MEAT RESEARCH CORPORATION

PROJECT CS.177

Control of Pimelea Poisoning in Cattle: Immunogen Feasibility Study

FINAL REPORT

CSIRO Tropical Agriculture

Queensland Department of Primary Industries

and

Central Queensland University
Tropical Beef Centre
Rockhampton

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1.0 ABSTRACT

Pimelea poisoning of cattle occurs when animals come in contact with *Pimelea species* plants. *Pimelea spp* plants are small sebaceous annuals that are native to Australia and occur predominently in the Maranoa Region of Queensland, Cobar District of New South Wales, and Marree District of South Australia. Particularly vigorous growth of *Pimelea spp* plants occurs after a relatively dry summer and early, light winter rain. The coincidence of this rainfall pattern and reduced availability of pastures, provides conditions for the most severe outbreaks of *Pimelea* poisoning. Incidences of *Pimelea* poisoning can, however, occur throughout the year.

Pimelea spp plants contain toxins that cause constriction of blood vessels in the lungs of cattle. The constriction of pulmonary venules, together with pressure exerted by the right ventricle of the heart, leads to the extrusion of plasma from cardio-pulmonary blood vessels. The blood plasma accumulates in the head and brisket of cattle, forming oedemas which characterise the "big-head" condition of cattle affected by *Pimelea* poisoning. Continued exposure to *Pimelea* toxins leads to an increase in systemic blood pressure and damage to kidney and liver function. *Pimelea spp* toxins appear to also act directly on the intestinal tract of cattle to induce diarrhoea. Hence, *Pimelea* poisoning of cattle is typically associated with diarrhoea, oedema of the head (in particular lower jaw) and brisket, and a rapid decline in live weight and body condition. This can result in death or sustained morbidity.

At the commencement of this project there was no treatment available to reduce the risk of *Pimelea* poisoning in cattle. Likewise, there was no specific treatment to facilitate the recovery of cattle affected by *Pimelea* poisoning.

One objective in this project was to determine whether cattle could be vaccinated against toxins found in *Pimelea spp* plants and, if this was possible, to then establish whether cattle vaccinated against toxins were less susceptible to *Pimelea* poisoning. The rationale for this approach was that vaccinated cattle should have anti-toxins antibodies which would bind and neutralise toxins in circulation, and block the normal pathophysiological effects of toxins on pulmonary venules and other tissues. Vaccinated cattle should therefore be less susceptible to *Pimelea* poisoning. The second objective was to determine whether vaccination against *Pimelea* toxins, or other strategies, would facilitated the recovery of cattle from *Pimelea* poisonig.

Toxins were isolated from *Pimelea spp* plants and formulated into vaccines using novel organic chemistry. Cattle vaccinated with prototype vaccines produced anti-toxins antibodies. Antibodies were purified from the blood of vaccinated cattle and, in laboratory organ-bath experiments, the antibodies were shown to neutralise the normal constricting actions of toxins on isolated pulmonary venules. It was concluded from this finding that anti-toxins antibodies had the capacity to bind and neutralise toxins and, potentially, could prevent the pathophysiological effects of toxins in vaccinated cattle. However, cattle vaccinated with prototype *Pimelea* vaccines and exposed to *Pimelea spp* plants were not immune, and developed all symptoms characteristic of *Pimelea* poisoning. Vaccinated cattle also did not show a faster recovery from *Pimelea* poisoning. Recovery of cattle from *Pimelea* poisoning could, however, be facilitated by treatment with Gastric Stimulant Powder, a commercial preparation for re-alimentation in animals.

A recommendation from this project is that, in areas susceptible to *Pimelea* poisoning, pastures should be managed to ensure that, wherever possible, there is sufficient pasture available to cattle to counter periods of increased growth of *Pimelea spp* plants. Cattle affected by *Pimelea* poisoning should receive supplementary feed (natural pasture or other supplement). In advanced cases of *Pimelea* poisoning and rumen stasis, cattle may be treated with Gastric Stimulant Powder to encourage re-alimentation; this has already been applied successfully.

2.0 EXECUTIVE SUMMARY

2.1 DEFINITION OF INDUSTRY ISSUE AND PROJECT STRATEGY

Outbreaks of *Pimelea* poisoning of cattle can have devastating consequences for individual or collective groups of producers in the Maranoa Region of Queensland, Cobar District of New South Wales and Marree District of South Australia. Germination and growth of *Pimelea spp* plants has been associated with various seasonal patterns of rainfall; however, it has not been possible to reliably predict outbreaks of *Pimelea* poisoning. At the commencement of this project, therefore, there was no strategy or technology to effectively prevent or minimise risks associated with the occurrence of *Pimelea* poisoning in cattle.

Pimelea spp plants are native to Australia and it is unlikely that biological control can be developed as a strategy to prevent outbreaks of *Pimelea* poisoning. Also, growth of *Pimelea* plants does not occur on a regular basis which could make it difficult to maintain critical populations of a biological control agent. Furthermore, *Pimelea* poisoning occurs in areas of rangeland and other extensive cattle production, further reducing the likelihood of biological control.

Because it is highly unlikely that *Pimelea spp* plants will be eliminated from environments where they are established, strategies to reduce the risk of *Pimelea* poisoning will need to focus on cattle and/or management.

Our charter in this project was to determine whether cattle could be vaccinated against toxins found in *Pimelea spp* plants, and then establish whether vaccination reduced susceptibility to *Pimelea* poisoning. This required: (i) the development of efficient procedures for isolation and purification of toxins; (ii) synthesis of novel immunogenic toxins-carrier protein conjugates; (iii) formulation of prototype vaccines; (iv) development of vaccination schedules; (v) development of laboratory procedures to evaluate anti-toxins antidodies; and (vi) field evaluation of prototype vaccines.

The present project was a continuation of an earlier immunogen (vaccine) feasibility study in project DAQ.072. During the course of the present project, 2 sub-projects were conducted in the Marree District of South Australia (Appendix D and Appendix E). The present project should therefore be read in conjunction with previous reports:

(i)	Project DAQ.072	*Pimelea poisoning in beef cattle: Plant ecology, epidemiology, therapeutic control and immunogen feasibility study.*
(ii)	Sub-project CS.177(1)	*Field study with an experimental Pimelea vaccine in the Marree District of South Australia (September-December 1995)* (Appendix D).
(iii)	Sub-project CS.177(2)	*Further field studies on Pimelea poisoning in cattle: Testing of an experimental Pimelea vaccine, gastric stimulant and experimental antidote (March-August 1996)* (Appendix E).

2.2 STRUCTURE OF REPORT

The studies conducted in this project constituted, in part, experimentation for two Master of Applied Science Theses and one Honours in Applied Science Thesis, at Central Queensland University. The theses incorporated the major aspects of this project:

(i) Isolation and purification of toxins from *Pimelea spp* plants;

(ii) Formulation of *Pimelea* toxins vaccines;

(iii) Immunisation of laboratory animals and cattle with prototype *Pimelea* toxins vaccines;

(iv) Determination of immune responses in laboratory animals and cattle to vaccination with prototype vaccines;

(v) Characterisation of anti-*Pimelea* toxins antibodies, *in vitro*;

(vi) Field evaluation of prototype *Pimelea* toxins vaccines;

(vii) Structure-function studies of *Pimelea* toxins and alternate strategies to prevent and/or treat Pimelea poisoning of cattle; and

(viii) Development of a bioassay for *Pimelea spp* plant toxins.

Because the theses embodied the research and technology transfer componets of the project, the body of this report comprises the theses as a series of Appendices:

Appendix A	***Pimelea Poisoning of Cattle***	Covers ecological, epidemiological and management aspects of *Pimelea* poisoning; toxins isolation and conjugation to immunogenic carrier proteins; vaccination of laboratory animals and cattle; purification of anti-toxins antibodies and *in vitro* studies with antibodies; conclusions.
Appendix B	***Methods for Reducing Pimelea poisoning of cattle***	Covers general aspects of *Pimelea* poisoning of cattle; isolation and derivatisation of toxins; synthesis of novel toxins-carrier protein conjugates, vaccination and evaluation of anti-toxins antibody responses; in vitro characterisation of anti-toxins antibodies and field evaluation of prototype vaccines; structure-activity studies; conclusions.

Appendix C	***Development of Protein Kinase C Assay Methodologies for the Quantification of Daphnane Toxins in Pimelea trichostachya***	Covers general background; binding of toxins to protein kinase C receptors; development of a competitive protein kinase C radioligand binding assay for *Pimelea spp* plant toxins; application of radioreceptor assay to measure seasonal changes in toxins content in *Pimelea spp* plants; conclusions.

3.0 MAJOR OUTCOMES

3.1 Efficient procedures were developed for extraction, isolation and purification of toxins from *Pimelea spp* plants.

3.2 Conjugation methods were developed for achieving high rates of incorporation (binding) of purified toxins to immunogenic carrier proteins.

3.3 Toxins-protein conjugates were formulated into vaccines which induced immune responses and production of anti-toxins antibodies in rabbits and cattle.

3.4 Anti-toxins antibodies were purified and, in laboratory studies, were shown to neutralise the normal constricting effects of toxins on isolated sections of pulmonary venules maintained in organ baths; these studies demonstrated the specificity and relatively high affinity of anti-toxins antibodies.

3.5 Cattle vaccinated in the field using prototype vaccines had significant circulating anti-toxins antibodies.

3.6 Vaccinated cattle with anti-*Pimelea* toxins antibodies in circulation remained susceptible to *Pimelea* poisoning.

3.7 Cattle vaccinated with prototype vaccines did not show any apparent enhancement of recovery from *Pimelea* poisoning.

3.8 In Sub-project CS.177(2), treatment of cattle with Gastric Stimulant Powder (Parnell Laboratories (Aust) Pty Ltd, Alexandria, New South Wales) facilitated the recovery of cattle suffering Pimelea poisoning; Gastric Stimulant Powder has subsequently be used successfully by producers (***Section 4.***0)

Hence, the immune responses induced by the prototype vaccines tested in this project, did not reduce the susceptibility of cattle to *Pimelea* poisoning and also did not facilitate the recovery of cattle from *Pimelea* poisoning.

A significant finding, however, was that in cattle suffering advanced *Pimelea* poisoning with rumen stasis, treatment with Gastric Stimulant Powder encouraged re-alimentation and facilitated recovery.

4.0 SUCCESSFUL APPLICATION OF GASTRIC STIMULANT POWDER IN CATTLE SUFFERING *PIMELEA* POISONING

Wilpoorinna Station	Marree, S.A.	Gordon and Lyn Litchfield
Boondara Station	Roma, QLD	Colin Faulkener
Wythburn Station	Taroom, QLD	Lorraine McGugan
Bundong Station	Coolabah, N.S.W.	Noel and Lynette Dunn

5.0 CONCLUSIONS

Toxins occurring in *Pimelea spp* plants can be isolated from plant material and formulated into vaccines that give rise to anti-toxins antibodies that are present in the circulation of vaccinated cattle. However, vaccinated cattle with circulating anti-toxins antibodies do not have any apparent reduced susceptibility to *Pimelea* poisoning.

The rationale of vaccination to "protect" cattle against *Pimelea* poisoning, assumes that the major effects of *Pimelea* poisoning require uptake of toxins into the general circulation and transport of toxins in blood to target tissues such as the lungs. Hence, it was considered that anti-toxins antibodies present in the blood of vaccinated cattle would bind toxins, and thereby prevent toxins from binding to target tissues and inducing normal pathophysiological responses of *Pimelea* poisoning. The failure of vaccination to reduce susceptibility to *Pimelea* poisoning in the present project could be interpreted to suggest:

(i) The levels of anti-toxins antibodies induced in vaccinated cattle were not sufficient to neutralise toxins in circulation;

(ii) The affinity of anti-toxins antibodies was less than the affinity of toxin receptors in target tissues, and hence toxins were preferentially bound by target tissues (e.g. pulmonary venules) and still induced pathophysiological responses;

(iii) The effects of toxins at target organs does not require prior uptake into the blood, but rather toxins can act directly at certain tissues; for example, toxins may act on pulmonary venules after inhalation rather than ingestion of toxins; similarly, the effects of toxins on the gastrointestinal tract to induce diarrhoea may involve a direct action on intestinal mucosa, rather than systemic.

Therefore, based on the findings in the present project, vaccination against *Pimelea* toxins does not provide a strategy for reducing susceptibility to *Pimelea* poisoning.

6.0 RECOMMENDATIONS

6.1 Practical

6.1.1 Pasture Management

A general industry observation, although not exclusive, is that the likelihood of occurrence of *Pimelea* poisoning is increased if pasture availability is limited. It is not known if this is because, in the absence of sufficient pasture, cattle graze *Pimelea spp* plants, or whether cattle inadvertently come into contact more often with *Pimelea spp* plants (standing plants or dried, powdery plant material on the ground) when grazing closer to the ground. Irrespective, an emerging recommendation is that pasture management could be an important feature for reducing susceptibility to outbreaks of *Pimelea* poisoning. Unfortunately, pasture management can be relatively difficult in the rangelands and other extensive production systems where *Pimelea* poisoning occurs. Stocking rates can, however, be controlled even in these environments. In a preferred situation, therefore, it would be desirable to have stocking rates that allowed retention of a necessary pasture condition throughout the year. It is possible that a reduction in cattle numbers, with an associated short-term reduction in revenue, could be justified bylonger-term protection against significant outbreaks of *Pimelea* poisoning, and cattle loses.

6.1.2 "Hospital Paddock"

As an alternative to whole-property pasture management for *Pimelea* poisoning, an area of pasture could be set aside to serve as a "hospital paddock" for cattle that are suffering *Pimelea* poisoning. This may be more practical in smaller holdings (e.g. Maranoa Region) than in rangelands and other extensive environments (e.g. Marree District).

6.1.3 Gastric Stimulant Powder

The use of Gastric Stimulant Powder is recommended for cattle with *Pimelea* poisoning that are emaciated and may have rumen stasis. In several situations, Gastric Stimulant Powder induced re-alimentation and facilitated the recovery of cattle suffering severe *Pimelea* poisoning (*Section 4.0*). The Gastric Stimulant Powder can be administered as a drench or incorporated into a molasses mix and fed to cattle.

6.2 Research

6.2.1 Modelling Stocking Rates and Pasture Management

Research should be considered in developing predictive models for evaluating short-term and longer-term economic implications of reducing stocking rates to protect against outbreaks of *Pimelea* poisoning. Issues of environmental sustainability would be integrated into such predictive models.

6.2.2 Pharmacological Treatment Strategies for Cattle with Pimelea Poisoning

6.2.2.1 Antidotes

The strategy with use of an antidote to treat for *Pimelea* poisoning, is to bind toxins in circulation, render toxins water soluble, and accellerate the rate of clearance of toxins in urine. In Sub-project CS.177(2) we evaluated whether an antidote developed for Annual Ryegrass Toxicity (ARGT-antidote) facilitated recovery of cattle with *Pimelea* poisoning. There are some chemical similarities between toxins that cause ARGT and *Pimelea* toxins. The ARGT-antidote did not have any apparent beneficial effect. However, this should be considered as a very preliminary experiment and the potential of an antidote for *Pimelea* poisoning has sufficient merit to justify further research.

6.2.2.2 Receptor Blockers

Pimelea toxins induce constriction of pulmonary venules by binding to protein kinase C (PKC) receptors. It might be feasible to treat cattle suffering *Pimelea* poisoning with compounds that displace toxins from PKC receptors, but do not induce pathophysiological responses. This strategy assumes that the action of Pimelea toxins on pulmonary venules is a major component of *Pimelea* poisoning; however, similar receptor displacement strategies could presumable be considered for other target tissues of *Pimelea* toxins.

6.2.2.3 Rumen Degradation of Pimelea Toxins

A longer-term strategy to protect against *Pimelea* poisoning could be to identify organisms that degrade *Pimelea* toxins and also survive in the rumen of cattle. This would be an optimistic goal and it is more likely that current rumen micro-organisms might be altered using molecular biology techniques to render them capable of degrading *Pimelea* toxins in the rumen. A precidence for genetic altering of rumen micro-organisms to serve specific degradation functions has been established. This strategy asumes that the effects of *Pimelea* toxins on pulmonary venules requires uptake into the blood and delivery of toxins to the lungs via systemic circulation; however, the relative contribution of ingested versus inhaled toxins in pulmonary venule constriction has yet to be established. We have demonstrated, however, that cattle drenched with *Pimelea* plant material develop typical oedema of the lower jaw and brisket, presumable secondary to pulmonary venule constriction.

7.0 PUBLICATIONS

7.1 Invited Book Chapter

1. Pegg GG, Oberoi G, Aspden WJ and D'Occhio MJ (1994) *Pimelea* poisoning of cattle. In: Vaccines in Agriculture: Immunological Applications to Animal Health and Production. Eds. Wood PR, Willadsen P, Vercoe JE, Hoskinson RM, Demeyer D, pp 155-159. CSIRO Publications, Victoria, Australia.

7.2 International Scientific Journal Paper

1. Pitcher DJ, Pegg GG and D'Occhio MJ (1998) Development and application of a protein kinase C radioligand binding assay for the quantification of daphnane orthoester toxins in *Pimelea spp* plants. Toxicon (in press).

7.3 Scientific Meeting Abstracts

1. Oberoi G, Hoey A, Aspden W, Pegg G and D'Occhio M (1993) *Nux vomica* attenuates constriction of bovine pulmonary venules induced by *Pimelea* toxins. In: Proceedings of the 27th Annual Meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists, Clinical and Experimental Pharmacology and Physiology Suppl 1, p 53.

2. Pegg G, Oberoi G, Aspden W, Hoey A and D'Occhio M (1993) Development of antibodies to toxins found in *Pimelea* plant species. In: Proceedings of the 27th Annual Meeting of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists, Clinical and Experimental Pharmacology and Physiology Suppl 1, p 56.

3. Lambert BA, Hoey AJ, Aspden WJ, Pegg GG and D'Occhio MJ (1995) Antibodies against *Pimelea* plant toxins have neutralising actions *in vitro*. Proceedings of the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists 2, 167.

4. Pegg GG, Lambert B, Nayyar G, Pitcher D, Hoey A and D'Occhio MJ (1995) Activation of protein kinase C by daphnane orthoesters. Proceedings of the 14th Annual Conference of the Molecular Graphics and Modelling Society, 27 Aug-1 Sep, Cairns, Australia, p C21.

5. Pitcher DJ, D'Occhio MJ, Pegg GG (1995) A protein kinase C competitive binding assay for the quantification of daphnane toxins in *Pimelea* species. Proceedings of the Australasian Society of Clinical and Experimental Pharmacology and Toxicology 2: 116.

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Peter Litchfield	*Mundowdna* Station
Shane, Kevin and Debbie Oldfield	*Clayton* Station
Paul Broad	*Etadunna* Station
Wayne and Larry Williams	*Carrieton* Station

Vaccination trials were also conducted at *Borah* Station, *Currawarra* Station and *Lilypool* Station (*Appendix B*).

9.0 ACKNOWLEDGMENTS

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We are grateful to Mr Stuart Nunn and Mr Robert Hunter, Kidman Holdings Pty Ltd (Managing Partner of S. Kidman and Company) for provision and management of some of the cattle used in this project; and Dr Fenella Cochrane (Parnell Laboratories (Aust) Pty Ltd, Alexandria, NSW) for generous supplies of Gastric Stimulant Powder.

The Maranoa Grazier's Association (MGA) merits special mention for their support of research on *Pimelea* poisoning. The MGA directly contributed $10,000 to support postgraduate students at Central Queensland University. Individuals of MGA who have made major contributions include Mr Alan Hirsch, Mr Don Compononi and Mr Greg Kruger. In his role as Chairman of the MGA *Pimelea* Sub-committee, Mr Greg Kruger facilitated trials in both the Maranoa Region of Queensland and Marree District of South Australia. He also contributed to the establishment of a *Pimelea* Working Group in the Marree District. Mr Daryl and Mrs Sharon Bell, *Dulkaninna* Station, Marree, were also major driving forces in raising awareness of *Pimelea* poisoning and in the establishment of trials in the Marree District. Mr Gordon and Mrs Lyn Litchfield were particularly generous in allowing a series of trials to be conducted at *Wilpoorinna* Station. Cattle, resources and time were also generously donated by Mr Shane and Mrs Debbie Oldfield (*Clayton* Station), Mr Peter Litchfield (*Mundowdna* Station), Mr Wayne and Mr Larry Williams (*Carrieton* Station) and Mr Paul Broad (*Etadunna* Station).

APPENDIX A

PHARMACOLOGICAL AND IMMUNOLOGICAL STUDIES AIMED AT PREVENTION OF PIMELEA POISONING OF CATTLE

1.1 ECOLOGICAL, EPIDEMIOLOGICAL AND MANAGERIAL ASPECTS OF *Pimelea* POISONING

Pimelea poisoning is an often fatal sickness in cattle caused either by the ingestion of fresh or dried plant material and/or inhalation of the plant dust of poisonous *Pimelea* species (Cantello 1969; McClure and Farrow 1971, Roberts and Healy 1971, Clark 1971). It is a seasonal problem, usually occurring in the spring-early summer but the lasting toxic nature of the weed and plant debris presents a risk throughout the year. *Pimelea* poisoning is estimated to cost the Queensland beef industry between $7.25 million and $10 million (1990 values) per year in lost production (McKenzie,1985; Pressland and Dadswell, 1992). *Pimelea* poisoning is also known as St. George disease, big head, Marree disease, *Pimelea* or flax weed poisoning. It was first recorded in Australia in 1938 at St. George, Queensland, (Maunder, 1947) and the first outbreak occurred in South Australia in 1951. It was common then for cattle to be brought from the Northern Territory, delivered to Marree by rail and then walked to their destinations. The disease tended to affect cattle shortly after their departure from Marree, with drovers reporting up to half of the mob dying (Trengover, 1982; Dodson, 1965).

In Queensland, *Pimelea* poisoning is primarily caused by three herbaceous plants namely, *Pimelea simplex (P. simplex* comprises two subspecies - *simplex* and *continua) Pimelea trichostachya* and *Pimelea elongata.* These plants are native to Australia and are small annual herbs which usually grow less than 50 cm high at maturity. *P. simplex* is commonly known as the desert rice flower and *P. trichostachya* as flax weed or poverty weed (Anon, 1991. Figure. 1.1). These plants predominantly grow in the Longreach, Blackall, Charleville, Cunnamulla, Quilipie, Thargomindah, Mitchell, Roma and St George areas, in an area below the Tropic of Capricorn and in inland areas of Queensland. In the Roma district of South Western Queensland, *P. trichostachya* commonly grows to 0.5 m in height and in some pastures contributes more than 30 % of the biomass (Pressland and Dadswell, 1992).

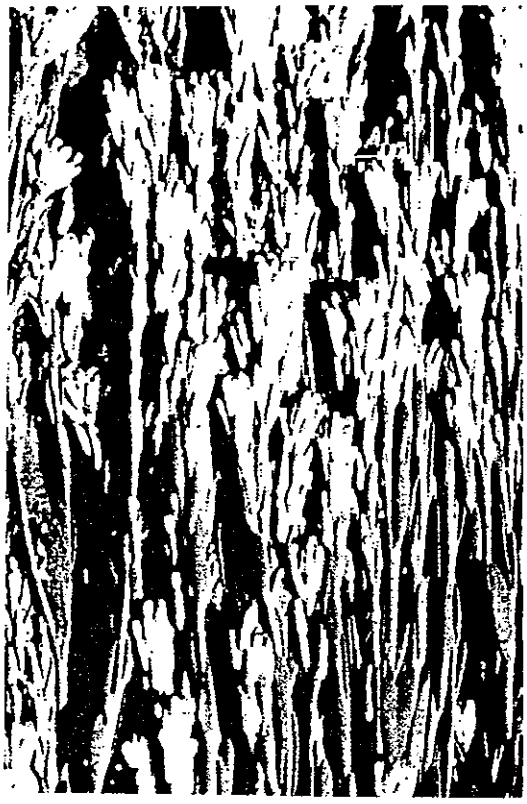
Germination

Pimelea generally thrives on light red, sandy, less fertile soils where pasture grasses are often severely over grazed, thus offering less competition. *Pimelea* species are hard-seeded. Evidence indicates that the seeds can remain viable in the soil for years. Thus it has high potential for survival even under very adverse conditions. Laboratory results from the Queenland Department of Primary Industries (QDPI) indicated that germination of *Pimelea* seeds occurred with night temperatures of 15° C to 20° C, and day temperatures of 20° C to 25° C. These optimum germination conditions coincide with the late summer/autumn period. Good rain in late summer/autumn stimulates the germination process. Hence establishment and survival of the plant is more likely in the south than the north, because of higher temperatures and lower probability of rain at the optimum time for germination in the north compared to southern districts (Pressland and Dadswell, 1992; Graham and Schefe, 1991).

Figure 1.1

Pimelea species

Pimelea elongata (whole plant)



Pimelea elongata (seed heads)

Pimelea trichostachya (whole plant)

Pimelea trichostachya (seed heads)

Outbreaks of *Pimelea* poisoning generally occur towards the end of the year (August to December). Anecdotal evidence (Cunningham *et al.*, 1981) suggests that poisoning tends to be more common in years where a previous dry summer has left areas in the pasture bare of perennial grass due to overgrazing. A wet autumn or winter then stimulates germination of the *Pimelea* seed reserve. Thus, overgrazing and winter rain following a period of drought are conducive to outbreaks of *Pimelea* poisoning. Under these conditions, the density of *Pimelea* species in the pasture increases, leading to a higher consumption of *Pimelea* by cattle and resultant poisoning (Pressland and Dadswell, 1992).

Susceptibility

All cattle are susceptible to *Pimelea* poisoning, regardless of genotype, sex and age. It seems that 18 month to 2 year old beasts are most commonly affected. The condition of the animal is not related to susceptibility, fat healthy cattle being equally likely to succumb to the disease as those in poor condition. Poisoning occurs in Brahmans as well as British breeds and crossbreds, and in home bred as well as introduced cattle; poisoning occurs less frequently in bullocks, steers and calves than in bulls, cows, heifers and weaners (Pressland and Dadswell, 1992).

Due to the unpalatable nature of *Pimelea* especially when green, native station cattle generally avoid grazing it. This explains the observation that stock introduced into *Pimelea* infested areas are more likely to develop signs of the disease than cattle native to these areas. *Pimelea* poisoning generally occurs when feed has become scarce and cattle are foraging less palatable feed; or when rainfall has produced a short stand of green feed, encouraging close grazing of associated green or dead flax weed (Trengove, 1982).

Confinement of affected animals with abundant good feed, such as wheaten or lucerne hay, supplementary vitamins and minerals and water usually overcomes the problem over a 3-4 week period, provided the condition is not too advanced. Farmers report that it is important to avoid stressing affected animals because this may result in sudden death. For example, badly affected animals need to be transported to the home paddock, as even the stress of walking can kill the animals.

Management

Producers find management of *Pimelea* difficult. Property sizes in the region often exceed 10,000 ha, making physical and/or chemical destruction of the plants prohibitive from an economic perspective. Some property owners have experimented with burning the plants, but available evidence seems to suggest that this practice encourages germination of seeds in the subsequent growing season. The seeds of *Pimelea* apparently remain viable in the soil for more than two years and these seeds are also light and fluffy and are likely to be easily carried by wind and dust. Farmers who attempt cultivation of the soil in badly affected areas find that this practice encourages growth of *Pimelea* .

The impact of *Pimelea* poisoning can be minimised by reserving an area of pasture with little or no *Pimelea* so that stock can be shifted from infested areas as soon as signs of the syndrome appears. *Pimelea* growth can be controlled by ensuring pasture seed (for example, buffel grass) is free of seeds of *Pimelea* and also by avoiding over-grazing of pasture land by cattle as *Pimelea* thrives when competition with other plants species is reduced. Grazing *Pimelea* infested areas when the plant is green is advocated by some producers since they believe the plant is less toxic at this stage. Since sheep are less sensitive to *Pimelea* toxicity than cattle, producers with mixed

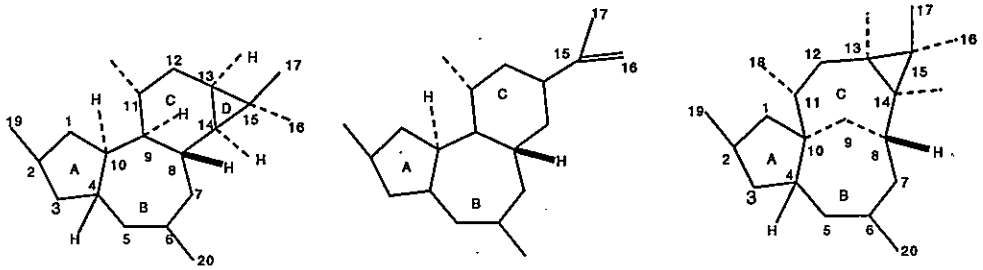
cattle/sheep operations tend to run their sheep in the paddocks most affected by *Pimelea* infestation (Pressland and Dadswell, 1992).

1.2 *Pimelea* TOXINS

Representatives of the genus *Pimelea* (family Thymelaeaceae) occur in South Africa, New Zealand, Timor and New Guinea. Thymelaeaceae are a medium sized plant family of 650 species distributed among some 50 genera. Representatives of the Thymaleaceae family occur in South Africa, Australia, New Zealand, the Mediterranean region, South America and the Steppes of Asia. Diterpene toxins have been isolated from several genera of Thymaleaceae (Borris *et al.*, 1988). Similar diterpene toxins have also been isolated from several genera of the closely related Euphorbiaceae family. Systemic toxicity symptoms resulting from the ingestion of plant materials is well established for humans as well as for several animal species (sheep, cattle, horse, goats etc) which is essentially constant throughout the Thymelaeaceae (Borris *et al.*, 1988).

The diterpene toxins of the Thymaleaceae are collectively known as tiglianes, daphnanes and ingenanes (Figure 1.2, adapted from Evan and Soper, 1978). These have been shown to occur in several genera of the families Euphorbiaceae and Thymaleaceae. These compounds are structurally related hydrocarbons consisting of tri- or tetracyclic rings which occur in higher plants as their O-acyl esters or more rarely as ortho-ester forms. Phytochemical evidence available indicates that these diterpene esters are responsible for toxic reactions and skin-irritant properties observed (Evans and Soper, 1978).

Figure 1.2
Structure of different Diterpenes found in Thymaleaceae species.



Interest in these compounds has centred upon two toxicological actions which are exhibited to a greater or lesser degree in test animals:

1. Intense inflammation produced on application to the skin (Kinghorn *et al.*, 1975).
2. Tumour-promoting activity arising from continued application to mice following a sub-threshold dose of a carcinogen (Berenblum *et al.*, 1947).

Two of the more conspicuous changes that occur in skin treated with diterpene esters, particularly those possessing tumour-promoting activity, are cell proliferation and hyperplasia. Some investigators have considered hyperplasia and proliferation to be critical events in tumour promotion (Frei *et al.*, 1968) but others have rejected this view (Raick *et al.*, 1972). Diterpene esters are able to modify the differentiation of epidermal cells, returning them to a less differentiated state. These ultrastructural changes induced by diterpene esters are, however, not permanent. For example, Raick and coworkers (1972), described histological changes that occur after a single application of 0.016 µg

of a phorbol ester such as TPA to normal adult mouse epidermis skin. Within 48 hours, such TPA-stimulated cells acquire a secretory activity not evident in normal adult mouse epidermis. These TPA-stimulated dark epidermal cells are smaller, contain large mitochondria rich in cristae, and their nuclear and cytoplasmic matrices are highly electrodense compared to normal untreated mouse epidermal cells. It has been suggested that these less differentiated dark cells are precursors of neoplastic cells. These dark cells revert almost completely after four weeks (Evans and Soper, 1978).

Diterpene esters are amphipathic in nature. The tumour promoting (Baird *et al.*, 1971) and irritant effects are dependent upon the presence of a lipophilic side chain. There is much current interest in the chemical constituents of the Thymelaeaceae as either potential therapeutic agents or new tools for cancer research. Many members of the family have been used in traditional medicines and in hunting. Six of the genera belonging to Thymelaeaceae (eg. Daphne) contain about 40 species to have found extensive (China and India to Europe) application in the treatment of cancer. A substantial number of species are used in other primitive medical treatments (such as epilepsy, malaria, snake-bite, and certain viral infections) (Pettit *et al.*, 1983). Of clinical significance is the observation that certain of these esters have an anti-leukemic action and further structure activity studies are required to ascertain the structural features necessary for tumour promotion on one hand and anti-leukemic action on the other.

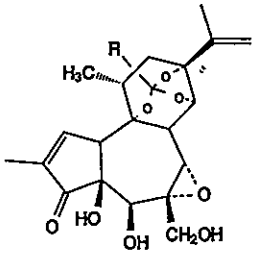
Toxins isolated from Pimelea species causing Pimelea poisoning

Simplexin is a diterpenoid orthoester which has been isolated from several *Pimelea* species and is known to be the primary cause of *Pimelea* poisoning in cattle in conjunction with other diterpenoid toxins present in *Pimelea* species. The first of the daphnane diterpenoid toxins isolated from *Pimelea simplex* (simplexin) was reported by Roberts *et al* (1975). Simplexin is a highly toxic compound, the LD_{50} for mice being 1 mg/kg. Simplexin is highly irritant in the mouse ear test and is moderately active as a co-carcinogen. In one instance intravenous injection of 9 mg simplexin into a calf (100 kg) caused death within 0.5 h. (Freeman *et al.*, 1979). Simplexin was identified from its similar spectral characteristics to the piscicide huratoxin (Sakata *et al.*, 1971).

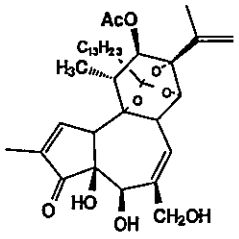
Robert *et al* (1975) reported that intravenous administration of simplexin produced a three fold increase in pulmonary arterial pressure within 100 secs at a dose of 400 µg (4 µg/kg) and oral dosing with simplexin produced the range of symptoms characteristic of *Pimelea* poisoning. Simplexin has been isolated from diverse *Pimelea* species such as *P. prostrata* (Pettit *et al.*, 1983); *P. trichostachya* and *P. simplex* (Freeman *et al.*, 1979). The latter two species are associated with *Pimelea* poisoning of cattle in Queensland.

A family of daphnane diterpenes related to simplexin (Figure 1.3) was isolated from *P. simplex* and *P. trichostachya* and reported by Freeman *et al*., (1979). Interestingly they reported 12b -acetoxyhuratoxin, tigliane derivatives related to mancinellin and loliolide in extracts of *P. simplex* and *P. trichostachya.* Further daphnane orthoesters were isolated from *Pimelea species* by Hafez *et al.* (1983) and Tyler and Howden (1985).

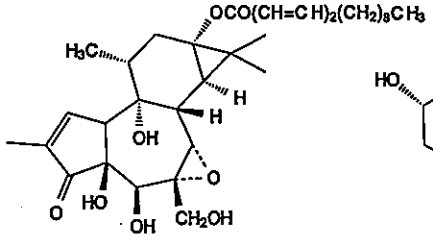
Figure 1.3
Various terpenoid compounds isolated from *P. trichostachya.*



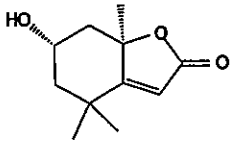
SIMPLEXIN (R =C_9H_{19})
HURATOXIN (R= $C_{13}H_{23}$)



12-ACETOXY
HURATOXIN



TIGLIANE ESTER (Mancinellin analogue)



LOLIOLIDE

An interesting observation is that crude extracts of *P. simplex* (Horward and Howden, 1975) and *P. prostata* (Cashmore *et al.,* 1976) exhibited antileukaemic activity . These authors also report that extracts of *P. linifolia* and *P. ligustrina* possess antineoplastic activity.

