1 M hydrochloric acid solution (5 mL) was added and warmed to 75-80°C for 1.5 h to hydrolyse conjugated sapogenins to free sapogenins. The mixture was cooled and extracted with chloroform (5 × 5 mL) to give a hydrolysed conjugated extract. The free and conjugated extracts were analysed using the SIM GC-MS methodology as described in Section 5.1.2, with the addition of 50 μL of standard sarsasapogenin propionate solution as internal standard for the free extract and 100 μL of standard sarsasapogenin propionate solution for the conjugated extract.

Results determined using protocol B for bile samples are presented in Table 3-3.

5.3.2 Trial 2 – Dosing Trial with a Mixture of Sarsasapogenin β-D-Glucoside and 20,23,23-D_3-Sarsasapogenin

Two approximately 12 week old 20 Kg Romney lambs (sheep 1 and 2) were given sedative and local anaesthetic and operated on to insert cannulae in the bile duct, rumen and jejunum respectively. The sheep were allowed to recover for a week before the commencement of dosing. Animal surgery was performed by Dr Barry Smith, Veterinary Scientist, AgResearch, Ruakura, Hamilton, New Zealand.

Mixtures of sarsasapogenin β-D-glucoside (2 g) and 20,23,23-D_3-sarsasapogenin (2 g), were suspended in 20% ethanol (100 mL) were dosed to sheep 1 and sheep 2 by intraruminal intubation, respectively. A series of the bile, rumen, jejunum and faeces samples (ca 5 g each for bile samples and ca 20 g each for rumen, jejunum and faeces samples) were recovered from these sheep after 0, 1, 3, 5, 7, 11, 16, 21, 29, 49, 54, 62, 74, 79 and 97 h.

Bile, rumen, jejunum and faeces samples were extracted and analysed using the SIM GC-MS as described in Section 5.3.1 (Trial 1). Results are presented in Tables 3-12, 3-13, 3-14, 3-15 and 3-16.

5.3.3 Trial 3 – Dosing Trial with 3β-D-Epismilagenin β-D-Glucoside

Two approximately 12 week old 20 Kg Romney lambs (sheep 3 and 4) were given sedative and local anaesthetic and operated on to insert cannulae in the bile duct, rumen and jejunum respectively. The sheep were allowed to recover for a week before the commencement of dosing. Animal surgery was performed by Dr Barry Smith, Veterinary Scientist, AgResearch, Ruakura, Hamilton, New Zealand.

Suspensions of 3β-D-Epismilagenin β-D-glucoside (2 g) in 20% ethanol (100 mL) were dosed to sheep 3 and sheep 4 by intraruminal intubation, respectively. A series of bile, rumen, jejunum and faeces samples (ca 5 g each for bile samples and ca 20 g each for rumen, jejunum and faeces samples) were recovered from these sheep 0, 1, 3, 5, 7, 11, 16, 21, 29, 49, 54, 62, 74, 79 and 97 h after dosing commenced.

Bile, rumen, jejunum and faeces samples were extracted and analysed using the SIM GC-MS as described in Section 5.3.1 (Trial 1). Results are presented in Tables 3-25, 3-27 and 3-28.

5.3.4 Trial 4 – Dosing Trial with 20,23,23-D_3-Sarsasapogenin

Two sheep (sheep 2 and 3), which had previously been dosed with a mixture of sarsasapogenin β-D-glucoside and 20,23,23-D_3-sarsasapogenin (sheep 2), or 3β-D-epismilagenin β-D-glucoside (sheep 3), were allowed to recover for ten days before the commencement of dosing.

Suspensions of 20,23,23-D_3-sarsasapogenin (2 g) in 20% ethanol (100 mL) were dosed to sheep 2 and sheep 3 by intraruminal intubation, respectively. A series of the bile, rumen, jejunum and faeces samples (ca 5 g each for bile samples and ca 20 g each for rumen, jejunum and faeces samples) were recovered from these sheep 0, 3, 20, 27, 44, 51 and 69 h after dosing commenced.

Bile, rumen, jejunum and faeces samples were extracted and analysed using the SIM GC-MS as described in Section 5.3.1 (Trial 1). Results are presented in Tables 3-5, 3-6 and 3-7.

5.4 Experimental, Chapter Four

5.4.1 *Narthecium ossifragum*

5.4.1.1 Isolation of *N. ossifragum* Saponins

(A) Freeze-dried, ground, *N. ossifragum* (6.55 g) collected from pastures in the Faroe Islands, Norway in 1995, supplied by Dr Arne Flåøyen, Department of Reproduction and Forensic Medicine, Norwegian College of Veterinary Medicine, Oslo, Norway, was extracted with dichloromethane (200 mL) in a Soxhlet extractor for 24 h, and then with ethanol-water (4:1, 200 mL) for 24 h. The ethanol-water extract was concentrated to ca 30 mL *in vacuo*, diluted with water (100 mL), washed with petroleum spirit (2 × 25 mL) and extracted with water-saturated 1-butanol (4 × 25 mL). The 1-butanol extract was evaporated to dryness *in vacuo*, and separated by radial chromatography using a 22.5 cm diameter × 2 mm silica gel (E. Merck 938) plate, which was developed using 100 mL portions of 1:0, 9:1, 4:1, 7:3, 3:2, 1:1, 3:7 and 0:1 of chloroform-5% aqueous methanol as eluents. A total of 35 × 20 mL fractions were collected. Fractions 16, 18, 21 and 24 afforded (25*S/R*)-5β-spirostan-3β-ol 3-*O*-[*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside] (faroecin) (**4-22**), (25*R/S*)-5β-spirostan-3β-ol 3-*O*-[*O*-β-D-glucopyranosyl-

(1→2)-β-D-galactopyranoside] (asiaticin) (**4-15**), (25*R*/*S*)-5β-spirostan-3β-ol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranoside} (narthecin) (**4-13**), and (25*S*/*R*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (narthecioside) (**4-24**).

Faroecin

Faroecin (**4-22**), the first eluting saponin fraction, had ^{1}H NMR (δ ppm, C_5D_5N) 0.82 (3H, s, H-18), 0.83 (3H, s, H-19), 1.07 (3H, d, J = 7.0 Hz, H-27), 1.16 (3H, d, J = 6.9 Hz, H-21), 4.75 (1H, d, J = 7.7 Hz, H-1'), 5.24 (1H, d, J = 7.8 Hz, H-1"); other ^{1}H and ^{13}C NMR assignments are given in Tables 4-2 and 4-3; ES-MS (-110 V): *m/z* 739.5 $(M-H)^-$.

Asiaticin

Asiaticin (**4-15**), the second eluting saponin fraction, had m.p. >360°C, ^{1}H NMR (δ ppm, C_5D_5N): (25*S* isomer) 0.82 (3H, s, H-18), 0.98 (3H, s, H-19), 1.08 (3H, d, J = 6.9 Hz, H-27), 1.16 (3H, d, J = 7.1 Hz, H-21), 4.92 (1H, d, J = 7.7 Hz, H-1'), 5.29 (1H, d, J = 7.7 Hz, H-1"); additional low field ^{1}H NMR assignments are given in Table 4-3; (25*R* isomer) 0.70 (3H, d, J = 5.6 Hz, H-27), 0.83 (3H, s, H-18), 0.76 (3H, s, H-19), 1.15 (3H, d, J = 6.9 Hz, H-21); ^{13}C NMR assignments are given in Tables 4-2 and 4-3; ES-MS (-110 V): *m/z* 739.6 $(M-H)^-$.

Narthecin

Narthecin (**4-13**), had m.p. 297-298°C [lit. 270-273°C (decomp) (Ceh and Hauge, 1981)], ^{1}H NMR (ppm, C_5D_5N): (25*S* isomer) 0.81 (3H, s, H-18), 0.96 (3H, s, H-19), 1.07 (3H, d, J = 6.9 Hz, H-27), 1.15 (3H, d, J = 7.1 Hz, H-21), 4.89 (1H, d, J = 7.7 Hz, H-1'), 5.17 (1H, d, J = 7.6 Hz, H-1'''), 5.53 (1H, d, J = 7.8 Hz, H-1"); additional lowfield ^{1}H NMR assignments are given in Table 4-3); (25*R* isomer) 0.69 (3H, d, J = 5.6 Hz, H-27), 0.82 (3H, s, H-18), 0.96 (3H, s, H-19), 1.14 (3H, d, J = 6.9 Hz, H-21); ^{13}C NMR assignments are given in Tables 4-2 and 4-3; ES-MS (-110 V): *m/z* 871.6 $(M-H)^-$.

Narthecioside

Narthecioside (**4-24**) had ^{1}H NMR (δ ppm, C_5D_5N) 0.88 (3H, s, H-18), 0.97 (3H, s, H-19), 1.03 (3H, d, J = 6.6 Hz, H-27), 1.32 (3H, d, J = 6.8 Hz, H-21), 4.81 (1H, d, J = 7.7 Hz, H-1""), 4.89 (1H, d, J = 7.7 Hz, H-1'), 5.17 (1H, d, J = 7.6 Hz, H-1'''),

5.53 (1H, d, J = 7.8 Hz, H-1"); other ^{1}H and ^{13}C NMR assignments are given in Tables 4-2 and 4-3; ES-MS (-110 V): *m/z* 1051.8 (M-H)$^{-}$ and 1087.9 (M+Cl)$^{-}$.

(B) Freeze-dried *N. ossifragum* (6.35 g), collected during July-August 1993 from pastures in the vicinity of Hemne Selbu, Sør-Trøndelag County, Norway, was extracted with dichloromethane (200 mL) in a Soxhlet apparatus for 16h. The dichloromethane extracted plant material was air-dried overnight, then extracted with methanol (200 mL) for 16 h. The methanol extract was evaporated to dryness *in vacuo*. The residue was dissolved in water (70 mL), washed with petroleum spirit (2 × 25 mL), and extracted with water-saturated 1-butanol (4 × 25 mL). The 1-butanol extract was evaporated to dryness *in vacuo* and separated by radial chromatography using a 22.5 cm diameter × 2 mm silica gel (E. Merck 938) plate, which was developed using 100 mL portions of 1:0, 9:1, 4:1, 7:3, 3:2, 1:1, 1:3 and 0:1 of chloroform-5% aqueous methanol as eluents. A total of 28 × 20 mL fractions were collected. Fractions 11, 13, 17, 21 afforded asiaticin (**4-15**), narthecin (**4-13**), narthecioside (**4-24**) and 22-*O*-methyl-narthecioside (**4-17**), respectively.

22-*O*-Methyl-narthecioside

22-*O*-Methyl-narthecioside (**4-17**) had ^{1}H NMR (δ ppm, C_5D_5N) 0.80 (3H, s, H-18), 0.97 (3H, s, H-19), 1.03 (3H, d, J = 6.6 Hz, H-27), 1.17 (3H, d, J = 6.8 Hz, H-21), 3.26 (3H, s, H-OCH_3), 4.81 (1H, d, J = 7.7 Hz, H-1""), 4.89 (1H, d, J = 7.7 Hz, H-1'), 5.17 (1H, d, J = 7.6 Hz, H-1"'), 5.53 (1H, d, J = 7.8 Hz, H-1"); ^{13}C NMR (δ ppm, C_5D_5N) 47.4 ppm (22-OCH_3); other ^{1}H and ^{13}C NMR assignments are given in Tables 4-2 and 4-3; ES-MS (-110 V): *m/z* 1065.8 (M-H)$^{-}$.

Asiaticioside

Separation of fraction 15 by radial chromatography using a 22.5 cm diameter × 2 mm silica gel (E. Merck 938) plate, which was developed using 100 mL portions of 1:0, 4:1, 7:3, 3:2, 1:1, 1:3 and 0:1 of chloroform-5% aqueous methanol as eluents. A total of 35 × 20 mL fractions were collected. Fraction 15-33 gave asiaticioside (**4-23**), ^{1}H NMR (δ ppm, C_5D_5N): 0.88 (3H, s, H-18), 0.94 (3H, s, H-19), 1.01 (3H, d, J = 6.9 Hz, H-27), 1.30 (3H, d, J = 7.0 Hz, H-21), 4.79 (1H, d, J = 7.8 Hz, H-1'''), 4.92 (1H, d, J = 7.6 Hz, H-1'), 5.29 (1H, d, J = 7.6 Hz, H-1"); other ^{1}H and ^{13}C NMR assignments are given in Tables 4-2 and 4-3; ES-MS (-110 V): *m/z* 919.8 (M-H)$^{-}$.

5.4.1.2 GC-MS Analyses of *N. ossifragum*

Three samples (samples 1, 2 and 3) of *N. ossifragum* collected from pastures in the Faroe Islands, Norway in 1995, were extracted and analysed using the GC-MS methodology as described in Section 5.1.2. Results are presented in Table 4-1.

5.4.2 *Panicum virgatum*

5.4.2.1 Isolation of *P. Virgatum* Saponins

Freeze-dried *P. virgatum* (9.98 g), collected by Dr R. L. Reid from Morgantown, West Virginia, USA in 1993, was extracted with dichloromethane (200 mL) in a Soxhlet apparatus for 24 h. The dichloromethane extracted plant material was air-dried overnight, then extracted with methanol (200 mL) for 24 h. The methanol extract was concentrated to dryness *in vacuo*, then the residue was fractionated on a 50 × 2 cm silica gel (60-120 mesh, BDH 30061) column, eluting with 100 mL portions of 1:0, 9:1, 3:1, 7:3, 1:1, and 0:1 of chloroform-5% aqueous methanol. A total of 18 × 20 mL fractions were collected. Fraction 12 was identified as a 10:1 mixture of (25*R/S*)-furost-5-ene-3β,22α,26-triol 3-*O*-{*O*-[α-L-rhamnopyranosyl-(1→4)-*O*-α-L-rhamnopyranosyl-(1→4)]-*O*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranoside (**4-25**), [25*R*-isomer (dichotomin), Munday et al., 1993); 25*S*-isomer (coloratin), Wilkins, pers commun).