1.3 SYMPTOMS OF *Pimelea* POISONING IN CATTLE

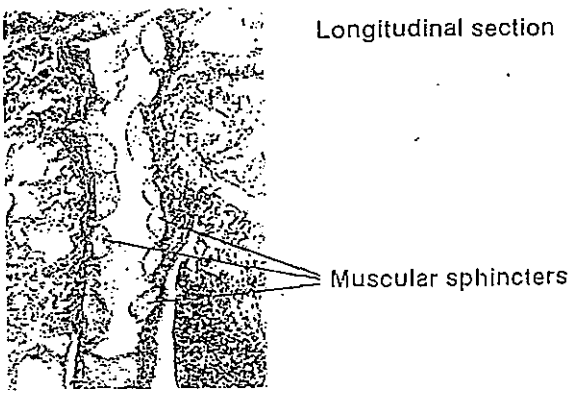
Correlation of the disease state with the occurrence of *Pimelea* species in the pasture was recognised in the late 1960's. Clark (1973) studied the pathogenesis of the condition through intoxication of cattle with dried, finely milled *Pimelea* plant material (introduced into the rumen by stomach tube). Animals receiving more than 50 mg kg^{-1}body weight day^{-1} of the dried material developed jugular distension, diarrhoea and mucous discharge from the eyes and nostrils within 3 days (Figure 1.4). Continuation of this dosage led to oedema of the neck and brisket and death of the animals within one week. Intoxication of animals with lower doses (25 mg kg^{-1} body weight day^{-1} and 15 mg kg^{-1}body weight day^{-1}) gave rise to recognisable signs of the disease state within 2 weeks and 28 weeks respectively.

The clinical and gross post-mortem findings in field cases vary considerably (Maunder 1947, Seawright and Francis 1971). Symptoms of *Pimelea* poisoning usually are diarrhoea, wasting, dependent oedema, reduced exercise tolerance, anaemia, hydrothorax, cardiac dilation, expanded hepatic sinusoids and portal venules (peliosis hepatis) and oedema of lymph nodes, depending upon the extent of the poisoning in each individual case.

One of the early symptoms (Clark, 1971; Roberts and Healy, 1971; McClure and Farrow, 1971) is profuse diarrhoea generally accompanied with loss of condition, roughening of the coat and jugular distension and pulsation. The animal becomes lethargic and has a peculiar, dejected walk, giving the impression of abdominal or thoracic pain. As the disease progresses, the oedema extends to the base of the neck, brisket and forelegs. Sometimes jugular distension and pulsation may occur before diarrhoea is evident. During oral dosage experiments, diarrhoea generally commences after 4-6 days. Diarrhoea persists with undigested blood appearing intermittently in the faeces. The severity and occurrence of diarrhoea and oedema are variable. Severe diarrhoea has been a feature of many of the outbreaks, although not every affected animal in the mob may necessarily scour. Generally, most of the affected cattle display varying degrees of a combination of diarrhoea and oedema rather than either extreme condition. The natural disease is caused by ingestion or inhalation of the poisonous plant material, or a combination of both. The extent and occurrence of the diarrhoea is dependent upon whether inhalation or ingestion predominates. Clark (1973) reported inhalation experiments where animals did not ingest the toxic plant material and these animals showed oedema without diarrhoea. The consistent trend is that diarrhoea is associated with ingestion of the toxic plant material. It is also possible to produce oedema with minimal diarrhoea through intravenous injection of the plant extract (Clark, 1973).

Figure 1.4
Cattle showing clinical signs of *Pimelea* poisoning: oedematous swelling of the head, jaws and brisket. Also note the prominent jugular vein.

Figure 1.5
Longitudinal section of a normal bovine pulmonary venule (x 200).



Cattle with *Pimelea* poisoning develop phlebectatic peliosis hepatitis (Seawright, 1984; Seawright and Francis, 1971). The term "peliosis" means blue-black and gross post-mortem findings in the advanced cases of the disease showed an enlarged swollen, bluish purple liver with liver lesions evident. This swelling of the liver is essentially due to massive dilation of the intrahepatic portal capillary bed. Distension was observed in most of the smallest branches of the portal vein, and the sinusoids into which they flowed were also dilated. Dilation of the portal venules caused formation of huge cavities lined by hepatocytes and Kuffer cells. Such distension of the sinusoids may lead to a breakdown of parenchymal structure and extreme atrophy of the hepatocytes. The extent of these pathological symptoms were observed to vary from case to case.

As the disease progresses, the oedema extends to the base of the neck, brisket and forelegs. The dilated right heart, distended systemic veins and dependent oedema then leads to the right sided heart failure (Rogers and Roberts, 1976; Clark,1973; Kelly,1975a). Heart failure is the primary cause of death in poisoned animals due to increased pulmonary vascular resistance.

In the progressive stage of the disease Kelly, (1975b) noted a fall in haemoglobin concentration, red cell count, packed cell volume and plasma protein concentration resulting from haemodilution. The effect of *Pimelea* toxins on total plasma protein was more varied and Kelly concluded that hypoproteinaemia was not responsible for the subcutaneous oedema characteristic of *Pimelea* poisoning as suggested earlier by McClure and Farrow (1971).

Increased pulmonary resistance

Alexander and Jensen (1963) investigated the structure of the bovine pulmonary vasculature in normal cattle using serial histologic sections and corrosion cast preparations. They reported that the bovine pulmonary vasculature is unusual in that both the arteries and veins have a heavy muscular coat even down to a vascular diameter of 20μm . A distinct variation in venule and arteriole muscular media was observed in vessels of 300-400μm or less in diameter. The muscular media of veins less than approximately 300-400μm was characterised by abrupt disruptions in continuity (Figure 1.5). The thick interrupted muscular media were classified as sphincter-like structures. These were observed in veins down to a size of approximate 20 μm. In arteries these abrupt sphincter-like discontinuities in the media were not observed. Cross sections of both arteries and arterioles revealed uniform wall thickness with no sphincter-like structures as observed in venules. Castigili (1958) referred to the thick muscular veins in the lungs of cattle as sphincter veins. Best and Heath (1961) also reported similar disruptions in the histological study of longitudinal sections of bovine pulmonary venules. These sections had a beaded appearance due to fibromuscular masses protruding into the lumen of the vessel. In transverse sections, the lumens of the smaller pulmonary veins appeared almost occluded by these muscular masses.

The distinct muscular sphincters present along the length of the pulmonary venules of cattle appear to be absent in sheep and horses (Alexander and Jensen, 1963). Sheep and horses can graze areas where *P. trichostachya* grows without developing dependent oedema. Therefore constriction of these sphincters in the bovine not only offers a pathogenesis for the condition but also an anatomical reason why only cattle are affected. Clark (1973) reported that intravenous injection of an ethanolic extract of the plant (at doses above a dried-plant equivalent of 130 mg kg^{-1}body weight threshold) caused rapid cardiovascular effects. Within 10 seconds of administration of the dose, systemic arterial pressure halved while right ventricular pressure doubled. These results

were suggestive of immediate constriction of the pulmonary venous system. Autopsy examination of severely poisoned animals revealed hydrothorax and dilation of both the pulmonary artery and the right side of the heart.

Marked constriction of pulmonary venule sphincters occurs during *Pimelea* poisoning. This leads to an increase in the pressure of the pulmonary capillary bed, pulmonary arterial system and right ventricle, accompanied by variable pulmonary oedema. If the constriction continues, dilation of the right ventricle causes the right atrio-ventricular valve to close incompletely, allowing regurgitation during ventricular systole. This increases the systemic venous pressure, and is expressed clinically first as distension and pulsation of the jugular veins and eventually as dependent oedema. This correlates well with the cardiovascular changes observed in a series of autopsies on natural and experimental *Pimelea* poisoning cases. After comparison of animals killed at various stages of the disease it appears likely that hydroperciardium and any pulmonary oedema develop when the hypertension is still contained within the pulmonary circulation, and there is as yet no change in heart sound or jugular vein appearance. Then after right atrio-ventricular valve insufficiency has developed, as evidence by heart sound changes, jugular distension and pulsation, dependent oedema and hydrothorax appear as a secondary systemic venous hypertension increases (Clark, 1973).

Mason (1976) showed that *in vitro* alcohol extracts of the plant caused contraction of the bovine venule tissue in organ bath studies which was in accordance with cardiovascular results obtained by Clark (1973) described above. These observations are discussed more fully in the introduction to Chapter 4.

1.4 PROTEIN KINASE C (PKC)

The primary cause of *Pimelea* poisoning is assumed to be prolonged activation of various isoenzymes of protein kinase C (PKC) by the slowly metabolised lipophilic diterpene toxins present in *Pimelea* plants. PKC plays a very important role in various physiological processes and the enzyme is normally transiently activated in the body by receptor mediated formation of diacylglycerol (DAG).

1.4.1 Receptor mediated activation of PKC

Stimulation of a_1-adrenergic receptors induces inositol phospholipid (PI) breakdown. Phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-biphosphate (PIP2) are produced by sequential phosphorylation of the myo-inositol moiety (PI) initiated by extracellular signals such as certain hormones, neurotransmitters, antigens, growth factors and many biologically active substances. PIP2 is further hydrolysed to yield inositol-1,4,5-triphosphate and diacylglycerol (DAG) (Figure 1.6, adapted from Nishizuka, 1984). Inositol 1,4,5-triphosphate serves as mediator of Ca^{+2} mobilisation from an internal store, probably located in the endoplasmic reticulum. 1,2 - Diacylglycerol remains in the membrane and initiates the activation of PKC. Kinetic analysis indicates that a small amount of DAG dramatically increases the apparent affinity of PKC for Ca^{+2}. PKC activation and Ca^{+2} mobilisation play an important role (often synergistically) in control of various cellular functions and in cellular proliferation (Nishizuka, 1986; Berridge and Irvine, 1984).

Inositol-1,4,5 triphosphate once produced, disappears very rapidly, and a major mechanism for terminating this signal flow is thought to be removal of the 5-phosphate by the action of a specific phosphatase. Both Ca^{+2} transport adenosine triphosphatase (ATPase) and Na^{+}/Ca^{+2} exchange protein are known to be responsible for the extrusion of Ca^{+2} to maintain homeostasis (Nishizuka, 1984).

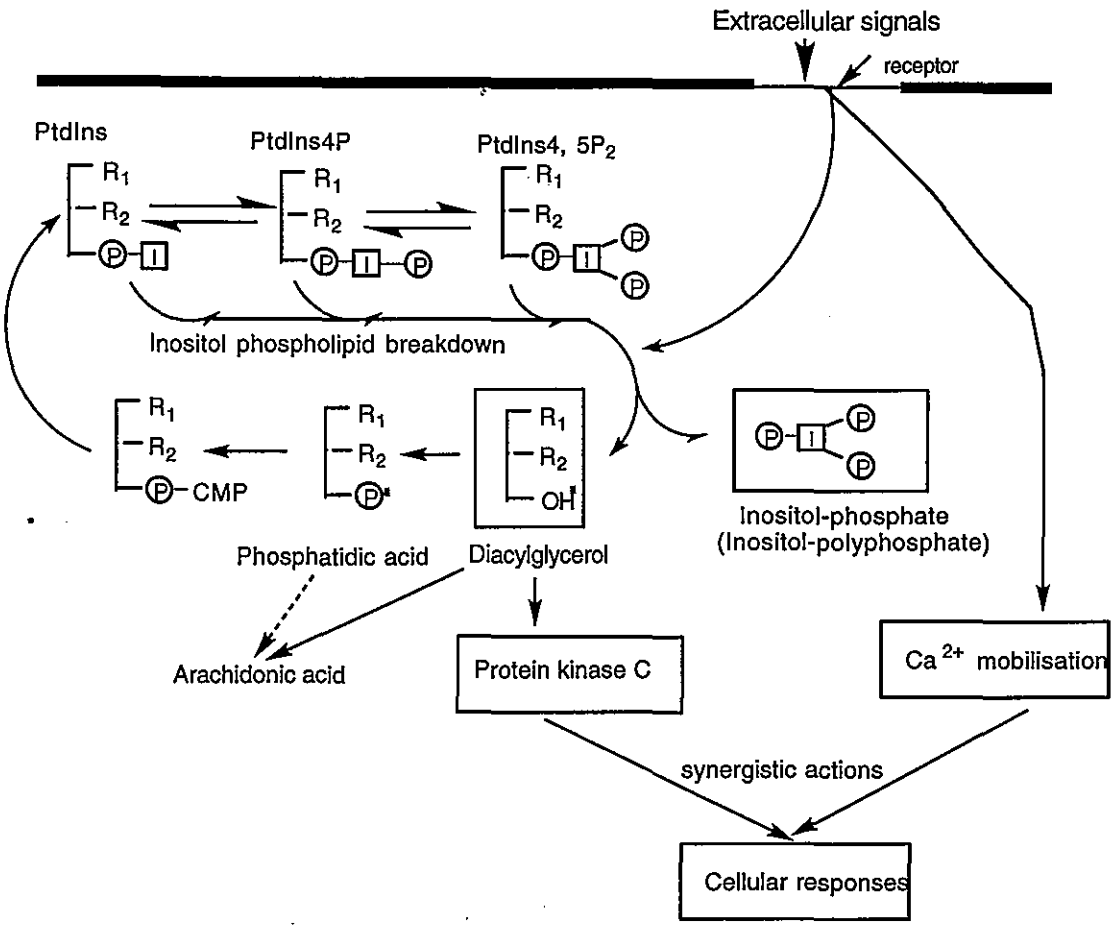
Under normal physiological conditions, free DAG is almost absent from membranes. Within a minute of formation it disappears, either for resynthesis of inositol phospholipid or becoming further degraded to acid for prostaglandin synthesis. Thus when cells are stimulated, PKC is only transiently activated by DAG. The active PKC in turn phosphorylates a range of cellular proteins involved in various cellular functions including proliferation. The protein phosphorylation catalysed by PKC may exert profound modulation of various Ca^{+2} mediated processes, such as release reactions and exocytosis, cell proliferation and differentiation, membrane conductance and transport, potentiation and desensitisation of other receptor system, smooth muscle contraction and other metabolic processes. A summary of PKC associated cellular functions is given in Table 1.1 and Table 1.2 (information adapted from Nishizuka, 1986).

In the resting condition in most tissues, PKC is largely present in a soluble inactive form but when for example 12-O-tetradecanoylphorbol-13-acetate (TPA) or phorbol-12,13-dibutyrate (PDBu) is added to intact cells, PKC is recovered in a form tightly associated with the cell membrane (Nishizuka, 1984).

1.4.2 PKC Structure

PKC was identified in 1977 as a proteolytically activated protein kinase in many tissues (Inoue *et al.*, 1977). PKC exists as a single polypeptide (approximate molecular weight 77,000 g/mol) that appears to be composed of two functionally different domains namely; a regulatory domain (amino-terminal half) and a catalytic or protein kinase domain (carboxyl-terminal half). Molecular cloning and biochemical analysis have revealed the enzyme to exist as a family of multiple sub-species having closely related structures (Coussens *et al.*, 1986). The PKC sub-species thus identified show a slightly different mode of activation, kinetic properties and substrate specificities (Ono *et al.*, 1989[a]). Most cell types contain more than one subspecies of the enzyme. The various PKC subspecies can be classified into two major groups as described below (Figure 1.7, adapted from One *et al.* , 1988.)

Figure 1.6
Inositol phospholipid turnover and signal transduction.



Abbreviations : PtdIns : phosphatidylinositol; PtIns4P : phospatidylinositol-4-phosphate; PtdIns4,5P2 : phosphatidylinositol-4,5-bisphosphate; R1 and R2 : fatty acyl groups; I : inositol; and P: phosphoryl group.

Table 1.1
Possible roles of protein kinase C in cellular responses

Tissues and cells	Responses
Endocrine systems:	
Adrenal medulla	Catecholamine secretion
Adrenal cortex	Aldosterone secretion
	Steroidogenesis
Pancreatic islets	Insulin release
Insulinoma cells	Insulin release
Pituitary cells	Pituitary hormone release
	Growth hormone release
	Luteinizing hormone
	Prolactin release
	Thyrotropin release
Parathyroid cells	Parathyroid hormone release
Thyroid C cells	Calcitonin release
Leydig cells	Steroidogenesis
Exocrine system :	
Pancrease	Amylase secretion
Parotid cells	Amylase and mucin secretion
Submandibular gland	Mucin secretion
Gastric gland	Pepsinogen secretion
	Gastric acid secretion
Alveolar cells	Surfactant secretion
Nervous systems :	
Ileal nerve endings	Acetylcholine release
Neuromuscular junction	Transmitter release
Caudate nucleus	Acetylcholine release
PC 12 cells	Dopamine release
Neurons	Dopamine release
Muscular systems :	
Vascular smooth muscle	Muscle contraction
	Muscle relaxation
Inflammation and immune systems :	
Platelets	Serotonin release
	Lysosomal enzyme release
	Arachidonate release
	Thromboxane synthesis
Neutrophils	Superoxide generation
	Lysosomal enzyme release
	Hexose transport
Basophils	Histamine release
Mast cells	Histamine release
Lymphocytes	T-lymphocyte activation
	B-lymphocyte activation
Metabolic and other cell systems :	
Adipocytes	Lipogenesis
	Glucose transport
Hepatocytes	Glycogenolysis

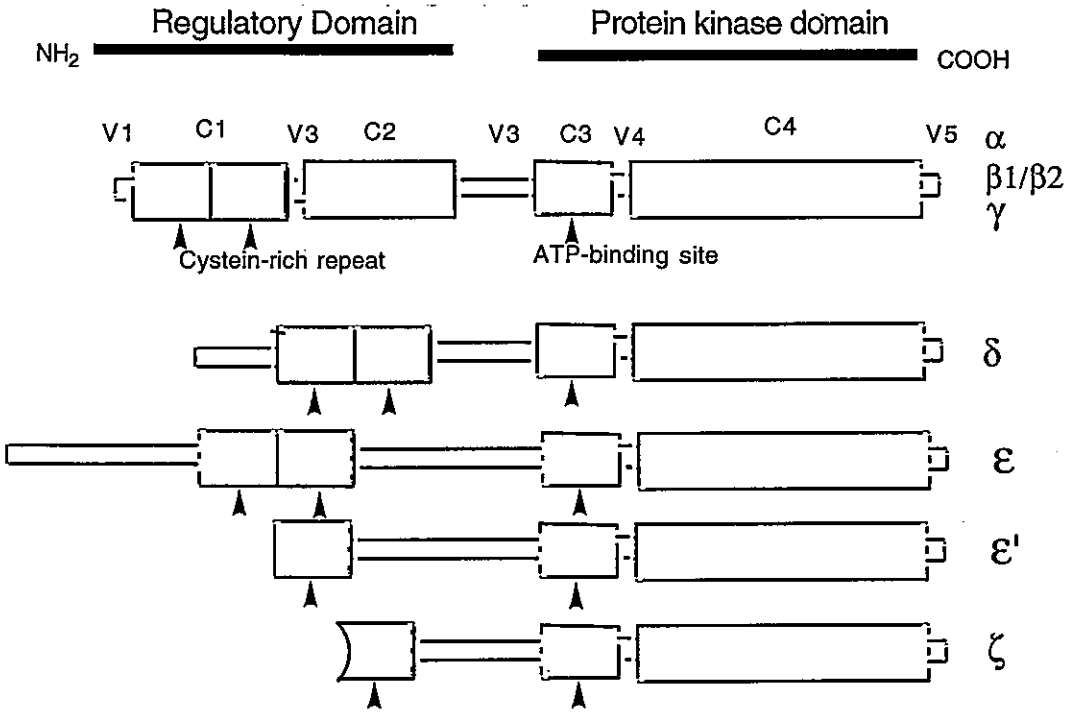
Epidermal cells	Inhibition of gap junction
Fibroblasts	Inhibition of gap junction
Hepatocytes	Inhibition of gap junction

Table 1.2
Proposed substrate proteins of protein kinase C.

Receptor proteins
Epidermal growth factor receptor
Insulin receptor
Somatomedin C receptor
Transferrin receptor
Interleukin-2 receptor
Nicotinic acetylcholine receptor
Immunoglobulin E receptor
Membrane proteins :
Ca+2 transport ATPase
Na+/K+ ATPase
Na+ channel protein
Na+/H+ exchange protein
Glucose transporter
GTP-binding protein
HLA antigen
Chromaffin granule-binding protein
Synaptic B50 (F1) protein
Contractile and cytoskeletal proteins :
Myosin light chain
Troponin T and I
Vinculin
Filamin
Caldesmon
Cardiac C-protein
Microtubule-associated proteins
Enzymes :
Glycogen phosphorylase kinase
Glycogen synthase
Phosphofructokinase
beta -Hydroxy-beta-methylglutaryl-coenzyme A reductase
Tyrosine hydroxylase
NADPH oxidase
Cytochrome P450
Guanylate cyclase
DNA methylase
Myosin light chain kinase
Initiation factor 2
Other proteins :
Fibrinogen
Retinoid binding protein
Vitamin D binding protein
Ribosomal S6 protein
GABA modulin
Stress proteins
Myelin basic protein

High mobility group proteins
Middle T antigen

Figure 1.7
Schematic representation of the structures of the PKC family.



Abbreviations : Conserved regions, C1-C4 are homologues in PKC family. V1-V5 are variable regions. Cysteine-rich and ATP-binding sites are indicated by traingles.

1. Conventional PKC

PKC -a, -bI, -bII and -z isoforms belonging to the conventional PKC subclass have been identified. These are single polypeptides with four conserved (C_1-C_4) and five variable (V_1-V_5) regions. They are characterised enzymatically by their requirement for Ca^{+2}, phospholipid and diacylglycerol for activation (Parker *et al.*, 1986; Ono *et al.* , 1988).

Regulatory domain :

The amino-terminal half of each polypeptide, containing regions C1 and C2, is the regulatory domain . C1 is a phospholipid, diacylglycerol and phorbol ester binding domain, which contains a tandem repeat of cysteine-rich sequences. The C2 region is a Ca^{+2} binding domain (Gschwendt *et al.*, 1991).

Catalytic or Kinase Domain :

The carboxyl terminal half of polypeptide with conserved C3 and C4 regions constitutes the catalytic domain that resembles many other protein kinases. The conserved C3 region also has an ATP binding sequence. While the conserved region C4 contains a similar sequence to the ATP binding site, the significance of this repeat remains

unknown (Kikkawa *et al.*, 1989). The catalytic moiety can be generated in *vitro* by limited trypsinolysis which generates a catalytically active fragment that is no longer dependent on Ca^{+2} and phospholipid.

2. Novel PKC

Isoforms of novel PKC that have been identified are PKC -d, -e, -e' -z, -h, and -l. PKC-h, and PKC-l are predominantly observed in skin and lung, supporting the concept that different members of the PKC family might play different cellular roles. Novel PKC isoforms differ structurally from conventional PKC in that the regulatory domain of these isoforms lacks the C2 conserved region (i.e the Ca^{+2} binding site). All kinase assays with the novel isoforms PKC have shown them to be independent of Ca^{+2} giving the same kinetic results in the presence or absence of Ca^{+2}. Novel PKC isoforms also contain the characteristic region C1 of the conventional PKC encoded by a, -bI, -bII and -g sequence (Nishizuka, 1986). Through a direct parallel comparison of several enzymes it rather appears that histone and other conventional PKC substrates are poor substrates for novel PKC's (Liyange *et al.*, 1992 ; Shin-ichi *et al.*, 1990).

It is important to note that the *in vitro* dependency of PKC on Ca^{+2}, phospholipid and DAG varies markedly with the phosphate acceptor protein. A typical example is protamine, the phosphorylation of which by PKC requires neither Ca^{+2}, phospholipid nor DAG. In fact, kinetic properties of PKC have repeatedly been shown to vary greatly with the substrate used (Berridge *et al.*, 1984). PKC -g sequence shows less activation by DAG, but is significantly activated by free arachidonic acid at micromolar concentration. Activation by arachidonic acid does not require Ca^{2+}, nor does it depend on phospholipid and DAG. Conventional PKC bI and bII show substantial activity without added Ca^{2+} in the presence of DAG and phospholipid, but respond much less to arachidonic acid. The PKC -a subform shows properties apparently similar to the g-subspecies and responds to high concentrations of free arachidonic acid only when Ca^{2+} is increased. It is possible that some PKC subspecies may be activated successively by a series of phospholipid metabolites, such as DAG, arachidonic acid and lipoxin A, that successively appear subsequent to stimulation of the receptor (Kikkawa *et al.*, 1989).

The PKC Regulatory domain

The regulatory domain of all PKC isoenzymes contain a so-called pseudo-substrate motif and a tandem repeat of a cysteine-rich sequence in their regulatory domain. These cysteine rich motifs resemble, the "cysteine-zinc DNA binding finger" which are found in many metallo-proteins and DNA binding proteins (Berg, 1990). No evidence is currently available to suggest that any PKC isoforms bind to DNA under physiological conditions. Through mutagenesis antibody inhibition studies, the zinc finger structures in the N-terminal part of PKC molecules have been recognised as the sites of action of phospholipid and phorbol ester activators (Ono *et al.*, 1989[b]). PKC z is unique among the PKC enzymes in that it contains a single zinc finger-like structure and has been claimed not to bind phorbol ester tumour promoters.

The pseudo-substrate motif of PKC was first recognised by House and Kemp (1987) in isoenzymes a, b and g . The pseudo-substrate motifs are polypeptide sequences which resemble a substrate phosphorylation site. It is believed that the pseudo-substrate motif interacts with the active site and renders the enzyme inactive in the absence of activating factors. In all of the PKC forms, the pseudo-substrate motif and cysteine rich regions are separated by either 21 or 22 amino acids, whereas the region between the amino terminus and the cysteine-rich regions of the various isoforms are separated by

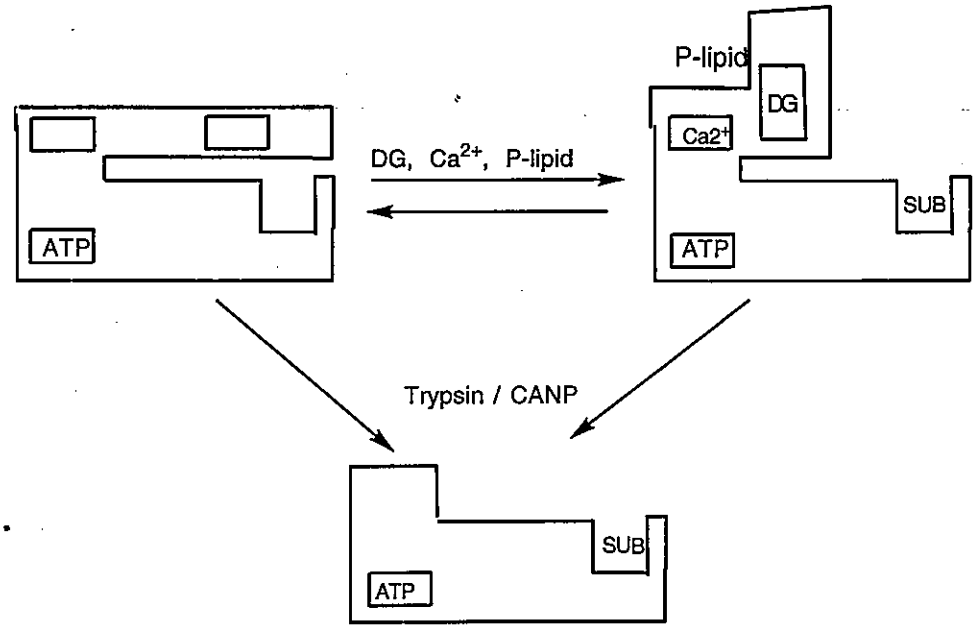
distances ranging from 43-182 amino acids. The distance from the pseudosubstrate motif to the cysteine rich regions appears to be crucial for the inactivation of the enzyme (Gschwendt *et al.*, 1991).

1.4.3 Proposed mechanism of activation of PKC by DAG and Phorbol esters

It has been suggested that the cysteine-rich regions of PKC are essential for phorbol-ester binding and that at least one of the cysteine rich finger-like sequences is needed for the tumour promoting phorbol ester to activate PKC (Ono *et al* ., 1989b). It seems likely that the phorbol esters and also DAG are hydrogen bonded to the thiol groups in these cysteine-rich regions. DAG dramatically increases the affinity of PKC for Ca^{2+} and thereby renders it fully active without a net increase in the Ca^{2+} concentration. DAG models having a 1,2,-Sn configuration with various fatty acids of different chain length, were capable of activating PKC. Analogues containing an unsaturated fatty acyl group were found to be most active. The hydrophobic domains of DAG (i.e. the acyl chains at C-1 and C-2) and of phorbol esters (acyl chains in C-12 and C-13) are believed to be required for non-specific interactions with the adjacent lipid microenvironment. In contrast, highly specific interactions involving the CH_2OH group (at C-3 of DAG and C-20 of phorbol esters) as well as additional residues in the case of phorbol esters (eg.3-keto, 4-OH) are essential for binding to the cysteine rich regulatory moiety of PKC (Castanga, 1987). Upon signal-receptor interaction, PKC tightly binds to the inner plasma membrane and becomes associated with DAG and phospholipid. The negatively charged phospholipids are the most efficient in supporting PKC activation (Catagna, 1987).

The allosteric conformational change induced by the activator, together with membrane phospholipids, appears to be sufficient for removal of the pseudosubstrate motif from the catalytic centre of all PKC isoenzymes. Binding of calcium to a region located between the cysteine-rich region and the ATP-binding site of the conventional isoenzymes possibly increases the conformational change induced by DAG (Figure 1.8, adapted from Parker *et al.*, 1986). However, the isoenzymes that lack the calcium binding region are probably activated by DAG alone (Gschwendt *et al* ., 1991; Parker *et al.*, 1986).

Figure 1.8
Model for activation of PKC.



Abbreviations : DG : diacylgycerol, P-lipid: phospholipid, CANP : calpain, SUB: substrte binding site.

1.4.4 Prolonged activation of PKC by b-phorbol compared to DAG

While the natural activator of PKC is DAG, it is especially interesting that the enzyme also serves as the receptor for phorbol esters, a class of tumour promoters. It is likely that inappropriate PKC activation induced by these compounds results in their tumour-promoting characteristics. Structural similarities exist between DAG and the 12,13-diesters of b -phorbol molecules in the region of the ester bonds. However, the conformer of 1,2-diacetyl-sn-glycerol that has a molecular geometry similar to the ester region of ß -phorbol is 3.2 kcal/mol above the lowest energy conformer and has the ester residues in a gauche conformation orientated at about -60 ° relative to one another (Leli *et al.*, 1989). This implies that substantial energy must be put into DAG for it to achieve the correct three dimensional structure for activation of PKC. This would be consistent with the much lower potency of DAG compared with phorbol esters. The half-life of phorbol esters is very long compared to DAG whereas the half life of DAG is less than one minute in platelets. Phorbol esters such as TPA are slowly metabolised and exert a sustained activation of PKC (Castanga, 1987). Autoradiographs of treated skin have demonstrated that TPA has a half-life of 23 hours in mouse skin (Witte and Hecker, 1971).

1.4.5 PKC activation and *Pimelea* poisoning

As evidenced by the data presented in Tables 1.1 and 1.2, PKC plays a very important role in controlling a plethora of cellular functions and is present in different tissues. Normally, the enzyme is transiently activated, and then immediately reverts to its inactive form. Phorbol esters and related daphnane diterpenes present in *Pimelea* toxins are likely to activate PKC for a long period of time (Witte and Hecker, 1971). This leads to abnormal activation of various cellular functions which are controlled by PKC, and precipitates as *Pimelea* poisoning symptoms in cattle. These toxins are lipophilic and metabolism of phorbol esters in the body is known to be slow. They may

be metabolised by several enzymatic pathways including oxidation, reduction and hydrolysis (Segal *et al.*, 1975).

Phorbol esters increase phosphorylation of smooth muscle myosin heavy and light chains through activation of PKC (Kamm *et al.*, 1989). These compounds also potently down-regulate endothelin (ET-1) binding sites in vascular smooth muscle cells by a mechanism involving PKC (Roubert *et al.*, 1989). Phorbol ester-induced morphological changes in living cultured cells were accompanied by reorganisation of filamentous actin (Miyata *et al* ., 1988). The adherence of polymorphonuclear leucocytes to the pulmonary vascular endothelium may contribute to the acute lung injury (Gudewicz *et al* ., June 1989).

TPA has been shown to induce reorganisation of actin filaments and calspectin in 3T3 cells. Possible mechanisms for these cytoskeletal changes produced by TPA are discussed by Sobue *et al.* (1988). Stimulation of tyrosine phophorylation by phorbol diesters suggests that initial stimulation of PKC activates a tyrosine kinase cascade.

In vascular smooth muscle phorbol esters cause a slowly developing contraction and an associated transmembrane calcium flux, both of which are inhibited by dihydropyridine calcium antagonists (Fish *et al.*, 1988). There is a possible involvement of reorganisation of actin filaments induced by tumour promoting phorbol esters, in changes in colony shape and enhancement of proliferation of cultured epithelial cells. Related tumour promoters such as phorbol-12,13-didecanoate and mezerein caused effects similar to TPA (Sastrodihardjo *et al.*, 1987).

Lung injury induced by TPA is closely associated with toxic oxidants released from activated granulocytes. The available data indicates that the hydroxyl radical, a toxic oxidant derived from stimulated granulocytes, is deeply involved in the pathogenesis of TPA-induced lung injury (Kuroda *et al.*, 1987).

1.5 OUTLINE OF THE PRESENT STUDY

The aim of the research described in this thesis was to investigate the feasibility of raising antibodies in cattle and rabbits against diterpenoid toxins isolated from *Pimelea trichostachya*. The protective efficacy of any antibodies raised in cattle was to be determined through toxin challenge experiments and the effects of the toxins on bovine pulmonary venule preparations were to be examined *in vitro* using modern organ bath techniques. A major part of the study was to be devoted to establishing the organ bath methodology since it was anticipated that the technique would offer the opportunity to validate the efficacy of purified antibodies in protecting the bovine target tissue from the toxins and also allow studies of potential antagonists of the toxins.

There are two examples of Australian research which indicate that an immunogen approach to plant toxicity problems in livestock is worthy of investigation. A successful vaccine has been developed against lupinosis in sheep. This disease is caused by ingestion of infected lupin stubbles infected by the fungus *Phomopsis leptostromiformis*. This fungus produces mycotoxins (phomopsins) which are lethal to animals. Phopmopsin A is the most toxic compound produced from the fungus and is a potent inhibitor of tubulin polymerisation and microtubule formation.

Phomopsin A toxicity in animals is characterised by severe liver damage, jaundice, loss of appetite, emaciation and ultimate death. Edgar and co-workers have developed an effective anti-phomopsin vaccine for sheep which protects vaccinated animals against phomopsin toxicity. The exact details of their conjugation and vaccination methodology has not been disclosed because of its potential commercial value, however the general principles have been described by Ralph (1990). The effectiveness of the

lupinosis vaccine was discovered only by chance, since the aim of the initial research was to produce anti-phomopsin antibodies in sheep which could be purified for use in an ELISA method for detecting phomopsins. It was found that vaccinated animals were protected against lupinosis toxicity and antibody titres could be augmented in the following season through a single booster injection of antigen.

In the case of the lupinosis vaccine, successful protection against the toxin may be attributable to phomopsin A being a peptide and amenable to destruction by the enzymes released by the macrophages that bind to the toxin-antibody complex. Other (non-peptide) toxins, approached in a similar way, may only be held temporarily by the antibody; but may not be digested by the macrophage; thereby releasing the toxin back into the general circulation. This would mean that toxin specific antibodies may extend the half-life of the toxin, exacerbating the poisoning event (Cockrum and Edgar 1985; Edgar *et al.*, 1982).

The other example is perhaps more relevant to the present study since it involves a toxicity in cattle caused by triterpenoid toxins. Lantana poisoning is a disease that affects ruminant animals and is characterised by jaundice, cholestasis, liver and kidney damage, photo sensitisation, ruminal stasis, constipation, anorexia and in very acute cases death (Pass *et al.*, 1981; Pass, 1986).

Lantana poisoning is caused by pentacyclic triterpene toxins called lantadenes which are present in introduced *Lantana camara* plant species. Stewart and Pass (1988) vaccinated sheep with conjugates of lantadenes A and B isolated from *Lantana camara.* .Because the lantadenes absorbed U.V. light very poorly, it was not possible for these workers to accurately quantify the degree of toxin incorporation into the carrier protein. Despite the modest antibody titres obtained in the vaccinated animals, Stewart and Pass (1988) observed protective effects against hepatotoxicity when vaccinated and control animals were fed *Lantana camara* . Serum bilirubin levels were significantly lower in the vaccinated group.