Saponin 4-25

Saponin **4-25**, an amorphous solid; ^{1}H NMR (δ ppm, C_5D_5N): 25*R* isomer: 0.87 (3H, s, H-18), 0.96 (3H, d, J = 6.5 Hz, H-27), 1.03 (3H, s, H-19), 1.30 (3H, d, J = 6.7 Hz, H-21), 1.54 (3H, d, J = 5.7 Hz, H-6'''), 1.56 (3H, d, J = 5.8 Hz, H-6''''), 1.73 (3H, d, J = 6.7 Hz, H-6''), 4.78 (1H, d, J = 7.9 Hz, H-1'''''), 5.77 (1H, s, H-1'''), 6.21 (1H, s, H-1''''), 6.32 (1H, s, H-1''); 25*S* isomer: 0.90 (3H, s, H-18), 1.04 (3H, s, H-19), 1.06 (3H, d, J = 6.8 Hz, H-27), 1.33 (3H, d, J = 6.7 Hz, H-21); other ^{1}H and ^{13}C NMR assignments are given in Tables 4-13 and 4-14; ES-MS (-110 V): *m/z* 1229 [$(M+Cl)^-$], 1193 [$(M-H)^-$], 1047 [$(M\text{-rham})^-$] and 753 [$(M- 3 \times \text{rham})^-$]; ES-MS (110 V): *m/z* 1217 [$(M+Na)^+$].

5.4.2.2 GC-MS Analyses of *P. Virgatum*

Thirteen USA collections of *P. virgatum* , supplied by Dr R. L. Reid, were extracted and analysed using the GC-MS methodology described in Section 5.1.2. The results are presented in Table 4-12.

5.4.3 *Panicum miliaceum*

5.4.3.1 Isolation of *P. miliaceum* Saponins

A freeze-dried specimen of *P. miliaceum* (9.62 g), collected by Dr C. O. Miles from Hamilton, New Zealand in 1994, was extracted with dichloromethane (200 mL) in a Soxhlet apparatus for 24 h. The dichloromethane extracted plant material was air-dried overnight, and extracted with methanol (200 mL) for 24 h. The methanol extract was concentrated to dryness *in vacuo*, and separated on a 50 × 2 cm silica gel (60-120 mesh, BDH 30061) column using 100 mL portions of 1:0, 9:1, 4:1, 7:3, 3:2, 1:1, 1:3 and 0:1 of chloroform-5% aqueous methanol to give 35 × 20 mL fractions. Fractions 8 and 17 gave (25*R/S*)-spirost-5-en-3β-ol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranoside} (**4-26**) and (25*R/S*)-furost-5-ene-3β,22α,26-triol 3-*O*-{*O*-α-L-rhamnopyranosyl-(1→2)-*O*-[*O*-α-L-rhamnopyranosyl-(1→4)-α-L-rhamnopyranosyl-(1→4)]-β-D-glucopyranosyl}-26-*O*-β-D-glucopyranoside (**4-25**) [25*R*-isomer (dichotomin) Munday et al., 1993); 25*S*-isomer (coloratin), Wilkins, pers commun].

Saponin 4-26

Saponin **4-26**, an amorphous solid had ^{1}H NMR (δ ppm, C_5D_5N): 25*R*-isomer: 0.84 (3H, s, H-18), 0.90 (3H, d, *J* = 6.6 Hz, H-27), 1.05 (3H, s, H-19), 1.14 (3H, d, *J* = 6.7 Hz, H-21), 4.96 (1H, d, *J* = 7.8 Hz, H-1'), 5.82 (1H, s, H-1'''), 6.26 (1H, s, H-1''''), 6.37 (1H, s, H-1''); 25*S*-isomer: 0.84 (3H, s, H-18), 1.05 (3H, s, H-19), 1.08 (3H, d, *J* = 6.7 Hz, H-27), 1.15 (3H, d, *J* = 6.7 Hz, H-21); other ^{1}H and ^{13}C NMR assignments are given in Tables 4-18 and 4-19; ES-MS (-110 V): *m/z* 1013 $[M-H]^{-}$].

Saponin 4-25

Saponin **4-25**, an amorphous solid had ^{1}H NMR (δ ppm, C_5D_5N): 25*R*-isomer: 0.90 (3H, s, H-18), 0.99 (3H, d, *J* = 6.7 Hz, H-27), 1.06 (3H, s, H-19), 1.33 (3H, d, *J* = 6.5 Hz, H-21), 1.58 (3H, d, *J* = 5.9 Hz, H-6'''), 1.59 (3H, d, *J* = 6.6 Hz, H-6''''), 1.76 (3H, d, *J* = 6.1 Hz, H-6''), 4.81 (1H, d, *J* = 7.8 Hz, H-1'''''), 5.81 (1H, s, H-

1'''), 6.26 (1H, s, H-1''''), 6.37 (1H, s, H-1''); 25*S*-isomer: 0.90 (3H, s, H-18), 1.03 (3H, d, J = 6.8 Hz, H-27), 1.06 (3H, s, H-19), 1.33 (3H, d, J = 6.7 Hz, H-21); other ^{1}H and ^{13}C NMR assignments are given in Tables 4-18 and 4-19; ES-MS (+100 V): *m/z* 1233 [$(M+K)^+$].

5.4.3.2 GC-MS Analyses of *P. miliaceum*

Freeze-dried seeds (1.9310 g) and stems (2.5512 g) of *P. miliaceum*, collected from a domestic garden, Hamilton, New Zealand, 23rd April, 1994, were extracted and analysed by the GC-MS methodology as described in Section 5.1.2. Results are presented in Table 4-17.

5.4.4 *Nolina texana*

5.4.4.1 Characterisation of *N. texana* Sapogenins

A freeze-dried blossom sample of *N. texana* (10.2 g), collected by Professor Murl Bailey from USA in 1995, was extracted with hexane (200 mL) in a Soxhlet apparatus for 24 h. The *n*-hexane extracted plant material was air-dried overnight, then extracted with ethanol-water (4:1, 200 mL) for 24 h. The ethanol-water extract was concentrated *in vacuo*, and dissolved in a 1 M hydrochloric acid solution (200 mL) and stirred at 80-85°C for 2 h. After cooling, hydrolysed sapogenins were extracted with chloroform (4 × 30 mL). The chloroform extract was evaporated to dryness *in vacuo*, then separated by radial chromatography on a 22 cm diameter × 2 mm silica gel (E. Merck 938) plate developed with mixtures of hexane-ethyl acetate (9:1, 4:1, 3:1, 3:2, 1:1, 3:7 and 1:4) and finally with methanol to give 44 × 20 mL fractions. Fraction 8 was shown by TIC GC-MS to be comprised mainly of esters of unsaturated fatty acids (*m/z* 41, 55, 67, 79, 95, 108), fraction 12 was a mixture of steroids including cholesterol (*m/z* 368), campesterol (*m/z* 382), stigmasterol (*m/z* 394) and β-sitosterol (*m/z* 396) and fraction 21 contained free fatty acids.

Sapogenins were present in fractions 31, 32, 33 and 34 (*m/z* 118, 137, 139, 280, 282, 392, 394). Fractions 31-34 were combined, and were shown by GC-MS and NMR analyses to be mainly comprised of ruscogenin (25*R*) (**4-11**), neoruscogenin (25*S*) (**4-12**) and $\Delta^{25(27)}$-ruscogenin (**4-40**) (1 : 2 : 2.5 ratio).

Ruscogenin (4-11)

Ruscogenin (**4-11**), the minor component, had ^{1}H NMR (δ ppm in $CDCl_3$) 0.77 (3H, d, *J* = 5.7 Hz, H-27), 0.80 (3H, s, H-18), 0.99 (3H, d, *J* = 6.8 Hz, H-21), 1.03 (3H, s, H-19), 3.35 (1H, t, *J* = 10.8 Hz, H-26a), 3.44 (1H, d, *J* = 4.6 Hz, H-26b), 3.41 (1H, d, *J* = 4.5 Hz, H-1), 3.53 (1H, m, H-3), 4.39 (1H, m, H-16), 5.53 (1H, d, *J* = 4.2 Hz, H-6); ^{13}C NMR assignments (in $CDCl_3$ and in C_5D_5N) are given in Table 4-21; GC-MS (acetate) *m/z* 118, 139, 280, 394, 514 (M^+).

Neoruscogenin (4-12)

Neoruscogenin (**4-12**), the intermediate component, had ^{1}H NMR (δ ppm in $CDCl_3$) 0.78 (3H, s, H-18), 0.98 (3H, d, *J* = 6.0 Hz, H-21), 1.03 (3H, s, H-19), 1.07 (3H, d, *J* = 7.1 Hz, H-27), 3.28 (1H, br d, *J* = 11.0 Hz, H-26b), 3.93 (1H, dd, *J* = 8.3 and 2.7 Hz, H-26a), 3.41 (1H, d, *J* = 4.5 Hz, H-1), 3.53 (1H, m, H-3), 4.39 (1H, m, H-16), 5.53 (1H, d, *J* = 4.2 Hz, H-6); ^{13}C NMR assignments (δ ppm in $CDCl_3$ and in C_5D_5N) are given in Table 4-21; GC-MS (acetate) *m/z* 118, 139, 280, 394, 514 (M^+).

$\Delta^{25(27)}$-Ruscogenin (4-40)

$\Delta^{25(27)}$-Ruscogenin (**4-40**), the major component, had ^{1}H NMR (δ ppm in $CDCl_3$) 0.80 (3H, s, H-18), 0.96 (3H, d, *J* = 7.0 Hz, H-21), 1.04 (3H, s, H-19), 3.41 (1H, d, *J* = 4.5 Hz, H-1), 3.53 (1H, m, H-3), 3.84 (1H, dd, *J* = 10.9 and 1.3 Hz, H-26b), 4.29 (1H, d, *J* = 12.1 Hz, H-26a), 4.43 (1H, m, H-16), 4.74 (2H, d, *J* = 13.4 Hz, H-27), 5.53 (1H, d, *J* = 4.2 Hz, H-6); ^{13}C NMR assignments (δ ppm in $CDCl_3$ and in C_5D_5N) are given in Table 4-21; GC-MS (acetate) *m/z* 118, 137, 280, 392, 512 (M^+).

5.4.4.2 Characterisation of *N. texana* Saponins

A freeze-dried blossom sample of *N. texana* (12.0 g) was extracted with hexane (200 mL) in a Soxhlet apparatus for 24 h. The *n*-hexane extracted plant material was air-dried overnight, then extracted with ethanol-water (4:1, 200 mL) for 24 h. The ethanol-water extract was concentrated to ca 20 mL, then diluted with water (100 mL) and extracted with water-saturated 1-butanol (4 × 20 mL). After the solvent was removed *in vacuo*, the residue was separated by radial chromatography on a 22 cm diameter × 2 mm silica gel (E. Merck 938) plate developing with mixtures of chloroform-5% aqueous methanol

(9:1, 4:1, 3:1, 3:2, 1:1, 3:7, 1:4) and finally with 5% aqueous methanol. A total of 42 × 20 mL fractions were collected. The fractions containing the same components were combined according to TLC R_F values to give five saponin fractions (fractions I-V) which were shown by GC-MS and NMR analyses to be mixtures of saponins.

A solution of fraction I (the least polar fraction) (50 mg) in methanol-water (4:1, 1 mL) was separated by HPLC (see Section 5.1.5) using a 8 mm id × 10 cm ODS silica-gel column and methanol-water (4:1, 0.5 mL/min) as eluent. A 100 μL of the sample solution was injected and the fractions were collected according to the detector signals, iteratively. A total of six fractions were collected. Three fractions (Ia, Ib and Ic) were identified containing saponins.

Fraction Ic was showed by one- and two-dimensional NMR and ES-MS data to be comprised mainly of a mixture of **4-40** and **4-41**. Likewise, fraction Ib was exhibited to be comprised mainly of **4-41** and **4-42**, and fraction Ia was revealed to contain mainly **4-42** and **4-43**.

Difference NMR analyses and ES-MS analyses of fractions Ia, Ib and Ic showed the saponins to be spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-fucopyranoside (**4-41**), spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-arabinopyranoside (**4-42**) and spirosta-5,25(27)-diene-1β,3β-diol 1-*O*-β-galactopyranoside (**4-43**).

Saponin 4-41

Saponin **4-41** had ^{1}H NMR (δ ppm, in C_5D_5N) 0.90 (3H, s, H-18), 1.04 (3H, d, J = 7.0 Hz, H-21), 1.33 (3H, s, H-19), 1.55 (3H, d, J = 6.4 Hz, H-6'), 3.74 (1H, br d, J = 7.4 Hz, H-5'), 3.94 (1H, br d, J = 5.0 Hz, H-1), 3.99 (1H, br s, H-3), 4.29 (1H, t, J = 7.8 Hz, H-2'), 4.44 (1H, br d, J = 12.7 Hz, H-26a), 4.52 (1H, m, H-16), 4.71 (1H, d, J = 7.7 Hz, H-1'), 4.77 (2H, br d, J = 16.4 Hz, H-27), 5.59 (1H, m, H-6). Other ^{1}H and ^{13}C NMR assignments (in C_5D_5N) are given in Table 4-24. Negative ion ES-MS (-100 V): *m/z* 573.5 [(M-H)$^-$] and positive ES-MS (100 V): *m/z* 597.5 [(M+Na)$^+$].

Saponin 4-42

Saponin **4-42** had ^{1}H NMR (δ ppm, in C_5D_5N) 0.84 (3H, s, H-18), 1.03 (3H, d, J = 6.7 Hz, H-21), 1.23 (3H, s, H-19), 3.72 (1H, d, J = 11.9 Hz, H-5'b), 3.83 (1H, d, J = 4.3 Hz, H-1), 3.86 (1H, d, J = 4.5 Hz, H-3), 4.01 (1H, br d, J = 12.6 Hz, H-26b), 4.11 (1H, dd, J = 3.6 and 5.6 Hz, H-3'), 4.26 (1H, br s, H-4'), 4.32 (1H, d, J = 14.7 Hz, H-5'a), 4.39 (1H, t, J = 7.7 Hz, H-2'), 4.45 (1H, br d, J = 11.8 Hz, H-26a), 4.52

(1H, m, H-16), 4.74 (1H, d, J = 7.6 Hz, H-1'), 4.79 (2H, br d, J = 16.9 Hz, H-27), 5.60 (1H, m, H-6). Other ^{1}H and ^{13}C NMR assignments (in C_5D_5N) are given in Table 4-24. Negative ion ES-MS (-100 V): *m/z* 559.4 [$(M-H)^-$] and positive ion ES-MS (100 V): *m/z* 583.4 [$(M+Na)^+$].

Saponin 4-43

Saponin **4-43** had ^{1}H NMR (δ ppm, in C_5D_5N) 0.82 (3H, s, H-18), 1.01 (3H, d, J = 6.9 Hz, H-21), 1.21 (3H, s, H-19), 4.39 (1H, br d, J = 12.0 Hz, H-26b), 4.43 (1H, br d, J = 12.2 Hz, H-26a), 4.91 (1H, d, J = 7.8 Hz, H-1'), 5.57 (1H, m, H-6). Other ^{1}H and ^{13}C NMR assignments (in C_5D_5N) are given in Table 4-24. Negative ion ES-MS (-100 V): *m/z* 589.4 [$(M-H)^-$] and positive ion ES-MS (100 V): *m/z* 613.3 [$(M+Na)^+$].

5.4.4.3 GC-MS Analyses of *N. texana*

Freeze-dried leaves (1.0510 g), stems (1.9919 g) and blossoms (2.0463 g) of *N. texana*, collected from sites in USA in 1995 by Professor Murl Bailey, College of Veterinary Medicine, Texas A&M University, were analysed by the GC-MS methodology as described in Section 5.1.2. The results are presented in Table 4-22.

5.4.5 *Tribulus terrestris*

Four samples, collected by Prof A. Rezakhani from two sites in Northern Iran in 1994, were analysed using the GC-MS methodology as described in Section 5.1.2. Results are presented in Table 4-26.

5.4.6 *Brachiaria decumbens*

A total of 82 samples, harvested by Ms. S. Low from research plots in the grounds of The Papua New Guinea University of Technology, Lae, Papua New Guinea, 1996 were air-dried and ground to a fine powder in Papua New Guinea, and forwarded to Hamilton, New Zealand, where the samples were extracted and analysed by the GC-MS methodology as described in Section 5.1.2. The results are presented in Table 4-27.

APPENDIX

Figure I. ^{1}H NMR spectra of sarsasapogenin β-D-glucoside tetraacetate (A) and 3β-D-epismilagenin β-D-glucoside tetraacetate (B).

Figure II. ES-MS spectra of saponin **4-24** (A) and saponin **4-23** (B) isolated from the ethanol-water extract of *N. ossifragum.*

Figure III. ^{1}H NMR spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside **4-24** isolated from the ethanol-water extract of *N. ossifragum.*

Figure IV. COSY spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum.*

Figure V. ^{13}C NMR spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum.*

Figure VI. HSQC spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum.*

Figure VII. HMBC spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum.*

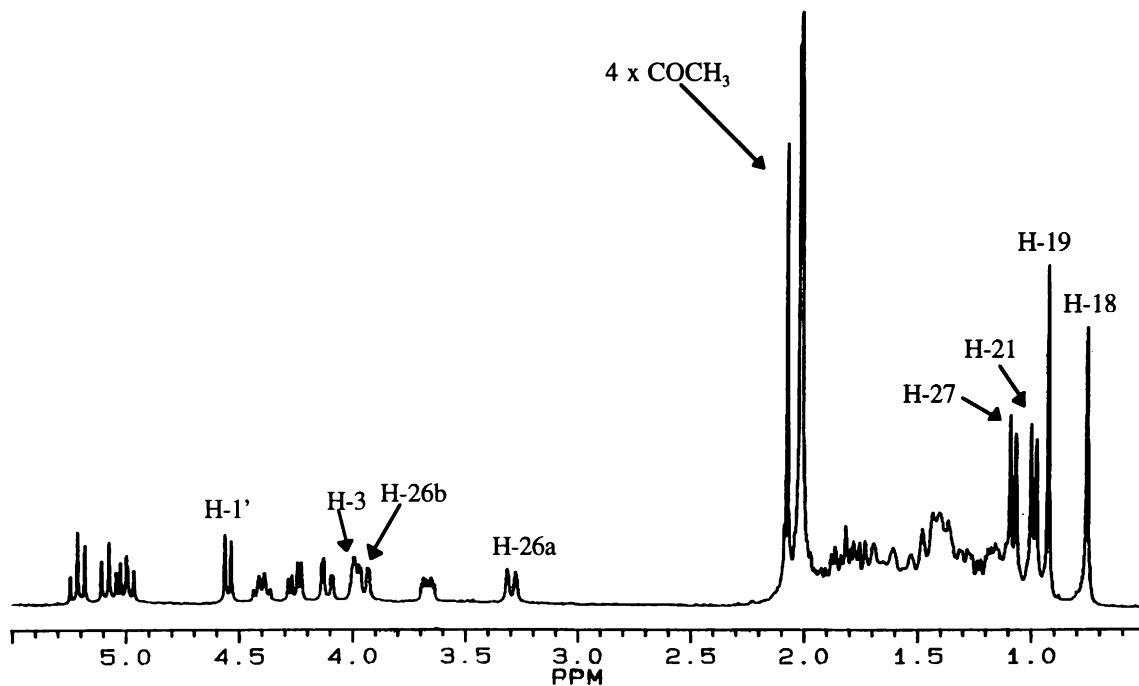
Figure VIII. Partial HOHAHA spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum.*

Figure IX. ROESY spectrum of saponin **4-24** isolated from the ethanol-water extract of *N. ossifragum.*

Figure X. Sample Excel spreadsheet used to calculate the levels of free and conjugated sapogenins and sterols in animal and plant samples

Figure I. ^{1}H NMR spectra of sarsasapogenin β-D-glucoside tetraacetate (A) and 3β-D-epismilagenin β-D-glucoside tetraacetate (B).

A sarsasapogenin β-D-glucoside tetraacetate



B 3β-D-epismilagenin β-D-glucoside tetraacetate

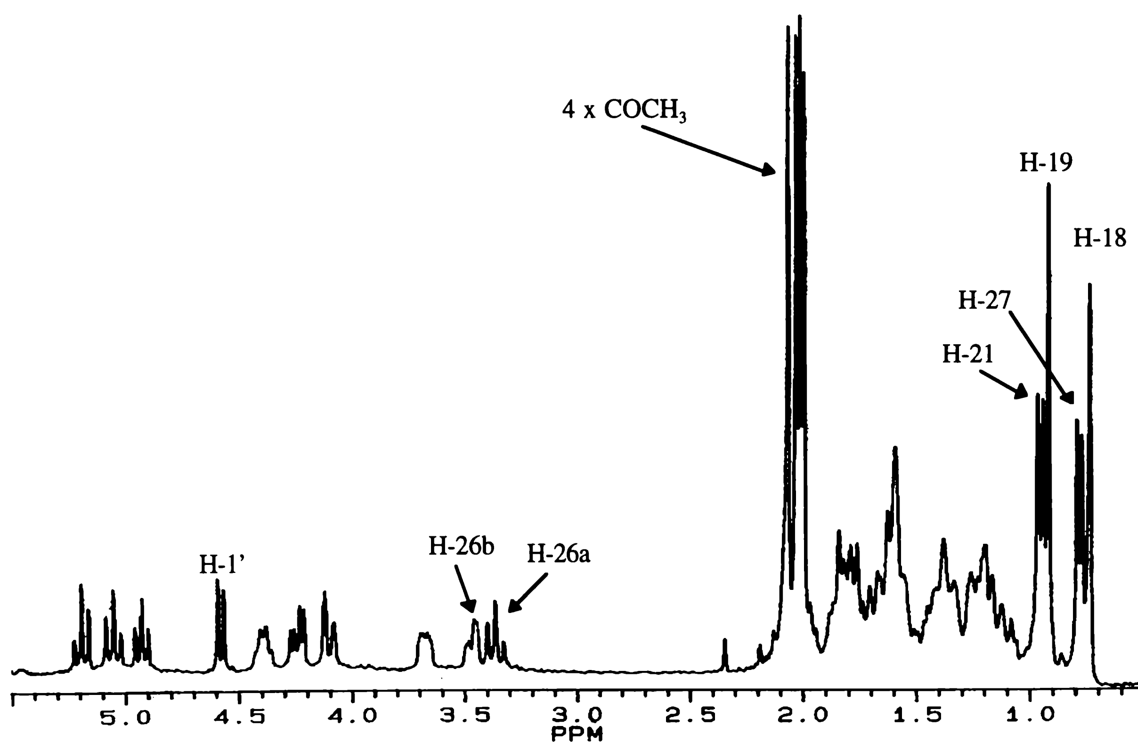
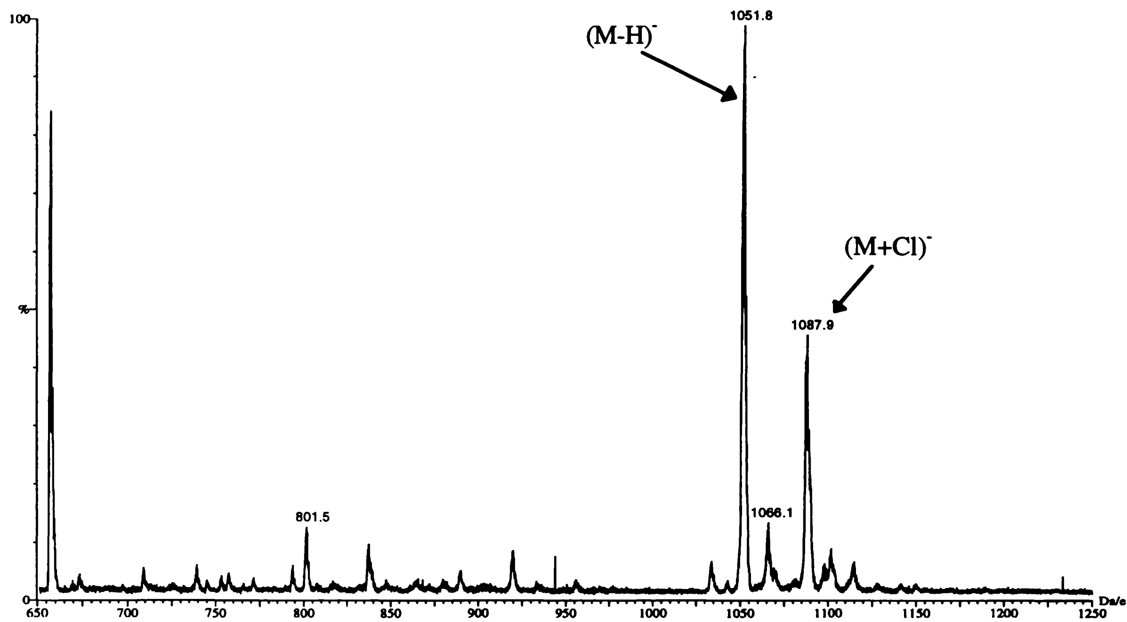


Figure II. ES-MS spectra of saponin **4-24** (A) and saponin **4-23** (B) isolated from the ethanol-water extract of *N. ossifragum*.

A



B

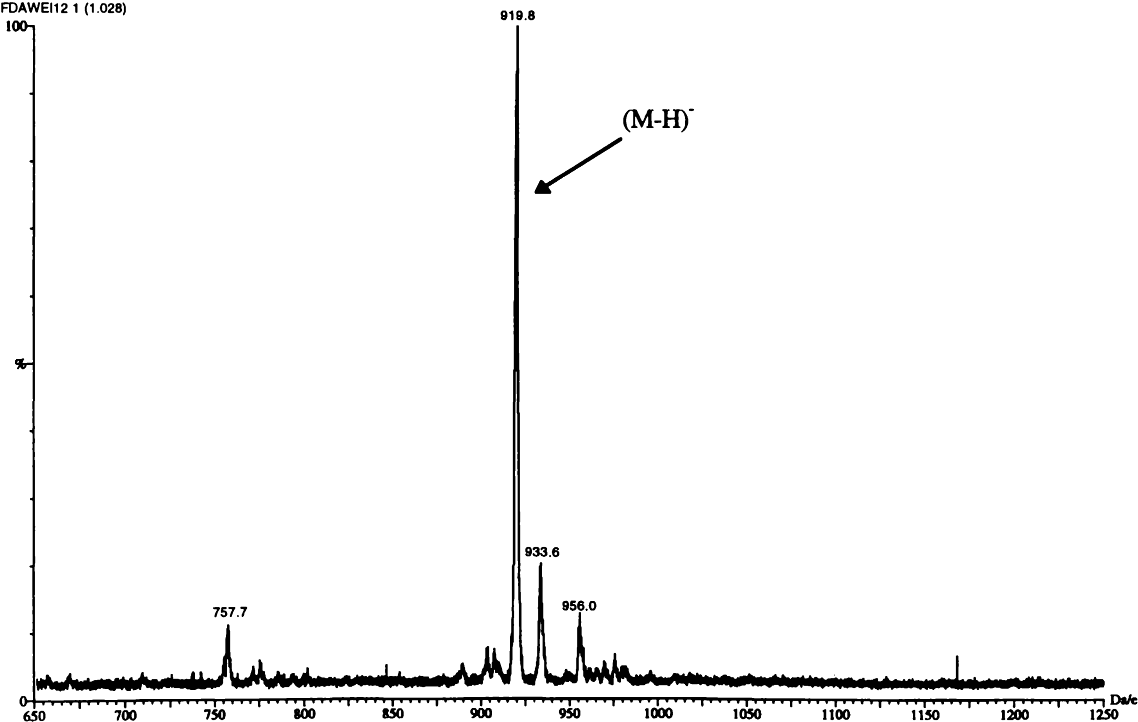


Figure III. ^{1}H NMR spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**) isolated from the ethanol-water extract of *N. ossifragum*.