In the case of *Pimelea* toxicity the toxins involved are tricyclic diterpenes related to phorbol esters which also contain an unusual orthoester functionality in the C - ring. These compounds are much more highly functionalised than the lantadenes and therefore possess potentially more antigenic determinants. The work of Tashjian *et al,* (1985) had shown that specific antibodies could be raised against phorbol 12,13 dibutyrate in mice and it therefore seemed reasonable to expect that specific immune responses to the structurally related daphnane toxins present in *Pimelea* could also be achieved. Whether these antibodies would offer any protection to *Pimelea* toxicity was a central question to be addressed through the present study.

2.0 CHAPTER 2
2.1 INTRODUCTION

The aims of the work described in this Chapter were: (i) to develop an efficient methodology for purification of the daphnane orthoester toxins from *Pimelea* ;and (ii) to conjugate these compounds to carrier proteins to enable preparation of experimental vaccines.

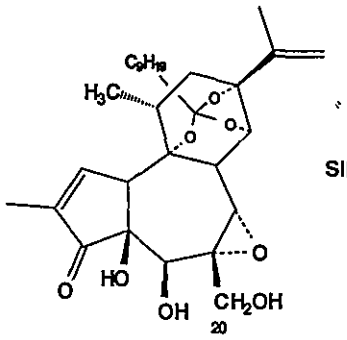
There had been a number of earlier reports describing isolation of daphnane and tigliane orthoesters from *Pimelea* species (Freeman *et al.*, 1979, Tyler and Howden 1985). In the present study, we sought to utilise preparative HPLC methodology for toxin isolation. The toxic fractions obtained from conventional column chromatography were to be identified using a fish toxicity assay and then these fractions further purified using preparative reverse phase HPLC techniques.

The *Pimelea* daphnane and tigliane toxins are relatively small molecules with molecular weights ranging from 500-700 mass units. To induce an immune response to the toxins in test animals, a toxin- protein conjugate needed to be prepared. Previous work in this laboratory had established some general procedures for the conjugation of daphnane type compounds to carrier proteins (Pegg, Hellqvist and D'Occhio, unpublished).

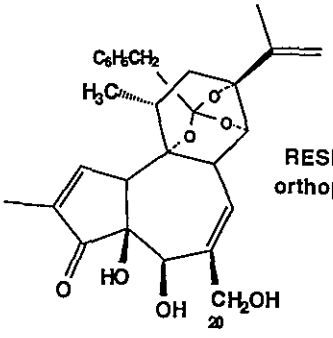
In the present study, conjugates of *Pimelea* A, B and C fractions, mezerein and resiniferinol were prepared using this methodology. As shown in Figure 2.1, the two commercially available daphnanes employed (namely mezerein and resiniferinol orthophenyl acetate) bear many structural and stereochemical similarities to simplexin and huratoxin. The rationale for the conjugation strategy was that the *Pimelea* daphnane and tigliane toxins and the two commercially available daphnanes all possessed a common C-20 hydroxymethyl grouping that was not sterically hindered and offered potential for chemical modification.

In preliminary work related to this project, antisera to phorbol-12,13-dibutyrate (PDBu) did not appear to recognise either pure simplexin (Dr W. Taylor, University of Sydney) or resiniferinol orthophenyl acetate. These findings suggested that the tricyclic ortho-ester functionality in the C-ring of simplexin and resiniferinol orthophenyl acetate (and absent in PDBu) was preventing molecular recognition by antibodies raised against PDBu. As a result of these experiments, all subsequent immunogen work directed at raising antibodies to *Pimelea* toxins has been undertaken with analogues possessing the unusual ortho-ester function in the C-ring.

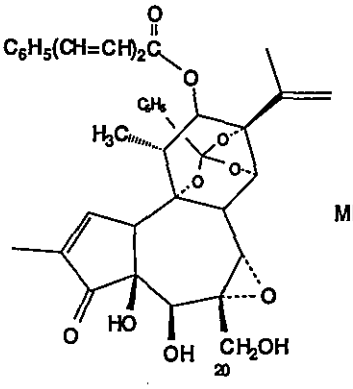
Figure 2.1
Structure of simplexin and three commercially available structural analogues.



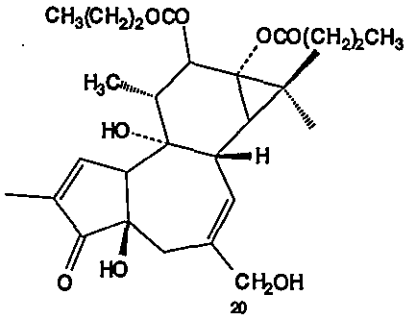
SIMPLEXIN



RESINIFERINOL
orthophenyl acetate

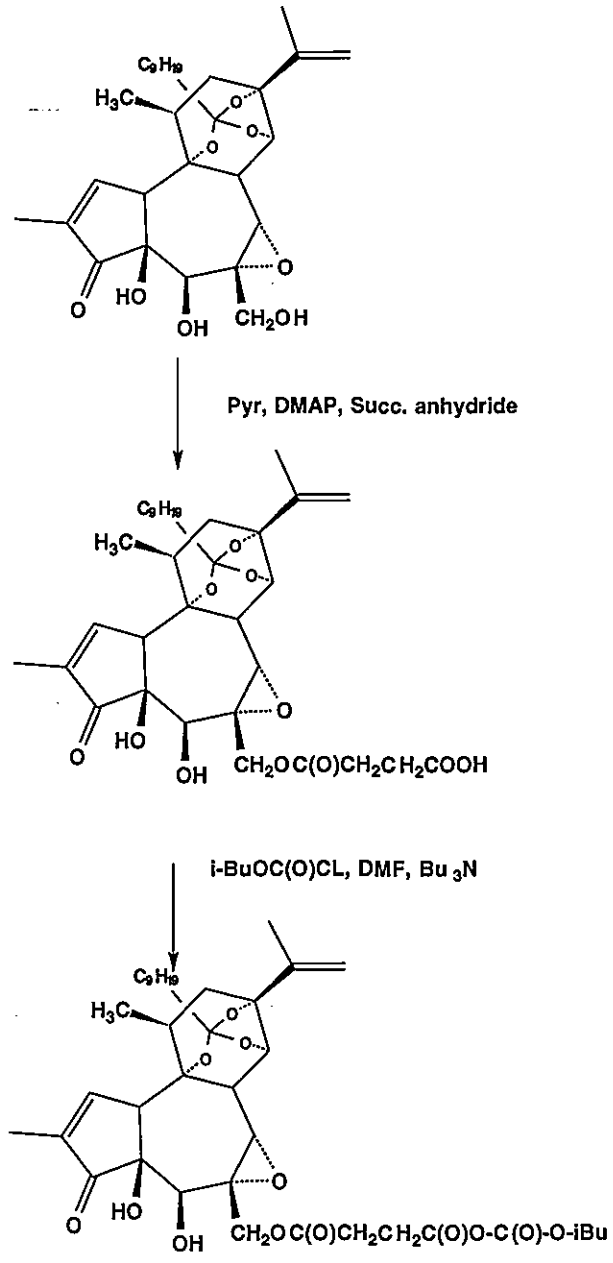


MEZEREIN



PHORBOL-12,13-DIBUTYRATE

Figure 2.2
Chemical transformation of simplexin.



The strategy employed for the conjugation of simplexin is depicted in Figures 2.2 and 2.3. As shown in Figure 2.2, the daphnane ortho-ester was first reacted with succinic anhydride in pyridine at 80 ^{o}C, in the presence of a catalytic amount of dimethylaminopyridine (DMAP). This reaction was carried out in a sealed glass ampoule containing an inert nitrogen atmosphere. It was presumed that most acylation

would occur at the relatively unhindered C-20 hydroxymethyl grouping, although there also existed the possibility of some reaction occurring at the C-5 secondary hydroxyl grouping.

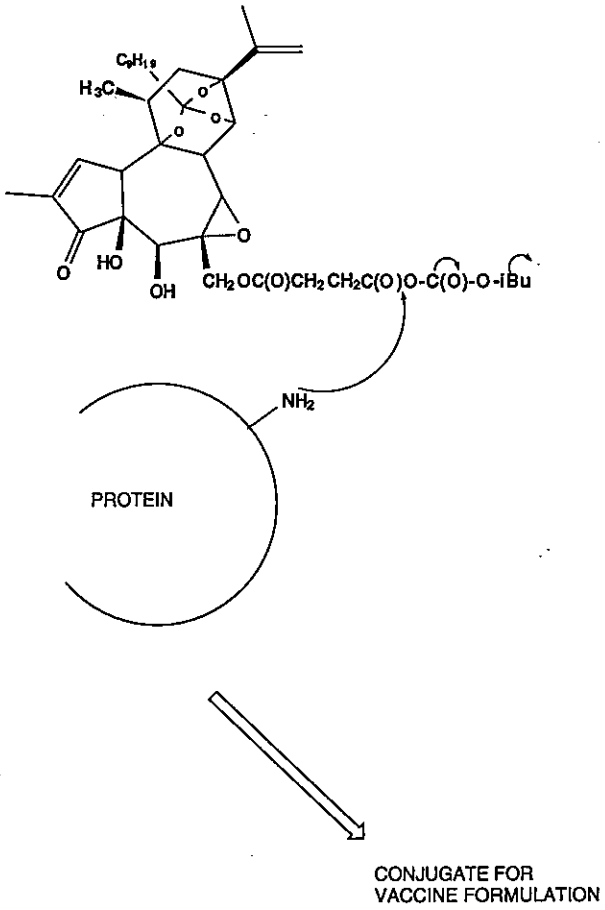
Earlier work showed that under these reaction conditions, simplexin was converted into a single product having a shorter retention-time on reverse phase HPLC (Pegg and Duivenvorden, unpublished). This increase in polarity was consistent with the incorporation of an additional polar free carboxyl functionality in the succinylated product. It was found that the reaction was sluggish in the absence of the DMAP acylation catalyst.

The next step in the procedure depicted in the lower portion of Figure 2.2 was the activation of the incorporated free carboxyl grouping by reaction with iso-butylchloroformate (IBCF) in anhydrous dimethylformamide (DMF). This reaction was carried out at 0° C in the presence of tributylamine to react with any hydrogen chloride liberated. HPLC analysis showed that complete conversion to a less polar mixed anhydride had occurred within 15 minutes. In this case, the mixed anhydride was very reactive and has been set up to liberate carbon dioxide and iso-butanol upon attack of a suitable nucleophile.

The final step of the procedure shown in Figure 2.3 requires the preparation of a solution of the carrier protein in 50:50 DMF : water, to which the "activated" toxin in DMF is added at 0° C. Free lysine amino groups in the carrier protein are sufficiently nucleophilic to effect a displacement reaction on the activated mixed anhydride intermediate, thereby linking the modified toxin to the protein via a new amide bond. The common observation in these reactions is evolution of carbon dioxide and a drop in pH of the reaction mixture. Particularly with ovalbumin, one must be careful to maintain the pH of the reaction mixture near 8.0 to prevent precipitation of the carrier protein from the DMF/ water mixture.

The same conjugation methodology was to be used in the present study except that a mixture of daphnane and tigliane toxins isolated from *Pimelea* was to be conjugated to the carrier protein rather than a single toxin. The rationale for this approach was to prepare a conjugate which possessed the full range of *Pimelea* toxin epitopes. The added advantage of using the mixture of toxins was that more material was available to work with, since even the major toxin simplexin makes up only 0.005% of the dry weight of *Pimelea simplex* (Freeman *et al.*, 1979).

Figure 2.3
Protein toxin conjugation



2.2 METHODOLOGY

2.2.1 Toxin Extraction from *Pimelea trichostachya*

The technique employed for toxin isolation was adapted from a report by Tyler and Howden (1985) in which those authors described the isolation of toxins from *Pimelea simplex*. It has been reported by Freeman *et al.*, (1979) that dried *Pimelea* plant material retains toxicity for several years and that significant amounts of the active toxins can still be isolated from the plant after this time. It appears that the diterpenoid toxins are stable when protected from light and oxygen within the plant, however after isolation these compounds are readily degraded. Storage at -20°C in the dark under an inert nitrogen atmosphere is required. Schmidt and Hecker (1975) have described autoxidation breakdown products of phorbol esters stored in common solvents, such as dimethyl sulphoxide and chloroform and it was expected that the daphnane orthoesters isolated from *Pimelea* might show similar autoxidation tendencies.

Whole dry *Pimelea trichostachya* (100 g) was powdered using an electric hammer mill. The plant material used included stems, leaves, flowers, and roots. The powdered plant material was exhaustively extracted with methanol at room temperature (3 x 500 mL, 12 h) and the combined methanol filtrates evaporated to dryness using rotary evaporation. The resultant dark gum-like residue (5.4 g) was stored under nitrogen at -20°C in the dark.

The gummy residue was then partitioned between chloroform (150 mL) and water (300 mL) in a separatory funnel and the water layer then repeatedly extracted with chloroform (3 x 50 mL). Most piscicidal activity (*vide infra*) was found in the chloroform layer. The aqueous layer containing the water soluble non-toxic component was discarded. The combined chloroform extracts were dried over anhydrous sodium sulfate, filtered and then evaporated to dryness using a rotary evaporator. The resultant gum like residue (1.40 g) was stored under nitrogen at -20°C in the dark. Piscicidal activity of the crude residue was tested using a fish bioassay technique.

2.2.2 Fish Bioassay

Sakata *et al.* (1971) have reported a fish bioassay technique to test huratoxin toxicity and these workers found that 0.003 ppm huratoxin concentration causes 100 % death in killie fish within 24 h. Since the daphnane toxins found in *Pimelea* spp. have very similar structures to huratoxin, it was decided to test the piscicidal activity of the crude chloroform extract directly using a fish bioassay.

Freeman, *et al.* (1979) reported that 0.005 % of the dry weight of *Pimelea simplex* is simplexin. On this basis it was assumed that the 1.40 g of residue obtained from 100 g of dry *Pimelea trichostachya* plant might contain 5 mg of simplexin. Since the diterpene toxins are immiscible in water but readily dissolve in acetone, a portion of the crude extract was dissolved in acetone by dissolving 0.14 mg of the crude extract (equivalent to 5 µg of simplexin) in 500 µL of acetone to approximate a 0.003 ppm simplexin concentration in 200 mL of water.

Six mosquito fish (*Gambusia* spp.) of similar size and weight (three test and three controls) were kept off food overnight and allowed to acclimatise in an eight chambered perspex tank. Each chamber contained 200 mL of continuously aerated water. Test fish were challenged with the test solution (500 µL of the toxin solution in acetone) while control fish were challenged with an equivalent volume of pure acetone. The fish were then observed for a period of 24 h.

2.2.3 Silica gel column chromatography

The crude extract (1.4 g) was further purified using vacuum assisted silica gel column chromatography. The toxic material was dissolved in 2 mL of benzene and chromatographed on silica gel (Merck Kieselgel G type 60) packed in a Buchner funnel. The column was eluted under reduced pressure with 100 mL aliquots of the following solvents :

1. Benzene
2. Benzene - ethyl acetate (9:1)
3. Benzene - ethyl acetate (6: 4)
4. Ethyl acetate
5. Methanol

TLC analysis

Thirty five fractions (each approximately 15 mL) were collected from the chromatography and small samples were spotted on silica TLC plates (Merck DE-Plasticfolien Kieselgel 60 F_{254}). Separation of components was achieved using benzene : ethyl acetate (6:4) as the mobile phase. The plates were first observed under short wave U.V. light (Extech Equipment Pty. Ltd.) and later were sprayed with a solution of concentrated sulphuric acid/vanillin. Colour development was achieved by heating the plates for three minutes at 100°C.

2.2.4 Modification of the silica gel column chromatography solvent system

Since benzene is a hazardous solvent, and that scale-up of the procedure described above would require handling a large quantity of benzene (3 L) over 6-7 h, we sought to substitute petroleum spirit for benzene in the chromatography.

To determine suitable mixtures of petroleum spirit/ethyl acetate, the following procedure was adopted.

1. A series of five control plates were spotted with the crude extract dissolved in chloroform. Each control plate was run against one of the series of eluting solvents used for the initial silica gel column chromatography described above. Separated compounds were observed by UV fluorescence and then each plate was sprayed with the sulphuric acid/vanillin spray.

2. Test silica plates were spotted with the crude extract in chloroform. Each test plate was then run in a petroleum spirit : ethyl acetate solvent of known ratio and each test plate was observed for UV fluorescence and sprayed with the concentrated sulphuric acid/vanillin spray for visualisation of components. Each test plate was compared with the series of control plates. This procedure was then repeated, each time with a fresh test plate and a new solvent mixture until the separation of the components on the test plate was almost identical with the corresponding control plate.

2.2.5 HPLC Purification

Sample preparation

C-18 reverse phase cartridges (Waters Associates) were used routinely for sample clean-up prior to HPLC analysis. Since the diterpene toxins from *Pimelea* have some polarity, a methanol solution of the toxins was eluted through C-18 sep-pak cartridges to remove highly hydrophobic impurities present in the sample. The recovered solution was then evaporated to dryness using a rotatory evaporator and the residue thus obtained was stored under nitrogen at -20°C in the dark. Using the fish bioassay technique described above, this material was found to be highly toxic to fish.

HPLC Separation

The *Pimelea* A,B fraction was then further purified using reverse phase HPLC. The components were separated using a methanol-water gradient on a C-18 reverse phase column with UV detection at 240 nm wavelength. The HPLC system used comprised a Waters 600E quaternary pumping system, a U6K injector and a Waters Model 486 Tunable Absorbance Detector. Chromatographic data were recorded via Baseline 8 software (Waters) on an IBM compatible 486 computer.

After preliminary sep-pak purification, *Pimelea* A,B fraction was dissolved in methanol before injecting into column. Good separation of components was achieved on analytical, semi-preparative and preparative scale C-18 reverse phase columns. For each of these columns, the optimum methanol-water gradient was established by trial and error application of different methanol-water gradients, until acceptable separation of components was obtained. HPLC grade methanol and Millipore filtered water were used for all separations. All solvents were filtered through a 0.45 µm filter before use and de-gassed by intermittent sparging with helium gas (10 mL/minute). Optimum conditions for effecting separation on a analytical column was first established, and then equivalent separation was successively achieved on semi-preparative scale followed by larger scale preparative column HPLC.

Details of the columns and guard columns used, the methanol-water gradients employed and the quantity of *Pimelea* A,B sample that could be adequately separated by each technique is given below.

(a). Analytical scale reverse phase HPLC :

Analytical column :
Waters Nova-pak C18 reverse phase analytical column
Size : 3.9 mm x 150 mm
Packing Material : dimethyloctadecylsilyl bonded amorphous silica, pore size- 60 Å, particle size - 4µm
Guard column :
Nova-pak C18 cartridge type
Sample quantity used :
Approximately 3-5 mg of *Pimelea* A,B fraction was dissolved in 50 µL of methanol
Methanol gradient :
The methanol/water solvent gradient employed is detailed in Table 2.1

Table 2.1
Methanol : Water gradient for HPLC separation of *Pimelea* toxins on analytical scale.

Time (min.)	Flow rate (mL/min.)	Methanol (%)	Water (%)
0	2.0	60	40
10	2.0	65	35
15	2.0	70	30
20	2.5	75	25
30	2.5	85	15
35	2.5	90	10
80	2.5	90	10

(b) Semi-preparative scale reverse phase HPLC :
Semi preparative column :
Activon Gold Pak C18 reverse phase column.
Size : 25 x 1 cm

Packing material : Exil 100/5 Octadecylsilica
Guard column :
Activon Gold Pak C18 cartridge.
Sample quantity used :
Approximately 15-25 mg of *Pimelea* A,B fraction was dissolved in 50 µL of methanol.
Methanol Water Gradient used :
The methanol/water solvent gradient that was employed is detailed in Table 2.2.

Table 2.2
Methanol : Water gradient for separation of *Pimelea* toxins on a semi-preparative scale.

Time (min)	Flow rate (mL/min)	Methanol (%)	Water (%)
0	3.5	60	40
10	3.5	65	35
15	3.0	70	30
20	3.0	75	25
30	3.0	85	15
35	3.0	90	10
80	3.0	90	10

(c) Preparative scale reverse phase HPLC :
Preparative column :
Waters Prep-pak cartridge reverse phase preparative column.
Size : 25 X 100 mm
Packing material : Prep Nova Pak HR C18.
Pore size - 60 Å, particle size : 6 µm.
Guard Column :
Guard Pak TM Cartridge Prep Nova-Pak HR C18
Size : 25 x 10 mm
Packing Material : Prep Nova Pak HR C18.
pore size- 60 Å, particle size - 6µm.
Sample quantity used :
Approximately 30-40 mgs of *Pimelea* A,B fraction was dissolved in 200 µL of methanol.
Solvent Gradient :
The solvent gradient employed was dependent on the sample size. For separation of up to 40 mg, the gradient detailed in Table 2.3 was employed.

Table 2.3
Methanol : Water gradient used for preparative separation of *Pimelea* toxins (up to 40 mg sample size).

Time (min.)	Flow rate (mL/min.)	Methanol (%)	Water (%)
0	4.0	60	40
13	4.0	65	35
21	4.0	70	30
29	4.0	75	25
45	4.0	85	15
53	3.5	90	10
83	3.5	90	10

Sample quantity used :

Approximately 40 mg of *Pimelea* A,B fraction was dissolved in 500 µL of methanol.

Table 2.4 below details the solvent gradient employed for preparative scale separation of sample sizes of 40-150 mg of *Pimelea* A,B toxic fraction.

Table 2.4
Methanol : Water gradient used for separation of *Pimelea* toxins on preparative scale (40-150 mg sample size).

Time (min)	Flow rate (mL/min)	Methanol (%)	Water (%)
0	7.0	60	40
3	7.0	65	35
7	7.0	70	30
10	7.0	75	25
13	7.0	80	20
19	7.0	85	15
23	7.0	90	10
60	7.0	90	10

Sample quantity used :
Approximately 150 mg of *Pimelea* A,B fraction was dissolved in 700µL of methanol

As the preparative run took approximately 80 minutes, fractions obtained each minute were collected using a Gilson Model 203 Micro Fraction Collector.

2.2.6 Conjugation Methodology

General

Mezerein and resiniferinol orthophenyl acetate were purchased from LC Services Corporation, Woburn, MA, USA. The best analytical or HPLC grade solvents available in the laboratory were used routinely. Pyridine (A.R.) was dried over potassium hydroxide pellets and distilled just prior to use. The dried distilled pyridine was stored over 3A molecular sieves. DMF (A.R.) was distilled from calcium hydride and stored over 3A molecular sieves. Solvent and liquid chemical transfers were generally accomplished with oven-dried glass syringes. Where required, a positive pressure of dry nitrogen gas was maintained in reaction flasks from nitrogen-filled balloons connected through Suba-Seal rubber septa.

Conjugates

For the present study, the following conjugates were prepared for immunisation experiments in rabbits and cattle :

Pimelea A - human serum albumin
Pimelea B - human serum albumin
Pimelea C - ovalbumin
Mezerein - ovalbumin
Resiniferinol - ovalbumin

Methodology

The conjugation methodology is described in detail for preparation of the *Pimelea* A-HSA conjugate. The same methodology was then applied for preparation of the other

four conjugates. *Pimelea* A and B toxic fractions were obtained after silica gel column chromatography as described previously (Section 2.2.4). *Pimelea* C fraction was obtained after preparative HPLC purification of *Pimelea* A and B fraction as explained earlier (Section 2.2.5). Because the *Pimelea* toxic fractions are a cocktail of different toxins, an average molecular weight of toxin molecules was assumed to be 500 g/mol for calculation purposes. The molecular weights of the *Pimelea* toxins range from 500-700 g/mol, depending on the substituent groupings on the daphnane or tigliane molecular backbone (Freeman *et al.*, 1979).

Pimelea A fraction (350 mg, 0.7 mmol) obtained from silica column chromatography was dissolved in 2 mL of dry pyridine and succinic anhydride (0.15 g, 1.5 mmol) and a catalytic amount of DMAP was then added. The reaction mixture was transferred to a pyrex ampoule and the contents sparged with nitrogen and sealed. The reaction ampoule was then heated overnight (15 h) at 80°C.

After cooling the sample to room temperature, the pyridine solvent was removed using a vacuum pump and dry ice/acetone trap. TLC (silica) comparison of the starting material and crude product from the reaction was undertaken to establish that succinylation of the toxin had occurred.

A sample for TLC analysis was prepared by removing the pyridine from a 0.3 mL aliquot of the reaction mixture. The residue thus obtained was partitioned between saturated ammonium chloride (1 mL) and ethyl acetate (1 mL) in a small glass tube. The ethyl acetate layer was removed by aspiration. The aqueous layer then was extracted a further 2-3 times with ethyl acetate (1 mL) washes. The combined ethyl acetate layers were then evaporated to a small volume (0.25 mL) and small aliquots spotted on the plate. Elution with benzene : ethyl acetate (1:1) showed that the reaction products (Rf approximately 0.3) were more polar than the starting material (Rf approximately 0.5).

The gummy residue (0.50 g) obtained from the succinylation reaction was dissolved in dry DMF (5 mL) and the solution cooled to 4° C. To the cooled solution, tributylamine (500 µL, 0.02 mmol) followed by isobutylchloroformate (300 µL, 0.023 mmol) was added. The reaction generated HCl which was neutralised by the tributylamine base. The activation reaction was normally run for 40 minutes at 4°C, although previous work in this laboratory has shown that these reactions are often complete within 5 minutes.

The activated mixed anhydride product was not isolated and immediately was reacted with HSA at 4° C to form the toxin-HSA conjugate. Firstly, HSA (2.5 g) was dissolved in distilled water (200 mL). The solution was cooled in an ice-bath and DMF was added in small portions until a 50:50 DMF : water mixture was achieved. The activated toxin was added dropwise to the protein solution with constant stirring at 4°C. Carbon dioxide was evolved, resulting in a fall in pH. The pH of the reaction mixture was maintained at 8-9 through the dropwise addition of 2 M NaOH as required. The conjugation reaction mixture was then stirred for 15 hours at 4° C before being dialysed.

The crude conjugate in DMF/water solvent was transferred into dialysis tubing and then dialysed at 4°C over a period of 48 hours against 5 litres of chilled 50 mM $NaHCO_3$. The bicarbonate solution was replaced 5 times during this period. After dialysis, the final volume of the protein solution was 1.37 L. Before freeze drying, the U.V. spectrum was recorded to establish that toxin molecules had been incorporated into HSA molecules.

The same methodology was used for preparation of *Pimelea* -B-HSA; *Pimelea*- C-ovalbumin; mezerein-ovalbumin and resiniferinol ortho phenylacetate-ovalbumin conjugates. The amounts of reagents used in each case are detailed in Table 2.5.

2.2.7 Estimation of Toxin Incorporation

A solution of HSA for U.V spectral analysis was made by dissolving 4 mg of HSA in 100 mL of 50 mM $NaHCO_3$. Correspondingly, a 4 mg protein/100 mL solution of the dialysed conjugate was prepared by appropriate dilution in 50 mM $NaHCO_3$. U.V. spectra were recorded over the range 220-330 nm and the extent of toxin incorporation estimated by spectral difference measurements at 244 nm.

Table 2.5
Preparation of other toxin-protein conjugates.

Toxin (mg)	Succinic anhydride (mg)	isobutyl-chloro-formate (µL)	tributyl-amine (µL)	protein (mg)
Pimelea B (450) 0.9 mmol	(2000.0) 2.0 mmol	(300.0) 0.23 mmol	(500.0) 0.2 mmol	(2000.0) BSA 30.3 µmol
Pimelea C (25) 50 µmol	(6.0) 60 µmol	(8.0) 62 µmol	(18.3) 77 µmol	(90.0) OVAL 2 µmol
Mezerein (20) 30 µmol	(5.0) 50 µmol	(5.0) 36 µmol	(10.7) 44.8 µmol	(44.0) OVAL 0.98 µmol
Resiniferinol (20) (43 µmol)	(5.0) 50 µmol	(7.0) 53.19 µmol	(15.0) 66 µmol	(63.0) OVAL 1.4 µmol

2.2.8 Estimation of protein recovery for the HSA conjugates

As expected, the weights of the freeze-dried conjugate material recovered exceeded the combined weights of the protein, toxic fraction and succinic anhydride used, due to incorporation of sodium bicarbonate during dialysis. A precipitation method was used to estimate the amount of protein in the freeze dried material recovered from the *Pimelea* A-HSA and *Pimelea* B-HSA conjugates.

Method

(i) The *Pimelea*-HSA conjugate (100 mg) was dissolved in 10 mL of distilled water.
(ii) To this solution 10 mL of 20 % trichloroacetic acid (TCA) was added and the mixture allowed to stand 20 minutes before it was centrifuged (5000 g, 5 minutes, 5°C).
(iii) The pellet was resuspended in 10 % TCA and the precipitate recovered by centrifugation as above.
(iv) The protein precipitate was washed three times with warm distilled water.
(v) The pellet thus obtained was freeze-dried and weighed.

The other conjugates prepared for this study involved lesser amounts of material than the *Pimelea* A and B conjugates. For this reason, protein recovery by the TCA precipitation method was not undertaken.

2.3 RESULTS AND DISCUSSION

2.3.1 Identification of Toxic Components by the Fish Bioassay Technique

After initial solvent extraction and chloroform /water partitioning, 1.40 g of a green gummy residue was obtained from 100g of dried *Pimelea trichostachya.* This material was stored under nitrogen at -20°C in the dark. Piscicidal activity of the crude residue was tested using the fish bioassay technique described in Section 2.2.2.

It was found that all three test fish were dead within 30 minutes of adding the crude toxin extract (at an estimated 0.003 ppm simplexin concentration), whereas the three control fish remained healthy after 24 hours. The fish bioassay experiment confirmed that piscicidal activity was concentrated in the crude organic extract from *Pimelea trichostachya* . The gummy residue was found to be freely soluble in both methanol and chloroform.

After conventional silica column chromatography as decribed in Section 2.2.3, the 35 fractions obtained were analysed by T.L.C and then grouped into five major fractions corresponding to the five solvent systems used. Each of the combined fractions was evaporated to dryness using a rotary evaporator and the residues thus obtained were stored under nitrogen at -20°C in the dark. Piscicidal activity of each fraction was determined using the fish bioassay system.

It was observed that piscicidal activity was greatest in fractions eluted with benzene - ethyl acetate (6:4) solvent, followed by the fraction collected from ethyl acetate. No piscicidal activity was observed in the other fractions. This activity pattern correlated with the UV absorption observations on the TLC analysis. For ease of identification within the laboratory, the toxic residues from the benzene - ethyl acetate and ethyl acetate fractions were named as *Pimelea* A toxic fraction and *Pimelea* B toxic fraction, respectively. These data are summarised in Table 2.6.

Table 2.6
Recovery, piscicidal activity and U.V. activity of fractions eluted from silica gel chromatography of the crude chloroform *Pimelea* extract.

SOLVENTS	WEIGHT (g)	FISH BIOASSAY	U.V. ABSORPTION
Benzene	0.89	Not Active	minimal
Benzene - ethyl acetate (9:1)	0.39	Not Active	minimal
Benzene - ethyl acetate (6:4)	0.05	Active	strong
Ethyl acetate	0.10	Active	strong
Methanol	0.40	Not Active	minimal

2.3.2 Modification of the Solvent System Employed for Silica Column Chromatography.

The original solvent system used for the silica column chromatography required the use of large amounts of benzene. Because of the hazardous nature of this solvent, its replacement with appropriate mixtures of petroleum spirit and ethyl acetate were investigated as described in Section 2.2.4. The data given in Table 2.7 shows the solvent mixtures identified which have equivalent eluting characteristics for separation of *Pimelea* toxins on silica TLC plates.

The chromatographic purification of the crude toxin extract was repeated using the petroleum spirit : ethyl acetate solvent mixtures specified in Table 2.7 below. The five

solvent fractions obtained from the column were evaporated to dryness using rotatory evaporation. The residues thus obtained were stored under nitrogen at -20°C in the dark.

Table 2.7
Equivalent solvent systems for separation of *Pimelea* toxins by TLC.

Benzene : ethyl acetate	Petroleum : ethyl spirit : acetate
90 : 10	100 : 0
75 : 25	90 : 10
50 : 50	60 : 40
0 : 100	0 : 100

Piscicidal activity was found to be greatest in the fraction eluted with petroleum spirit - ethyl acetate (50:50) solvent, followed by the fraction eluted with ethyl acetate solvent. The residues eluted from petroleum spirit - ethyl acetate (50 : 50) and ethyl acetate were named as *Pimelea* A and B fractions respectively. The three other non-toxic fractions obtained were discarded.

2.3.3 HPLC Purification

For subsequent purification of the *Pimelea* toxins by preparative HPLC, the *Pimelea* A & B fractions were combined. The combined material was called *Pimelea* A,B toxic fraction. The various toxic and non-toxic components in the combined fraction were then separated by reverse phase HPLC using a methanol/water gradient. Appropriate solvent regimes for analytical, semi-preparative and preparative scale separations were developed as described in Section 2.2.5 and representative chromatograms are given in Figures 2.4 - 2.7.

From the preparative scale HPLC separation of 150 mg of *Pimelea* A,B toxic fraction as shown in Figure 2.4, 80 fractions were collected and these fractions were grouped into 9 major fractions as described in Table 2.8. Each of the 9 fractions were evaporated to dryness and the residues tested for piscicidal activity using the fish bioassay technique. It was observed that fractions 1-3 obtained from the first 45 minutes of the run were non-toxic to fish. All test fish remained alive after 24 hours.

Residues from fraction 4-9, i.e. fractions eluted between 55-80 minutes were toxic to fish. Residues derived from fractions 6 and 7 were found to be most toxic to fish , causing the death of the test fish within 15 minutes. The toxic fractions were combined together and evaporated. The residue thus obtained was called *Pimelea* C toxic fraction. *Pimelea* C fraction was stored under nitrogen at -20° C in the dark until required for the conjugation procedure.

Table 2.8
Grouping of fractions obtained from preparative HPLC purification of *Pimelea* toxins.

Time (min)	Fraction No.	Toxicity
0-19	1	non toxic
20-40	2	non toxic
41-45	3	non toxic
46-54	4	toxic
55-59	5	toxic
60-63	6	highly toxic
64-68	7	highly toxic
69-73	8	toxic
74-80	9	toxic

2.3.4 Toxin : Protein Molar Incorporation Ratios

Freeman *et al.*, (1975) reported molar absorbtivity coefficients at 244 nm for the main daphnane toxins in *Pimelea simplex* , namely: simplexin (8100); simplexin diacetate (5900); and 12-acetoxy huratoxin (18,600). For the purposes of calculating molar incorporation ratios after completing the conjugation procedure, the molar absorbtivity coefficient for simplexin was used and the absorbance contributed by the 4 mg/100 mL protein component at 244 nm subtracted as described in Section 2.2.7. Molar incorporation ratios ranging from approximately 7.0 to 17.0 were obtained and these data are detailed in Table 2.9.

Figure 2.4
A representative chromatogram for separation of *Pimelea* A,B Fraction
by analytical scale reverse phase HPLC

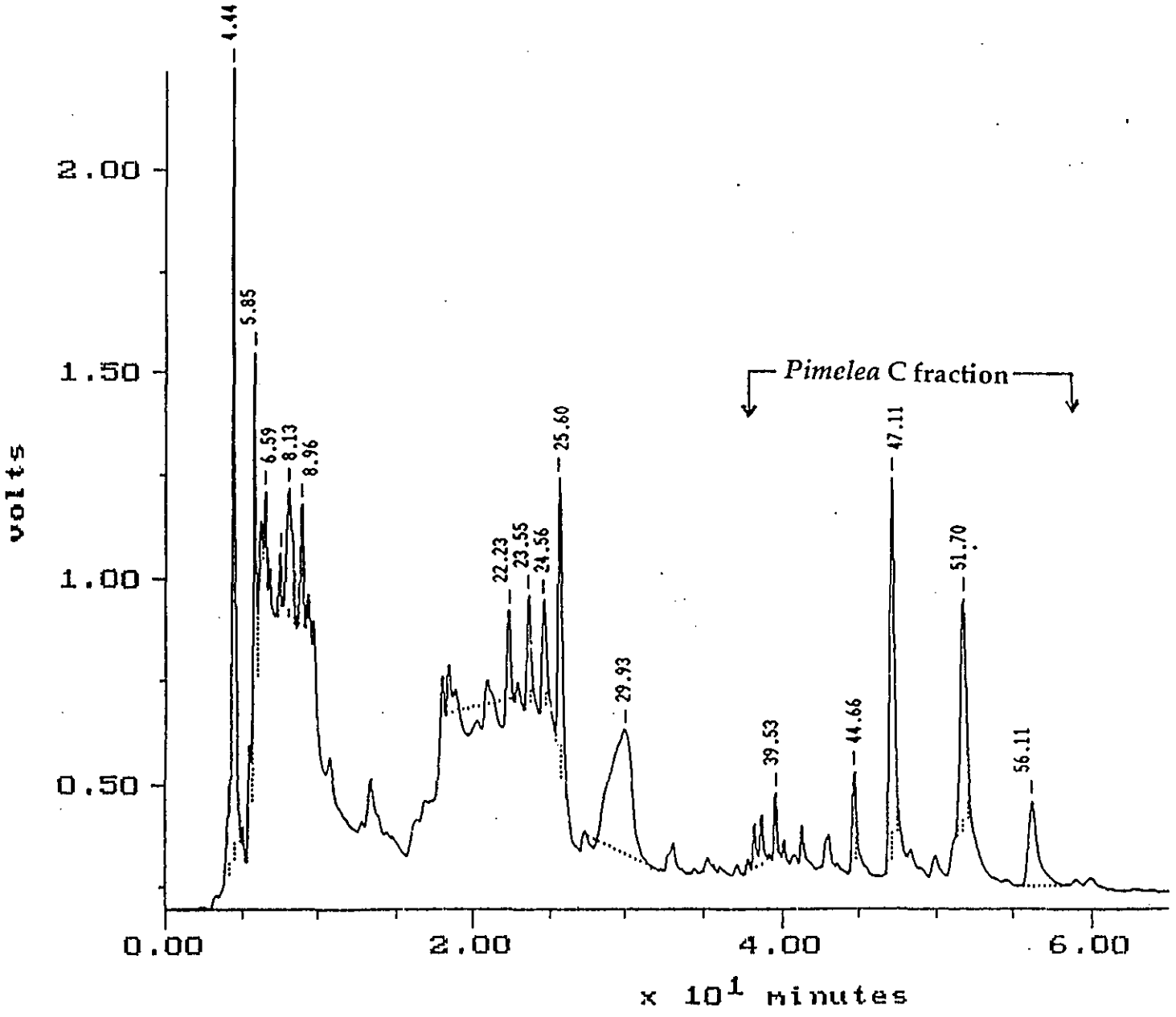


Figure 2.5
A representative chromatogram for separation of *Pimelea* A,B Fraction
by semi-preparative scale reverse phase HPLC

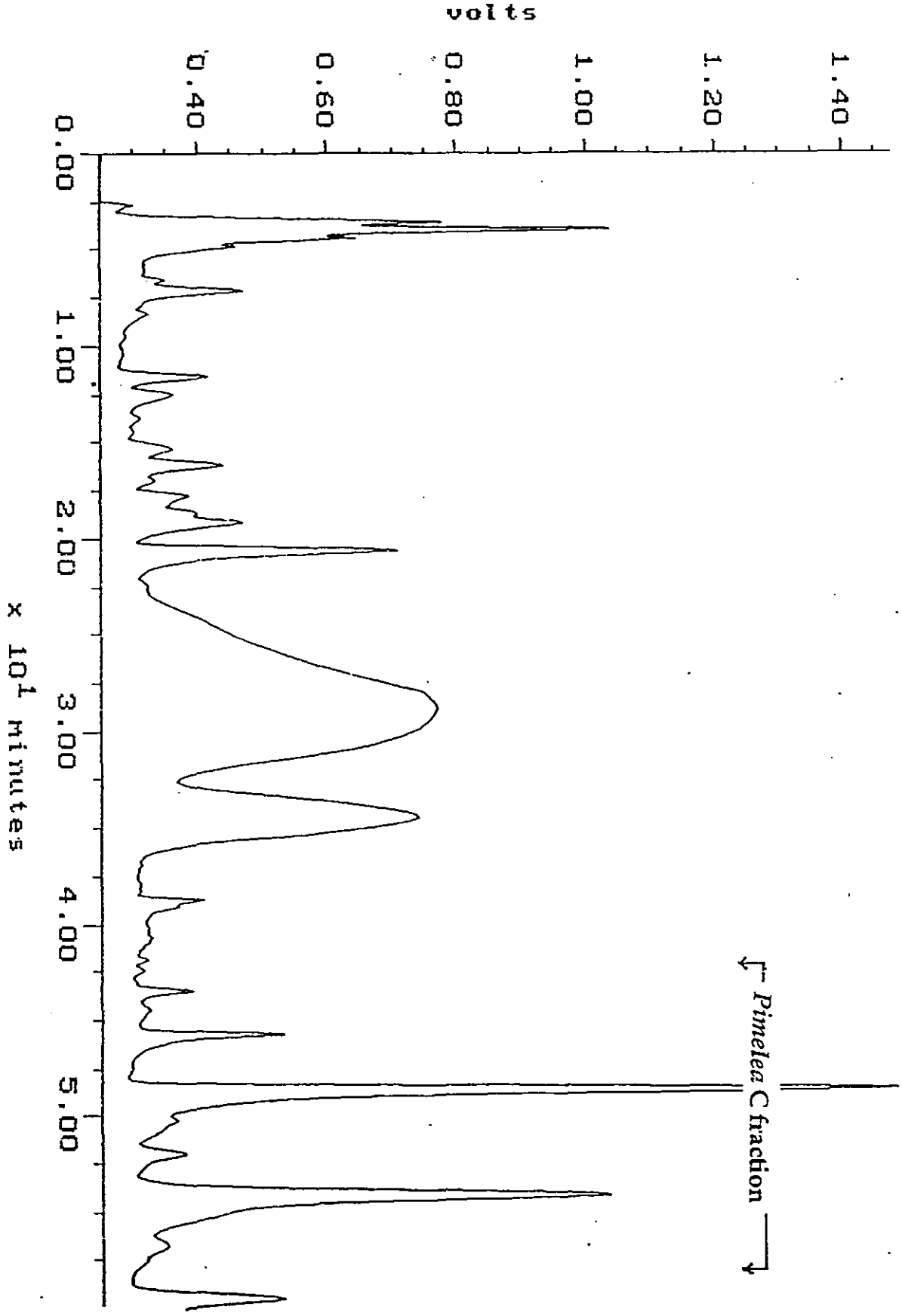


Figure 2.6
A representative chromatogram for separation of *Pimelea* A,B Fraction
by preparative scale (40 mg) reverse phase HPLC

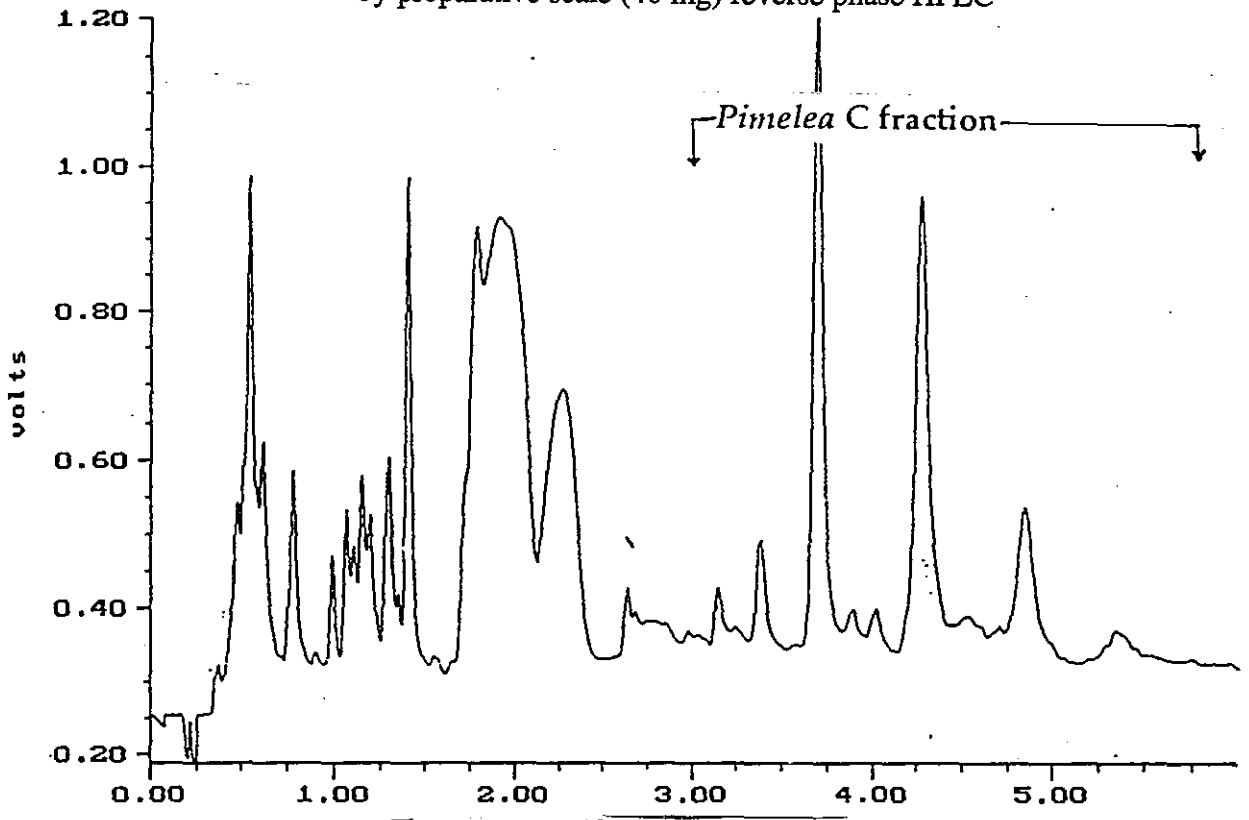


Figure 2.7
A representative chromatogram for separation of *Pimelea* A,B Fraction
by preparative scale (150 mg) reverse phase HPLC

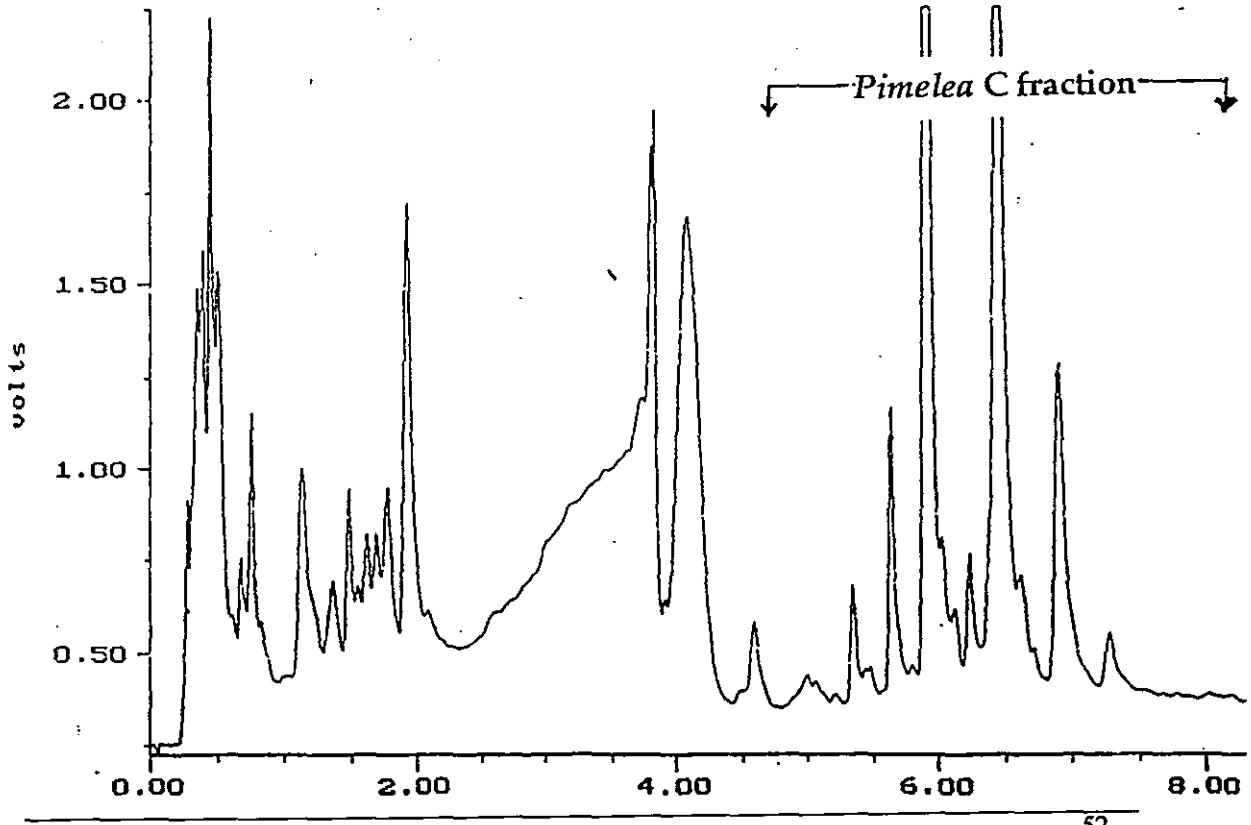


Table 2.9
Determination of toxin/protein incorporation ratios by U.V. analysis at 244 nm. for 4 mg/100 mL protein solutions.

Conjugate	Δ Abs. between conjugate and protein solutions.	Protein (μM)	Toxin (μM)	Incorporation ratio.
A-HSA[a]	0.083	0.580	10.2	17.6
B-HSA[a]	0.033	0.580	4.07	7.0
C-OVAL[b]	0.14	0.89	12.4	13.9

[a]HSA molecular weight 69,000. [b]OVAL molecular weight 45,000.

2.3.5 Protein Recovery

The protein recovery for the freeze dried *Pimelea* A -HSA and *Pimelea* B - HSA conjugates was determined using a TCA precipitation method as described in Section 2.2.8. The data presented in Table 2.10 indicates a high level of $NaHCO_3$ incorporation during the dialysis step. These data were used for determining the amount of conjugate required for the vaccination experiments described in Chapter 3.

Table 2.10
Protein recovery in freeze dried conjugates of *Pimelea* A and B fractions.

Pimelea -HSA conjugate	Wt of freeze dried conjugate (mg)	Wt of protein (mg)	Wt. of salt (mg)
A	100	27	72
B	100	35	65

The *Pimelea* C-, mezerein- and resiniferinol ortho phenyl acetate -ovalbumin conjugates were prepared on a much smaller scale than the corresponding HSA conjugates of the *Pimelea* A and B fractions. TCA precipitation was not attempted for these smaller scale preparations and for the purposes of vaccine preparation described in Chapter 3, the amount of protein employed initially in each case was used to estimate the amounts of freeze dried conjugate required. The recovery of each of the ovalbumin conjugates obtained is given in Table 2.11 below.

Table 2.11
Recovery of freeze-dried conjugates

Phorbol	wt of protein conjugate (mg)	Toxin (mg)	wt. of freeze dried (mg)
Pimelea C	67.5	30	161
Mezerein	45	20	250
Resiniferinol	45	20	216

2.4 CONCLUSIONS

Pimelea toxins were isolated successfully from *Pimelea trichostachya* whole dry plant through modification of the methodology described by Tyler and Howden (1979). Significant improvements were made to the silica column chromatography step by replacing benzene/ethyl acetate mixtures with equivalent polarity petroleum spirit/ ethyl

acetate solvent mixtures. The fish bioassy technique used by Sakata *et al.*, (1971) to establish the toxicity of huratoxin like compounds was applied to test the piscicidal activity of fractions isolated from *Pimelea*. It was reasoned that the daphnane and tigliane toxins present in *Pimelea* should also be potent piscicides and this was confirmed experimentally.

Preparative scale HPLC methodology for separation of components in toxic fractions obtained from silica column chromatography was developed. This approach could be further scaled up if required for future work. The method developed allows preparative separation of each of the major toxin components. Toxic fractions (A and B) obtained from silica gel chromatography and toxic fraction C obtained from preparative HPLC were conjugated either to HSA or ovalbumin. U.V analysis of the dialysed conjugates showed molar incorporation ratios of toxins to protein ranging from 7 to 17.0.

3.0 CHAPTER 3
3.1 INTRODUCTION

The isolation of *Pimelea* toxins was described in the previous chapter, along with the conjugation chemistry used for linking the toxins to immunogenic carrier proteins. This Chapter describes the preparation of prototype vaccines derived from these conjugates, and presents the results of vaccination trials in rabbits and cattle. The aims were first to confirm the presence of specific antibodies against *Pimelea* toxins in rabbits and cattle and then to evaluate the potential of vaccinated cattle to combat *Pimelea* poisoning in toxin challenge experiments. In addition, we wished to purify total serum IgG from vaccinated cattle and rabbits for use in organ bath experiments utilising bovine pulmonary venule preparations (see Chapter 4). The first laboratory task to initiate these studies was to prepare water-in-oil emulsions of the freeze-dried toxin-protein conjugates. These preparations also contained adjuvants to promote the immune response in test animals (Harlow and Lane, 1988).

Adjuvants are non-specific stimulators of the immune response. Most adjuvants incorporate two components. One is a substance designed to form a deposit protecting the antigen from rapid catabolic breakdown. The second component needed for an effective adjuvant is a substance that will stimulate the immune response non-specifically. These substances act by raising the level of a large set of soluble peptide growth factors known as lymphokines. Lymphokines stimulate the activity of antigen processing cells directly and cause a local inflammatory reaction at the site of injection. In the present study, Freund's adjuvant was used to prepare vaccines for rabbits, whilst a commercial vaccine formulation was used for cattle (Hoskinson, 1990).

Freund's adjuvant is a water-in-oil emulsion prepared with non-metabolisable oils. If the mixture contains killed *M. tuberculosis*, it is referred to as complete Freund's adjuvant (CFA), whereas without the bacterial component it is known as incomplete Freund's adjuvant (IFA). CFA is most often used for the primary vaccination only, while IFA is employed for secondary and any subsequent vaccinations. Freund's adjuvant has proven to be one of the best adjuvants available for stimulating strong and prolonged responses in experimental animals. Major disadvantages of Freund's adjuvant is that it often invokes very aggressive and persistent granulomas and also, it cannot be used for commercial livestock applications. For these reasons, the commercial formulation was used for the cattle experiments.

In the present study, two groups of rabbits were to be vaccinated with either the *Pimelea* A-HSA or *Pimelea* B-HSA conjugate. Three groups of cattle were to be vaccinated with either *Pimelea* C-ovalbumin, mezerein-ovalbumin or resiniferinol-ovalbumin conjugates. Vaccination of cattle with the mezerein and resiniferinol conjugates would determine whether the antibodies raised against these two commercially available daphnane orthoester compounds would cross react with toxins present in the *Pimelea* C fraction. The rationale for this approach was to establish whether the commercially available compounds might form the basis of a vaccine to combat *Pimelea* toxicity in cattle.

An additional requirement of this part of the project would be the development of an enzyme linked immunosorbent assay (ELISA) to detect specific antibodies against *Pimelea* (or commercially available) toxins from vaccinated animals.

3.2 METHODOLOGY

3.2.1 Vaccination of Rabbits with *Pimelea* A-HSA and *Pimelea* B-HSA Conjugates

This section describes: the formulation of vaccines derived from freeze-dried *Pimelea* toxin-protein conjugates; the vaccination schedule for primary and subsequent booster vaccinations; injection and bleeding protocols; and the collection of serum for ELISA studies. Rabbits A-C were vaccinated with the *Pimelea* -A conjugate, while rabbits D-F were vaccinated with *Pimelea* -B conjugate.

Vaccine Preparation :

Oil-in-water emulsion vaccines were formulated from the *Pimelea* toxin-HSA conjugates as follows :

(i) Freeze-dried material equivalent to 250 µg of conjugate (*Pimelea* A-HSA or *Pimelea* B-HSA) was used per vaccine per animal.

(ii) The weighed freeze dried conjugate (9.24 mg of *Pimelea* A-HSA conjugate and 7.14 mg of *Pimelea* B conjugate) was dissolved in 5 mL of saline. This preparation represents the aqueous phase.

(iii) CFA (Sigma Chemical Co.) was used as the adjuvant for primary vaccination and IFA (Sigma Chemical Co.) was used for subsequent booster injections.

(iv) To 5 mL of the appropriate adjuvant solution, the aqueous phase prepared in (ii) above was added dropwise. An emulsion was formed by homogenisation of the mixture with an Ultra-Turrax homogeniser employing a 527 N head attachment at 135000 rpm. Care was taken to avoid denaturing the protein due to heat of mixing. A drop of the emulsion thus f ormed was tested on water to establish that it did not disperse.

(v) Each rabbit was vaccinated with a total of 1 mL of the emulsified preparation, usually via multisite intra dermal (I.D.) injection.

Vaccination Schedule

The following vaccination schedule was employed:

Day	Vaccine formulation
0	250 µg conjugate, CFA adjuvant, multisite I.D.
27	250 µg conjugate, IFA adjuvant, multisite I.D.
118	250 µg conjugate, IFA adjuvant, multisite I.D.
140	1 mg conjugate, 1 mL saline containing 100 µg commercial adjuvant, I.P.
76	1 mg conjugate, 1mL saline containing 100 µg commercial adjuvant, I.P.

Mode of Injection

Two 5.0 cm x 5.0 cm sections on the lower back of the rabbit were shaved. Then given multiple site intradermal injections up to 10-12 sites (0.1 mL/site) on the rabbit's back in an effort to limit granuloma formation. The injected material was expected to drain into the local lymphatic system and become concentrated in the lymph nodes closest to the sites of injection. In an effort to boost the antibody responses in animals late in the experiment, two intraperitoneal injections (I.P.) of the conjugates in saline (containing the commercial adjuvant) were administered on days 140 and 176. Antigens injected into the peritoneum drain into the thoracic lymphatic system.

Bleeding Animals via the Marginal Ear Vein

Samples of blood from vaccinated animals were usually taken 7-14 days after injection. This timing corresponds with the peak antibody response for most injection routes. The blood is usually collected from the ear vein of rabbits since this site is easily accessible and does not have high numbers of nerve endings. Between 5-10 mL of blood was collected from each animal using the procedure described below.

The rabbit was wrapped in a towel and the ear shaved about two-thirds of the distance from the head to the tip of the ear around the marginal ear vein . The marginal vein was clearly visible on the inner edge of the ear. The easiest method found for collecting blood from the ear was to make a transverse cut at a 45° angle to the vein with a sterile scalpel and then collect the blood by allowing it to drip into a clean glass test tube. After collecting 5-10 mL, the blood flow was stopped by gentle application of pressure to the cut with a sterile piece of gauze or bandage for 10-20 seconds. It is important that the rabbit is kept warm and relaxed throughout this procedure. If the rabbit is stressed, the fright response is to constrict the ear artery.

Bleeding schedule

Animals were bled according to the following schedule:

(i) pre-immunisation bleed;
(ii) 28 days post-2° vaccination;
(iii) 10 days post-3° vaccination;
(iv) 10 days post-4° vaccination;
(v) 7 days post-5° vaccination.

Isolation of serum from blood

Blood was allowed to clot in the collection container at room temperature for 2-3 h. Serum was then removed by pipette and the clot was then centrifuged to allow collection of residual serum. Serum aliquots were stored in plastic tubes at -20° C for future use

3.2.2 Immunisation of Cattle with *Pimelea* C - Ovalbumin, Mezerein -Ovalbumin and Resiniferinol - Ovalbumin Conjugates

Three experimental groups comprising six cattle (3 males and 3 females) each were vaccinated with either *Pimelea* C-ovalbumin, mezerein-ovalbumin or resiniferinol-ovalbumin conjugates. A group of six control cattle were also vaccinated with the carrier protein (ovalbumin). As the conjugates were all dialysed for several days against 50 mM $NaHCO_3$, the freeze dried material also incorporated a significant amount of sodium bicarbonate along with the toxin-protein component. For each vaccination, an amount of the freeze-dried conjugate material approximating 2 mg of conjugate was used per animal. The original weight of protein used in the conjugation and recovery of freeze-dried material is given in Table 3.1 below. Estimation of the amount of freeze-dried conjugate needed to prepare each dose of the vaccine was based on the amount of protein originally used to prepare the conjugate. (The rationale for this simple approach was that while a 20-fold incorporation of toxin molecules per protein molecule would raise the molecular weight of the protein conjugate by approximately 10,000 mass units to 55,000, this increase in molecular weight would be almost exactly compensated for if only 80% of the protein fraction was recovered.)

Table 3.1
Recovery of freeze-dried toxin-ovalbumin conjugates used for vaccination of cattle

Toxin	wt of protein (mg)	freeze-dried recovery (mg)	amount of freeze-dried material for 7 vaccines (mg)
Pimelea C	67.5	161.0	38
Mezerein	45.0	250.0	81
Resiniferinol	45.0	216.0	70

Vaccine Preparation

(i) A mixture of ondina 15 oil (94.5 mL, Shell company of Australia Ltd.) and emulsifier, arlacel A (10.5 mL, Sigma) was stirred vigorously on a magnetic stirrer.

(ii) The amount of freeze-dried conjugate required (see Table 3.1) was dissolved in a mixture of saline (5 mL) and then made up to 10.5 mL with 15 % DEAE-Dextran solution with pH between 6.5-7.5. (The pH of the 15 % DEAE-Dextran solution was adjusted with conc TRIS). Hereafter this solution is called the aqueous phase.

(iii) The aqueous phase (10.5 mL) was added slowly via a 10 mL syringe to a vigorously stirred 24.5 mL aliquot of the oil/arlacel A solution prepared in (i) above. (The mixture turned milky at this stage.)

(iv) The loose emulsion thus formed was then further emulsified using an Ultra-Turrax homogeniser T25 (527 N head attachment at 13500 to 20500 rpm). This gave a creamy emulsion which did not disperse when tested on water.

(v) Animals were given subcutaneous vaccinations on both sides of the shoulder with 5 mL of the emulsion.

(vi) The same protocol as described in (i)-(v) above was used for formulation and administration of vaccines for secondary and subsequent vaccinations. The only modification being that half the amount of conjugate was used (approximately 1 mg/ vaccination) for 3° and 4° vaccinations.

Vaccination Schedule

The vaccination schedule employed is given below.

Day	Vaccination
0	1°
27	2°
55	3°
85	4°

Bleeding schedule

The following bleeding schedule was used to collect serum from immunised cattle for ELISA studies. Blood (20 ml) was collected from the jugular vein using disposable plastic (25 mL) syringes and sterile needles.

Day	*Collection*
0	1°
27	2°
42	2 weeks post 2°
55	3°

70	2 weeks post 3°
85	4°
99	2 weeks post 4°
104	post toxin challenge experiment

3.2.3 Enzyme-Linked Immunosorbent Assay (ELISA) Analysis of Antibody Responses

ELISA is a powerful technique applied routinely for the detection and quantitation of a wide variety of analytes. For determining the presence and amount of antibody, a diluted serum sample is incubated with a solid phase (ELISA plate) coated with excess antigen (i.e. more than enough antigen to bind all of the antibody present in a given sample). Any antigen-specific antibodies present bind to the antigen coated on the solid phase. After washing, bound antibody is detected by an enzyme-labelled anti-immunoglobulin- conjugate. Unbound conjugate is washed away, and an enzyme substrate added to yield a coloured product. The amount of colour developed is proportional to the amount of antibody in the sample.
In the present study, the daphnane and tigliane diterpene toxins being investigated had very limited water solubility so a toxin-protein conjugate was required to coat the plate with antigen. The protein used for coating should be unrelated to the carrier protein used in the vaccine. Hence the ELISA plate would need to be coated with a toxin-ovalbumin conjugate in the case where animals had been vaccinated with a toxin-HSA conjugate.

Antigen attachment and the stability of that attachment to the solid phase is important in ELISA. Experience with a number of monoclonal antibodies, polyclonal antibodies and other proteins has shown that 1-10 µg/mL is usually the optimum range for coating of antigens. It is important not to coat the plates with highly concentrated antigen since high levels of coating protein usually leaves behind a significant amount of loosely adsorbed material which desorbs during subsequent assay steps and interferes in the assay. The conformation of the antigen also may be altered at high binding densities and antibody binding sites may be sterically obscured because of multi-layered packing. The most widely used coating buffers are 50 mM carbonate at pH 9.6, 20 mM Tris-HCl at pH 8.5 and 10 mM PBS at pH 7.2. When coating proteins, it is best to use buffers with a pH value 1-2 units above the pI of the protein. Wash solutions are required to remove all extraneous and loosely bound material from the surface of the solid phase. Commonly used wash solutions are PBS or TBS containing 0.05 % Tween 20 (to lower surface tension and assist the flushing process).

After the plate has been coated with antigen, a blocking step is used to minimise non-specific binding of antibody to the solid phase support. Casein or non-fat dry milk, which contains a large proportion of casein, are most effective blocking agents. The rationale proposed for the efficacy of casein is that it is a heterogeneous mixture of large protein complexes (casein micelles), individual casein molecules and smaller peptide fragments of various charges and hydrophobicity. It is believed that this mixture of proteinaceous material inhibits both hydrophilic and hydrophobic binding of antibody molecules which would otherwise cause background problems.

In the present work, an antibovine IgG-HRP (horse radish peroxidase) conjugate was to be used for cattle antibody detection, while an analogous antirabbit-HRP conjugate would be required for the rabbit ELISA. Normally, a 1:4000 dilution of the commercially available antibody-HRP conjugate in assay buffer is a useful starting concentration , although experiments to determine the optimum dilution of the second antibody need to be undertaken for each batch.

On an antigen coated microtitre plate, different concentrations of sample are added to each row of wells. After incubation and washing, different concentrations of anti-IgG-HRP conjugate are added to each column of wells. The plate is incubated and washed, then enzyme substrate is added to facilitate colour development. The results allow the selection of the conditions which provide optimum substrate colour development in the desired concentration range of the analyte. Diluents for horseradish peroxidase conjugates should never contain sodium azide, since azides are powerful peroxidase inhibitors.

A number of different HRP substrates are routinely used in ELISA. TMB was chosen for this study as it is known to be efficient for the detection of low levels of enzyme (approximately 4 times more sensitive than o-toluidine and ABTS and 2 times more sensitive than OPD) and also has a higher absorbance plateau than the other substrates. The incubation time required for colour development to be complete is 25 minutes. For most ELISA assays, phosphate or Tris buffered saline of pH 7.0 - 7.5 is a suitable buffer. The buffer should be supplemented with some type of non-interfering protein, to help block non-specific binding. Commonly used proteins are BSA, SMP, gelatine or normal animal sera. (If an assay only uses antibodies from one species only, then non-immune serum from the same species would be the best one to use.)

Reagents and Materials

Immunol 4 (Flat bottom) 96 well polystyrene microtiter immunoassay plates (Dynatech Laboratories, USA), were used in all experiments. The coating buffer used to dissolve the antigen was 50 mM carbonate buffer (pH 9.6) which was prepared by dissolving 4.2 g of $NaHCO_3$ in 1 litre of distilled water and the pH was then adjusted by the addition of 2M NaOH. Stock solutions (0.5 mg/mL) of the freeze-dried conjugate materials (including salts) were prepared in coating buffer .

Working solutions for coating plates were prepared by diluting 100 µL of the 0.5 mg/mL stock solutions in 10 mL of coating buffer which gives 0.5 µg/mL actual conjugate concentration. The wash solution (0.01 M PBS/0.05 % Tween 20) was prepared by dissolving Na_2HPO_4 (7.1 g), EDTA (1.9 g), NaCl (40.9 g) in 2 L of distilled water. The pH of the mixture was adjusted to 7.5; 2.5 mL of Tween 20 added and then the final volume made up to 5 L. The blocking solution was prepared by dissolving 1 g of skim milk powder in 100 mL of assay buffer. The blocking solution was then filtered and the pH adjusted to 7.5. The colour reagents used was either TMB or ABTS (Kikegard and Perry laboratories Inc.).

A stock solution of TMB (0.5 mg/mL) in DMSO was stored at 4 ^{o}C. A working TMB solution was prepared just before use by mixing 2 mL of the stock solution and 1.5µL of 30 % H_2O_2 in 8 mL of 0.1 M acetate buffer (pH 5.6; 100 µL required per well). The acetate buffer was prepared by dissolving sodium acetate (13.6 g) in 1 L of double glass distilled water and the pH adjusted to 5.6 by the addition of glacial acetic acid. The stop solution employed for the TMB colour reagent system was 0.5 M H_2SO_4 (100 µL added per well at the completion of the substrate incubation period). For ABTS colour reagent the stop solution used was 1 % SDS (100 µL per well). The assay buffer (0.01 M PBS /0.2 % BSA, pH 7.5) was prepared by dissolving Na_2HPO_4 (1.42 g), EDTA (0.37 g) , NaCl (8.17 g) and BSA (2.0 g) in approximately 500 mL of distilled water. The pH of the mixture was adjusted to 7.5 and the volume then made up to 1L. The assay buffer was filtered before use. Unless otherwise specified, assay plates were sealed with sticky plate sealer after each step in the procedure to prevent evaporation of solutions from the wells.

Coating Plates with Antigen

(i) Immunol 4 microtiter immunoassay plates were coated overnight at 4°C with 50 µL per well of the conjugate coating solutions. (Care was taken throughout the procedure to ensure that all samples were incubated for the same length of time.)

(ii) The plates were washed 5 times on an automatic washer (Titrek's Microplate Washer 120) with wash solution to remove the unbound antigen. Plates were then flicked dry on a blotting paper to ensure that the wells were as dry as possible.

Blocking

After coating the wells with antigen, non-specific binding sites in the wells were blocked with 300 µL of blocking solution added via a multi-channel pipette. The plate was then incubated for 1h at room temperature before being washed 5 times using the automatic washer. Plates were then flicked dry to ensure that the wells were as dry as possible.

Sample Addition and Incubation

Appropriate dilutions of serum in assay buffer (50 µL/well) in duplicate or triplicate were added to the wells according to a pre-determined layout arrangement. The well contents were mixed using a automatic shaker (IKA-Schuttler MTSG shaker, Flow Laboratories), the plates covered with aluminium foil and then incubated at room temperature for two hours. After incubation, the plates were washed 5 times using the automatic washer and then flick-dried as previously described. Serum samples were added quickly to the wells to minimise the variations in incubation times.

Addition of the second antibody

Fresh antibody-HRP conjugate (Silenus Laboratories, diluted 1:4000) was prepared in assay buffer and 50 µL of this solution then added to each well. (Anti-bovine IgG-HRP was used for analysis of cattle sera while anti-rabbit-HRP conjugate was used for detecting antibodies in the rabbit ELISA.) The solutions in the wells were mixed and the plates then incubated at room temperature for 1 hour. As the HRP conjugate is light sensitive, plates were always covered with aluminium foil. After incubation, the plate was washed 5 times using the automatic washer and then flicked-dry as previously described.

Colour development

The TMB working solution (100 µL/well) was added via a multi-channel pipette. The plate was then covered with aluminium foil and incubated for 30 minutes at room temperature. Colour development was stopped by adding 100 µL of the stop solution to each well using a multi-channel pipette. This changes the colour from blue to yellow and this absorbance was read at 450 nm using a Biorad 3550 Microplate Reader via Microplate Manager software on an IBM compatible 386 microcomputer.

3.2.4 *Pimelea* Challenge Experiment with the *Pimelea* C -Ovalbumin Vaccinated Cattle

Control cattle (n=6) and cattle vaccinated against *Pimelea* C fraction (n=6) received an oral drench daily of milled *Pimelea* plant material based on body weight (Table 3.2). The dose of milled dried plant material was mixed in water (500 mL) and the slurry put directly into the rumen using a stomach tube and funnel. Each animal had to be

restrained in a crush and the tube inserted carefully through the mouth until the rumen was reached (as evidenced by the odour of ruminal fluid).

Table 3.2
Body weights and sex of cattle used in the vaccination trial.