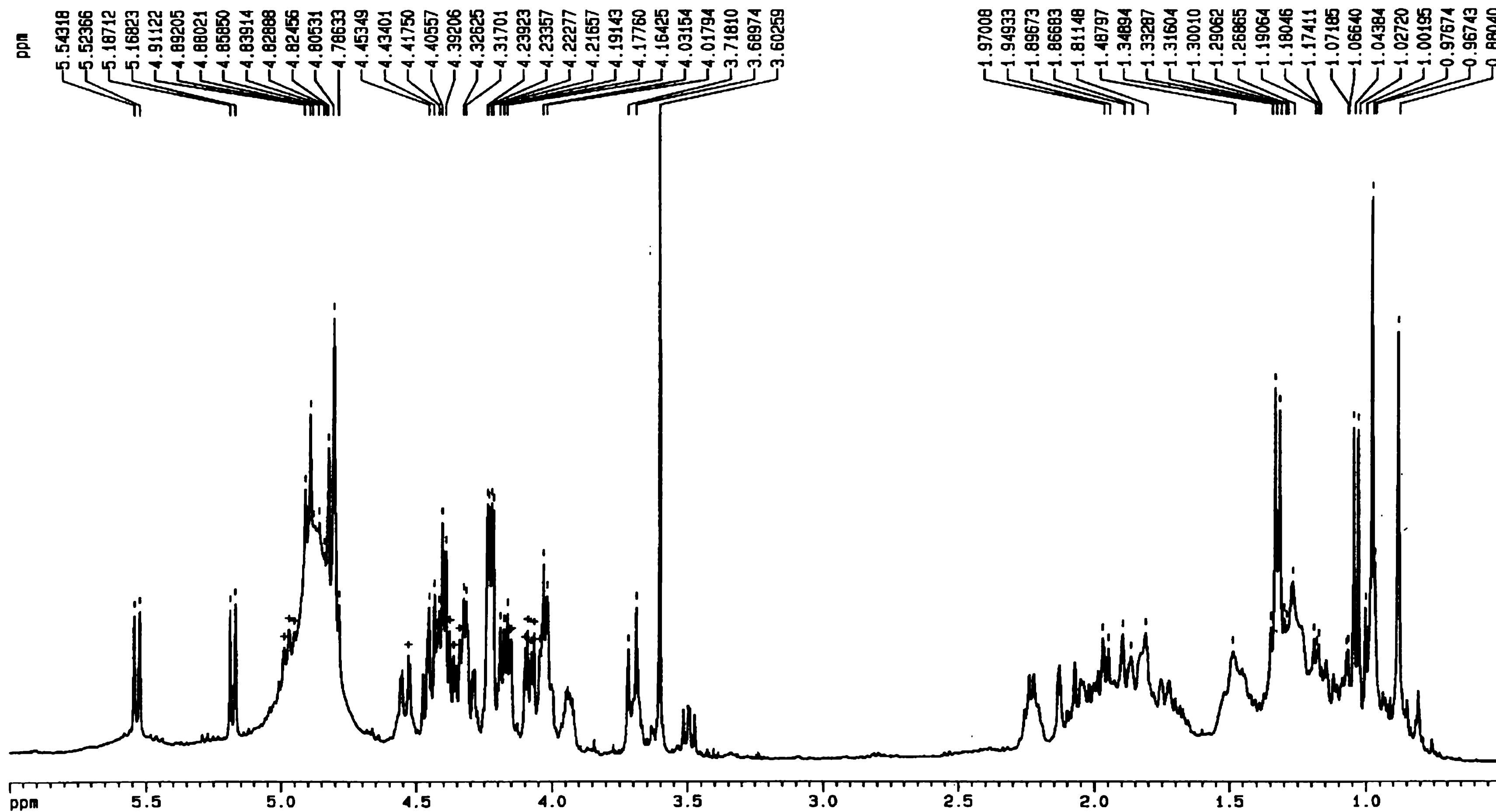


Figure IV. COSY spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**) isolated from the ethanol-water extract of *N. ossifragum*.

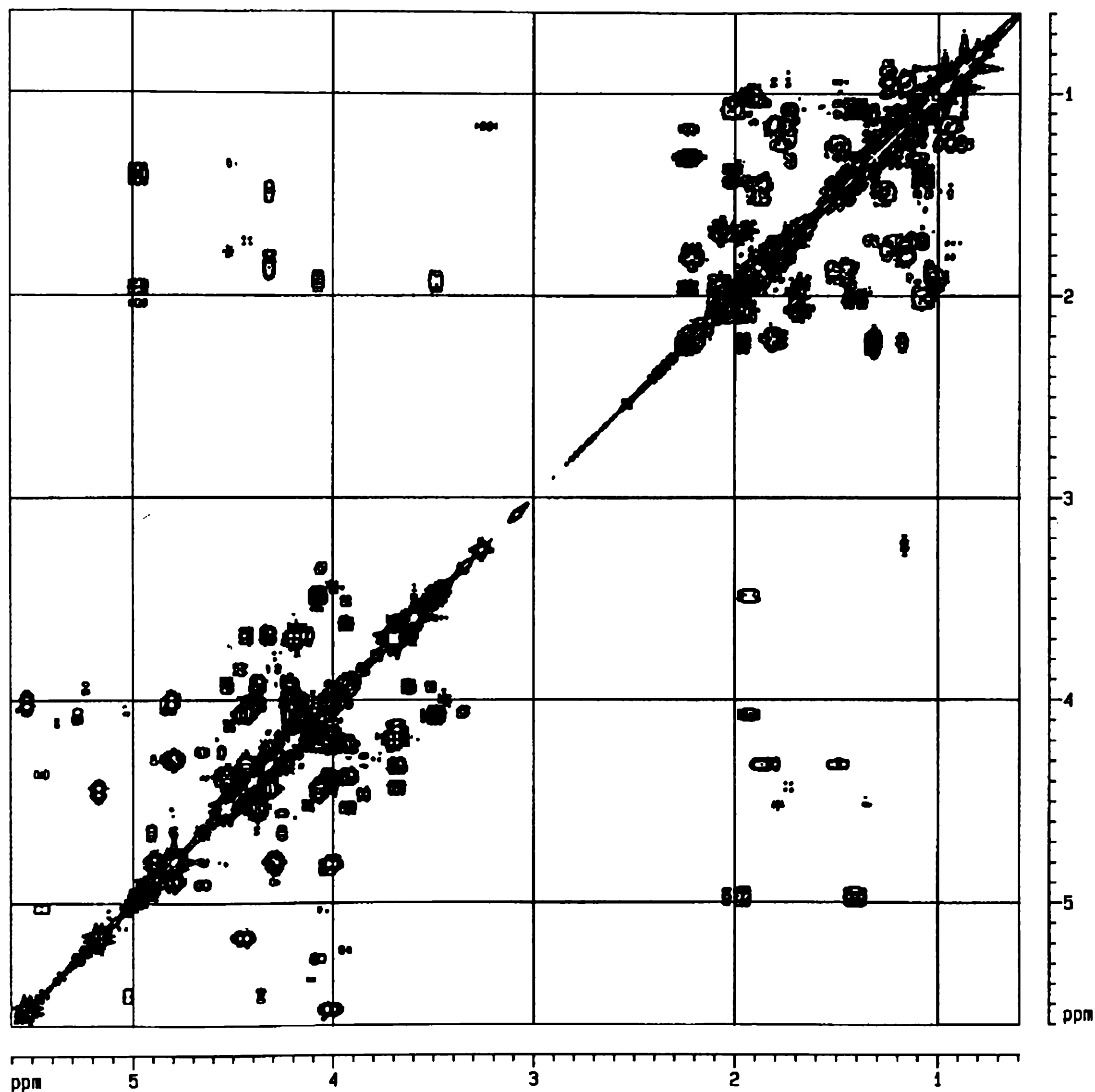


Figure V. ^{13}C NMR spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**) isolated from the ethanol-water extract of *N. ossifragum*.

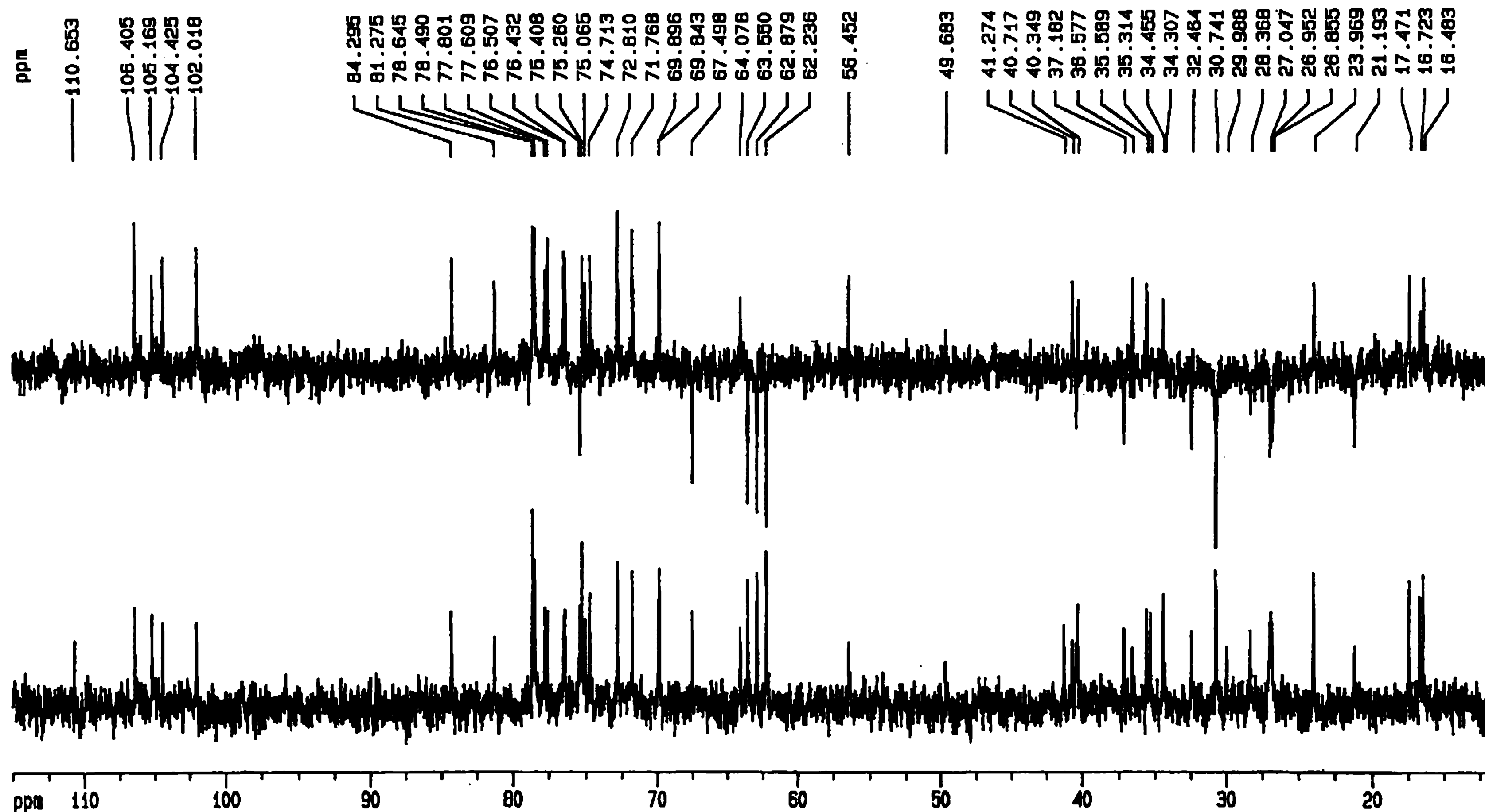


Figure VI. HSQC spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**) isolated from the ethanol-water extract of *N. ossifragum*.

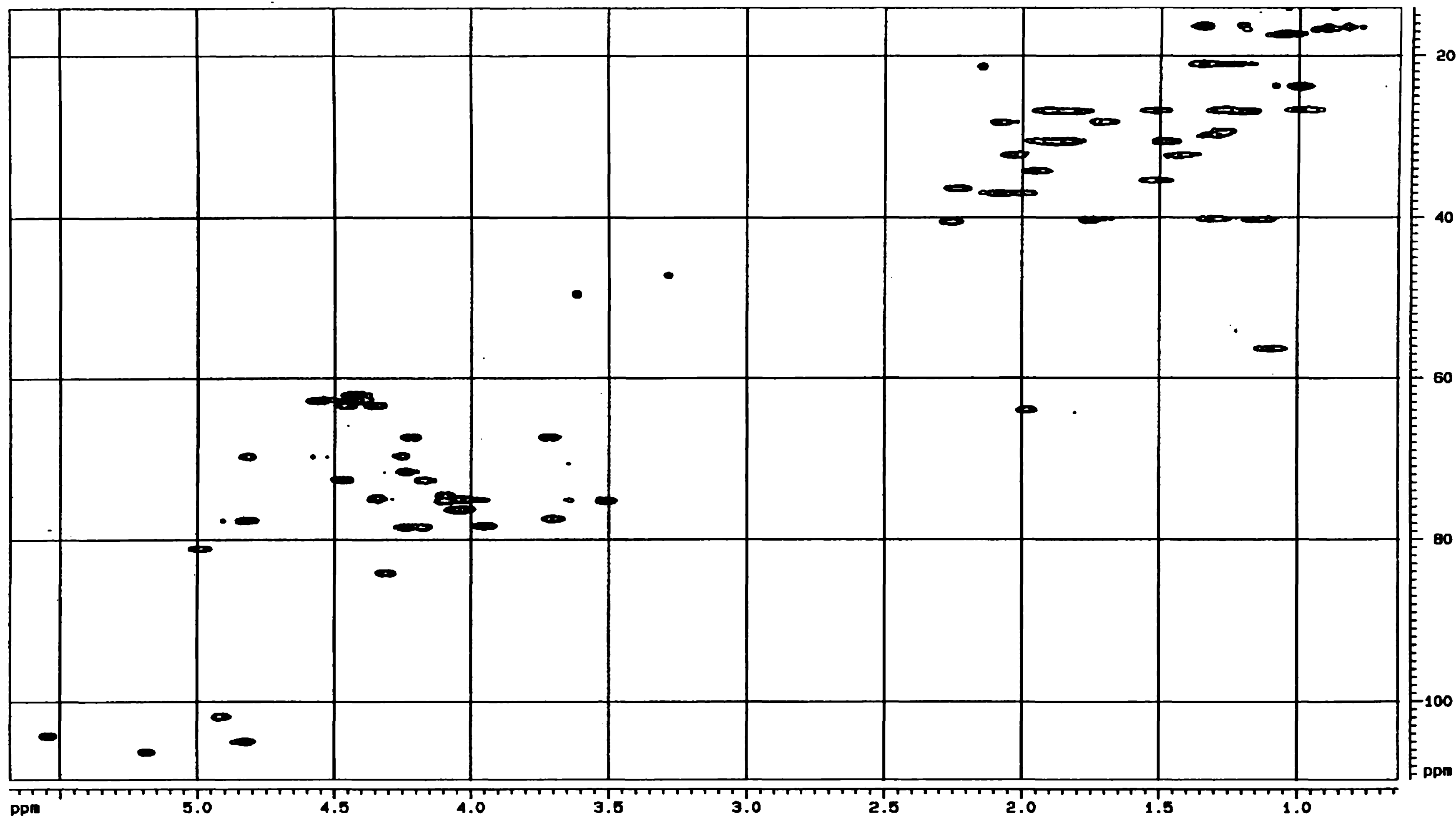


Figure VII. HMBC spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**) isolated from the ethanol-water extract of *N. ossifragum*.