Control Cattle			*Pimelea*-C -OVAL vaccinated cattle		
CattleNo.	Sex	Weight (kg)	CattleNo.	Sex	Weight (kg)
1001	F	200	1008	F	190
1002	F	222	1010	F	166
1006	F	190	1011	F	200
1016	M	174	1015	M	186
1019	M	188	1021	M	212
1023	M	246	1022	M	190

Doses were administered on a body weight basis as follows :

50 mg/kg live weight	Day 0 to 9
100 mg/kg live weight	Day 10 to 14
250 mg/kg live weight	Day 15 to 17

Blood samples were collected each day and the physical appearance and pathology of each animal monitored. Blood parameters such as haemoglobin levels, haematocrit, leucocytes, neutrophils, cortisol, transaminases, total protein, urea and red cell count were measured at Dr T. B. Lynch Pathology Laboratory, Rockhampton.

3.2.5 Purification of IgG from Vaccinated Cattle and Rabbits

General

Econo-Pac Serum IgG purification columns (3 mL serum capacity; Bio-Rad Laboratories, Richmond, CA., USA) were used for purification of IgG from rabbit and cattle serum. Serum was collected from two of the vaccinated cattle (1008 and 1016) showing high antibody responses on ELISA and also from rabbit F vaccinated with the *Pimelea* B- HSA conjugate. Econo-Pac 10 DG desalting columns were used for sample preparation. The purified serum sample was chromatographed on pre-packed DEAE Affi-Gel Blue agarose gel columns (Econo-Pac serum IgG purification columns) to produce a purified IgG fraction expected to be free from other serum proteins and plasminogen with only residual contamination from transferrin.

Reagents

Application and regeneration buffers were supplied as premixed solids. Buffer solids were stored at room temperature. To prepare the application buffer (0.02 M Tris-HCl, pH 8.0, 0.028 M NaCl), 1.3 g of the supplied buffer solids were dissolved in 300 mL of Millipore filtered water and the solution then filtered through a 0.45 µm filter. The pH of the buffer was adjusted to 8.0 ± 0.2 with either NaOH or HCl as required and the solution stored at 4° C.
The regeneration buffer (2M guanidine.HCl in application buffer) was prepared by dissolving 12.2 g of the supplied buffer solids in 100 mL of Millipore filtered water and the solution filtered through a 0.45 µm filter. No pH adjustment was necessary and the buffer was stored at 4° C until use.

Chromatography

For preparation of 3 mL of serum for purification on serum IgG purification columns the following procedure was used. (If the serum sample was less than 3 mL, application buffer was added to achieve a starting volume of 3 mL) The buffer above the top frit of Econo Pac 10 DG column was discarded and the bottom tip of the column was snapped off. Application buffer (20 mL) was added to column and the buffer allowed to drain to the top frit. The serum sample (3 mL) was added to the column and the first 3 mL eluted was discarded. A further 4 mL of application buffer was used to elute the serum from the column. The desalting columns were washed with 20 mL of application buffer before re-use. The desalted serum sample was then further purified as described below.

The buffer above the top frit of the Econo-Pac serum IgG purification column was discarded and the bottom tip of the column snapped off. Each column was then prewashed with 40 mL of regeneration buffer, followed by 40 mL of application buffer. (This initial wash removes residual blue dye in the columns which otherwise might be eluted with serum proteins.) The 4 mL fraction eluted from the desalting column was applied to the Econo-Pac serum IgG purification column in one portion. After allowing the sample to pass into the column, the IgG fraction was eluted with 20 mL of application buffer. Albumins were then eluted by washing the column with application buffer containing 1.4 M NaCl. Columns were then regenerated with 20 mL of regeneration buffer to ensure removal of bound proteins and to prevent cross-over contamination from one run to the next.

Spectrophotometric absorbance at 280 nm was measured for each IgG fraction so that the actual freeze dried recovery could be compared with that expected on the basis of absorbance. To remove salts, the IgG fractions were dialysed against distilled water for 24 h at 4°C. After dialysis, the liquid was freeze dried and the lyophilised material used for *in vitro* experiments described in Chapter 4.

Calculations of expected IgG recovery based on absorbance at 280 nm were performed as detailed below.
Rabbit :

Volume of rabbit serum used = 5 mL
Volume of the IgG fraction obtained = 25 mL
Absorbance at 280 nm = 0.812
Freeze dried IgG obtained = 29.7 mg
Standard absorbance of IgG is 1.35/1 mg
Therefore, calculated IgG present in IgG fraction (25 mL) = 33 mg which compares favourably with the amount actually recovered.

Cattle :

Volume of serum used = 150 mL
Volume of IgG fraction obtained = 850 mL
Absorbance at 280 nm = 1.920
Freeze dried IgG material = 1.4 g
Standard absorbance of IgG is 1.35/1mg
Therefore, calculated IgG present in IgG fraction (850 mL) = 1.1 g which approximates the amount actually recovered.

The Effect of Freeze Drying Purified IgG from Rabbits and Cattle.

To investigate the possibility for loss of antibody binding as a result of IgG purification and freeze drying, ELISA analysis was conducted with fresh serum and an equivalent

amount of the lyophilised IgG fraction. Serum dilutions of 1:500 for vaccinated rabbit, vaccinated cattle and normal rabbit serum (unvaccinated) were tested for antibody binding. These were compared with freeze dried IgG material obtained from the same quantity of serum in each case. Quantities of freeze dried material required were calculated as described below.

Cattle :

1.4 g of purified IgG was obtained from 150 mL of vaccinated cattle serum. Therefore, 128 µL of cattle serum is equivalent to 1.2 mg of IgG and this amount of the solid was dissolved in 128 µL of assay buffer. A 2.5 µL aliquot of each solution was further diluted in 5 mL of assay buffer to give equivalent 1: 500 dilutions.

Rabbit :

30 mg of purified IgG was obtained from 5 mL of vaccinated rabbit serum. Therefore, 167 µL of rabbit serum is equivalent to 1 mg of IgG and this amount of the solid was dissolved in 167 µL assay buffer. A 2.5 µL aliquot of each solution was further diluted in 5 mL of assay buffer to give equivalent 1: 500 dilutions.

3.3 RESULTS AND DISCUSSION

3.3.1 Antibody Responses in Rabbits Vaccinated with *Pimelea* A-HSA and *Pimelea* B-HSA Conjugates

Generally, an increase in B-cells bearing surface antibodies specific for the inoculated antigen should be detectable 5-6 days after the primary vaccination. Antibody is usually detected in the serum from around 7 days and persists at a low level for a few days, typically reaching a peak around day 10. Rabbits should remain effectively primed for at least a year after receiving the first vaccination. The response to a second injection of the same antigen given after 3-4 weeks is dramatically different. The number of B cells bearing antigen-specific cell-surface antibodies increases exponentially after the secondary vaccination, usually peaking between days 3 and 4. Antibodies in the serum are also detectable at this time but peak levels are usually observed in the second post-secondary week. High antibody levels persist for about 2-4 weeks after the secondary vaccination and then gradually diminish over time.

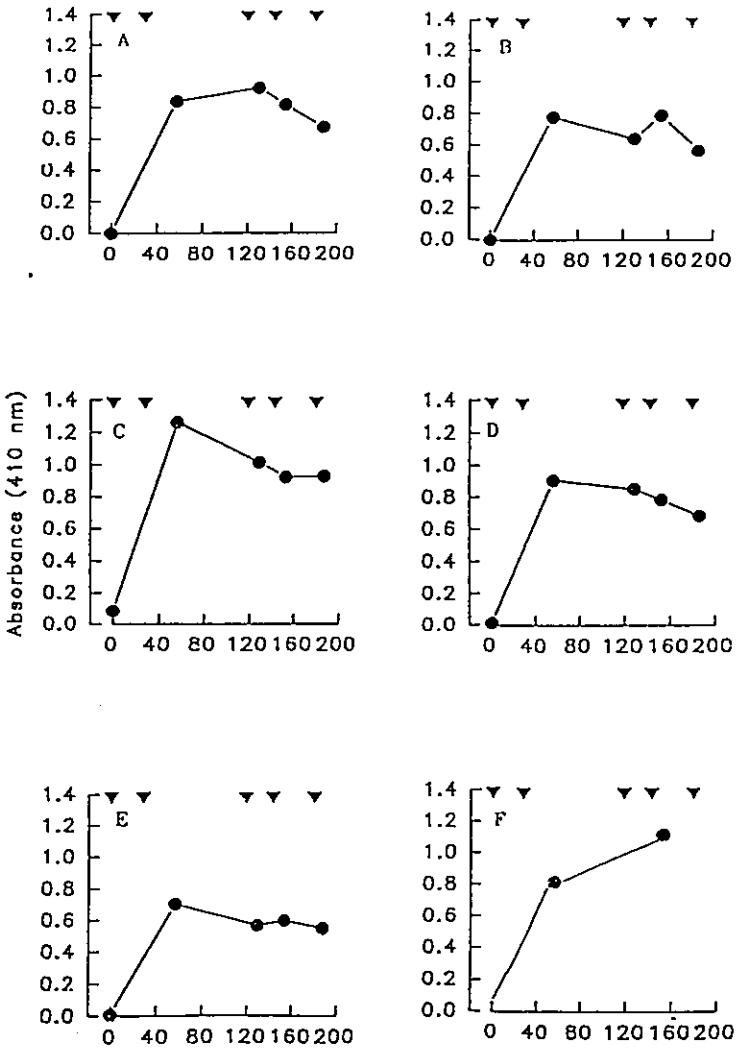
Rabbits A- C and rabbits D-F were vaccinated with the *Pimelea* A-HSA conjugate and the *Pimelea* B-HSA conjugate respectively, five times over a period of 129 days. Serum was collected as detailed in the methodology section and ELISA analysis was undertaken to monitor the time course of antibody responses for each rabbit. Serum dilutions of 1:500, 1:1000 and 1:5000 were analysed and the serum of an unvaccinated rabbit was used as a control blank. The absorbance of the control was subtracted from the test data.

Results for individual rabbits are given in Figure 3.1 and Table 3.3 and the group data is summarised in Table 3.4. The control serum and serum from test animals prior to immunisation (Day 0) showed no specific IgG which recognised the *Pimelea*- C -BSA conjugate coated on the plate. In contrast, significant antibody responses were detected 28 days post 2° vaccination for all six experimental animals at 1/5000 serum dilution. The data shows that the antibody responses remained high for the duration of the experiment. Subsequent booster vaccinations did not appear to cause further peaks in

antibody responses. (The data presented for rabbit F is incomplete due to a shortage of serum).

The grouped data presented in Table 3.4 reinforces the observation that the antibodies developed against the less pure *Pimelea* fractions, A and B conjugates gave good recognition to the more highly purified daphnane and tigliane fraction C conjugate coated on the plate. Moreover, these data vindicated our first approach to immunise animals with a conjugate derived from a mixture of compounds. It was reasoned that only those compounds containing a relatively unencumbered hydroxyl grouping would covalently link to the protein. Fortunately, all of the main toxic components in *Pimelea* have a free hydroxymethyl grouping in the B-ring and the results suggest that these compounds were selectively conjugated from the mixture.

Figure 3.1
Time course of antibody responses in rabbits vaccinated with *Pimelea* A-HSA (animals A-C) or *Pimelea* B-HSA (animals D-F)



Abbreviation : ▼ indicates the day of vaccination.

Table 3.3

ELISA absorbance results[a] for rabbits A-F against a *Pimelea* C-BSA conjugate plate coating.

Rabbit	Serum dilution	Day 0	28 days post 2°	10 days post 3°	10 days post 4°	7 days post 5°
Control	1:500	0.127				
	1:1000	0.076				
	1:5000	0.008				
A	1:500	0.115	1.384	1.447	1.374	1.324
	1:1000	0.045	1.291	1.368	1.230	1.194
	1:5000	0.001	0.839	0.922	0.817	0.674
B	1:500	0.038	1.369	1.286	1.362	1.247
	1:1000	0.013	1.253	1.139	1.220	1.106
	1:5000	0.002	0.779	0.643	0.789	0.563
C	1:500	0.267	1.517	1.501	1.470	1.488
	1:1000	0.086	1.555	1.442	1.333	1.369
	1:5000	0.018	1.262	1.014	0.920	0.925
D	1:500	0.115	1.387	1.422	1.377	1.304
	1:1000	0.038	1.323	1.276	1.206	1.160
	1:5000	0.017	0.909	0.854	0.788	0.687
E	1:500	0.056	1.236	1.253	1.187	1.199
	1:1000	0.018	1.170	1.105	1.055	1.072
	1:5000	0.011	0.704	0.569	0.595	1.550
F	1:500		1.426		1.446	
	1:1000		1.269		1.415	
	1:5000		0.814		1.114	

[a] average of duplicate determinations

Table 3.4
Grouped ELISA data[a] for rabbits A-C and D-F [b]

Group	Serum dilution	Day 0	28 days post 2°	10 days post 3°	10 days post 4°	7 days post 5°
Cont-rol	1:500 1:1000 1:5000	0.127 0.076 0.008				
A -C	1:500	0.14±0.063	1.42 ± 0.004	1.41 ± 0.06	1.40 ± 0.028	1.35 ± 0.06
	1:1000	0.02±0.005	1.36 ± 0.090	1.31 ± 0.08	1.26 ± 0.034	1.22 ± 0.07
	1:5000	0.29±0.023	0.96 ± 0.150	0.85 ± 0.10	0.84 ± 0.034	0.72 ± 0.10
D-F	1:500	0.08 ±0.028	1.34 ± 0.050	1.33 ± 0.063	1.33 ± 0.075	1.25 ± 0.049
	1:1000	0.028±0.007	1.25 ± 0.040	1.19 ± 0.069	1.22 ± 0.10	1.11 ± 0.042
	1:5000	0.014 ± 0.002	0.80 ± 0.050	0.71 ± 0.11	0.83 ± 0.15	1.11 ± 0.43

[a] Results are presented as means ± SEM.
[b] Data compiled from Table 3.3

3.3.2 Antibody Responses in Cattle Immunised with *Pimelea* C - Ovalbumin, Mezerein -Ovalbumin and Resiniferinol - Ovalbumin Conjugates

Three groups of cattle (n=6) were vaccinated with ovalbumin conjugates prepared from *Pimelea* C fraction, resiniferinol and mezerein. Control cattle were vaccinated only with the carrier protein ovalbumin. The purposes of this experiment were to determine if cattle would mount a specific immune response to the daphnane-protein conjugates and also to gain preliminary information on the degree of cross-reactivity of the antibody response. A central question was whether antibodies raised against resiniferinol or mezerein would cross react with daphnanes isolated from the plant.

ELISA analysis of serum samples collected one week post secondary vaccination was undertaken with plates coated with BSA conjugates of *Pimelea* C, mezerein and resiniferinol, and also with BSA alone. (The cross reactivity tests with *Pimelea* C-BSA toxin coated plates were carried out subsequent to the other analyses but care was taken to ensure that all analytical conditions were identical so that the data obtained could be compared.) The average absorbance derived from control serum (cattle vaccinated with ovalbumin only) was subtracted from all test absorbance values. In general, the cattle responded less strongly than the rabbits and the data given below are for 1/500 dilution of serum samples.

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Binding of anti-ovalbumin antisera to BSA

All of the cattle groups had been immunised with ovalbumin conjugates, whereas the plate coating chosen to detect specific antibody responses was a BSA conjugate. The first task was to demonstrate that antisera from vaccinated animals did not bind to the wells when BSA alone was coated on the plate. Seven samples were analysed as detailed in Table 3.5. These data demonstrated very low binding levels to the BSA coating and confirmed that anti-ovalbumin antibodies did not significantly cross react

with BSA. (Note that individual animals were numbered from 1001 - 1024 for the purposes of the experiment.)

Table 3.5
ELISA data for sera from animals immunised with ovalbumin carrier protein against a BSA plate coating.

Control group	Abs.	"Mez"- Oval group	Abs.	"Res" - Oval group	Abs.	*"Pim* - C " group	Abs.
1001	0.032	1003	0.043	1007	0.003	1008	
1002		1004		1009		1010	
1006		1005	0.028	1012	0.034	1011	
1016		1013		1014		1015	
1019		1017		1020		1021	
1023	0.019	1018	0.016	1024		1022	

Cross Reactivity

The data presented in Table 3.6 shows that all experimental groups raised antibodies which recognised a mezerein-BSA plate coating, indicating a considerable degree of cross - reactivity.

In an analogous experiment, plates were coated with a resiniferinol ortho phenyl acetate-BSA conjugate and cross-reactivity of antibody responses between the experimental groups established. The data obtained (Table 3.7) confirmed that antibodies raised in all three experimental groups did bind to the resiniferinol conjugate.

The cross-reactivity results presented in Tables 3.6 and 3.7 for individual animals 28 days post 2^{o} vaccination is summarised in Table 3.8 for each experimental group .

Table 3.6
ELISA analysis (means ± s.e.m.) of sera from vaccinated test animals with a mezerein-BSA conjugate plate coating (Average absorbance of controls has been subtracted.)

Control group	Abs.	"Mez"- Oval group	Abs.	"Res"- Oval group	Abs.	*"Pim* - C" group	Abs.
1001	0.156	1003	0.695	1007	0.452	1008	0.975
1002	0.106	1004	1.115	1009	0.533	1010	0.728
1006	0.118	1005	0.844	1012	1.024	1011	0.551
1016	0.121	1013	0.833	1014	0.476	1015	0.930
1019	0.116	1017	1.101	1020	0.486	1021	0.942
1023	0.144	1018	1.66	1024	0.889	1022	0.599

Table 3.7
ELISA analysis (means ± s.e.m.) of sera from vaccinated test animals with a resiniferinol-BSA conjugate plate coating.[a]

Control group	Abs.	"Mez" - Oval group	Abs.	"Res" -Oval group	Abs.	*"Pim* - C" group	Abs.
1001	0.148	1003	0.400	1007	0.353	1008	0.775
1002	0.103	1004	0.865	1009	0.302	1010	0.473
1006	0.11	1005	0.534	1012	0.814	1011	0.354
1016	0.110	1013	0.512	1014	0.343	1015	0.724
1019	0.102	1017	0.762	1020	0.270	1021	0.718
1023	0.148	1018	0.836	1024	0.641	1022	0.382

[a]Average absorbance of the control group has been subtracted from values given for experimental test groups.

Table 3.8
Summary of ELISA absorbance data[a] for 1/500 serum dilution in cross-reactivity assays

Vaccination group (n=6)	Mezerein -BSA plate coating	Resiniferinol-BSA plate coating
Pimelea C-Oval	0.79 ± 0.07	0.57± 0.07
Mezerein - Oval	1.04 ± 0.13	0.65 ± 0.07
Resiniferinol-Oval	0.65 ± 0.09	0.45 ± 0.08
Control	0.12 ± 0.007	0.12 ± 0.009

[a] Average absorbance of the control group has been subtracted from values given for experimental test groups. Values are given as means ± s.e.m.

These data supported our initial hypothesis that antibodies developed to commercially available daphnane orthoesters such as resiniferinol orthophenyl acetate and mezerein should also recognise the native daphnane toxins from *Pimelea* species. Of the two compounds, the data suggests that mezerein would be the preferred candidate for further studies since it gives a stronger antibody response and also provides better recognition of anti -*Pimelea* toxin antibodies than resiniferinol. A further advantage is that mezerein is the less expensive of the two compounds. [Unfortunately the *Pimelea* C - BSA conjugate (see Section 3.3.3) was not available at the time these antibody cross-reactivity assays were being conducted.]

In performing these cross-reactivity experiments, it would have been preferable to coat the toxin directly to ELISA plates by first dissolving the toxin in an organic solvent. Some preliminary experiments were conducted but coating these small organic toxin molecules in this manner proved capricious. There was a great deal of variability in the data, suggesting non-uniform retention of the toxin on the plate during the ELISA procedure. For this reason the BSA -toxin conjugates were used and reproducible results were obtained.

3.3.3 Time course of Antibody Responses in *Pimelea* C- Ovalbumin vaccinated Cattle.

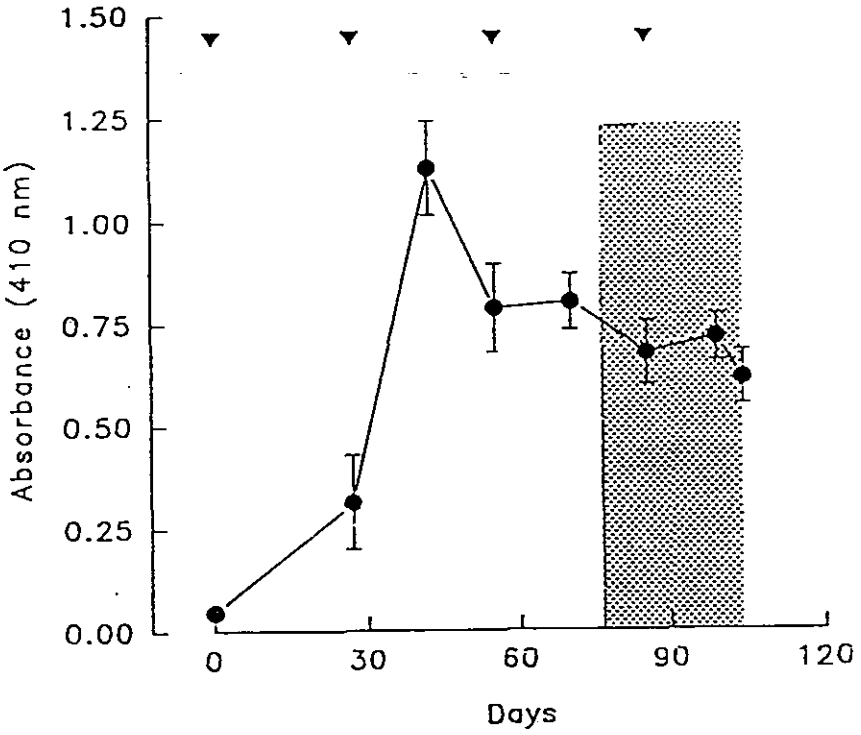
Since animals were vaccinated with the *Pimelea* C -ovalbumin conjugate, ELISA analysis of the antisera was conducted with a *Pimelea* C - BSA plate coating. It had

already been established that bovine anti-ovalbumin antibodies did not bind significantly to BSA.

In an effort to boost antibody responses in preparation for the toxin challenge experiment, the *Pimelea* C group (n=6) were given several booster injections and the time course of the antibody response over 104 days followed by ELISA analysis. The vaccination schedule for this experiment has been described in section 3.2.2 .

The data given in Figure 3.2 and Table 3.9 shows that all animals responded to the vaccination and that the highest antibody levels occurred after the second vaccination and then declined gradually despite additional booster vaccinations. Antibody titres were sufficiently strong to be detectable at 1:5000 serum dilution. Average absorbance of control animals (ovalbumin immunised) was subtracted from all test data. All analyses were conducted simultaneously using an identical protocol.

Figure 3.2
Time course of antibody responses in cattle vaccinated with *Pimelea* C-ovalbumin conjugate.



▼ : Day of vaccination.

Shaded area : Period during which cattle were intoxicated with *Pimelea* material.

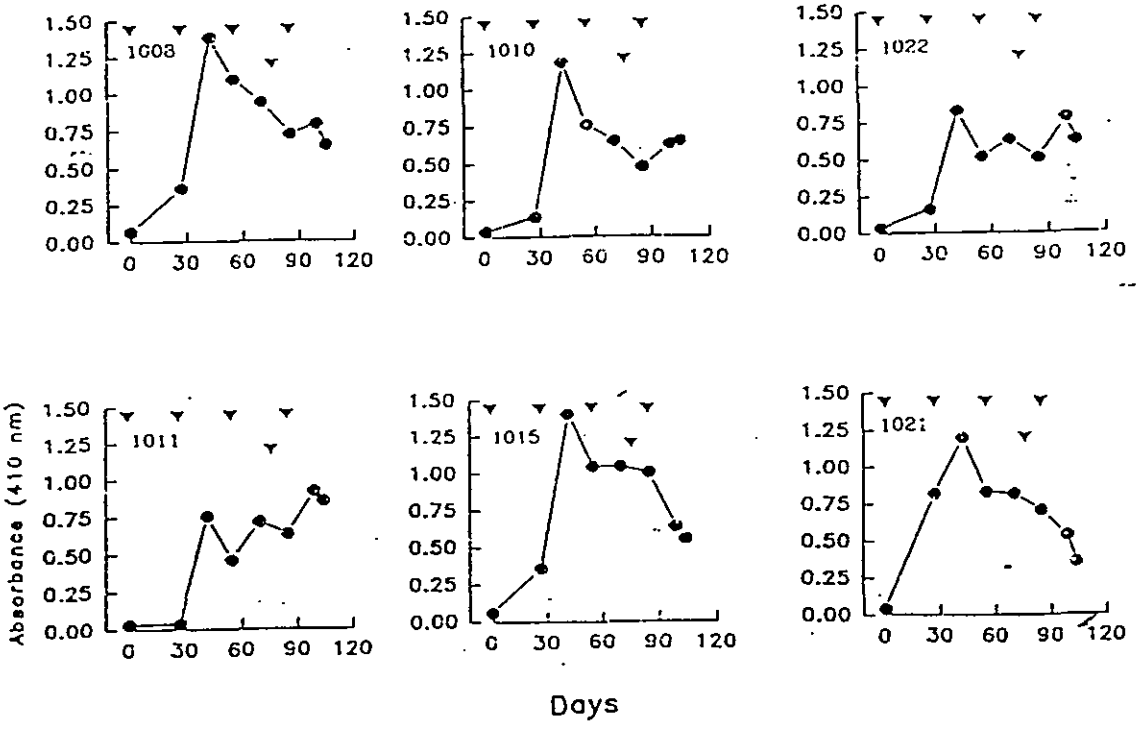


Table 3.9
Time course of antibody responses in *Pimelea* C- Ovalbumin vaccinated cattle.

Cattle no.	Serum dilution	Day 0	Day 27 (2°)	Day 42	Day 55 (3°)	Day 70	Day 85 (4°)	Day 99	Day 104
1008	1:500	0.067	0.365	1.388	1.098	0.723	0.941	0.789	0.649
	1:1000	0.031	0.124	0.999	0.727	0.385	0.541	0.351	0.366
	1:5000	0.014	0.025	0.233	0.123	0.023	0.042	0.033	0.029
1010	1:500	0.040	0.134	1.183	0.752	0.473	0.647	0.625	0.644
	1:1000	-	0.025	0.782	0.447	0.181	0.305	0.251	0.351
	1:5000	-		0.157	0.036	-	-	0.020	0.018
1011	1:500	0.031	0.039	0.753	0.458	0.634	0.717	0.920	0.855
	1:1000			0.376	0.186	0.298	0.412	0.566	0.544
	1:5000			0.027	0.006	-	0.017	0.072	0.064
1015	1:500	0.061	0.361	1.403	1.041	1.005	1.047	0.638	0.558
	1:1000		0.153	0.952	0.633	0.576	0.670	0.273	0.275
	1:5000			0.186	0.090	0.051	0.075	0.019	0.018
1021	1:500	0.037	0.820	1.201	0.834	0.708	0.818	0.538	0.355
	1:1000	0.081	0.334	0.790	0.501	0.272	0.431	0.223	0.129
	1:5000	0.121		0.121	0.045	0.004	0.031	0.019	0.004
1022	1:500	0.038	0.160	0.830	0.517	0.508	0.633	0.780	0.624
	1:1000		0.025	0.485	0.254	0.207	0.308	0.345	0.323
	1:5000		0.071	0.072	0.028	0.003	0.047	0.054	0.021

3.3.4 *Pimelea* Challenge Experiment with the *Pimelea* C -Ovalbumin Vaccinated Cattle

Control cattle (n=6) and cattle vaccinated against *Pimelea* C fraction (n=6) received an oral drench daily of milled *Pimelea* plant material as described in section 3.2.4. The dose was progressively increased after day 10 since the physical and clinical signs reported by Clark (1973) were not observed within the anticipated time. At the higher doses, both groups of cattle developed varying degrees of diarrhoea during the experiment. Several animals from both groups showed oedema of the lower jaw and upper brisket typical of *Pimelea* poisoning. By the end of experiment all the animals were severely emaciated, lethargic, had stopped eating and exhibited variable diarrhoea.

Overall, it would appear that vaccination against purified *Pimelea* C toxin fraction did not afford protection from ingested plant material at the doses administered. However, the response in vaccinated cattle requires further testing, since the 100 and 500 mg/kg live weight doses of *Pimelea* plant material were very high. According to Clark (1973), this dosage killed the cattle within 9 days. Although the ELISA results showed that specific antibodies against *Pimelea* toxins were present, the animals were probably overdosed compared to the levels of toxin they are likely to experience in the field. Therefore, any protective effects of the vaccines should be tested under field conditions.

Figure 3.3 compares the variations in red cell count RCC, haemoglobin Hb, lymphocytes and neutrophils for both the control and treated cattle group over the time course of the experiment. In earlier work, Kelly (1975) had reported falls in total leucocytes, lymphocytes, neutrophils and haemoglobin levels arising from the oral intoxication of cattle with *Pimelea* . Although the differences in these parameters between the control and vaccinated group were not significantly different at high toxin dosage, a consistent trend over the early part of the experiment (while toxin dosage was low) was that vaccinated cattle performed better than controls. This experiment needs

to be repeated with animals having a higher antibody response and with lower toxin dosage before any definite conclusions can be made.

As an adjunct to the toxin challenge experiment, the antibody response in the vaccinated group was compared before and after challenge with the toxin. Although it was considered unlikely that challenge with the plant would augment the antibody response, this had not been previously verified. The ELISA data given in Table 3.10 for 1:500 diluted serum shows that there was little difference in antibody binding for serum collected two weeks post secondary vaccination and serum collected after the toxin challenge experiment. It is therefore concluded that free *Pimelea* toxins are not immunogenic and do not invoke any further immune response in cattle previously vaccinated with protein-toxin antigen.

Figure 3.3
Neutrophil, red cell count, lymphocytes and haemoglobin levels (means ± s.e.m.) in vaccinated and control animals during the *Pimelea* challenge experiment

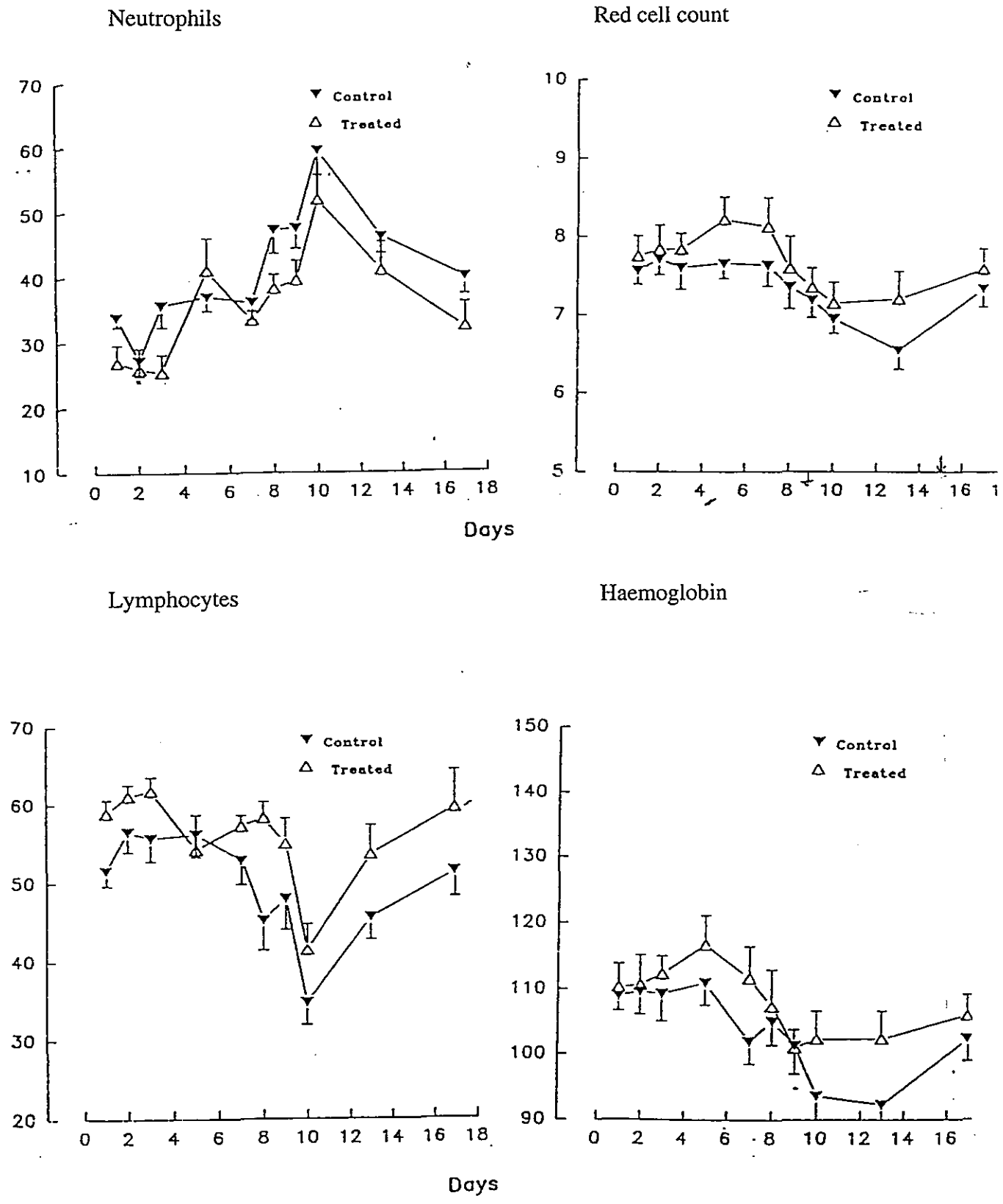


Table 3.10
Difference in IgG absorbances pre and post *Pimelea* challenge treatment.

Controls	Abs[a]. (2 wk post 2° vac)	Abs[a]. (Post treatment with *Pimelea* material)
0.237	1.169	1.168
0.167	1.443	0.991
0.312	1.113	1.530
0.252	1.726	1.388
0.418	1.531	0.904
0.174	1.239	1.282
0.26 ± 0.094	1.45 ± 0.10	1.21 ± 0.10

[a]Control average absorbance was subtracted from each of tested serums (from vaccinated cattle). Values are means ± s.e.m.

3.3.5 The Effect of Freeze Drying Purified IgG from Rabbits and Cattle

As explained earlier in this chapter, IgG was purified from serum collected from vaccinated cattle and rabbits and then freeze dried for use in the *in vitro* organ bath studies (Chapter 4). We were interested in comparing ELISA binding data for serum and an equivalent amount of the freeze dried IgG fraction. As shown in the data given in Table 3.11, antibody binding in fresh cattle serum was much higher than an equivalent amount of purified freeze dried IgG material. In contrast, there was minimal loss of specific binding observed for the purified freeze dried rabbit IgG. These findings may help to explain the results of *in vitro* studies reported in Chapter 4, where the rabbit IgG was shown to be protective against the toxins, whereas the bovine IgG lacked any efficacy.

Table 3.11
Comparision of ELISA absorbance data for serum and freeze dried IgG.

	Cattle IgG absorbance[a]	Rabbit IgG absorbance[a]
Purified freeze dried IgG	0.623	1.325
Serum	1.670	1.502

[a]average absorbance normal rabbit serum (0.259) has been subtracted from each of the tested serum.

3.4 CONCLUSIONS

ELISA results for serum obtained from vaccinated cattle and rabbits showed that specific antibodies were raised against *Pimelea* toxin- protein conjugates. There was a significant rise in IgG titres after secondary vaccination compared with primary vaccination. The data given in Figure 3.1 and 3.2 shows that all animals responded to the vaccination and that the highest antibody levels occurred after the second vaccination and then declined gradually despite additional booster vaccinations. Antibody responses were sufficiently strong to be detectable at 1:5000 serum dilution.

Attempts were made to prove that antibody binding could be displaced with free toxin. In general, these experiments achieved 20 -50% displacement and while this might reflect methodological difficulties (the toxins being insoluble in aqueous media), it might also help explain the lack of protection in cattle exposed to plant material.

The data given in Table 3.11 shows that antibody titres in fresh cattle serum are higher compared to purified freeze dried IgG material. There was a significant loss of antibody binding associated with purification of the IgG fraction from serum. In contrast, there was no apparent loss of binding observed for the purified rabbit IgG fraction. This difference may either have been due to unknown problems associated with the purification procedure or perhaps reflects a species difference in the immune responses.

Control cattle (n=6) and cattle vaccinated against *Pimelea* C fraction (n=6) received an oral drench daily of milled *Pimelea* plant material as described in section 3.2.4. Taken overall, it appeared that vaccination against purified *Pimelea* C toxin fraction did not afford protection from ingested plant material at the doses administered. However, the response in vaccinated cattle requires further testing, since the 100 and 500 mg/kg live weight doses of *Pimelea* plant material were very high. According to Clark (1973), this dosage should kill the cattle within 9 days. Although the ELISA results showed that specific antibodies against *Pimelea* toxins were present, the animals were probably overdosed compared to the levels of toxin they are likely to experience in the field.

4.0 CHAPTER 4

4.1 INTRODUCTION

As discussed in the introductory chapter, the primary mode of action of *Pimelea* toxins in cattle is sustained contraction of the pulmonary venule system. Clark (1973) showed that intravenous injection of an ethanolic extract derived from *Pimelea trichostachya* dried plant material (at doses above a dried-plant equivalent of 130 mg kg^{-1} bodyweight threshold) caused rapid cardiovascular effects in cattle. Within 10 seconds of intravenous administration of the dose, systemic arterial pressure halved while right ventricular pressure doubled. These results were suggestive of constriction of the pulmonary venous system.

An increase in right ventricular pressure and dilatation of the right side of the heart follows as a natural consequence of chronic *Pimelea* poisoning in cattle grazing in the field. Following logically from the *in vivo* cardiovascular observations of Clark (1973), Mason (1976) reasoned that constriction of the pulmonary venule system was the most immediate effect of the toxins. Kelly and Bick (1976) and Mason (1976) performed *in vitro* studies of bovine pulmonary venule preparations using rudimentary organ bath techniques available at the time and showed that an alcoholic extract derived from *Pimelea trichostachya* caused contraction of the venule tissue. These studies showed that the effects of the toxins were essentially irreversible, and the contraction could not be washed out by replacing the organ bath buffer solution. These observations are consistent with highly lipophilic toxins such as the daphnane orthoesters now known to be present in the plant.

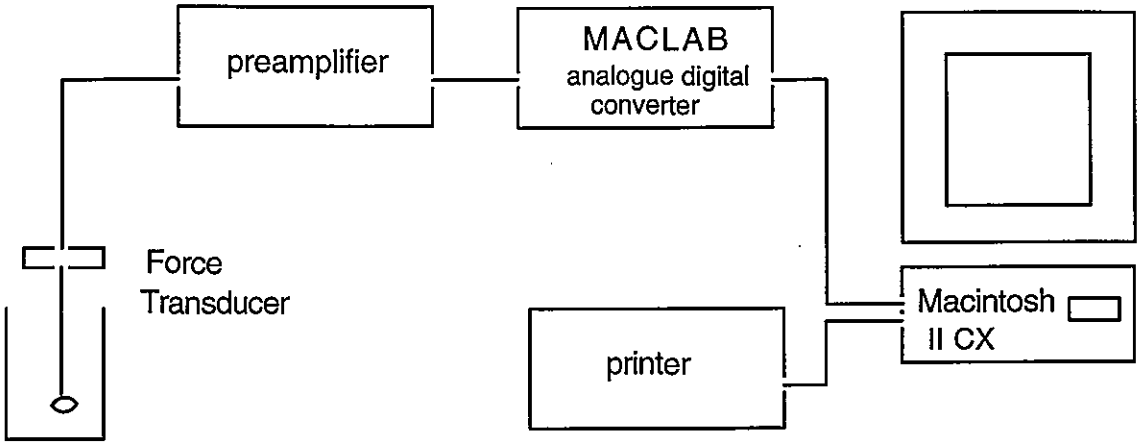
In the studies described in this Chapter, physiologically viable pulmonary venule preparations dissected from fresh lung tissue were used for organ bath studies. The first aim of these *in vitro* studies was to establish that purified *Pimelea* toxins (ie. Fraction C comprising simplexin and other structurally similar daphnanes) did cause contraction of pulmonary venule preparations. This had not previously been established in the literature since the only previous work had used a large amount of a crude alcoholic extract of the plant. Once the efficacy of Fraction C had been confirmed, a dose-response curve would need to be determined in order to establish the EC_{50} concentration of the toxins that caused half maximal contraction. The toxin concentration in the bath would then be fixed at the EC_{50} level and the efficacy of purified antibodies (from *Pimelea* vaccinated cattle and rabbits) to attenuate the contractile response would be investigated. It was also planned to use the organ bath technology to explore the potential of possible inhibitors of *Pimelea* toxicity such as PKC inhibitors and calcium channel blockers.

4.2 METHODOLOGY

4.2.1 General

In all experiments, the force of contraction was measured using Grass FT03 Force Transducers connected to a Transbridge TBM4 amplifier manifold (World Precision Instruments Ltd) and recorded for subsequent analysis via a MacLab 8 using Chart software (AD Instruments Ltd) running on a Macintosh II CX microcomputer. The layout of the four channel system and the 25 mL organ baths employed (manufactured by Mr Tom Jeston of James Cook University, Queensland, Australia) is shown in Figure 4.1. A schematic layout of the system is given in Figure 4.2. The design of the baths was such that bubbling of carbogen in the side chamber caused a continuous circulation of the buffer in the larger sample chamber.

Figure 4.2
The layout of the four channel system.



Preparation of Normal Tyrodes Solution and Tyrodes Solution Containing 100 mM KCl

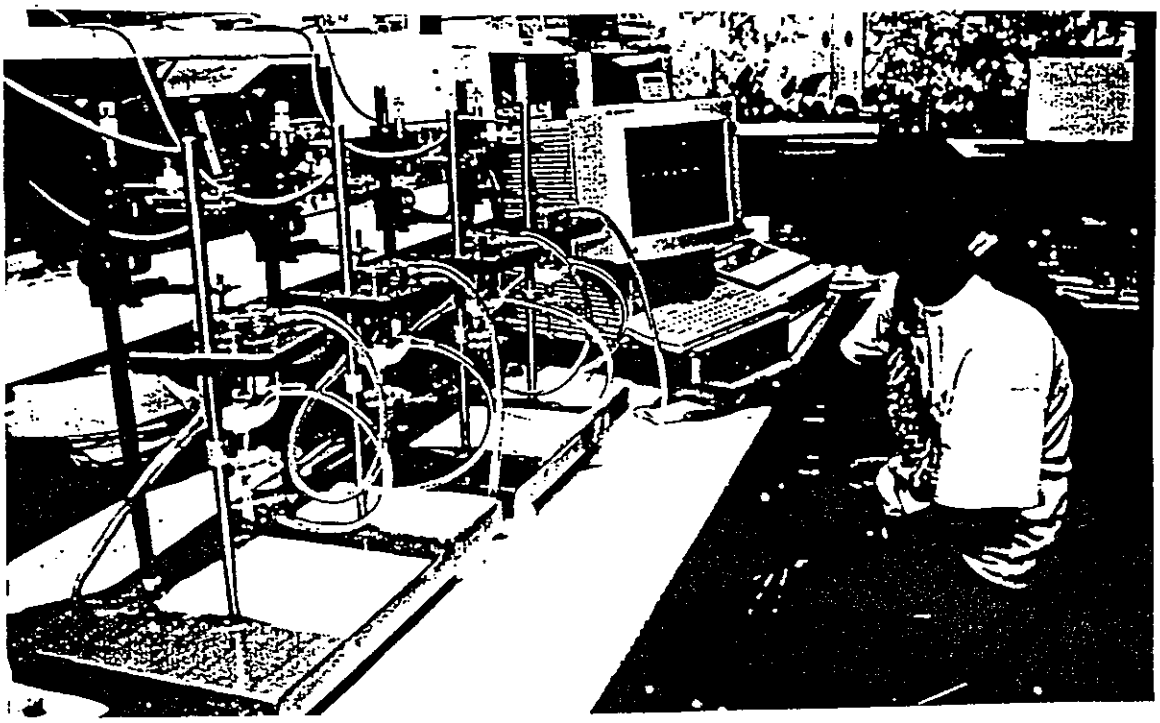
For preparation of Tyrodes solution and Tyrodes solution containing 100 mM KCl, Stock A, Stock B and Stock C solutions were prepared and then stored at 4° C. The same B and C stock solutions were used for both forms of the buffer. Fresh Tyrodes solutions were prepared each day and remaining stock solutions were always discarded after two weeks.

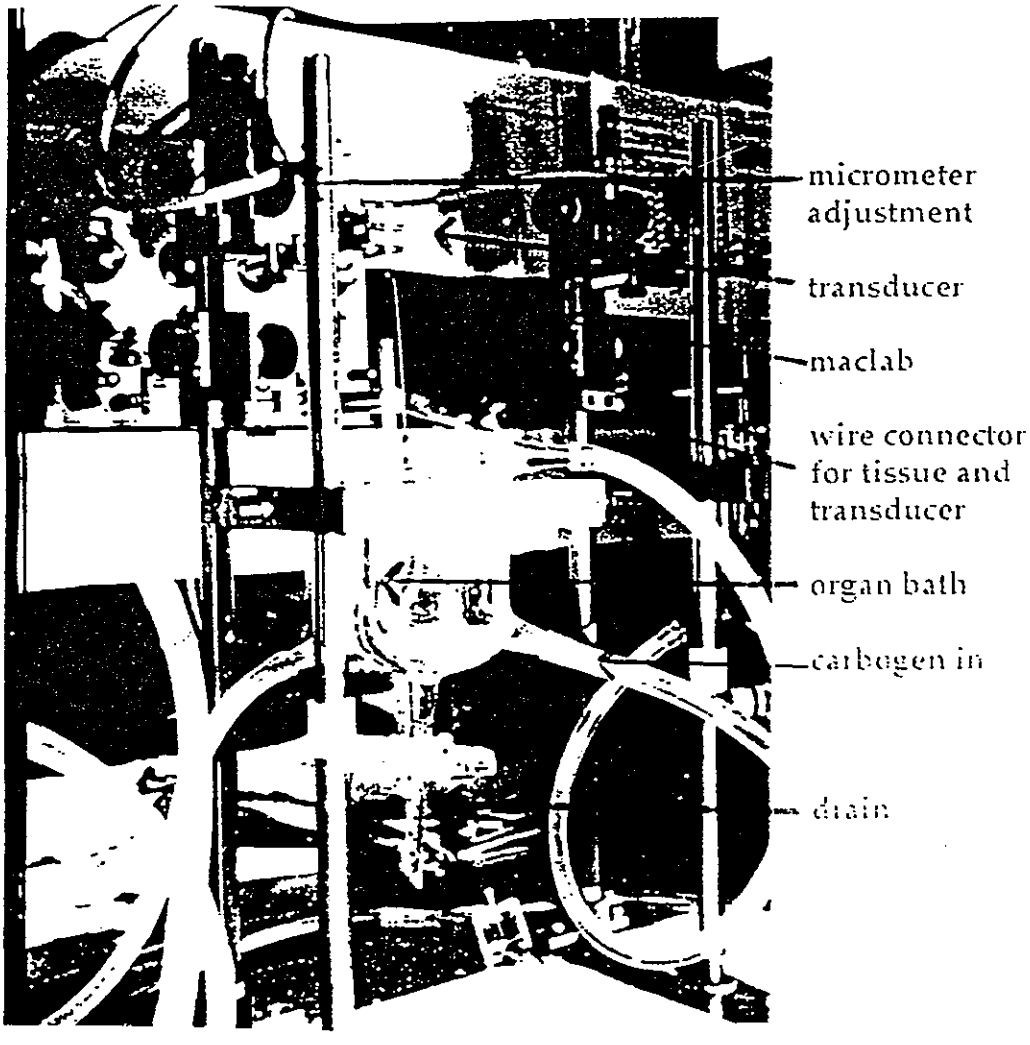
Stock A for Tyrodes solution containing 100 mM KCl :

The chemical composition for 2 L of modified Stock A solution is given below. The solution was prepared using demineralised water.

Chemical	Weight (g)	Concentration
NaCl	98.9	0.85 M
KCl	300	2.0 M
$MgCl_2.H_2O$	8.6	38 mM
$NaH_2PO_4.2H_2O$	2.6	8.3 mM

Figure 4.1
The four channel computerised organ bath system





Normal Stock A solution

Chemical composition and final component concentrations for 2 L of normal Stock A solution is given below. Solutions were prepared using demineralised water.

Chemical	Weight (g)	Concentration
NaCl	320	2.74 M
KCl	16.2	108 mM
$MgCl_2.H_2O$	8.6	38.0 mM
$NaH_2PO_4.2H_2O$	2.6	8.3 mM

Stock B

Stock B solution was 0.45 M sodium bicarbonate and was prepared by dissolving 76.0 g (0.45 mM) of $NaHCO_3$ in 2 L of demineralised water.

Stock C :

Stock C solution was prepared by dissolving 10.6 g (36 mM) of $CaCl_2.2H_2O$ in 2 L of demineralised water.

Working 100mM KCl Tyrodes Solution

To prepare a working 100 mM KCl Tyrodes solution, Stock A (100 mL), Stock B (100 mL) and Stock C (100 mL) and 2 g of glucose were made up to a 2 L final volume with demineralised water in a volumetric flask. (Final concentration of different component in 100 mM KCl tyrodes solution in mM; NaCl 42.3, KCl 100, $MgCl_2.H_2O$ 1.90, $NaH_2PO_4.2H_2O$ 0.42, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8)

Working Tyrodes Physiological Solution

The working Tyrodes (physiological) solution was prepared exactly as described above for the 100 mM KCl Tyrodes except that normal Stock A solution was used. The pH of this solution is normally 5.4 although after bubbling carbogen through the chilled solution for 30 minutes, the pH rises to approximately 7 because of dissolved CO_2. (Final concentration of different component in tyrodes solution in mM; NaCl 136.9, KCl 5.4, $MgCl_2.H_2O$ 1.90, $NaH_2PO_4.2H_2O$ 0.42, $NaHCO_3$ 22.6, $CaCl_2.2H_2O$ 1.8)

4.2.2 Dissection of pulmonary venules from bovine lung tissue

The tips of bovine lung lobes (approx 150 g) were removed from whole lungs within 30 minutes of slaughter of the animals at the abbatoir and after inspection by AQIS Officers. The lung tissue was then immersed in chilled, pre-carbogenated Tyrode solution and transported to the laboratory on ice. Small pulmonary venules were dissected free from the branches of bronchioles and cleared of fat and connective tissue.

4.2.3 Mounting tissue preparations in the organ baths

After dissection, the venules were cut into ring sections of approximately 3 mm in length and diameter, mounted on support posts and loosely connected to the Force Transducers via a wire connector. The tissue preparation needs to be thin enough to allow oxygenation of the innermost layers of the tissue and for adequate removal of

waste products by diffusion. Rings of between 0.5 to 5 mm thickness generally meet these requirements. The preparations were then suspended in 25 mL water jacketed organ baths (35° C) circulated with Tyrode solution and bubbled with carbogen (95% O_2 and 4% CO_2). The baths were designed so that carbogen was not bubbled directly on the tissue to avoid mechanical damage to the preparation. Gas flow was set at the beginning of the experiment and was maintained at the same level throughout, thereby preventing variations in gas tension which may effect the metabolism of the tissue.

After allowing the tissues to equilibrate in the baths, the preparations were gradually brought up to pre-load tension. Preload tension was applied using micro and macro adjustment mechanisms on the support posts. The precise engineering of the stands allowed adjustment of tension down to 0.1 mN increments. Care was taken to avoid damaging the tissue by applying excessive force at any time during the mounting procedure or during the pre-load adjustment phase. Tissues were equilibrated for 60-70 minutes, with exchange of the bath buffer every 20 minutes. Over this time the pre-load tension on each preparation was adjusted to the optimum 25 mN.

4.2.4. Determination of Optimum Pre-load Tension

Optimum pre-load tension to obtain maximum contractile response to 100 mM KCl for the preparations was determined by repeatedly challenging three pulmonary venule rings with 100 mM KCl Tyrodes solution at various pre-load tensions (1, 5, 10, 20 and 30 mN) and the maximum contraction produced in each case was measured. The weights of the tissue preparations used in these initial experiments ranged from 2.0 - 12.6 mg. (Once the techniques had been established, most tissue preparations used were in the 2-3 mg range.)

4.2.5 Contractile Responses to 5-HT (1μM and 3μM) and 100 mM KCl

The maximal contraction of the venule preparations in response to 5HT (5-hydroxytryptamine) and Tyrodes solution containing 100 mM KCl was determined as follows. Venule ring preparations were set to approximately 25 mN pre-load tension in normal Tyrodes buffer and the tissue then exposed to Tyrodes solution containing 100 mM KCl. The maximum tension produced in response to KCl was measured when the contraction of the tissue had stabilised. The maximum contraction was calculated by subtracting the pre-load tension from the maximum tension produced in response to 100 mM KCl.

As it took more than one hour for the maximal response to 100 mM KCl to be achieved in most cases, the maximal contractile response of the venule preparations to (5-HT) (Sigma) was also investigated. Mason (1976) had also used 5-HT in earlier experiments to demonstrate normal function and sensitivity of the preparation before experimentation. Both 1 μM and 3 μM 5-HT concentrations in the bath were trialed and it was found that maximal response was achieved with the lower concentration. The contractile response to 5-HT was readily reversible by wash-out and replacement of the chamber buffer with fresh Tyrodes solution. It was observed that preparations maintained for over 6 hours in Tyrodes solution at 37° C remained relaxed throughout this time but would still then respond normally to challenge with 1.0 μM 5-HT.

4.2.6 Preparation of *Pimelea* C fraction

Despite having been purified by solvent partition, silica column chromatography and preparative reverse phase HPLC, the *Pimelea* C toxin fraction (see Chapter 2) remained a complex mixture of four major components. For the purposes of the organ bath studies, it was assumed that the average molecular weight of the toxin components was

500 g/mol. The daphnane and tigliane components of *Pimelea* which have been described by other workers generally have molecular weights ranging over 500-700 mass units. A stock solution of the *Pimelea* C fraction (1 mM) was prepared by dissolving 70 mg in 1.4 mL of HPLC grade methanol and this solution was stored at -20°C in darkness. From this stock solution, fresh serial dilutions were prepared every two weeks and stored in freezer and protected from sunlight. Based on the assumed molecular weight of 500, aliquots of the stock solution were added to the organ bath directly in order to achieve the required concentration in the 25 mL total volume (given that the average molecular weight of the toxins in the *Pimelea* C fraction was expected to be greater than 500 mass units and that it was unlikely that all components in the mixture would cause contraction of the venules, the calculated molar concentrations used in these experiments are likely to be over-estimates).

4.2.7 Experimental Protocol

Preparations were equilibrated to 25 mN as described above and thereafter the Tyrodes solution was exchanged at approximately 20 minute intervals. After 75 minutes, the preparations were subjected to 1μM 5-HT to determine maximum contractile response for each individual tissue. The response usually stabilised after 30 minutes and the 5-HT was then washed away by exchanging the Tyrodes solution three times at 15 minute intervals, thereby allowing complete relaxation of the tissue. The preparations were then re-equilibrated to approximately 25 mN. Once the preparations were stable, a small volume of a methanolic solution of the toxin was added and the effect on the contraction of the venule was observed until the tissue was stabilised. The *Pimelea* toxins cause a slowly developing contraction after a latent period of 15-30 minutes and it usually took 300 minutes for maximum contraction to be achieved.

The concentration of methanol in the bath never exceeded 0.3% (v/v). Preliminary experiments had shown that methanol concentrations up to 1.0% (v/v) had no effect on the contractile response. Each tissue preparation was subjected to only one concentration of toxin. The experimental apparatus available allowed four tissue preparations to be studied simultaneously. Any tissue which responded abnormally in the pre-load sequence or to 5-HT was rejected. Occassionally, some tissues gave normal responses in the set-up phase but then gave a spasmodic contractile response to the toxin such that waves of contraction and then partial relaxation were observed. These preparations were also rejected.

Experiments involving different toxin concentrations were assigned randomly to the baths so that results from all baths contributed to the replicates for any one concentration. Usually, the response to a particular toxin concentration was measured 4-6 times. The baths were cleaned at the completion of each experiment and then filled with absolute alcohol and flushed with demineralised water. The baths were subsequently cleaned with nitric acid and flushed repeatedly with demineralised water so as to avoid cumulative contamination with toxins.

4.2.8 Experiments with Antibodies and Inhibitors

To test the attenuating effect of purified antibodies from vaccinated rabbit or cattle serum on the contraction produced by *Pimelea* toxins, venule preparations were incubated with the freeze dried IgG dissolved in water (200 μL) for 30 minutes before adding the EC_{50} concentration of *Pimelea* toxin. The contractile response was then compared to the response obtained from one or more control preparations exposed only to the EC_{50} toxin concentration. Wherever possible, the control and "treated" preparations were dissected from the same venule.

Determination of EC_{50} required for the contraction of Bovine Pulmonary Venules by Mezerein

Mezerein (a daphnane orthoester) is a tumour promoter similar in structure to the *Pimelea* toxins and is known to activate PKC both *in vitro* and *in vivo* (Miyake *et al.*, 1984). The EC_{50} Mezerein concentration required for contraction of bovine pulmonary venules was determined and then the effects of known PKC inhibitors in attenuating the contraction produced by mezerein (EC_{50}) was investigated. As with the *Pimelea* toxins, mezerein was dissolved in methanol for these experiments.

Preliminary experiments with the PKC inhibitors, D-*erythro*--sphingosine derived from bovine brain (200 µM dissolved in 200 µL ethanol) and polymyxin B sulfate (250 µM dissolved in 200 µL water), (Calibochem Corporation), were conducted in a similar manner to the antibody experiments. After wash out of the 5-HT response, the preparations were equilibrated for 30 minutes in the presence of the PKC inhibitor (D-*erythro*--sphingosine or polymyxin B sulfate) prior to adding the mezerein (EC_{50}).

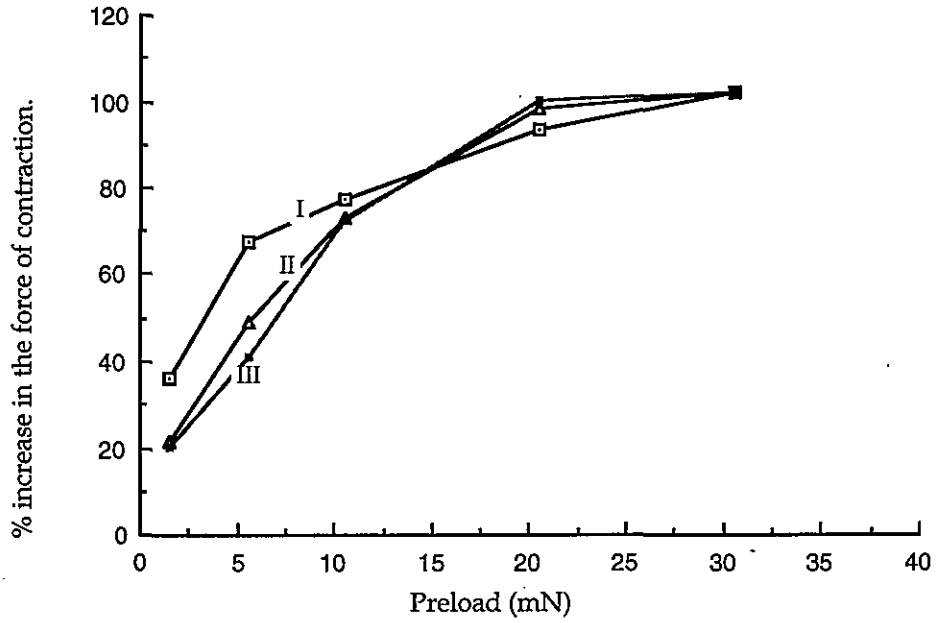
To test the attenuating effect of *Nux vomica* CM tincture (containing icajine, novacine, vomicine, pseudostrychnine, pseudobrucine) (Bratati and Bisset,1991) on the contraction produced by the EC_{50} concentration of *Pimelea* toxins, preparations were exposed to an aliquot of *Nux vomica* CM tincture (Homeopathic medicine, Brauer Biotherapies Pty Ltd) immediately followed by addition of the EC_{50} *Pimelea* toxin concentration. Control preparations were exposed only to the EC_{50} *Pimelea* toxin concentration. The contraction was monitored over 200-300 minutes as previously described. Experiments were also conducted to establish the response of the preparations to 200 µL of *Nux vomica* CM tincture in the absence of added toxin. After 300 minutes exposure, the response of the tissue preparations to 1.0 µM 5-HT was examined both before and after wash-out of the *Nux vomica* .

4.3 RESULTS AND DISCUSSION

4.3.1 Pre-load determination

]Pre-load is the tension applied to the preparation before testing it with the contractile agent. The force of contraction produced usually increases as the pre-load tension is increased until the optimum conditions are reached. Thus, optimum pre-load is the tension applied to the vessel at which maximum contraction is produced. At this tension, the tissue preparation is stretched so that myosin and actin filaments are at the optimum angle for initiation of the contractile force. The data given in Figure 4.3 and Table 4.1 are the results obtained from testing the responses of three separate tissue preparations to 100 mM KCl.

Figure 4.3
Determination of optimum preload.



Abbreviations : I : Preparation no. 1; II : Preparation no. 2; III : Preparation no. 3.

As there was only a slight increase in maximum response obtained for all tissues between the 20-30 mN pre-load range, the optimum pre-load tension was taken to be 25 mN. These results were obtained with tissue preparations ranging in weight from 2-12 mg so the physical size of the preparation did not appear to have a major effect on the optimum pre-load tension.

Table 4.1
Determination of optimum preload for contraction of bovine pulmonary venule preparations

	Preparation - 1		Preparation - 2		Preparation - 3	
Pre-load mN [a]	Δ mN [b]	% contraction [c].	Δ mN	% contraction.	Δ mN	% contraction.
1	34	34 %	14	19 %	29	18 %
5	67	65 %	35	47 %	64	39 %
10	77	75 %	53	71 %	115	70 %
20	94	91 %	72	96 %	160	98 %
30	103	100 %	75	100 %	164	100 %

[a] *Preload mN* — different tensions at which vessels were stretched before inducing contraction with 100 mM KCl .

[b] *Δ mN* — maximum force of contraction induced by 100 mM KCl calculated by subtracting the Pre-load applied from the measured maximum contraction.

[c] *% contraction* — For each preparation force of contraction produced at different preloads were calculated as a % of the maximum contraction produced at 30 mN pre-load.

4.3.2 Maximum Contractile Responses to 5-HT (1 μM and 3 μM) and 100 mM KCl

The contractile behaviour of each bovine pulmonary preparation was expected to be dependent on numerous factors such as the size of the tissue ring, the ratio of collagen and muscle present, genetic variations between animal breeds, the age of the animal, receptor densities, time variations from the death of the animal and dissecting out the tissue, and damage to the tissue during preparation. Hence to allow comparison between tissue preparations. the maximum contractile response for each individual tissue was determined at the commencement of the experiment and then any contraction measured during the experimental phase expressed as a percentage of this maximum response.

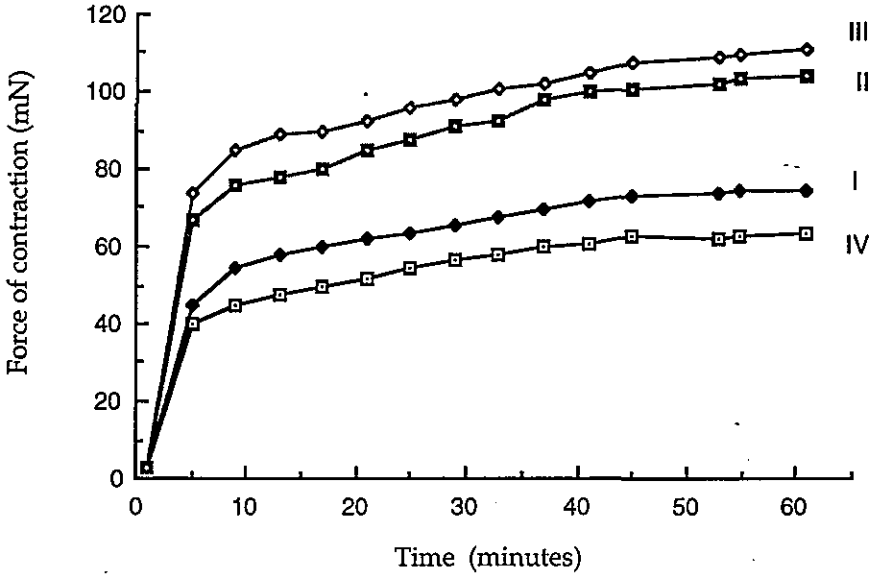
A requirement for the determination of maximal contractile response was that the agent used should be fast acting and also be conveniently washed out by replacement of Tyrodes solution. The tissue should then rapidly re-equilibrate to the pre-load tension prior to being contracted with the test agent. Tyrodes solution containing 100 mM KCl was first investigated as it is often used for this purpose in organ bath experiments. With bovine pulmonary venule preparations however, maximal response to 100 mM KCl was never achieved in less than 1 hour and this was considered too time consuming for our purposes. The data obtained from these preliminary experiments are given in Figure 4.4 and Table 4.2.

Table 4.2
Contractile responses of bovine pulmonary venule preparations to 100 mM KCl

Preparation No.	Preload (mN)	Δ mN [a]	Weight of tissue (mg)
1	24.5	72	2.8
2	23.9	101.5	2.9
3.	25.4	108.5	7.4
4	23.1	61.5	4.7

[a] Maximum contractile response minus pre-load tension.

Figure 4.4
Determintion of maximum contractile response to 100 mM KCl.



Abbreviations I : Preparation no. 1; II : Preparation no. 2;
III : Preparation no. 3 ; IV : Preparation no 4

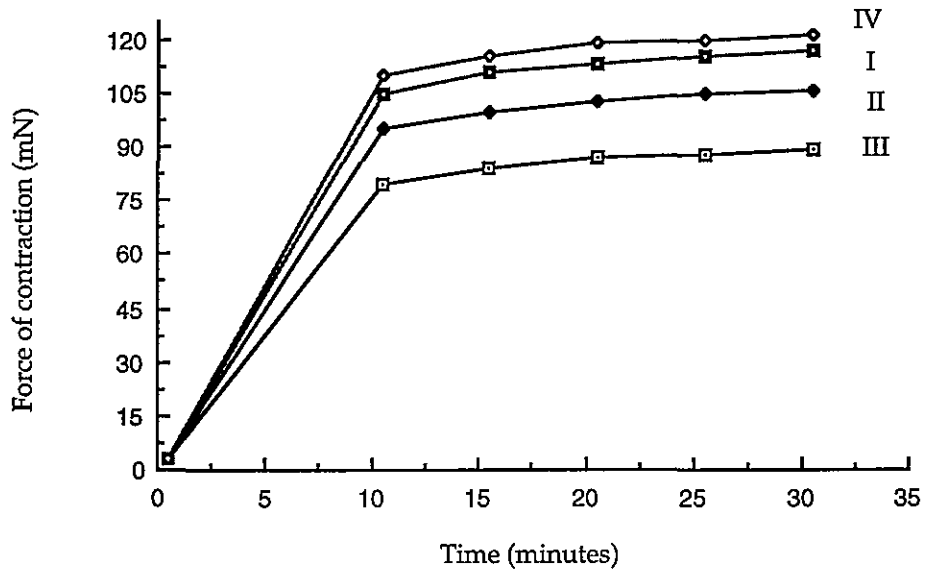
The maximal contractile response of the preparations to 1μM and 3μM concentrations of 5-hydroxytryptamine (5-HT) were determined. It was found that maximal response was achieved even with the lower concentration after 30 minutes (see Figure 4.5 and Table 4.3). The contractile response to 5-HT was observed to be readily reversible by wash-out and replacement of the chamber buffer with fresh Tyrodes solution. Tissue preparations which had been contracted with 1 μM 5-HT and then washed out remained relaxed over 6 hours in Tyrodes solution at 37° C, but then still gave the maximum response to further challenge with 1.0 μM 5-HT.

Table 4.3
Contractile responses of bovine pulmonary venules to 5-HT

Preparation No.	Preload (mN)	Δ mN [a] (1μM 5HT)	Δ mN [a] (3 μM 5HT)	weight of tissue (mg)
1	22.1	114.2	111.7	2.8
2	23.4	102.7	101.1	2.9
3	23.9	86.5	74.1	7.4
4	23.7	118	109.8	4.7

[a] Maximum contractile response minus pre-load tension.

Figure 4.5
Determination of maximum contractile force in response to 1μM 5HT.



Abbreviations I : Preparation No. 1; II : Preparation No. 2;
III : Preparation No. 3 ; IV : Preparation No 4

4.3.3 Concentration / Response Curve for the Contraction of Bovine Pulmonary Venules by *Pimelea* Toxins.

Mason (1976) reported an *in vitro* contraction of bovine pulmonary venules in response to 10 mg of a crude alcoholic extract of *Pimelea trichostachya* dissolved in 40 μL of ethyl alcohol. In the present study, the *Pimelea* -C toxic fraction employed had been purified from the crude alcoholic extract by solvent partition, silica column chromatography and preparative reverse phase HPLC. It was expected that the *Pimelea* -C fraction would prove much more efficacious in the *in vitro* studies compared with the crude extract used by Mason (1976).
Figure 4.6 shows typical slow developing contraction over a period of 300 minutes in bovine pulmonary venule in response to *Pimelea* toxins.

Repeated experiments were undertaken in order to establish the concentration-response relationship for the contraction of bovine pulmonary venules by the *Pimelea* -C toxic fraction. This data is presented in Table 4.4 and from the plot of the averaged results (Figure 4.7), the EC_{50} concentration was determined. The EC_{50} value was taken as the apparent toxin concentration required to produce half the maximal response produced by 1 μM 5-HT.

Figure 4.6
Slow developing contraction in bovine pulmonary venule in response to *Pimelea* toxins.

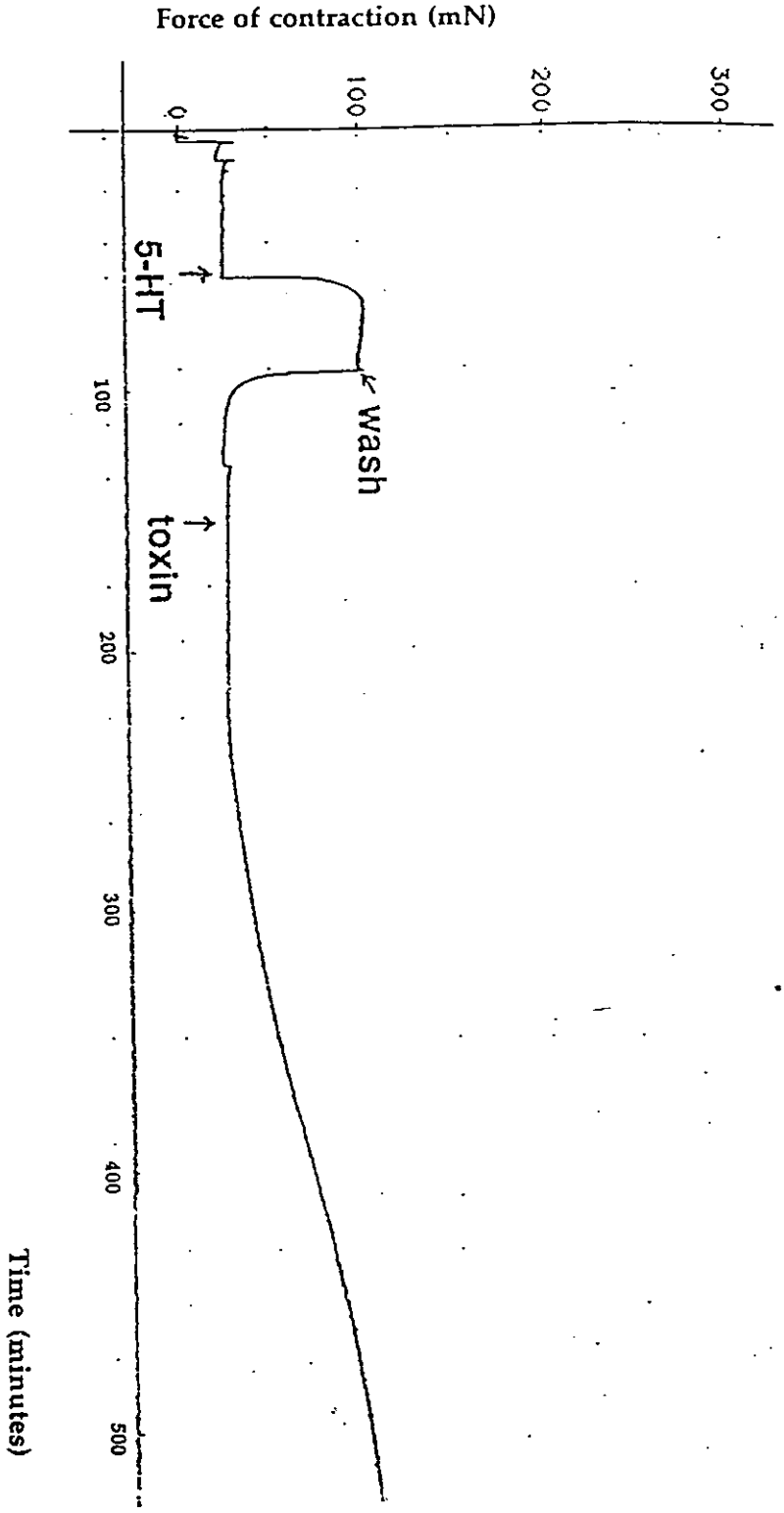


Figure 4.7
Concentration response data for *Pimelea* toxins on bovine pulmonary venule preparations. (Values are means ± s.e.m.)