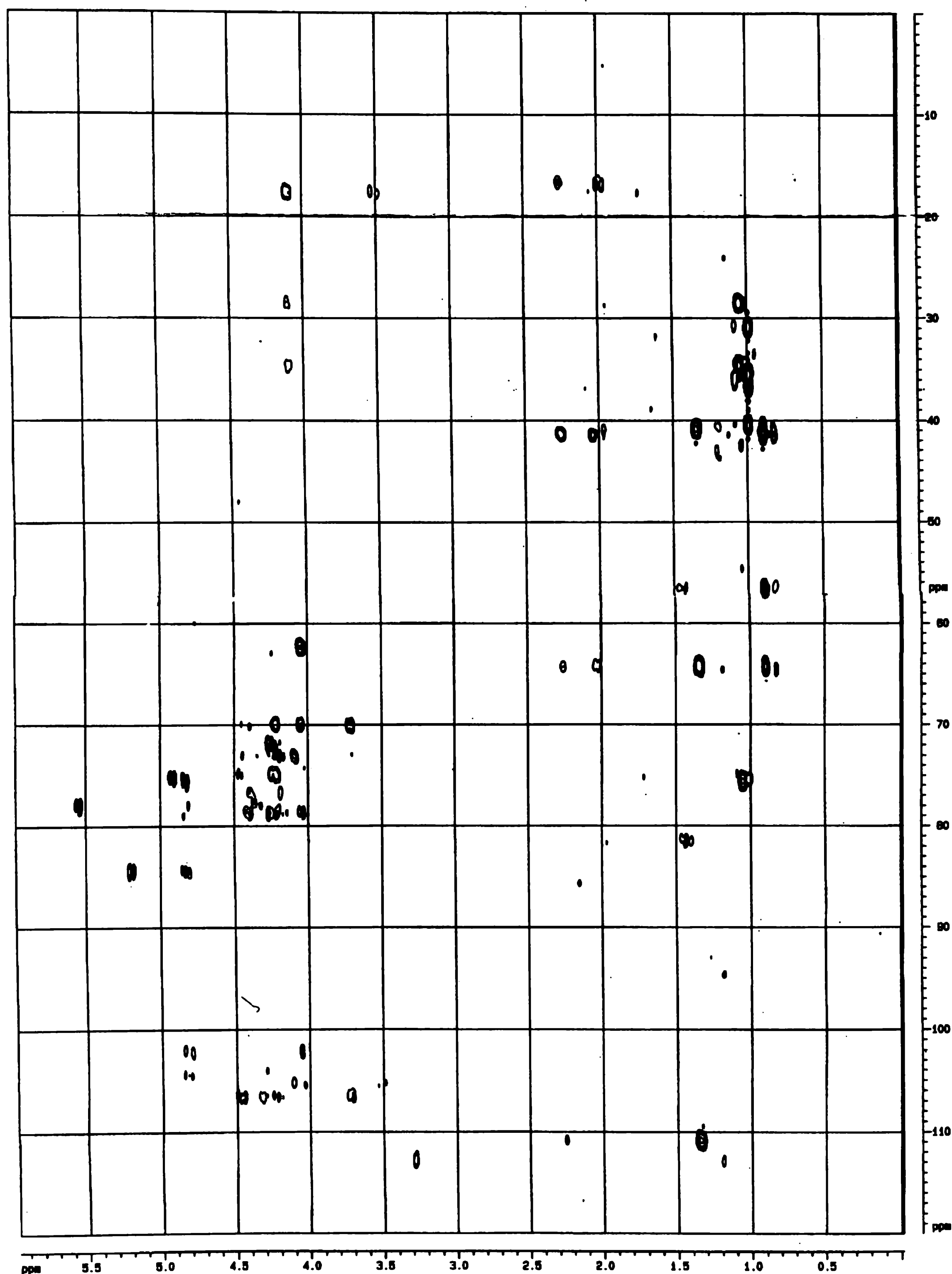


Figure VIII. Partial HOHAHA spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**) isolated from the ethanol-water extract of *N. ossifragum*.

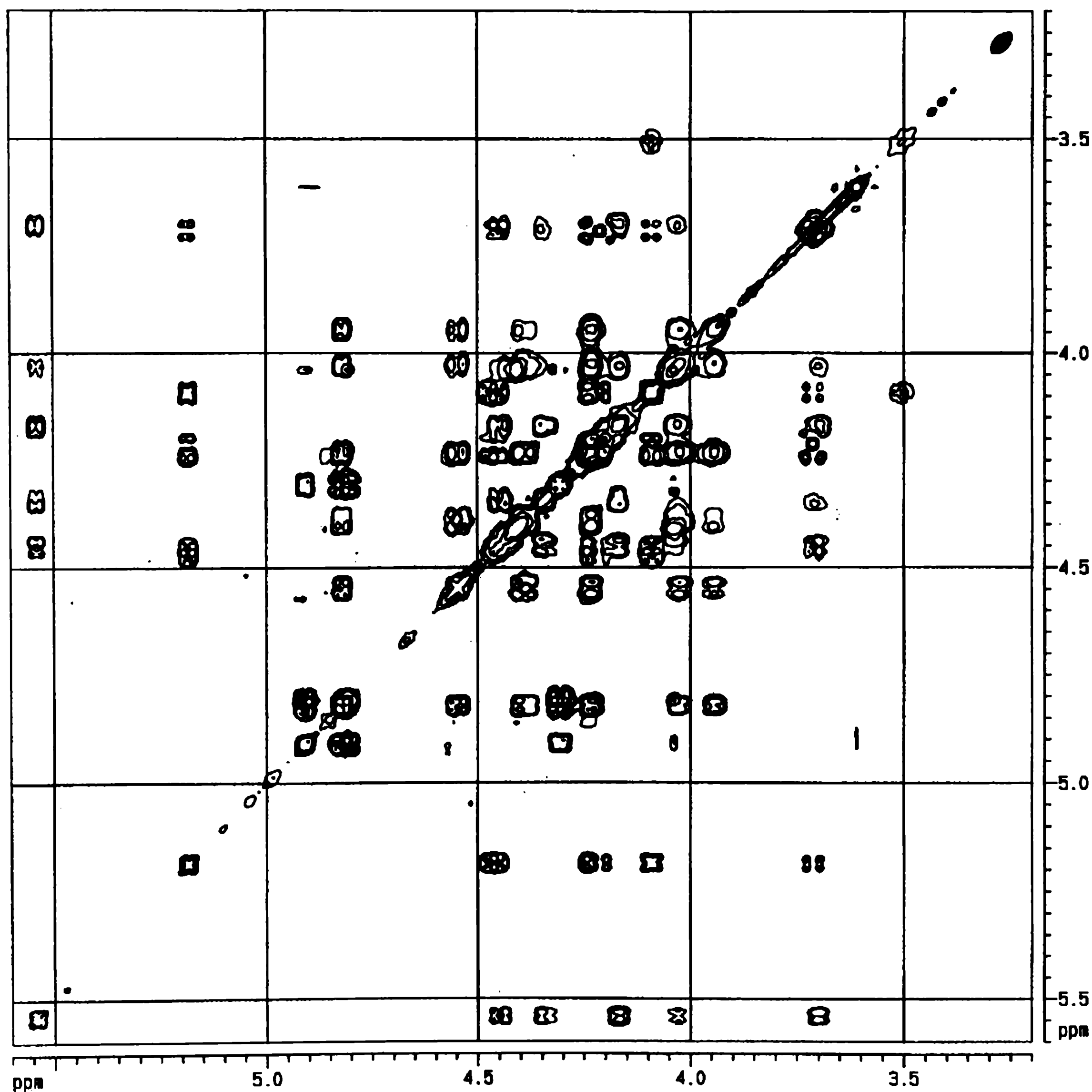


Figure IX. ROESY spectrum of (25*R/S*)-5β-furostane-3β,22α,26-triol 3-*O*-{*O*-β-D-glucopyranosyl-(1→2)-*O*-[α-L-arabinopyranosyl-(1→3)]-β-D-galactopyranosyl}-26-*O*-β-D-glucopyranoside (**4-24**) isolated from the ethanol-water extract of *N. ossifragum*.

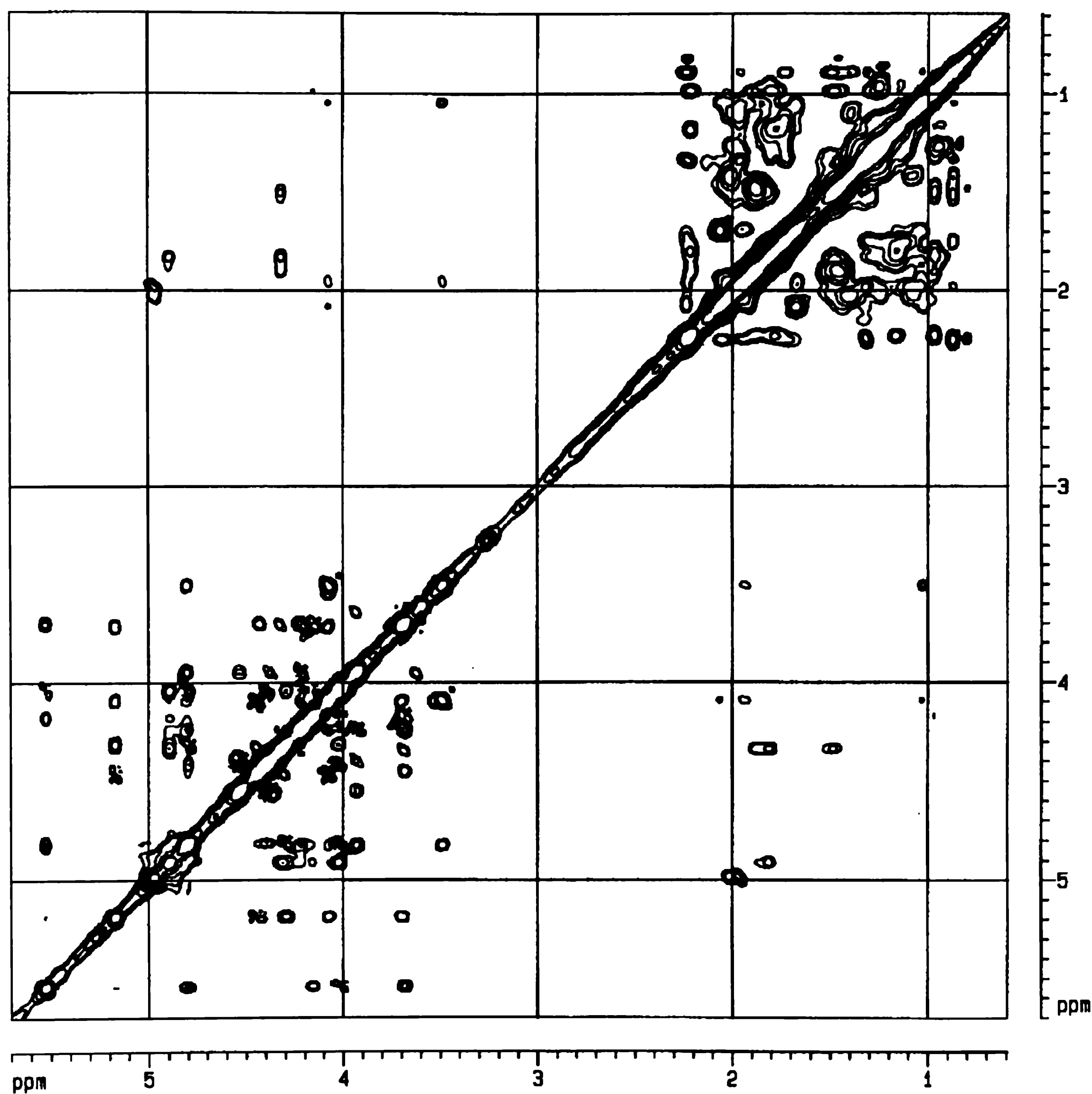


Figure X. Sample Excel spreadsheet used to calculate the levels of free and conjugated sapogenins and sterols in animal and plant samples.

This spreadsheet calculates response factors and concentrations ex plant extractions in mg/kg

The default response factor is 1 (entered on line 16) - otherwise type in the value calculated on line10

FILE: DDI2AxxA.D

INTERNAL STANDARD		
weight Sar-OPr	5.2	mg
Volume	25.0	mL
Conc	0.208	mg/mL

RESPONSE FACTOR CALCULATIONS

compound	Chol-OAc	Stig-OAc	Sito-OAc	Sar-OAc	Dio-OAc	Dio-OPr	Std Sar-OPr	camp
ion	368	394	396	139	139	139	139	(calc)
wght (mg)	1.9	2.2	1.9	2.0	2.1	2.1	2.5	
Area	38004151	36762400	32220001	22292047	17776230	15439800	25365410	
Ratio	20002185	16710182	16957895	11146024	8464871	7352286	10146164	
RF	1.971	1.647	1.671	1.099	0.834	0.725	1.000	1.821

SAMPLE PARAMETERS

PEAK AREAS - ex GC/MS

Compd	Chol-OAc	Camp-OAc	Stig-OAc	Sito-OAc	Smil-OAc	Sar-OAc	Dio-OAc	Tig-OAc	Neotig-OAc	Heco-OAc	Neoheco-OAc	std Sar-Pr
TIME	18.97	21.37	22.07	23.47	21.05	21.49	22.31	22.7	23.14			23.91
Ion	368	382	394	396	139	139	139	139	139			139
RF	1.971	1.821	1.647	1.671	1.099	1.099	0.834	0.834	0.834	0.834	0.834	1

Rack no	SAMPLE	COMMENT(s)		Sample Weight (gm)	ISTD Vol (uL)	ISTD Weight (mg)	Chol-OAc	Camp-OAc	Stig-OAc	Sito-OAc	Smil-OAc	Sar-OAc	Dio-OAc	Tig-OAc	Neotig-OAc	Heco-OAc	Neoheco-OAc	std Sar-Pr
1	Iran94/1	Conj	BtOH	1.0373	250	0.052	1090101	506073	2044967	5160786	1152735	758887	7776818	15733164	4466841	6454154	5480548	5318783
2	Iran94/1	Free	Hex	1.0373	250	0.052	7551302	1766656	8560342	15389557	618696	216251	0	0	0	0	0	6432864
3	Iran94/2	Conj	BtOH	0.5410	250	0.052	1802917	0	747810	2975836	8123217	3691174	1473193	1442740	0	1593036	1091284	13387330
4	Iran94/2	Free	Hex	0.5410	250	0.052	2126853	433089	2451162	5847704	1082677	359422	0	0	0	0	0	4205666
5	Iran94/3	Conj	BtOH	0.9861	250	0.052	4035788	1059582	6404604	11722461	0	0	787397	581879	0	489155	0	830723
6	Iran94/3	Free	Hex	0.9861	250	0.052	2933182	523992	3796048	6598072	604784	246866	0	0	0	0	0	4296859
7	Iran94/4	Conj	BtOH	1.0314	250	0.052	1645094	433773	2824738	4523329	0	0	3159002	1790971	0	951502	0	946968
8	Iran94/4	Free	Hex	1.0314	250	0.052	4749861	921391	6360067	9711043	1070074	481683	0	0	0	0	0	6893806

Saponin/sapogenin levels (mg/kg)

	Chol	Camp	Stig	Sito	Smil	Sar	Dio	Tig	Neotig	Heco	Neoheco	Sar-Pr
OAc	428	442	454	456	458	458	456	456	456	472	472	
OH	386	400	412	414	416	416	414	414	414	430	430	
Factor	0.902	0.905	0.907	0.908	0.908	0.908	0.908	0.908	0.908	0.911	0.911	

Rack no	SAMPLE	COMMENT(s)		Sample Weight (gm)	Chol	Camp	Stig	Sito	Smil	Sar	Dio	Tig	Neotig	Heco	Neoheco	ITSD Sar-Pr
1	Iran94/1	Conj	BtOH	1.0373	4.7	2.4	10.6	26.4	9.0	5.9	79.8	161.4	45.8	66.4	56.4	50.1
2	Iran94/1	Free	Hex	1.0373	26.9	6.8	36.8	65.2	4.0	1.4	0.0	0.0	0.0	0.0	0.0	50.1
3	Iran94/2	Conj	BtOH	0.5410	5.9	0	3.0	11.6	48.2	21.9	11.5	11.3	0.0	12.5	8.6	96.1
4	Iran94/2	Free	Hex	0.5410	22.2	4.9	30.9	72.6	20.5	6.8	0.0	0.0	0.0	0.0	0.0	96.1
5	Iran94/3	Conj	EtOH	0.9861	117.2	33.4	224.0	404.3	0.0	0.0	54.4	40.2	0.0	33.9	0.0	52.7
6	Iran94/3	Free	Hex	0.9861	16.5	3.2	25.7	44.0	6.1	2.5	0.0	0.0	0.0	0.0	0.0	52.7
7	Iran94/4	Conj	EtOH	1.0314	40.1	11.5	82.9	130.8	0.0	0.0	183.1	103.8	0.0	55.3	0.0	50.4
8	Iran94/4	Free	Hex	1.0314	15.9	3.3	25.6	38.6	6.5	2.9	0.0	0.0	0.0	0.0	0.0	50.4

REFERENCES

Abas-Mazni, O.; Mohd-Khusahry, Y.; Sheikh-Omar, A. R. Jaundice and photosensitization in indigenous sheep of Malaysia grazing on *Brachiaria decumbens*. *Malaysia Vet. J.* **1983**, *7*, 254-263.

Abas-Mazni, O.; Sharif, H.; Mohd-Khusahry, Y.; Vance, H. N. Photosensitization in goats grazed on *Brachiaria decumbens*. *Mardi. Res. Bull.* **1985**, *13*, 203-206.

Abdelkadar, S. V.; Ceh, L.; Dishington, I. W.; Hauge, J. G. Alveld-producing saponins. II. Toxicological studies. *Acta Vet. Scand.* **1984**, *25*, 76-85.

Abdullah, A. S.; Lajis, N. H.; Bremner, J. B.; Davies, N. W.; Mustapha, W.; Rajion, M. A. Hepatotoxic constituents in the rumen of *Brachiaria decumbens* intoxicated sheep. *Vet. Hum. Toxicol.* **1992**, *34*, 154-155.

Abdullah, A. S.; Nordin, M. M.; Rajion, M. A. Signal grass (*Brachiaria decumbens*) toxicity in sheep: Changes in motility and pH of reticule-rumen. *Vet. Human Toxicol.* **1988**, *30*, 256

Agrawal, P. K.; Jain, D. C.; Gupta, R. K.; Thakur, R. S. Carbon-13 NMR spectroscopy of steroidal sapogenins and steroidal saponins. *Phytochemistry* **1985**, *24*, 2479-2496.

Agrawal, P. K.; Jain, D. C.; Pathak, A. K. NMR spectroscopy of steroidal sapogenins and steroidal saponins: an update. *Magn. Res. Chem.* **1995**, *33*, 923-953.

Agrawal, P. K. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* **1992**, *31*, 3307-3330.

Allison, C. D. *Livestock poisoning plants of New Mexico rangelands*. Cooperative Extension Service Circular 531, New Mexico State University, Las Cruces, USA. **1991**.

Amjadi, A. R.; Ahourai, P.; Baharsefat, M. First report of geeldikkop in sheep in Iran. *Arch. de l'Inst. Razi.* **1977**, *29*, 71-78.

Anonymous. *Estimated cost of facial eczema to sheep production.* Paper No. T124 New Zealand Meat and Wool Boards' Economic Service, Wellington, NZ. **1990**.

Barrow, G. M. *Physical Chemistry.* McGraw-Hill Book Co. Inc., New York. **1981**, pp 341.

Bennett, R. D.; Helftmann, E.; Joly, R. A. Biosynthesis of diosgenins from 26-hydroxyl-cholesterol in Dioscorea floribunda. *Phytochemistry* **1970**, *9*, 349-353.

Berndt, S. A. *Aspects of the chemistry of saponins implicated in a form of hepatogenous photosensitization afflicting livestock.* M.Sc. thesis, the University of Waikato, Hamilton, New Zealand. **1997**.

Blunden, G.; Jaffer, J. A.; Jewers, K.; Robinson, J. M. Configurational assignments to spirostan-3-ols by mass spectrometry. *Steroids* **1980**, *36*, 299-303.

Bock, K.; Refn, S. The substrate specificity of amyloglucosidase (AMG). Part IV. Hydroxylcyclohexyl glucosides. *Acta Chem. Scand.* **1989**, *43*, 373-380.

Bollenback, G. N.; Long, J. W.; Benjamim, D. G.; Lindquist, J. A. The synthesis of aryl-D-glucopyranosiduronic acids. *J. Am. Chem. Soc.* **1955**, *77*, 3310-3315.

Bowden, K.; Heilbron, I. M.; Jones E. R. H.; Weedon, B. C. L. Researches on acetylenic compounds. Part I. The preparation of acetylenic ketoses by oxidation of acetylenic carbinols and glycols. *J. Chem. Soc.* **1946**, 39-45.

Bridges, C. H.; Camp, B. J.; Livingstone, C. W.; Bailey, E. M. Kleingrass (*Panicum coloratum*) Poisoning in sheep. *Vet. Pathol.* **1987**, *24*, 525-531.

Brown, J. M. M. Biochemical studies on geeldikkop and enzootic icterus. *Onderstepoort J. Vet. Res.* **1968**, *35*, 319-576.

Button, C.; Paynter, D. I.; Shiel, M. J.; Colson, A. R.; Pateson, P. J.; Lyford, R. L. crystal-associated cholangio-hepatopathy and photosensitization in lambs. *Aust. Vet. J.* **1987**, *64*, 176-180.

Camp, B. J.; Bridges DVM, C. H.; Hill, D. W.; Patamalai, B.; Wilson DVM, S. Isolation of a steroidal sapogenin from the bile of a sheep fed *Agave Lecheguilla. Vet. Human Toxicol.* **1988,** *30,* 533-535.

Ceh, L.; Hauge, J. G. Alveld-producing saponins I. Chemical studies. *Acta Vet. Scand.* **1981,** *22,* 391-402.

Cheeke, P. R. Nutritional and physiological implications of saponins: a review. *Can. J. Anim. Sci.* **1971,** *51,* 621-632.

Cheeke, P. R.; Shull, L. R. *Natural toxicants in feeds and poisonous plants.* AVI Publishing Co., Westport, CT, USA. **1985.**

Clare, N. T. *Photosensitization in diseases of domestic animals.* Commonwealth Agricultural Bureau, Farnham Royal, England. **1952,** pp 58.

Clare, N. T. Photosensitization in animals. *Adv. Vet. Sci.* **1955,** *2,* 182-211.

Coetzer, J. A. W.; Kellerman, T. S.; Sadler, W.; Nath, G. F. Photosensitivity in South Africa. V. A comparative study of the pathology of the ovine hepatogenous photosensitivity diseases, facial eczema and geeldikkop (*Tribulosis ovis*), with special reference to their pathogenesis. *Onderstepoort J. Vet. Res.* **1983,** *50,* 59-71.

Collett, M .G.; Spickett, A. M. Unusual hepatic parenchymal crystalloid material and biliary microliths in goats. *J. South African Vet. Assoc.* **1989,** *60,* 134-138.

Conrow, R. B.; Bernstein, S. Steroid conjugates. VI. An improved Koenigs-Knorr synthesis of aryl glucuronides using cadmium carbonate, a new and effective catalyst. *J. Org. Chem.* **1971,** *36,* 863-870.

CRC Handbook of Chemistry and Physics 59th Edition. Weast, R. C.; Astle, M. J. Ed. CRC press, Inc., Florida. 1978-1979, pp. D-186.

Dauben, W. G.; Fonken, G. J.; Noyce, D. S. The stereochemistry of hydride reductions. *J. Am. Chem. Soc.* **1956,** *78,* 2579-2582.

Davenport, H. W. *A digest of digestion.* Ed. 2. Chicago, Year Book Medical Publishers, Inc. **1978**.

De Kock, W. T.; Enslin, P. R. Chemical investigations of photosensitisation diseases of domestic animals. Part 1. Isolation and characterisation of steroidal sapogenins from *Tribulus terrestris. J. S. Afr. Chem. Inst.* **1958**, *11*, 33-36.

Di Giulio, R. T.; Benson, W. H.; Sanders, B. M.; Van Veld, P. A. Biochemical mechanisms: metabolism, adaptation, and toxicity. In *Fundamentals of aquatic toxicololgy*. 2nd Ed. Rand, G. M. ed., Taylor & Francis press, Washington, D. C. **1995**, pp 523-561.

Djerassi, C.; Engle, R. R.; Bowers, A. The direct conversion of steroidal Δ^5-3β-alcohols to Δ^5- and Δ^4-3-ketones. *J. Org. Chem.* **1956**, 1547-1549.

Ender, F. Undersøkelser over alveldsykens etiologi (Etiological studies on alveld - a disease involving photosensitization and icterus in lambs). *Nor. Vet. Med.* **1955**, *7*, 329-377.

Enslin, P. R.; Wells, R. J. Chemical investigations of photosensitisation diseases of domestic animals. *S. Afr. Ind. Chemist.* **1956**, *10*, 96-97, 101.

Everist, S. L. *Poisonous plants of Australia*, 2nd Ed. Angus and Robertson Pub, Sydney, Aust. **1981**, pp 761-764.

Ewart, A. J. The poisonous action of ingested saponins. *Commonw. Aust. Counc. Sci. Ind. Res. Bull.* **1931**, No. 50.

Familton, A. S. Animal disorders arising from consumption of pastures, In *Pastures their ecology and management*; Langer, R. H. M. ed., Oxford University Press, **1990**.

Faul, W. H.; Failli, A.; Djerassi, C. Side-chain transformations and deuterium labeling in the steroidal sapogenin series. *J. Org. Chem.* **1970**, *35*, 2571-2585.

Flåøyen, A. National Veterinary Institute, P.O.Box 8156 Dep. N-0033 Oslo, Norway. (pers commun).

Flåøyen, A. A difference in susceptibility of two breeds of sheep to the alveld toxin. *Vet. Res. Commun.* **1991**, *15*, 455-457.

Flåøyen, A. Do steroidal saponins have a role in hepatogenous photosensitization diseases of sheep? *Adv. Exp. Med. Biol.* **1996**, *405*, 395-403.

Flåøyen, A. *Studies on the aetiology and pathology of alveld with some comparisons to sporidesmin intoxication.* Dr. med. vet. thesis, Norwegian College of Veterinary Medicine, Oslo, Norway, **1993**.

Flåøyen, A.; Binde, M.; Bratberg, B.; Djønne, B.; Fjølstad, M.; Grønstøl, H.; Mantle, P. G.; Landsverk, T.; Schönheit, J.; Tønnesen, M. H. Nephrotoxicity of *Narthecium ossifragum* in cattle in Norway. *Vet. Rec.* **1995a**, *137*, 259-263.

Flåøyen, A.; Bratberg, B.; Frøslie, A.; Grønstøl, H. Nephrotoxicity and hepatotoxicity in calves apparently caused by experimental feeding with *Narthecium ossifragum. Vet. Res. Commun.* **1995b**, *19*, 63-73.