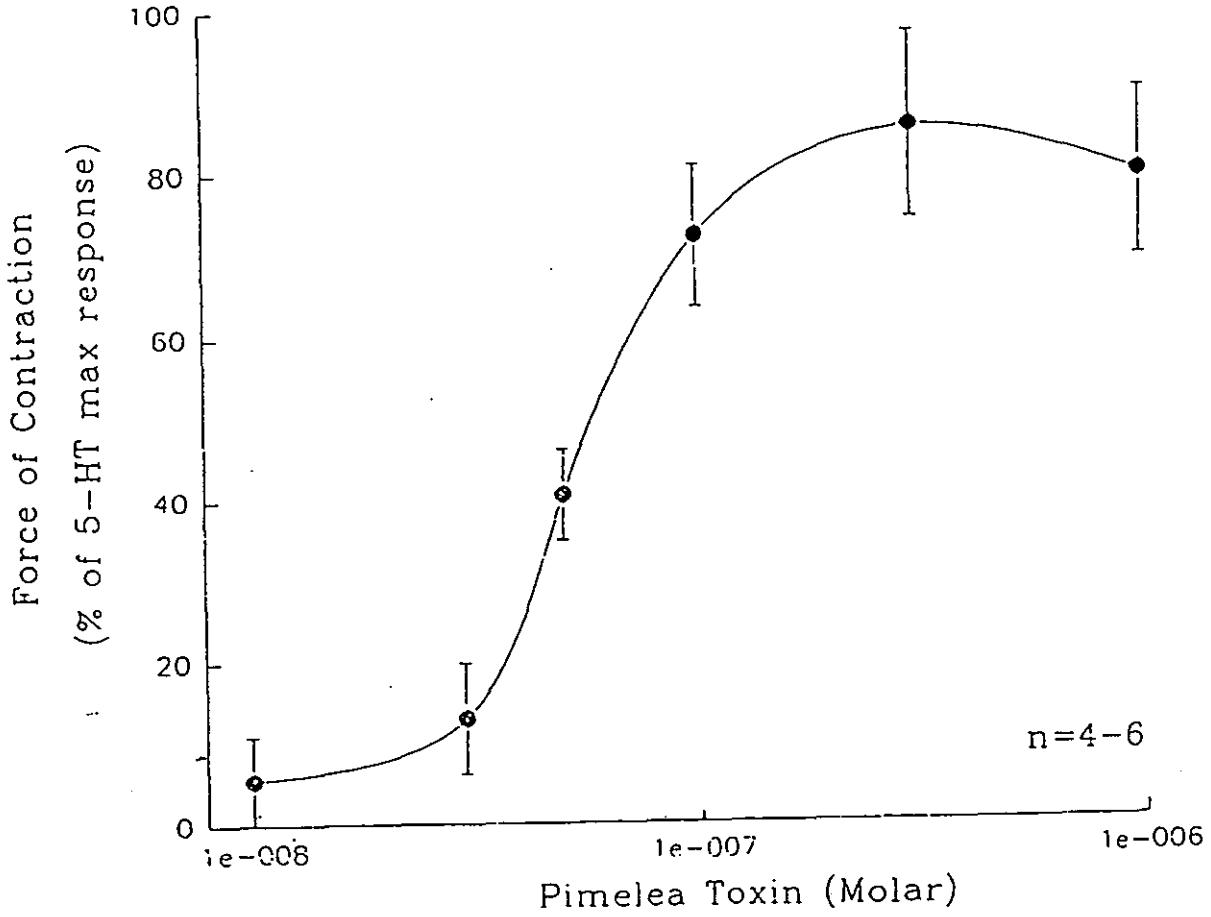


Table 4.4

In vitro contractile responses of bovine pulmonary venules to *Pimelea* -C fraction.

[*Pimelea* -C] µM [a]	Maximum contraction (Δ mN) [b]	*Pim.elea* contraction (Δ mN) [c]	% Contraction by toxin [d]	Weight of tissue (mg).
0.01	180.7	0	0	6.3
	192.6	78.3	40.7	6.3
	162	0	0	6.5
	108.6	0	0	6.9
0.03	192.3	57.7	30	6
	99.3	18.2	18.3	7
	108.5	3.8	4	5.7
	108	0	0	6.2
0.05	127	57.7	45.5	6.2
	179.3	89.6	51	6.0
	172	43.9	25.5	3.4
	132.8	53.4	40.4	5.4
0.10	155.2	78.9	50.83	8.3
	169.8	163.0	96	--
	129.9	80.9	62.3	7.6
	81.9	95.9	117	3.7
	150.3	139.8	93	4.4
	156.8	120.7	77	7.4
0.30	128.3	77.4	60.3	--
	150.8	149.3	99	5.7
	78	79.6	102	3
	112.7	142	126	6.2
	192.2	102.8	53.5	8
	95	66	69.5	5
1.0	144.2	103.8	72	7
	110.8	100.6	90.8	4.6
	145	162.4	112	4.6
	126.6	49	70	7.3
	221	112.7	51	7

[a] Based on assigned molecular weight of 500 g $mole^{-1}$. [b] Maximum contraction to 1.0 µM 5 -HT minus pre-load tension. [c] Maximum contraction in response to *Pimelea* toxin minus the pre-load tension. [d] Expressed as a % of the 5-HT maximal response.

From the concentration-response curve (Figure 4.7), the EC_{50} concentration was calculated to be 5.28×10^{-8} M (0.053 µM). The bovine pulmonary venule preparations did not respond to 0.01 µM toxin concentration (10.17 ± 10.17 %, n=4, mean ± s.e.m.) and minimum concentration required to produce maximum contraction was 0.3 µM (85.05 ± 11.55 %, n=6, mean ± s.e.m.). Although there is considerable variation in the results, it must be remembered that this data was derived over many weeks from tissue preparations of differing size from a large number of animals. For each toxin concentration increment, pulmonary venule tissue from at least four separate animals was investigated. The data is presented in summary form in Table 4.5.

Table 4.5
Summary of contractile responses of bovine pulmonary venules in response to *Pimelea* C fraction. (Values are means ± s.e.m.)

Pimelea -C µM	% Contraction relative to 1.0 µM 5-HT	Weight of tissue (mg).
0.01	10.2 ± 10.2, n=4.	6.50 ± 0.14
0.03	13.1 ± 6.9, n=4	6.22 ± 0.27
0.05	40.6 ± 4.9, n=5	5.25 ± 0.56
0.10	82.7 ± 9.9, n=6	6.28 ± 2.47
0.3	85.0 ± 11.5, n=6	5.93 ± 0.73
1.0	80.0 ± 10.3, n=5	6.1 ± 0.61

4.3.4 *In Vitro* Effects of Purified Rabbit Antibodies

In the previous chapter, ELISA data was presented which suggested that a specific antibody response had been developed in both rabbits and cattle after vaccination with *Pimelea* toxin -protein conjugates. To further confirm the presence of specific IgG against *Pimelea* toxins, it was proposed to examine the attenuating effect of different concentrations of purified rabbit IgG using the *in vitro* bovine pulmonary venule preparation.

For this purpose, a minimum of four pulmonary venules from four different animals were tested for each dose of IgG in the presence of the EC_{50} *Pimelea* C fraction concentration. Control tissue preparations from the same animal as the test preparations were incubated with the EC_{50} *Pimelea* C concentration only. The contractile response of the antibody-treated preparations and control tissues were compared to establish the efficacy of rabbit IgG in preventing the contraction response to *Pimelea* toxins (Table 4.6, Figure 4.8).

The results of these experiments are summarised in Figure 4.9. Contraction of bovine control pulmonary vein tissue incubated with EC_{50} concentration of *Pimelea* toxin (shaded region; mean ± s.e.m., n = 13) and attenuation of this contraction (mean ± sem, n = 4 to 6) with increasing amounts of purified rabbit anti-toxin IgG antibodies are shown. From these results it is clear that the purified rabbit IgG attenuates the toxin-induced contraction in a dose dependent manner. Mean contractions (± s.e.m.) produced by treated bovine pulmonary venules in the presence of 5 mg of rabbit IgG and EC_{50}

Table 4.6

Contractile responses of bovine pulmonary venule preparations in the presence of IgG and EC_{50} *Pimelea* toxin concentration.

Treated preparations (preparations exposed to IgG and *Pimelea* toxin (EC_{50})					Control preparations (preparations exposed to *Pimelea* toxin (EC_{50}) only)			
IgG (mg) [a]	Δ mN 1μM 5HT [b]	Δ mN *Pim.* [c]	% *Pim.* Con. [d]	Tissue (mg).	Δ mN 1μM 5HT	Δ mN *Pim.* [c]	%*Pim.* Con.[d]	Tissue (mg).
0.1	81.7	34.3	42	3.1	69	15.9	23	1.7
0.1	98	43.1	44	3.5	156	113.9	73	3.4
0.1	70	48.3	69	--	58	25	43	--
0.1	73.3	38.1	52	--	91	4.6	5	--
0.3	117.5	64.6	55	2.9	112	73.9	66	3
0.3	63.2	32.9	52	1.8	93	4.6	69	2.7
0.3	72	39.6	55	1.6	57.5	64.2	43	--
0.3	122.6	67.4	55	--	118.2	17.8	31	3.8
0.3	57.8	41.6	72	1.7	70.9	36.6	51	1.2
0.3	113.2	83.8	74	--	74.1	36.2	55	3
1	127	5.7	4.5	3.1	60.3	40.8	55	--
1	92.2	64.5	70	1.4	76.5	33.2	111	3.2
1	76.1	42.6	56	2.1	67.6	84.9	54	2
1	68	28.6	42	--	76.5	36.5	41	3.6
2	54	6.2	11.5	1.7	57.2	16.6	29	1.7
2	75.8	136.4	18	1.2				
2	71	39	55	2.2				
2	68	12.9	19	--				
5	99	5	5	2.7				
5	107	0	0	4				
5	126.4	10.1	8	3				
5	87.3	0	0	4.1				
5	86	13.8	16	2.6				
5	71	20.6	29	2.6				

[a] weight of the freeze dried rabbit IgG used in organ bath. [b] Maximum contraction to 1.0 μM 5 -HT minus pre-load tension. [c] Maximum contraction in response to *Pimelea* toxin minus the pre-load tension. [d] Expressed as a % of the 5-HT maximal response.

Figure 4.8
Attenuating effects of rabbit IgG (5mg) on contraction of bovine pulmonary venules in response to *Pimelea* toxin (EC_{50})

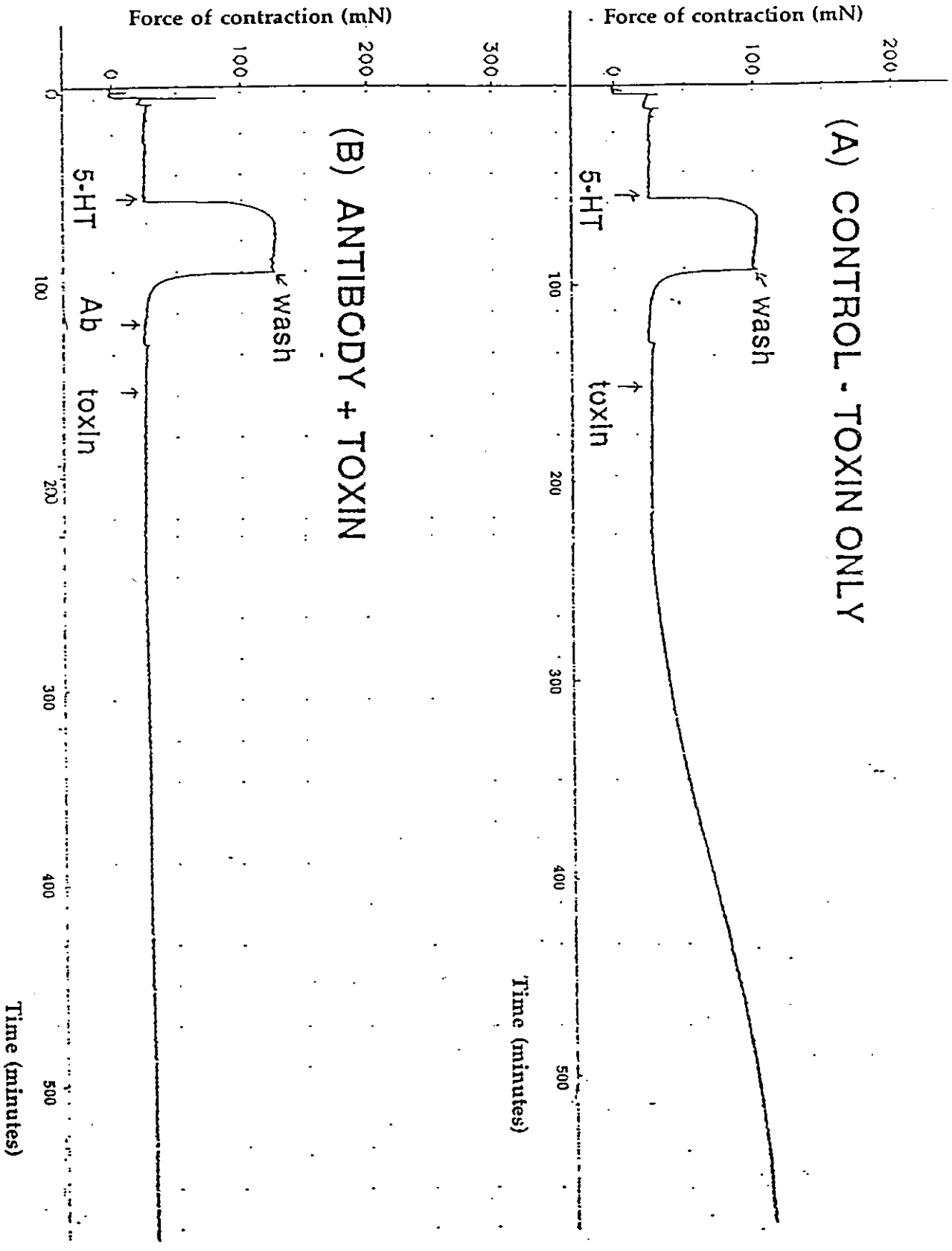
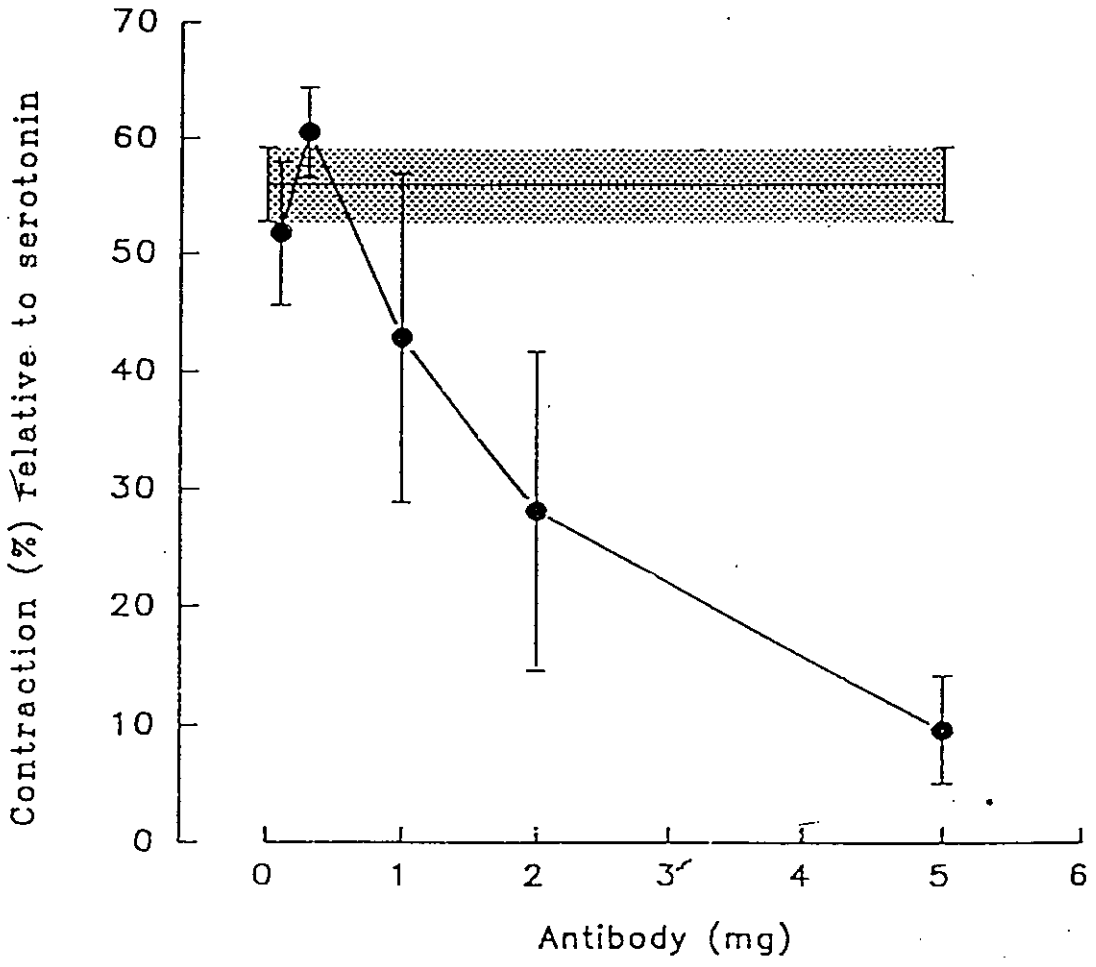


Figure 4.9

Purified rabbit IgG attenuates the contractile response of bovine pulmonary venules to *Pimelea* toxin (EC_{50}) in a dose-dependent manner. (The shaded region represents contraction with EC_{50} toxin concentration.)



Pimelea C toxic fraction was only 9.3 ± 11.2 %, n=6 compared to average contraction of control tissues, 42.0 ± 21.8 %, n=13.

These results (summarised in Table 4.7) suggest that a specific antibody-toxin association either in the organ bath or on the surface of the tissue prevents the action of the toxin. Control experiments conducted with equivalent amounts of non-specific rabbit IgG showed no attenuation of the contractile response (results not shown).

Table 4.7
Average contraction (± s.e.m.) of bovine pulmonary venules in response to purified rabbit IgG in the presence of EC_{50} *Pimelea* C concentration.[a]

Antibody added(mg)	% of 1.0 µM 5-HT contraction.
0.1	51.7 ± 12.2, n=4
0.3	60.5 ± 9.7, n=6
1.0	42.0 ± 28.0, n=4
2.0	25.8 ± 19.6, n=4
5.0	9.6 ± 11.1, n=6

[a] Average control contraction (n=13) was 47.1 ± 21.7 %

4.3.5 *In Vitro* Effects of Purified Cattle Antibodies

ELISA analysis revealed the presence of a specific immune response in cattle against *Pimelea* toxins; however, purified IgG from the vaccinated cattle had no neutralising action in the organ bath even at a 100 mg dose (treated tissue : 63.0 ± 14.7 %, n=4 and control tissue, 55.0 ± 13.2%, n=4). With such a large dosage of IgG in the bath, frothing was a significant experimental problem. The data for the 50 and 100 mg antibody dosage experiments are given in Table 4.8. Much lower concentrations of antibody (comparable with the rabbit data) were trialed initially but no effect was observed.

It is possible that the purified IgG antibody fraction prepared from cattle serum does not contain any neutralising anti-toxin antibodies, which may be present in another antibody subclass such as IgM. As reported in Chapter 3, the effect of freeze-drying the purified IgG was also investigated using ELISA analysis. There was the possibility that structural change or loss of IgG activity had occurred during purification and freeze drying of the bovine samples. The results indicated that there was substantial loss of binding as a result of freeze drying and that this effect was much more dramatic for bovine IgG compared with the purified rabbit IgG. However, even allowing for the loss of IgG activity with freeze drying, some response would have been expected at the very high doses used. Given that the purified rabbit IgG had shown neutralising actions in the organ bath work, the cattle IgG results were disappointing given that this was the target species.

Table 4.8
Contractile responses of bovine pulmonary venule preparations in the presence of bovine IgG and EC50 *Pimelea* toxin concentration.

Treated preparations (preparations exposed to IgG and *Pimelea* toxin EC50)					Control preparations (preparations exposed to *Pimelea* toxin EC50 only)			
IgG (mg)	Δ mN 1μM 5HT[b]	Δ mN *Pim.* [c]	% PIm Con.[d]	Tissue (mg).	Δ mN 1μM 5HT	Δ mN *Pim.*	% *Pim.* Con.	Tissue (mg).
50	95	65.6	69	2.6	70	43.4	62	1.5
50	88.7	40	45	--	75	39	52	2
50	93.3	43	46	4.6	120	36	30	4.6
100	116	70.8	61	3.1	93.1	54.9	59	4.8
100	129	104.5	81	5.5	117.8	69.5	59	4.1
100	156	101.4	65	2.8	155.1	105.4	68	5.2
100	112.9	50.8	45	5.2				

[a] weight of the freeze dried cattle IgG used in organ bath. [b] Maximum contraction to 1.0 μM 5 -HT minus pre-load tension. [c] Maximum contraction in response to *Pimelea* toxin minus the pre-load tension. [d] Expressed as a % of the 5-HT maximal response.

4.3.6 Preliminary Investigations with *Nux vomica* CM Tincture

Nux vomica CM tincture tincture is an extract obtained from the nut of *Strychnos nux vomica* which is a moderate sized tree native of the Cormandel coast and Cochin China. The fruit is very like an orange in appearance and contains numerous flattened circular seeds, each about the size of a 10 cent piece, ash grey in colour and covered with fine silky hair. The seeds are intensely bitter but the pulp is innocuous and is said to be eaten by birds. If nitric acid is added to the seeds, a deep orange yellow colour is produced. In India, tinctures of *Nux vomica* are administered in cases of intermittent fever and also for snake bite. A leaf extract of the plant is used externally in rheumatism. Dried seeds of *Strychnos nux vomica* L. (Loganiaceae) have been used in traditional Chinese medicine for treatment of blood circulation problems and relieving pain (Cai *et al.*, 1990).

In India *Nux vomica* CM tincture is used as an oral drench for treatment of animals suffering certain plant toxicities. Preliminary experiments were therefore carried out to examine whether *Nux vomica* may show promise as an antidote for *Pimelea* poisoning.

The experimental design was similar to that used in the antibody experiments. The maximum contraction of the venules was measured in response to 5-HT, preparations (n= 10) were then exposed to 200 μL, *Nux vomica* CM potency tincture followed immediately by addition of EC50 *Pimelea* toxin. Control preparations were exposed to EC50 *Pimelea* toxin only. Contraction was recorded for 200-300 minutes. It was observed that treated tissue remained totally relaxed even after 300 minutes (0.70 ± 0.5 %, n= 10) whereas control tissues always contracted (50 ± 10 %, n=7) in the normal way (Figure 4.10).

There was some concern that the *Nux vomica* extract might have adverse effects on the physiological viability of the tissue preparations. However, further experimentation showed that preparations which had remained relaxed after being exposed to *Nux*

vomica and *Pimelea* toxin for 300 minutes could be washed out by changing the Tyrode solution three times at intervals of 10 minutes, and these preparations still gave the normal rapidly developing contraction to 1μM 5-HT. After washing away the 5-HT in the usual way, these preparations were re-equilibrated to 25 mN pre-load and then exposed to EC_{50} *Pimelea* toxin concentration. The tissues showed a slow developing contraction (39.0 ± 12.1, n=3) over a further 300 minutes (Figure 4.11). It was concluded that the *Nux vomica* extract had not significantly affected the viability of the tissues since such significant contraction had been achieved in preparations which had been mounted in the organ baths for more than 12 hours.

In another set of experiments, tissues which had remained relaxed after being exposed to *Nux vomica* and *Pimelea* toxin for 300 minutes also gave normal contractile responses to 1.0 μM 5-HT without any wash-out of the bath buffer (Figure 4.12). These results suggested that*Nux vomica* tincture can inhibit the normal contraction response to *Pimelea* toxin (EC_{50}). The mechanism of inhibition remains unresolved; however, it is possible that *Nux vomica* may inhibit PKC activity or it may block the action of the toxins by affecting calcium mobilisation within cells (O'Neil and Bolger, 1990).

Figure 4.10
Attenuating effect of *Nux vomica* tincture (200 µL) on contraction of bovine pulmonary venule in response to *Pimelea toxin* (EC_{50}).

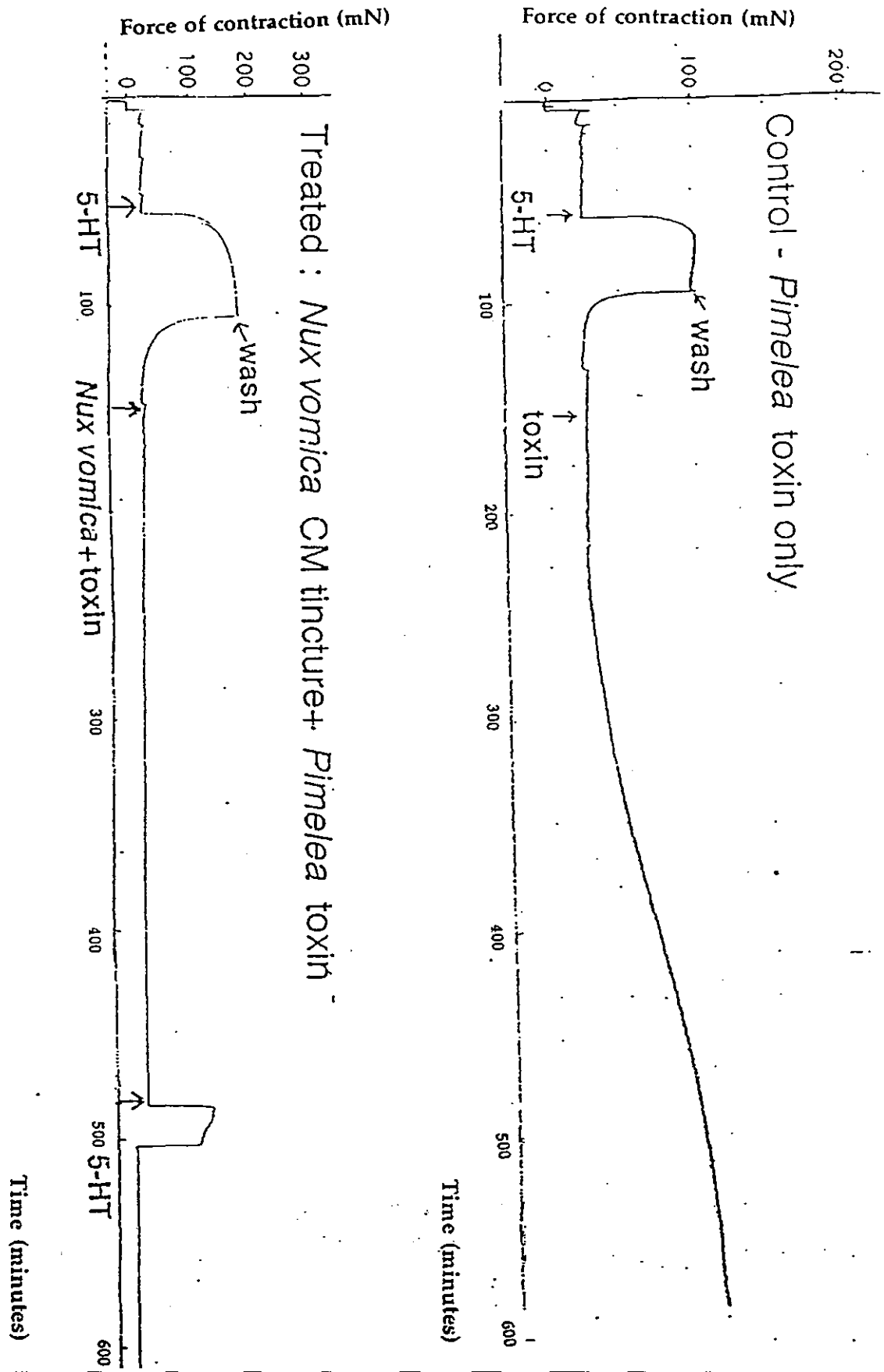


Figure 4.11

Attenuating effect of *Nux vomica* is reversible

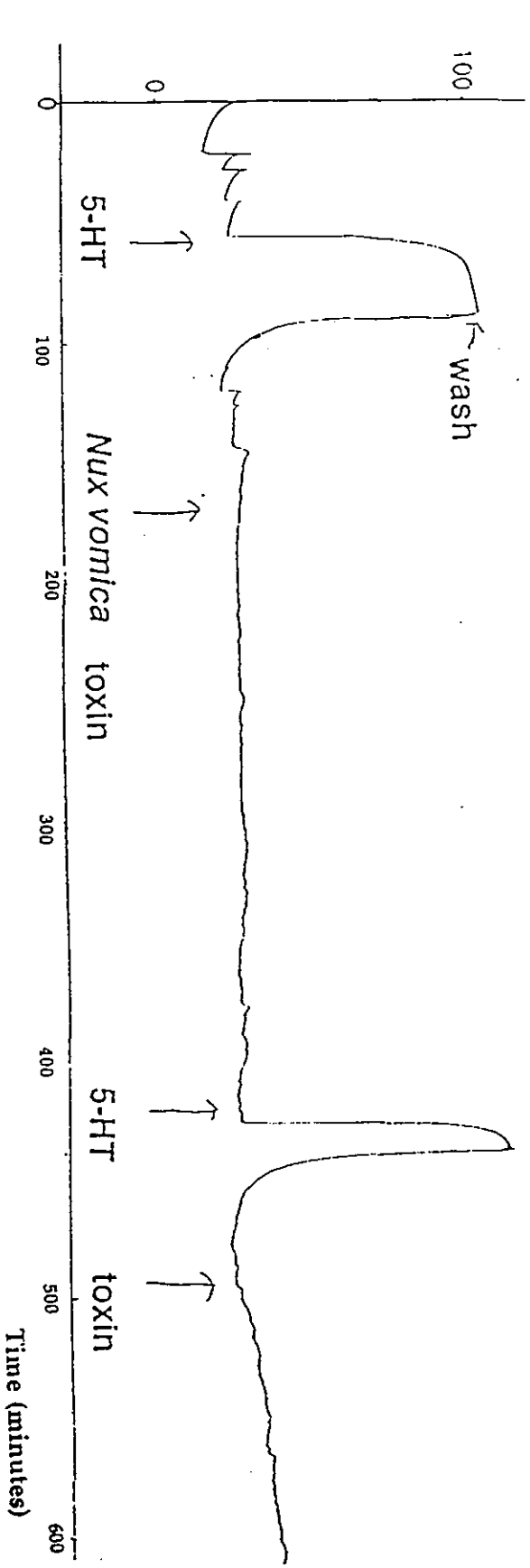
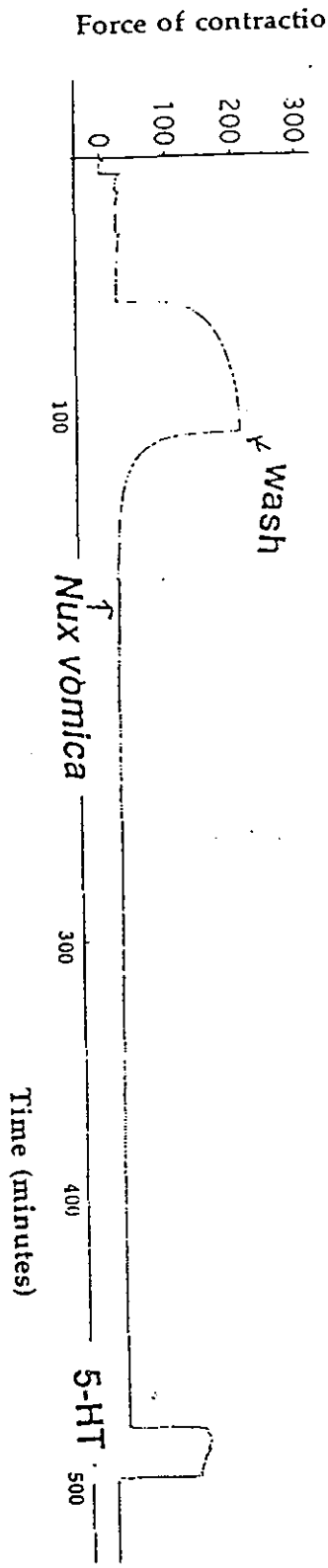


Figure 4.12

Non toxic effect of Nux vomica (200 uL) on bovine pulmonary



4.3.7 Preliminary Experiments with the Tumour Promoter Mezerein

Miyake *et al.*, (1984) report that mezerein activates PKC *in vivo* (platelets) and *in vitro* (rat brain tissue). Mezerein is found in *Daphne mezereum L* plant species which belong to the same family (Thymelaeaceae) as *Pimelea* species (Borris *et al*,, 1988).

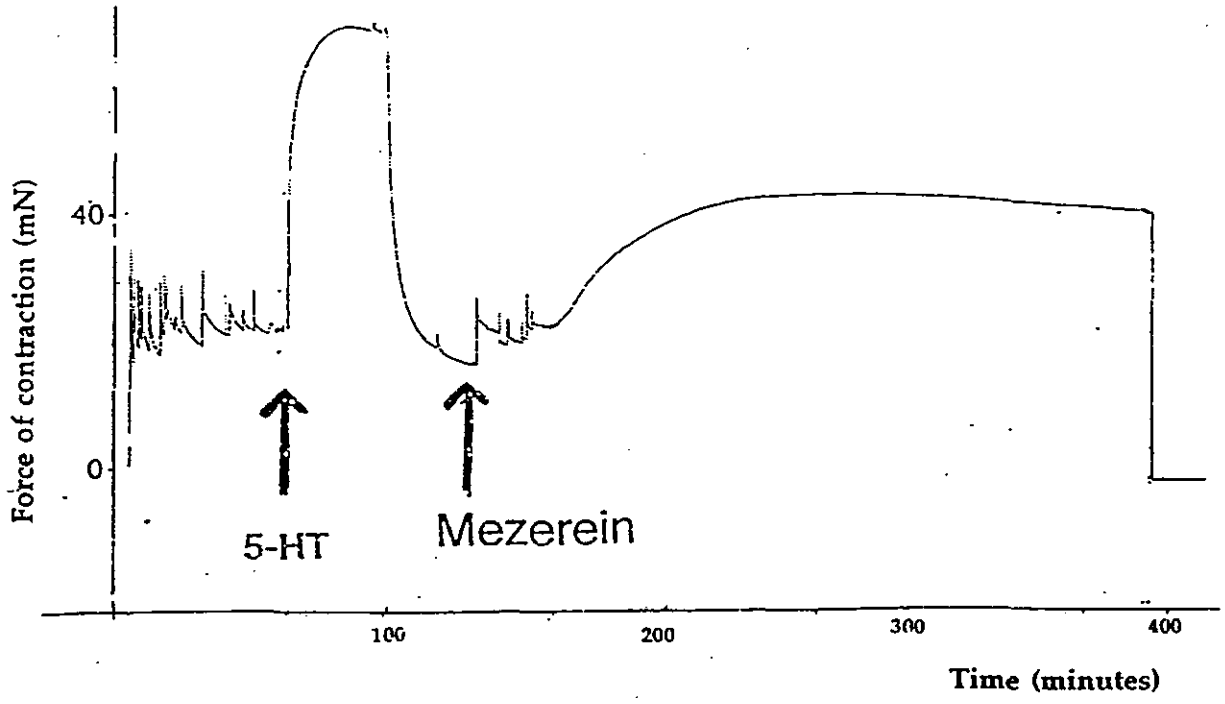
It is presumed that the mode of action of the *Pimelea* toxins on the bovine pulmonary venule system is by way of activation of PKC. The organ bath technique therefore provided a useful experimental tool to verify that a known PKC activator having close structural similarity to the *Pimelea* toxins also caused contraction of bovine pulmonary venule preparations.