Flåøyen, A.; Bratberg, B.; Frøslie, A.; Grønstøl, H. Nephrotoxicity in lambs apparently caused by experimental feeding with *Narthecium ossifragum. Vet. Res. Commun.* **1995c**, *19*, 75-79.

Flåøyen, A.; Bratberg, B.; Frøslie, A.; Grønstøl, H.; Langseth, W.; Mantle, P. G.; von Krogh, A. Nephrotoxicity in goats caused by dosing with a water extract from the stems of *Narthecium ossifragum* plants. *Vet. Res. Commun.* **1997a**, *21*, 499-506.

Flåøyen, A.; Bratberg, B.; Frøslie, A.; Grønstøl, H.; Langseth, W.; Mantle, P. G.; von Krogh, A. Further studies on the presence, qualities and effects of the toxic principles from *Narthecium ossifragum* plants. *Vet. Res. Commun.* **1997b**, *21*, 137-148.

Flåøyen, A.; Frøslie, A. Photosensitization disorders. In *Handbook of plant and fungal toxicants*. D'Mello, J. P. Felix ed., CRC Press, Inc., New York. **1997**.

Flåøyen, A.; Handeland, K.; Refsum, T.; Ryeng, K. A.; Stuve, G. Nephrotoxicity in moose (*Alces alces*), red deer (*Cervus elaphus*), reindeer (*Rangifer tarandus tarandus*) and fallow deer (*Cervus dama*) caused by experimental dosing with *Narthecium ossifragum. J. Wildlife Diseases.* **1997c**, in press.

Flåøyen, A.; Jòhansen, J.; Olsen, J. *Narthecium ossifragum* associated photosensitization in sheep in the Faroe Islands. *Acta Vet. Scand.* **1995d,** *36*, 277-278.

Flåøyen, A.; Miles, C. O.; Smith, B. L. Alveld in lambs in Norway. In *Plant Associated Toxins: Agricultural, Phytochemical and Ecological Aspects*; Colegate, S. M., Dorling, P. R. Eds.; CAB International, Sydney, Aust. **1994**, 293-296.

Flåøyen, A.; Smith, B. L.; Miles, C. O. An attempt to reproduce crystal-associated cholangitis in lambs by the experimental dosing of sarsasapogenin or diosgenin alone and in combination with sporidesmin. *N. Z. Vet. J.* **1993,** *41*, 171-174.

Flåøyen, A.; Tønnesen, H. H.; Grønstøl, H.; Karlsen, J. Failure to induce toxicity in lambs by administering saponins from *Narthecium ossifragum*. *Vet. Res. Commun.* **1991**, *15*, 483-487.

Flåøyen, A.; Wilkins, A. L. Metabolism of saponins from *Narthecium ossifragum* - a plant implicated in the aetiology of alveld, a hepatogenous photosensitization of sheep. *Vet. Res. Commun.* **1997**, *21*, 335-345.

Flowers, H. M.; Jeanloz, R. W. The Synthesis of 2-acetamido-2-deoxy-3-O-(β-D-galactopyranosyl)-α-D-glucose. *J. Org. Chem.* **1963,** *28*, 1377-1379.

Ford, E. J. H. A preliminary investigation of photosensitization in Scottish sheep. *J. Comp. Path.* **1964**, *74*, 37-44.

Glastonbury, J. R. W.; Boal, G. K. Geeldikkop in goats. *Aust. Vet. J.* **1985**, *62*, 62-63.

Glastonbury, J. R. W.; Doughty, F. R.; Whitaker, S. J.; Sergeant, E. A syndrome of hepatogenous photosensitization, resembling geeldikkop in sheep grazing *Tribulus terrestris*. *Aust. Vet. J.* **1984,** *61*, 314-316.

Grabley, S.; Gareis, M.; Böckers, W.; Thiem, J. Glycosylation of mycotoxins. *Synthesis* **1992**, 1078-1080.

Graydon, R. J.; Hamid, H.; Zahari, P.; Gardiner, C. Photosensitization and crystal associated cholangiohepatopathy in sheep grazing *Brachiaria decumbens*. *Aust. Vet. J.* **1991**, *68*, 234-236.

Griem, H.; Schwarz, L.; Czygan, P.; Popper, H. *Pathogenesis and mechanisms of liver cell necrosis*. Keppler, D. eds, MTP press, Lancaster. **1975**, pp 199.

Healy, A. J. Contributions to a knowledge of the naturalised flora of N. Z. No. 1 *Trans. Royal Soc.* **1946**, *75*, 399-400.

Helferich, B.; Bergar, A. The Synthesis of glucuronides. *Chem. Ber.* **1957**, *90*, 2492-2498.

Helferich, B.; Wedemeger, K. F. The preparation of glycosides from acetobromo-halogenoses. *Chem. Ber.* **1950**, *83*, 538-540.

Helferich, B.; Wedemeger, K. F. The Preparation of glycosides from acetobromo-glucose. *Ann* . **1949**, *563*, 139-145.

Helftmann, E. Biochemistry of steroidal saponins and glycoalkaloids. *Lloydia* **1967**, *30*, 209-230.

Helftmann, E. Biosynthesis of plant steroids. *Lloydia* **1968**, *31*, 293-317.

Henrici, M. Comparative study of the content of starch and sugars of *Tribulus terrestris*, lucerne and some gramineae, and *Pentzia incana* under different meteorological, edaphic and physiological conditions. II. Carbohydrate nutrition. *Onderstepoort J. Vet. Res.* **1952**, *25*, 45-92.

Hershey, A. L. Some poisonous plant problems of New Mexico. *New Mexico Agric. Exp. Sta. Bull.* **1945**, No. 322,.

Holland, P. T.; Miles, C. O.; Mortimer, P. H.; Wilkins, A. L.; Hawkes, A. D.; Smith, B. L. Isolation of the steroidal sapogenin epismilagenin from the bile of sheep affected by *Panicum dichotomiflorum* toxicosis. *J. Agric. Food Chem.* **1991**, *39*, 1963-1965.

Inoue, T.; Yoshihiro, M.; Sashida, Y.; Kobayashi, M. Structures of toxic steroidal saponins from *Narthecium asiaticum* Maxim. *Chem. Pharm. Bull.* **1995**, *43*, 1162-1166.

Kaneko, J. J.; Cornelius, C. E. (Eds). *Clinical biochemistry of domestic animals.* 2nd Ed. Academic Press, New York. **1970**.

Karjala, S.; Link K. P. Synthesis of glycol glucosides. *J. Am. Chem. Soc.* **1940**, *62*, 917-920.

Kellerman, T. S.; Coetzer, J. A. W. Hepatogenous photosensitivity diseases in South Africa. Technical Communication No. 193. pp 1-23. Veterinary Research Institute, Onderstepoort, **1984**.

Kellerman, T. S.; Van Der Westhuizen, G. C. A.; Coetzer, J. A. W.; Roux, C.; Marasas, W. F. O.; Minne, J. A.; Bath, G. F.; Basson, P. A. Photosensitivity in South Africa. II. The experimental prodyction of the ovine hepatogenous photosensitivity disease geeldikkop (*Tribulusis ovis*) by the simultaneous ingestion of *Tribulus terrestris* plants and cultures of pithomyes chartarum containing the mycotoxin sporidesmin. *Onderstepoort J. Vet. Res.* **1980**, *47*, 231-261.

Kellerman, T. S.; Miles, C. O.; Coetzer, J. A. W.; Brown, J. M. M.; Maartens, B. P. Photosensitivity in South Africa. VI. The experimental induction of geeldikkop in sheep with crude steroidal saponins from *Tribulus terrestris. Onderstepoort J. Vet. Res.* **1991**, *58*, 47-53.

Kellerman, T. S.; Miles, C. O.; Erasmus, G. L.; Wilkins, A. L.; Coetzer, J. A. W. The possible role of steroidal saponins in the pathogensis of geeldikkop, a major hepatogenous photosensitization of small stock in South Africa, in *Poisonous Plants of the World: Agricultural, Phytochemical and Ecological Aspects*; Colgate, S. M.; Dorling, P. R. Eds.; CAB International, Sydney, Aust. **1994**, 287-292.

Kinns, M.; Samders, J. K. M. Improved frequency selectivity in nuclear overhauser effect difference spectroscopy. *J. Magn. Reson.* **1984**, *56*, 518-520.

Kobayashi, M.; Suzuki, K.; Nagaasawa, S.; Mimaki, Y. Purification of toxic saponins from *Narthecium asiaticum* Maxim. *J. Vet. Med. Sci.* **1993**, *55*, 401-407.

Koenigs , W.; Knorr, E. *Ber.* **1901,** *34*, 957.

Krohn, K.; Thiem, J. Three synthesis of lacticolorin. *J. Chem. Soc. Perkin I.* **1977**, 1186-1190.

Lajis, N. H.; Abdullah, S. H.; Salim, S .J. S.; Bremner, J. B.; Khan, M. N. Episarsasapogenin and epismilagenin: two sapogenins isolated from the rumen of sheep intoxicated by *Brachiaria decumbens. Steroids* **1993,** *58*, 387-389.

Lancaster, M. J.; Vit, I.; Lyford, R. L. Analysis of bile crystals from sheep grazing *Panicum schinzii* (sweet grass). *Aust. Vet. J.* **1991,** *68*, 281.

Lemieux, R. U.; Bundle, D. R.; Baker, D. A. The properties of a "synthetic" antigen related to the human blood-group Lewis a. *J. Am. Chem. Soc.* **1975**, *97*, 4076-4083.

Lemieux, R. U.; Driguez, H. The chemical synthesis of 2-acetamido-2-deoxy-4-O-(α-L-fucopyranosyl)-3-O-(β-D-galactopyranosyl)-D-glucose. The Lewis a blood-group antigenic determinant. *J. Am. Chem. Soc.* **1975**, *97*, 4063-4069.

Lemos, R. A.; Salvador, S. C.; Nakazato, L. Photosensitization and crystal-associated cholangiohepatopathy in cattle grazing *Brachiaria decumbens* in Brazil. *Vet. Human Toxicol.* **1997**, *39*, 376-377.

Lindahl, I. L.; Davis, R. E.; Tertell, R. T. Alfalfa saponins. Studies on their chemical, pharmacological and physiolgical properties in relation to ruminant bloat. *U.S. Dep. Agric. Tech. Bull.* **1957**, No. 1161. Washington, D.C.

Linstead, R. P. The preparation of cholestanol glucosides with all four possible configurations in the glucoside link. *J. Am. Chem. Soc.* **1940,** *62*, 1766-1770.

Lorimer, S. D.; Mawson, S. D.; Perru, N. B.; Weavers, R. T. Isolation and synthesis of β-miroside an antifungal furanone glucoside from *Prumnopitys ferruginea.. Tetrahedron* **1995,** *51*, 7287-7300.

Low, S. G.; Bryden, W. L. Photosensitization of cattle grazing signal grass (*Brachiaria decumbens*) in Papua New Guinea. *N. Z. Vet. J.* **1993,** *41*, 220-221.

Low, S. G.; Jephcott, S. B.; Bryden, W. L. Weaner illthrift of cattle grazing signal grass (*Brachiaria decumbens*) in Papua New Guinea. In *Poisonous Plants of theWorld: Agricultural, Phytochemical and Ecological aspects*; Colegate, S. M., Dorling, P. R., Eds.; CAB International, Sydney, Aust. **1994**.

Lubineau, A.; Queneau, Y. Aqueous cycloadditions using glyco-organic substrates. 1. Stereochemical course of the reaction. *J. Org. Chem.* **1987**, *52*, 1001-1007.

Mahato, S. B.; Ganguly, A. N.; Sahu, N. P. Steroid saponins. *Phytochemistry* **1982**, *21*, 959-978.

Malone, F. E.; Kennedy, S.; Reilly, G. A. C.; Woods, F. M.; Bog asphodel (*Narthecium ossifragum*) poisoning in cattle. *Vet. Rec.* **1992**, *131*, 100-103.

Marker, R. E.; Rohrmann, E. Sterols. LIII. The structure of the side chain of sarsasapogenin. *J. Am. Chem. Soc.* **1939**, *61*, 846-851.

Mathews, F.P. Lechuguilla (*Agave lecheguilla*) poisoning in sheep and goats. *J. Am. Vet. Med. Assoc.* **1938**, *93*, 168-175.

Mathews, F.P. Lechuguilla (*Agave lecheguilla*) poisoning in sheep, goats and laboratory animals. *Texas Agr. Exp. Sta. Bull.* **1937**, No. 554.

Mathews, F.P. Poisoning in sheep and goats by sacahuiste (*Nolina texana*) buds and blooms. *Texas Agr. Exp. Sta. Bull.* **1940**, No. 585.

McCloskey, C. M.; Pyle, R. E.; Coleman, G. H. The preparation of β,β-trehalose octaacetate. *J. Am. Chem. Soc.* **1944**, *66*, 349-350.

Meagher, L. P. *Aspects of the chemistry of some agricultural myco- and phytotoxins.* Ph.D thesis, the University of Waikato, Hamilton, New Zealand. **1996**.

Meagher, L. P.; Wilkins, A. L.; Miles, C. O.; Collin, R.; Fagliari, J. J. Hepatogenous photosensitization of ruminants by *Brachiaria decumbens* and *Panicum dichotomiflorum* in the absence of Sporidesmin: Lithogenic saponins may be responsible. *Vet. Hum. Toxicol.* **1996**, *38*, 271-274.

Miles, C. O.; Munday, S. C.; Holland, P. T.; Lancaster, M. J.; Wilkins, A. L. Further analysis of bile crystals from sheep grazing *panicum schinzii* (sweet grass). *Aust. Vet. J.* **1992a**, *69*, 34.

Miles, C. O.; Wilkins, A. L.; Erasmus, G. L.; Kellerman, T. S.; Coetzer, J. A. W. Photosensitivity in South Africa. VII. Chemical composition of biliary crystals from a sheep with experimentally induced geeldikkop. *Onderstepoort J. Vet. Res.* **1994a**, *61*, 215-222.

Miles, C. O.; Wilkins, A. L.; Erasmuys, G. L.; Kellerman, T. S. Photiosensitivity in South Africa. VIII. Ovine metabolism of *Tribulus terrestris* saponins during experimentally induced geeldikkop. *Onderstepoort J. Vet. Res.* **1994b**, *61*, 351-359.

Miles, C. O.; Wilkins, A. L.; Munday, S. C.; Holland, P. T.; Smith, B. L; Lancaster, M. J.; Embling, P. P. Identification of the calcium salt of epismilagenin β-D-glucuronide in the bile crystals of sheep affected by *Panicum dichotomflorum* and *Panicum schinzii* toxicoses. *J. Agric. Food Chem.* **1992b**, *40*, 1606-1609.

Miles, C. O.; Wilkins, A. L.; Munday, S. C.; Holland, P. T.; Smith, B. L. Identification of insoluble salts of the β-D-glucuronides of episarsasapogenin and epismilagenin in the bile of lambs with alveld, and examination of *Narthecium ossifragum*, *Tribulus terrestris*, and *Panicum miliaceum* for sapogenins. *J. Agric. Food Chem.* **1993**, *41*, 914-917.

Miles, C. O.; Munday, S. C.; Holland, P. T.; Smith, B. L.; Embling, P. P.; Wilkins, A. L. Identification of a sapogenin glucuronide in the bile of sheep affected by *Panicum dichotomiflorum* toxicosis. *N. Z. Vet. J.* **1991**, *39*, 150-152.

Milgate, J.; Roberts, D. C. K. The nutritional and biological significance of saponins. *Nutrition Research.* **1995**, *15*, 1223-1249.

Miller, L. P. *Phytochemistry.* Vol II. Van Nostrand Reinhold Co., New York. **1973**.

Mimaki, Y.; Takaashi, Y.; Kuroda, M.; Sashida, Y.; Nikaido, T. Steroidal saponins from *Nolina recurvata* stems and their inhibitory activity on cyclic AMP phosphodiesterase. *Phytochemistry* **1996**, *42*, 1609-1615.

Morrison, S. M.; Scott, J. K. Variation in population of *Tribulus terrestris* (Zygophyllaceae).3. Isozyme analysis. *Aust. J. Bot.* **1996,** *44* , 201-212.

Mortimer, P. H.; White, E. P.; di Menna, M. E. Pithomycotoxicosis "facial eczema" in sheep. In *Mycotoxic fungi, Mycotoxins, mycotoxicoses. An encyclopaedic handbook*; Wyle, T. D.; Morehouse, G. L. Eds. Dekker, New York. **1978**, 2, 195-203.

Munday, R.; Fowkes, E. A.; Thompson, A. M.; Wesselink, C.; Towers, N. R. A slow-release intramural device for protection against facial eczema in sheep. In *Proceedings of the 17th International Grasslands Congress.* **1993a,** 1392-1393.

Munday, S. C.; Wilkins, A. L.; Miles, C. O.; Holland, P. T. Isolation and structure elucidation of dichotomin, a furostanol saponin implicated in hepatogenous photosensitization of sheep grazing *Panicum dichotomiflorum. J. Agric. Food Chem.* **1993b,** *41*, 267-271.

Nambara, T.; Imai, K. Syntheses of estriol monoglucuronides. *Chem. Pharm. Bull.* **1967**, *15*, 1232-1238.

Noller, C. R.; Rockwell, W. C. The preparation of some higher alkylglucosides. *J. Am. Chem. Soc.* **1938,** *60*, 2076-2077.

Norris, J. J.; Valentine, K. A. Principal livestock poisoning plants of New Mexico ranges. *New Mexico Agric. Exp. Sta. Bull.* **1954,** No. 390.

Opasina, B.A. photosensitization jaundice syndrome in West African dwarf sheep and goats grazed on *Brachiaria decumbens. Tropec. Grass.* **1985,** *19*, 120-123.

Patamalai, B. Identification of hepatotoxins in kleingrass and a study of their toxicity. *Dissertation Abst. Int.* **1988,** *49*, 2080.

Patamalai, B.; Hejtmancik, E.; Briges, C. H.; Hill, D. W.; Camp, B. J. The isolation and identification of steroidal sapogenins in kleingrass. *Vet. Hum. Toxicol.* **1990**, *32*, 314-318.

Paulsen, H. Advances in selective chemical synthesis of complex oligosaccharides. *Angew. Chem. Int. Ed. Engl.* **1982,** *21*, 155-224.

Paulsen, H. Haworth memorial lecture: synthesis of complex oligosaccharide chains of glycoproteins. *Chem. Soc. Rev.* **1984**, *13*, 15-45.

Puoli, J. R.; Reid, R. L.; Belesky, D. P. Photosensitization in lambs grazing switchgrass. *Agron. J.* **1992**, *84*, 1077-1080.

Rankins, D. J., Jr.; Smith, G. S.; Ross, T. T. Rat study of beargrass (sacahuista) toxicity. *Am. Soc. Anim. Sci.* **1986**, *37*, 224-226.

Rankins, D. J., Jr.; Smith, G. S.; Ross, T. T.; Caton, J. S.; Kloppenburg, P. Characterization of toxicosis in sheep dosed with blossoms of sacahuiste (*Nolina microcarpa*). *J. Anim. Sci.* **1993**, *71*, 2489-2498.

Redemann, C. E.; Niemann, C. Acetobromoglucose (2,3,4,6-tetraacetyl-α-D-glucopyranosyl bromide). *Org. Synth.* **1955**, *Coll. Vol. III*, 11-14.

Regnault, T. R. H. Secondary photosensitization of sheep grazing bambatsi grass (*Panicum coloratum* var makarikariense). *Aust. Vet. J.* **1990**, *67*, 419.

Robertson, A.; Waters, R. B. Syntheses of glucosides. Part VI. The preparation of β-glucosides of phenols. *J. Chem. Soc.* **1930**, 2728-2733.

Robinson, T. *The organic constituents of higher plants. Their chemistry and interrelationship.* Burgess Publishing Company, Minneapolis, Minn. **1963**.

Rodriguez, E. B.; Scally, G. D.; Stick, R. V. The synthesis of optically pure epoxyalkyl β-D-glucosides and β-cellobiosides as active-site directed inhibitors of some β-glucan hydrolases. *Aust. J. Chem.* **1990**, *43*, 1391-1405.

Samford, M. D. *Rat and sheep studies of beargrass blossoms (Nolina microcarpa): Toxicity diminished by storage.* M. S. Thesis. New Mexico State Univ., Las Cruces. **1985**.

Scheer, I.; Kostic, R. B.; Mosettig, E. The C-25 isomerism of smilagenin and sarsasapogenin. *J. Am. Chem. Soc.* **1955**, *77*, 641-646.

Schmidt, R. R. New methods for the synthesis of glycosides and oligosaccharides - are there alternatives to the Koenigs-Knorr method? *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 212-235.

Schneider, J. J.; Bjacca, N. S. Synthesis and characterization of cholesterol β-D-glucuronide and derivatives. *J. Org. Chem.* **1969**, *34*, 1990-1993.

Schneider, J. J.; Lewbart, M. L.; Levitan, P.; Lieberman, S. Isolation from urine and synthesis of tetrahydrocortisone glucuronoside. *J. Am. Chem. Soc.* **1955**, *77*, 4184-4185.

Schroeder, L. R.; Green, J. W. Koenigs-Knorr syntheses with mercuric salts. *J. Chem. Soc.* (C). **1966**, 530-531.

Shapiro, R. H.; Williams, D. H.; Budzikiewicz, H.; Djerassi, C. Mass spectrometry in sructural and stereochemical problems. LIII. Fragmentation and hydrogen transfer reactions of a typical 3-keto steroid, 5α-androstan-3-one. *J. Am. Chem. Soc.* **1964**, *86*, 2837-2845.

Shoppee, C. W.; Summers, G. H. R. Steroids and the walden inversion, part VI. reduction of cholest-5-en-3-one with lithium aluminium hydride. *J. Chem. Soc.* **1950**, 687-689.

Skerman, P. J.; Riveros, F. *Tropical grasses.* Food and Agric. Organ. of U. N., Rome, Italy. **1990**, pp 238.

Smith, B. L.; Miles, C. O. A role for *Brachiaria decumbens* in hepatogenous photosensitization of ruminants. A letter to the editor. *Vet. Hum. Toxicol.* **1993**, *35*, 256-257.

Smith, B. L.; Towers, N. R. Pithomycotoxicosis (facial eczema) in New Zealand and the use of zinc salts for its prevention. In: Seawright, A. A.; Hegarty, M. P.; James, L. F.; Keeler, R. F. (eds), *Plant Toxicology: Proceedings of the Australia-USA poisonous plants Symposium, Brisbane, Australia. May 14-18, 1984.* Queensland Poisonous Plant Committee, Yeerongpilly, Qld. **1985**, pp 70-79.

Sollman, T. H. *A manual of pharmacology and its applications to therapeutics and toxicology*. 8th Ed. W. B. Saunders Co., Philadelphia. **1957**.

Spector, W. S. *Handbook of biological data*. W. B. Saunders Co., London. **1956**, pp 61-64.

Stabursvik, A. A new sarsasapogenin glycoside from *Narthecium ossifragum* L. Huds. *Acta Chem. Scand.* **1954**, *8*, 1304-1305.

Stabursvik, A. A phytochemical study of *Narthecium ossifragum* (L.) Huds.; Norges Tekniske Vitenskapsakademi, Series 2, No 6, Oslo University Press, Oslo, Norway, **1959**, pp 89.

Staley, T. E.; Stout, W. L.; Jung, G. A. Nitrogen use by tall fescue and switchgrass on acidic soils of varying water holding capacity. *Agron. J.* **1991**, *83*, 732-738.

Stout, W. L.; Jung, G. A.; Shaffer, J. A.; Estepp, R. Soil water conditions and yield of tall fescue, switchgrass and Caucasian bluestem in the Appalachian Northeast. *J. Soil Water Conserv.* **1986**, *41*, 184-186..

Summerfield, R. J. Biological flora of British Isles, *J. Ecol.* **1974**, *162*, 325-339.

Suzuki, K.; Kobayashi, M.; Ito, A.; Nakagawa, M. *Narthecium asiaticum* Maxim. poisoning of grazing cattle: observations on spontaneous and experimental cases. *Cornell Vet.* **1985**, *75*, 348-365.

Takaashi, Y.; Mimaki, Y.; Kuroda, M.; Sashida, Y. Recurvosides A-E, new polyhydroxylated steroidal saponins from *Nolina recurvata* stems. *Tetrahedron* **1995**, *51*, 2281-2292.

Takechi, M.; Shimada, S.; Tanaka, Y. Structure-activity relationships of synthetic diosgenyl diglycosides. *Phytochemistry* **1992a**, *31*, 3280-3281.

Takechi, M.; Tanaka, Y. Structure-activity relationships of synthetic diosgenyl monoglycosides. *Phytochemistry* **1991**, *30*, 2557-2558.

Takechi, M.; Tanaka, Y. Structure-activity relationships of synthetic methyl oleanolate glycosides. *Phytochemistry* **1992b**, *31*, 3789-3791.

Tapia, M. O.; Giordano, M. A.; Gueper, H. G. An outbreak of hepatogenous photosensitization in sheep grazing *Tribulus terrestris* in Argentina. *Vet. Hum. Toxicol.* **1994**, *36*, 311-313.

Theiler, A. Geeldikkop in sheep (*Tribulus ovis*). Report on Veterinary Research, Union of South Africa. **1918**, *7/8*, 1-56.

Tunnicliff, E. A. Forty-second annual report. *Texas Agric. Exp. Sta.* **1929**, pp 139.

Wall, M. E.; Eddy, C. R.; McCleannan, M. C.; Klumpp, M. E. Detection and estimation of steoidal sapogenins in plant tissues. *Anal. Chem.* **1952**, *24*, 1337-1347.

Wall, M. E.; Serota, S.; Eddy, C. R. Steroidal sapogenins. XVII. Stereochemistry of the spiroketal side chain. *J. Am. Chem. Soc.* **1955a**, *77*, 1230-1237.

Wall, M. E.; Serota, S.; Witnauer, L. P. Steroidal sapogenins. XXIV. The hydrochloric acid catalyzed equilibration of 22ξ,25D- and 22ξ-25L-spirostanes. *J. Am. Chem. Soc.* **1955b**, *77*, 3086-3089.

Wall, M. E.; Warnock, B. H.; Willaman, J. J. Steroidal sapogenins. LXVIII. Their occurrence in *Agave lecheguilla*. *Economic Botany*. **1962**, *16*, 266-269.

Wang, Y.; Lu, Y. Study on the quantitative analysis for the saponin in *Tribulus terrestris*. *Yall Wa Fenzi Zazho* **1991**, *11*, 70-73.

Wilkins, A. L.; Miles, C. O.; De Kock, W. T.; Erasmus, G. L.; Basson, A. T.; Kellerman, T. S. Photosensitivity in South Africa. IX. Structure elucidation of a β-glucosidase-treated saponin from *Tribulus terrestris*, and the identification of saponin chemotypes of South African *T. terrestris*. *Onderstepoort J. Vet. Res.* **1996**, *63*, 327-334.

Wilkins, A. L.; Miles, C. O.; Smith, B. L.; Meagher, L. P.; Ede, R. M. GC-MS method for analysis of plant and animal samples associated with ovine photosensitization. In *Plant-associated toxins agricultural, phytochemical and ecological aspects*; Colegate, S. M.; Dorling, P. R. Eds.; CAB International, Sydney, Aust. **1994**, 263-268.

Wilkins, A. L. The University of Waikato, Hamilton, New Zealand (pers commun).

Wotiz, H. H.; Smakula, E.; Lichtin, N. N.; Leftin, J. H. D-Glucopyranosiduronates. I. Steroidyl-β-D-glucopyranuronosides. *J. Am. Chem. Soc.* **1959**, *81*, 1704-1708.

Zeven, A. C.; Zhukovsky, P. M. *Dictionary of cultivated plants and their centres of diversity, excluding onamentals, forest trees and lower plants.* Certre for Agricultural Publishing and Documentation, Wageningen. **1975**.

Zill, G.; Ziegler, W.; Engelhardt, G.; Wallnöfer, P. R. Chemically and biologically synthesized Zearalenone-4-β-D-glucopyranoside: comparison and convenient determination by gradient HPLC. *Chemosphere* **1990**, *21*, 435-442.