Table 4.9
Responses of bovine pulmonary venule preparations to mezerein

Preparation no.	1.0 µM 5-HT (mN)	% of 5-HT contraction to 1µM mezerein.	weight of tissue (mg)
1	94	42	2
2	60	48	1.6
3	141	49	2.4
4	63	39	3.6

In vitro studies showed that mezerein (LC Services, USA) causes a slow developing contraction in bovine pulmonary venule preparations, similar to the contraction induced by *Pimelea* C fraction (Figure 4.13). The EC_{50} concentration for Mezerein was found to be approximately 1 µM (average contraction 44.5 ± 4.7 % relative to 1.0 µM 5-HT; n= 4, Table 4.9). These findings are consistent with the suggestion that contraction of the bovine pulmonary venule system by *Pimelea* toxins may be mediated by PKC activation.

Figure 4.13
Slow developing contraction of bovine pulmonary venule induced by Mezerein



Further to the exploratory experiments with mezerein, some preliminary experiments were also undertaken to observe whether two commercially available PKC inhibitors, polymyxin B sulfate and D-*erythro*- sphingosine (Calbiochem), and also *Nux vomica* (200 µL), attenuated the contraction response to Mezerein (EC_{50}). Hannun *et al.* (1986) reported inhibition of PKC activity and of phorbol dibutyrate binding *in vitro* and in human platelets by sphingosine. Muller *et al.*, (1991), reported prevention of glucose induced insulin receptor tyrosine kinase resistance in rat fat cells by PKC inhibitors. Oishi *et al.*, (1988) have also shown *in vitro* inhibition of PKC activity by polymyxin B and sphingosine.

Control tissue preparations were exposed to mezerein (1µM) only, whereas test preparations were exposed to mezerein (1µM) in addition to either sphingosine (200 µM), polymyxin B (250 µM), or *Nux vomica* (200 µL). The average contraction of control preparations exposed to mezerein (EC_{50}) only was 36.5%, (n=2). The average contraction of treated preparations was : sphingosine 24.5%, (n=2); polymyxin B 22.5%, (n=2); *Nux vomica* 12%, (n= 1). Although the data is very preliminary, it was suprising that the PKC inhibitors were not more efficacious given other literature reports of K_D values.

4.4 CONCLUSIONS

It was shown that the contractibility of bovine pulmonary venule preparations in response to *Pimelea* toxins was concentration dependent. From the concentration response data, the EC_{50} concentration was determined to be 5.3×10^{-8} M (0.053 µM).

Experiments with purified rabbit anti-toxin IgG showed that these antibodies were able to neutralise the effects of *Pimelea* toxins on bovine pulmonary venules in the *in vitro* system. While these results were highly encouraging, the corresponding bovine anti-toxin IgG appeared to lack any measurable efficacy even at very high dosage. This probably reflects a lack of high affinity antibodies in the cattle IgG preparation and in part may be due to denaturation of the bovine antibodies during freeze drying. Further work needs to be done on improving the experimental vaccine.

The results obtained with *Nux vomica* suggest that this material may provide an antidote strategy to combat *Pimelea* poisoning, however this needs testing in live animals before any firm conclusions can be made. The organ bath results for mezerein show that this compound causes contraction of the pulmonary venule tissue, consistent with its known PKC activation characteristics.

5.0 CHAPTER 5.0

5.1 Achievement of Aims

A major aim of this project was to examine the feasibility of inducing protective immunity against *Pimelea* toxicity in cattle. This required isolation of the toxins, conjugation of these compounds to suitable carrier proteins and vaccine formulation. The experimental problem was that the lipophilic daphnane toxins in *Pimelea* species were low molecular weight diterpenes (molecular weight range 500-700 mass units) and in their native form were not immunogenic. The toxins were also present in very low levels in the plant, therefore the extraction procedure had to be highly efficient. For example, even the major toxin component (simplexin) made up only 0.005% of the dry weight of *Pimelea trichostachya* . The first experimental task was to separate out sufficient quantities of the toxic components from the myriad of other organic components present in methanol extracts of dried plant material. There was some information on suitable chromatographic methods in the literature, but most of these studies were undertaken prior to HPLC methodology becoming routine laboratory practice.

The chromatographic approach developed for this project involved a combination of solvent partition, silica column chromatography and preparative HPLC. Identification of toxic fractions was accomplished with a fish bioassay technique. It was reasoned that since the huratoxins were known piscicidal agents and that *Pimelea* daphnane toxins were structurally similar to huratoxin, then these toxins should also be active piscicides. This assumption proved correct and it was found that the *Pimelea* toxins were extremely potent in the bioassay. The HPLC methodology was scaled up from analytical through to semi-preparative and finally preparative scale to enable isolation of sufficient quantities of *Pimelea* toxins to conduct the vaccination trials in both experimental animals and cattle. The entire procedure could be scaled up further for commercial scale separation of *Pimelea* toxins.

The next task was to link the isolated toxins covalently to immunogenic carrier proteins so that experimental vaccines could be prepared. In previous work (G. Pegg and L. Hellqvist, unpublished) resiniferinol orthophenyl acetate and mezerein had been linked to HSA and BSA. The strategy involved succinylation of a C-20 hydroxymethyl group common to these compounds by reaction with succinic anhydride in pyridine solvent. The free carboxyl grouping was then activated by reaction with iso-butylchloroformate and the resultant mixed anyhydride then reacted with free lysine residues on the carrier protein. The initial activation chemistry was conducted in DMF solvent and the protein coupling reaction then accomplished in a 50:50 DMF: water mixture.

It was reasoned that since almost all of the published *Pimelea* daphnane and tigliane toxins also had a free C-20 hydroxymethyl group then this same chemistry could be applied in the present project to a mixture of *Pimelea* toxins isolated from the plant by chromatographic methods. The rationale for working with the natural mixture of toxins was that antibodies might then be developed to a range of toxin epitopes rather than to a single compound. Conjugation of toxin mixtures to ovalbumin and BSA proved successful. UV spectrophotometric analysis of solutions of the conjugates clearly showed that toxins had been linked to the carrier proteins in each case.

One concerning outcome of the conjugation chemistry was the apparent cross-linking of carrier protein molecules. This was a result of traces of unreacted *iso*-butylchloroformate activating free aspartate and glutamate sidechains in the carrier protein and these activated groups then cross linking with free lysines on adjacent protein molecules. Advice from immunologists associated with the group was that any

supramolecular protein complexes would still be processed by macrophages and could even prove advantageous for development of an immune response.

Oil-in-water emulsions of the conjugates were prepared using Freund's adjuvant for the rabbit studies and a commercial formulation for cattle. The rabbit experiments were conducted first to establish that the conjugates were immunogenic. ELISA analyses to recognise anti-toxin antibodies had to be conducted with conjugates of the toxins rather than free toxin coated to the plate. This approach raised some concerns about measuring the absolute specificity of the immune response but was unavoidable because the toxins were not water soluble and these molecules would not bind in their native form to the plate. Displacement of bound antibodies with free toxin was equivocal, since only 20-50% of the binding could be displaced. This may well reflect the insolubility of the toxins in aqueous media. While the inclusion of organic solvents such as DMSO in the ELISA improved the percent displacement, total displacement was never achieved (L. Hellqvist, unpublished).

The ELISA data consistently provided strong evidence that immune responses to the toxins had been obtained in both the rabbits and cattle. In rabbits, antibody levels rose dramatically after the secondary vaccination and were maintained by subsequent booster vaccinations. In cattle, a similar rise in antibody levels were observed after secondary vaccination, but the titre then continued to decline slowly, despite booster vaccinations. IgG was purified from the serum of immune animals for *in vitro* studies of the efficacy of the antibodies raised to the toxins.

The vaccinated cattle group and an equivalent number of control animals were challenged with a slurry of dried *Pimelea* plant material, administered directly into the rumen of each animal via a stomach tube. The dosage rate administered was based on literature reports and the experiment was initially conducted for more than a week using a dosage that should have affected the animals within a few days. Because of the lack of response, the dosage was increased in two stages over the second week. Unfortunately, the animals appeared to have been severely over-dosed by the completion of the experiment and both the control and vaccinated group had developed clear signs of *Pimelea* poisoning such as variable diarrhoea, distension of the jugular vein and oedema of the neck and brisket. Blood parameters were monitored daily and for a number of these parameters such as total haemoglobin and red cell volume, the vaccinated group seemed to be less affected than controls over the first week and half of the experiment. The result of the intoxication experiment was that vaccination did not appear to counteract *Pimelea* toxicity at the doses tested.

It might be argued that the highly lipophilic character of the daphnane toxins may prove the downfall of the immunological strategy to combat *Pimelea* poisoning. Antibodies may not have the opportunity to bind and neutralise the toxins in the circulatory system if these small lipophilic molecules are rapidly taken up into membranes and fat reserves. However, steroids are similar in size and hydrophobicity to *Pimelea* toxins and these molecules are found in the circulatory system in the free form and also associated with binding proteins. It has proven possible to vaccinate successfully against steroids, through inducing highly specific antibodies. For this reason, the experimental *Pimelea* vaccine formulation developed in this study is worthy of further research and development.

The primary site of action of *Pimelea* toxins in cattle is sustained constriction of the pulmonary venules in the bovine lung due to peculiar sphincter like structures present in the walls of these vessels. This constriction impedes the returning flow of blood from the lungs and also results in right-sided heart failure. There had been a single report of an *in vitro* study of bovine pulmonary venules which demonstrated that a 10 mg quantity of the tarry residue obtained by extracting the plant with alcohol caused

contraction of ring preparations (Mason, 1976). We were interested in conducting more refined *in vitro* studies aimed first at establishing the efficacy of the toxin fractions obtained from extensive chromatographic purification of plant extracts. The EC_{50} concentration for the purified toxin fraction was found to be in the sub-micromolar range. It was found that while 1.0 μM 5-HT caused rapid maximal contraction of the venule preparations within 30 minutes, a slow developing response to the toxins required 300 minutes to reach equilibrium.

The *in vitro* technique provided the opportunity to assess whether purified rabbit and cattle IgG resulting from the immunisation experiments had any protecting effect on the target tissue in cattle. It was observed that the rabbit IgG gave dose-dependent protection from the toxins, whereas the cattle IgG lacked efficacy. The rabbit IgG data showed that antibodies raised to the toxin-protein conjugates are capable of both toxin recognition and target tissue protection at least in the *in vitro* system.

There may be several reasons for the lack of efficacy observed for the purified bovine IgG in the organ bath experiments. It was considered that there might have been loss or structural change of toxin specific IgG during purification of the serum and in the freeze-drying process. ELISA binding data for reconstituted freeze-dried IgG was compared with an equivalent amount of serum for both rabbits and cattle. It was observed that there had been a significant loss of binding for the freeze dried bovine IgG material compared to the native serum, whereas the rabbit serum sample and the freeze dried IgG equivalent gave similar binding. However, such high concentrations of the purified cattle IgG were tested that attenuating effects should have been observed if the bovine IgG fraction contained even low levels of specific anti-toxin antibodies. It is also possible that the purified IgG antibody fraction prepared from cattle does not contain the toxin specific antibodies present in the serum and these may be present in another antibody subclass.

The finding that *Nux Vomica* CM tincture was effective in preventing contraction of bovine pulmonary venules in the organ experiments was an interesting observation. *Nux Vomica* may prevent PKC activation of muscle contraction by impeding calcium mobilisation (O'Neill and Bolger, 1990).

5.2 DIRECTIONS FOR FURTHER WORK

The problem of cross-linking of protein molecules during the conjugation of toxin to carrier protein may have compromised the efficacy of the prototype vaccines. Further development of the conjugation chemistry needs to be conducted. One possibility might be to activate the succinylated toxin by reaction with N-hydroxysuccinimide, rather than with *iso*- butylchloroformate. The resultant N-hydroxysuccinimide ester toxin derivative should be stable enough to tolerate chromatographic purification prior to the final cross-linking step and this approach should eliminate the possibility of protein aggregation.

Another aspect of the conjugation chemistry that requires investigation is to determine the optimum ratio of toxin to protein required to illicit a highly specific immune response. The present study has shown that incorporation of up to 20 molecules of toxin per protein molecule can be achieved. However, this incorporation ratio may have been too high as this raises the possibility of deleterious steric interactions between toxin molecules which might compromise the specificity of the antibody response. McAdam *et al.*, (1992) reported that in development of an immunoassay for pyrethroids, the optimum incorporation ratio of pyrethroid was 10. Higher incorporation ratios caused more antibody production, but these antibodies were less specific.

The extraction and isolation of *Pimelea* toxins from dried plant material is a time consuming and expensive process. A logical substitute compound for further work is mezerein, since this daphnane orthoester is commercially available and is a close structural homologue of simplexin and huratoxin. It was shown in the present work that cattle vaccinated with a mezerein conjugate developed an immune response and these antibodies also cross- reacted with *Pimelea* toxin-protein conjugates.

While this study has shown that it is possible to raise antibodies to *Pimelea* daphnane orthoester toxins and that the purified rabbit anti-*Pimelea* IgG has demonstrable efficacy at protecting the bovine target tissue in *in vitro* studies, the likelihood that the immunogen approach might result in effective protection of cattle against *Pimelea* toxicity remains uncertain. The study has produced some interesting results and further work aimed at improving the vaccine formulation should be undertaken.

An alternative approach may be to investigate the modulation of PKC activity by generation of sphingosine intracellularly by breakdown of sphingomyelin and related molecules by drugs or antibodies. Hannun *et al* ., (1986) reported inhibition of PKC activity by sphingosine in *in vitro* preparations and in human platelets. Sphingosine inhibition was modulated by Ca^{2+} and by the molar ratios of diacylglycerol and phospholipid present. Sphingosine prevents the formation of an active lipid-PKC complex by displacement of the activator (DAG or phorbol ester) from the complex. Sphingosine levels may be regulated in response to either intra or extracellular signals. Sphingomyelin was observed to undergo rapid N-acyl exchange when L-929 fibroblasts were stimulated with specific antibodies (Ulrich *et. al.*, 1984.). The deacylation of sphingosyl phosphorylcholine also leads to the generation of sphingosine through hydrolysis of the phosphorylcholine head group. Further literature investigation is required to determine if these catabolic pathways offer any opportunities for modulating PKC activity in the bovine lung.

Validation of an assay for measuring PKC activation by *Pimelea* toxins is a worthwhile future research endeavour. The PKC assay technique might prove the most efficient and cost effective approach to assaying toxin levels in plant and soil extracts. The present study has shown that direct HPLC assay is too insensitive and the ELISA analysis would likely be problematic because the toxins are not water soluble. It would be useful to be able to conveniently analyse toxin levels from different parts of the plant (roots, stems, leaves, flowers) as there is no information in the literature regarding the time course and location of toxin levels in the plant. Producers believe that the plant is more toxic as it flowers and dries; however, it could be that pasture grasses also become limiting at this time and foraging animals therefore consume more of the plant under these conditions.

Another potential future research direction is to investigate detoxification/ chemical transformation of the daphnane orthoesters by rumen microflora. For this work it would be advantageous to have a radiolabelled toxin component (perhaps derived from mezerein in the first instance) where the label was likely to be retained in the metabolites. For example, the methylene hydrogens on the C-20 hydroxymethyl group could be replaced by tritium through selective oxidation followed by reduction. Experiments could be conducted both with live animals and rumen broth fermentation studies in the laboratory. The labelled toxin would also be useful for determining the residence time of *Pimelea* toxins in the live animal and the uptake of toxins into the animal's organs and lipid reserves.

With regard to structure-activity studies of the requirements for PKC activation, there is information in the literature suggesting that reduction of the A ring 5-membered enone moeity might lessen the efficacy of the toxins towards PKC (Jeffery *et al.*, 1985). This

presents a research opportunity to specifically reduce the enone (and perhaps other reducible moieties) using modern organic chemistry methodologies, verify the structures by high field NMR and mass spectrometry, and then determine the ED_{50} of the reduced toxins in the organ bath studies and in the PKC assay system. The results from these experiments might then shed new light on what types of anaerobic microflora could be advantageous for detoxification of *Pimelea* toxins in the rumen and perhaps offer some hope for solving the problem of *Pimelea* toxicity to cattle by either rumen modification or introduction of new rumen microbes.

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APPENDIX B

METHODS FOR REDUCING *PIMELEA* POISONING OF CATTLE

1.0 *PIMELEA* POISONING OF CATTLE

1.1 INTRODUCTION

1.1.1 The impact of Pimelea Poisoning of Cattle

Pimelea species are herbaceous annuals belonging to the Thymelaeceae family and are found in regions of Australia which have low annual rainfall (less than 600 mm p.a.). *Pimelea* plants proliferate in pastures where competition is low (caused by over-stocking or drought).

Poisoning of cattle by plants from the *Pimelea* species contributes significant costs to the beef industry each year. The estimation of a total monetary value attributable to *Pimelea* poisoning is difficult, as other costs incurred as a result of the condition include: the reduced production capacity of survivors of poisoning; reduced carrying capacity of land caused by the presence of poisonous plants; and veterinary treatment and control methods[1]. The total cost due to *Pimelea* poisoning in Queensland was estimated to be between $7.25, and $10 million per annum consisting of mortalities ($2.1 million), production losses ($5.9 million) and reproduction losses ($2.09 million)[2]. However, *Pimelea* poisoning of cattle also occurs in South Australia, New South Wales and the Northern Territory, and as a national issue is considered to cause losses between $14 and $20 million per annum. Although this figure is relatively small compared to the value of the Australian beef industry, the impact caused to individual producers may be considered very significant.

1.1.2 The History of *Pimelea* Poisoning (St. George disease)

Pimelea poisoning (St. George disease) of cattle was first reported as a disease of unknown cause affecting cattle of all ages and types, with morbidity rates ranging from 1 to 100%[3]. The disease was reported from 1939 - 1943 on properties in the St. George district (thus the name St. George disease), as well as in the Cunnamulla, Chinchilla, and Roma districts of Queensland. These cases had occurred on inferior sheep country where cattle were also kept as an additional source of income. Cases were observed in the Rockhampton district, near Aramac, and on properties between Yalleroi and Barcaldine in 1946[3]. The disease was also known as Marree disease, affecting cattle driven from the Northern Territory to Marree in South Australia[3].

1.1.3 Symptoms

Animals affected by *Pimelea* poisoning suffer severe diarrhoea, thus rapidly lose condition. The temperature of the animal is normal (an increase may occur over time), while shallow and rapid respiration is observed along with a weak pulse. Jugular distension coupled with oedema, spreads to the head, neck, and brisket. The oedema extends beyond the brisket, however death may occur prior to this, which is within ten to fourteen days. The oedema is caused by the accumulation of excessive quantities of blood plasma in subcutaneous tissue, and in the thoracic cavity (caused by the filtration of

blood through vessel tissue under increased back-pressure). Extensive damage also occurs to the liver, and it adopts a mottled appearance with dark blue to bronze-purple discolouration[4].

1.1.4 Suggested Causes of *Pimelea* Poisoning

Initially, it was thought that the symptoms were caused by a protein deficiency due to adverse soil, pasture, and climate conditions. It was postulated that a disturbance in protein intake retarded the ability of rumen micro-flora to synthesise the thiamine (vitamin B) complex, thus causing a thiamine deficiency. There also appeared to be a striking similarity between the biological changes of cobalt deficiency, and St. George disease[3]. Therefore, thiamine therapy and cobalt feeding were investigated as trial solutions[3], and both these methods have proved unsuccessful for alleviating the symptoms.

1.1.5 The Pathogenesis of *Pimelea* Poisoning

The occurrence of St. George disease in the Bourke district of New South Wales was correlated with the presence of *Pimelea simplex* in the pastures[5]. Outbreaks of St. George disease in Queensland were also reported to coincide with the occurrence of *Pimelea trichostachya* as disintegrated remnants[6].

The cause of the oedema in affected cattle was suggested to be due to an increase in pulmonary vascular resistance as a result of pulmonary venule constriction[7]. Therefore, it was concluded that an agent in the *Pimelea* plant acted primarily on the lungs of cattle. The symptoms of *Pimelea* poisoning were shown to be inducible in cattle either through oral dosage or forced inhalation of dried *Pimelea trichostachya* plant material[8].

Intravenous injection of an ethanol extract of *Pimelea trichostachya*, (130mg/Kg liveweight) caused an immediate fall in systemic arterial pressure, left atrial pressure, and cardiac output, coupled with a rise in pulmonary arterial pressure, and a slight rise in heart rate[8].

The normal bovine pulmonary vasculature is lined with muscular tissue in sphincter-like arrangements[9], which are absent from the pulmonary veins of sheep and horses. These species commonly graze *Pimelea* affected areas without developing right ventricular distension (the diarrhoea is observed in sheep).

The effect of *Pimelea* extract on bovine pulmonary venule preparations *in vitro* was examined[10], and it was shown that tissues suspended under approximately 20mN pre-load tension in organ baths could be contracted reversibly by 5-hydroxytryptamine (5-HT), yet irreversibly by *Pimelea* plant extract. A slow developing contraction was observed even following several washes with fresh assay buffer, suggesting the toxic component to be highly lipophilic.

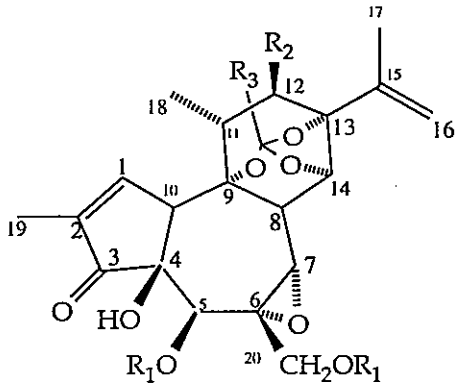
The toxic component of *Pimelea simplex* was isolated and identified as simplexin, a diterpene orthoester belonging to the daphnane family of compounds[11] (Figure 1.1). It

was similar in structure to huratoxin, a piscicidal constituent of *Hura crepitans L.* (Euphorbiaceae)[12]. Huratoxin was also found in *Pimelea Simplex* extract. Other compounds of the daphnane type include daphne toxin, a poisonous principle of *Daphne* species[13] and mezerein, a major toxic principle of *Daphne mezereum L* [14]. The systematic name for simplexin is 3a,3b,3c,4a,5,5a,8a,9,10,10a-decahydro-5,5a-dihydroxy-4a-hydroxymethyl-7,9-di methyl-10a-(1-methylethenyl)-2-(nonyl)-6*H*-2,8b-epoxyoxireno[6,7]azuleno-[5,4-*e*]-1,3-benzodioxol-6-one.

Native cultures have traditionally used plants containing daphnane type compounds for hunting and medicinal purposes. For example, the white sap from *Excoecaria agallocha* (Milky mangrove, or "Blind-your-eye") was traditionally used for catching fish in New Caledonia. The piscicidal constituent of this plant was found to be a daphnane compound similar to huratoxin[15], as shown in Figure 1.2. Other uses are very diverse, including arrow poisons, and treatment of disease[16].

Interest in the daphnane orthoesters was heightened by the observation that compounds of the tigliane, daphnane, and ingenane type, although structurally similar (Figure 1.3), displayed variable biological activities. For example, some compounds displayed tumour promoting properties, while others displayed anti-leukemic activity[17], indicating a subtle structure-function relationship.

Compounds possessing phorbol and daphnane type structures were shown to activate protein Kinase C (PKC)[18]. Therefore, a closer examination of the PKC pharmacophore and activation mechanism is required to gain insight into the mode of action of the *Pimelea* toxins.



R_1=R_2=H, R_3=C_9H_{19}; Simplexin

R_1=R_2=H, R_3= $(CH_2)_8CH_3$; Huratoxin

R_1=R_2=H, R_3= ; Daphnetoxin

R_1=H, R_2= , R_3 = ; Mezerein

Figure 1.1 - Daphnane toxins.

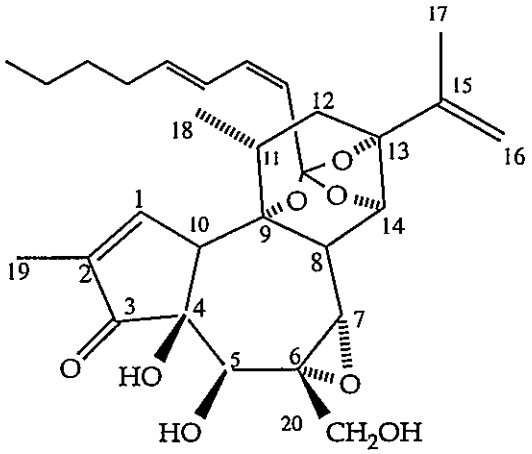


Figure 1.2 - The piscicidal constituent of *Excoecaria agallocha*.

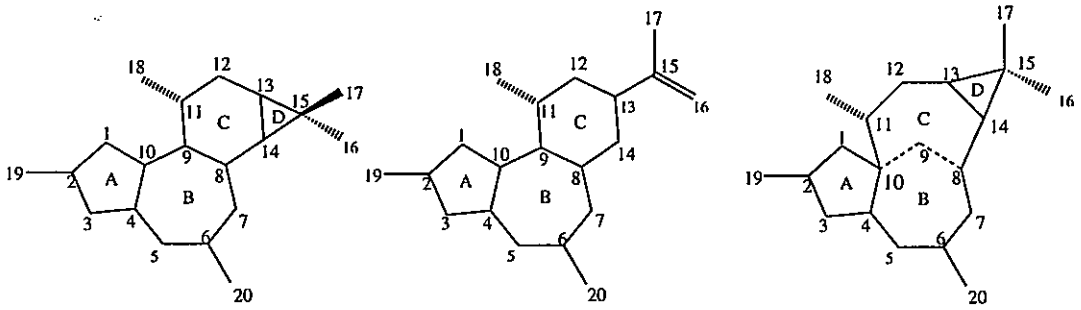
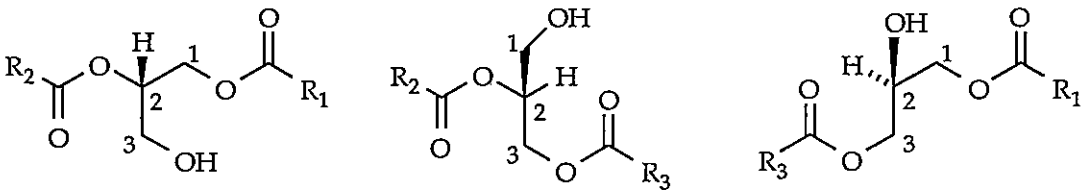


Figure 1.3 - The tigliane, daphnane and ingenane skeletons.

1.1.6 Activation of Protein Kinase C - The Suggested Mode of Action of *Pimelea* Toxins

The protein kinase C (PKC) family of isoenzymes was first identified as a proteolytically activated protein kinase[19], and it is now well accepted that PKC is ubiquitous in tissues and organs[20]. The mechanism of activation has more recently been discussed[20,21], and PKC catalyses the phosphorylation of a variety of substrate proteins. PKC is activated by diacylglycerol in the presence of receptor-mediated hydrolysis of inositol phospholipids, and in many cases calcium (II) ions.

It was suggested that a highly specific lipid-protein interaction is required for activation, as *sn*-1,2-diacylglycerols (DAG) with varying fatty acid chain lengths activate PKC, while *sn*-2,3-diacylglycerols and 1,3-diacylglycerols (Figure 1.4) neither activate nor inhibit PKC. The affinity of one molecule of PKC for calcium (II) ions is increased in the presence of greater than four, but less than ten molecules of phosphatidyl serine, therefore rendering it fully active without a net increase in calcium concentration. The property of activation by compounds with specific stereochemistry is evidence of receptor-like behaviour, analogous to a "lock and key" scenario (compounds not possessing the appropriate structure do not bind to or activate the enzyme).



sn-1,2-diacylglycerol 2,3-diacylglycerol 1,3-diacylglycerol

Figure 1.4 - Diacylglycerol structures

A number of PKC sub-types were characterised[22-27], and it is assumed that the different sub-types catalyse phosphorylation of specific substrates according to cell function. The PKC structure consists of two basic functional regions, the catalytic portion (carboxy-terminal half) and the regulatory domain (amino-terminal half)[28]. The regulatory domain of PKC a, b, and g contains a calcium binding region which is absent in the PKC d, e, and h subtypes (which are calcium unresponsive[28,29]). Phospholipid and diacylglycerol are still however required for the activation of the calcium unresponsive PKC d, e, and h subtypes. PKC z found in rat brain (which is predominantly PKC h) was shown to be calcium and DAG unresponsive[27], therefore would not be expected to allow phorbol ester or daphnane orthoester binding.

Calcium-induced contractile responses of isolated rabbit vascular smooth muscle were shown to be enhanced by the addition of phorbol-12-tetradecanoate-13-acetate (TPA), while the activation of adenylate cyclase (by addition of forskolin) produced an immediate inhibition of the induced contraction[30]. It was therefore concluded that the activation of PKC played a significant role in regulating vascular smooth muscle contraction[30]. The general consensus of the literature in this area indicates that the calcium and DAG dependence of PKC is governed by the availability of these

activators, coupled with the phosphate demand of the substrate proteins according to cell function. It has been discussed that the PKC structure dictates the binding of the calcium and DAG activators, resulting in the regulation of the kinetics of corresponding phosphorylation reactions[27]. That is, if calcium and DAG are in plentiful supply, and if the phosphate demand of the substrate protein is high (for example, in muscle tissue) the corresponding PKC would possess calcium and DAG binding sites, and display rapid catalytic properties when activated. In relation to *Pimelea* poisoning of cattle, the PKC found in bovine pulmonary venule is assumed to be calcium and DAG dependent, as these activators would be readily available from blood passing through the pulmonary venule system.

Therefore, the focus of this discussion will be that of calcium-dependent protein kinase C, as the contraction of sphincter-like bundles of smooth muscle in normal bovine pulmonary venule is recognised as a unique diagnostic symptom indicating poisoning of cattle by *Pimelea* plants. The cause of the diarrhoea and loss of condition is also assumed to be due to the activation of PKC in the intestinal tract, thus not allowing the absorption of nutrients across the membrane wall.

1.1.7 The Transient Role of Calcium Dependent Protein Kinase C

PKC is found in the cell cytoplasm, where its function is the regulation of signalling across the cell membrane to regulate calcium dependent processes where required. PKC plays a transient role in cellular responses in the endocrine, exocrine, nervous, muscular, inflammation and immune, metabolic, and other cell systems[20].

Positive signals in biological systems are normally followed by immediate negative feedback control regulation to allow responses to subsequent signals. A major function of PKC appears to be the feedback control of cell surface receptors. PKC catalyses the phosphorylation of a variety of substrate proteins which include receptor proteins, membrane proteins, contractile and cytoskeletal proteins, and enzymes[20]. More recently, it was shown that the activation of either cAMP-dependent protein kinase (cAPK) or PKC caused rapid desensitisation of the b2-adrenergic receptor (b2AR) stimulation of adenylyl cyclase in L cells. The stimulation of adenylyl-cyclase involves the cAPK/PKC consensus phosphorylation site in the third intracellular loop of the b2-adrenergic receptor[31].

1.1.8 The Transient Activation of Calcium Dependent PKC.

Phosphatidyl inositol-4,5,bis(phosphate), [Ins-4,5-]P_2 (Figure 1.5) is an inositol phospholipid produced by the sequential phosphorylation of the *myo*-inositol moiety of phosphatidylinositol, and phosphatidylinositol-4-phosphate, which is induced by stimulation of a_1-adrenergic receptors.

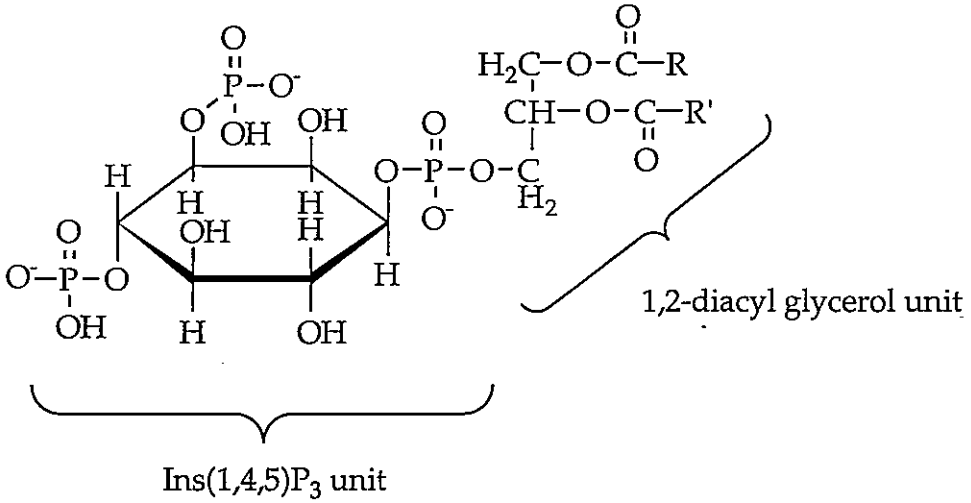


Figure 1.5 - Phosphatidyl inositol-4,5-bis(phosphate).

The cleavage of the phosphate ester linkage of [ins-4,5-]P_2 is catalysed by phospholipase C, and results in the formation of two second messengers, leading to PKC activation[32]. The first of these second messengers is inositol-1,4,5-tris(phosphate) ([ins-1,4,5-]P_3) which allows entry of extra-cellular calcium through its own receptor system (by the opening of calcium channels)[33], causing PKC to associate with membrane phospholipids.

Once produced, [ins-1,4,5-]P_3 disappears very rapidly, and termination of this signal flow is thought to be the removal of the 5-phosphate group by the action of a specific phosphatase. Both Calcium (II) transport adenosine phosphatase (ATPase) and Na^+/Ca^{2+} exchange protein are known to be responsible for extrusion of Ca^{2+} to maintain homeostasis[34]. The other second messenger produced by the hydrolysis of [ins-4,5-]P_2 is *sn*-1,2-diacylglycerol (DAG) which activates membrane associated PKC to phosphorylate serine and threonine residues on a variety of substrates, such as the GTPase activating protein (GAP)[35]. DAG also disappears rapidly after its appearance in the cell membrane, due to both its conversion back to inositol phospholipids by way of phosphate turnover, and its degradation to arachidonic acid, which in-turn generates other messengers such as prostaglandins. Thus, when cells are stimulated, PKC is only transiently activated by DAG. Although both calcium (II) ions and DAG signals are transient, the two pathways are essential, and often synergistic for evoking subsequent cellular responses [20].

The phorbol esters and daphnane orthoesters are thought to mimic the action of DAG by binding with a higher affinity than DAG to the PKC regulatory domain. The

activation of PKC by the *Pimelea* toxins is prolonged, thus it is assumed that the activation of this enzyme by these compounds is not transient, in other words the lipophilic daphnane compounds have a high affinity for the PKC receptor. Due to the high lipophilicity and high receptor affinity of the daphnane orthoesters, the toxins are likely to be accumulated in membrane tissues. This may result in accumulated toxic effects such as prolonged PKC activation causing increased bovine pulmonary venule resistance, and ultimately right sided heart failure. The cause of the other symptoms of *Pimelea* poisoning is likely to be through the prolonged activation of PKC in different tissue sites (eg. the diarrhoea is likely to be caused by prolonged PKC activation in the intestinal tract). Figure 1.6 schematically represents the normal activation of calcium dependent PKC.

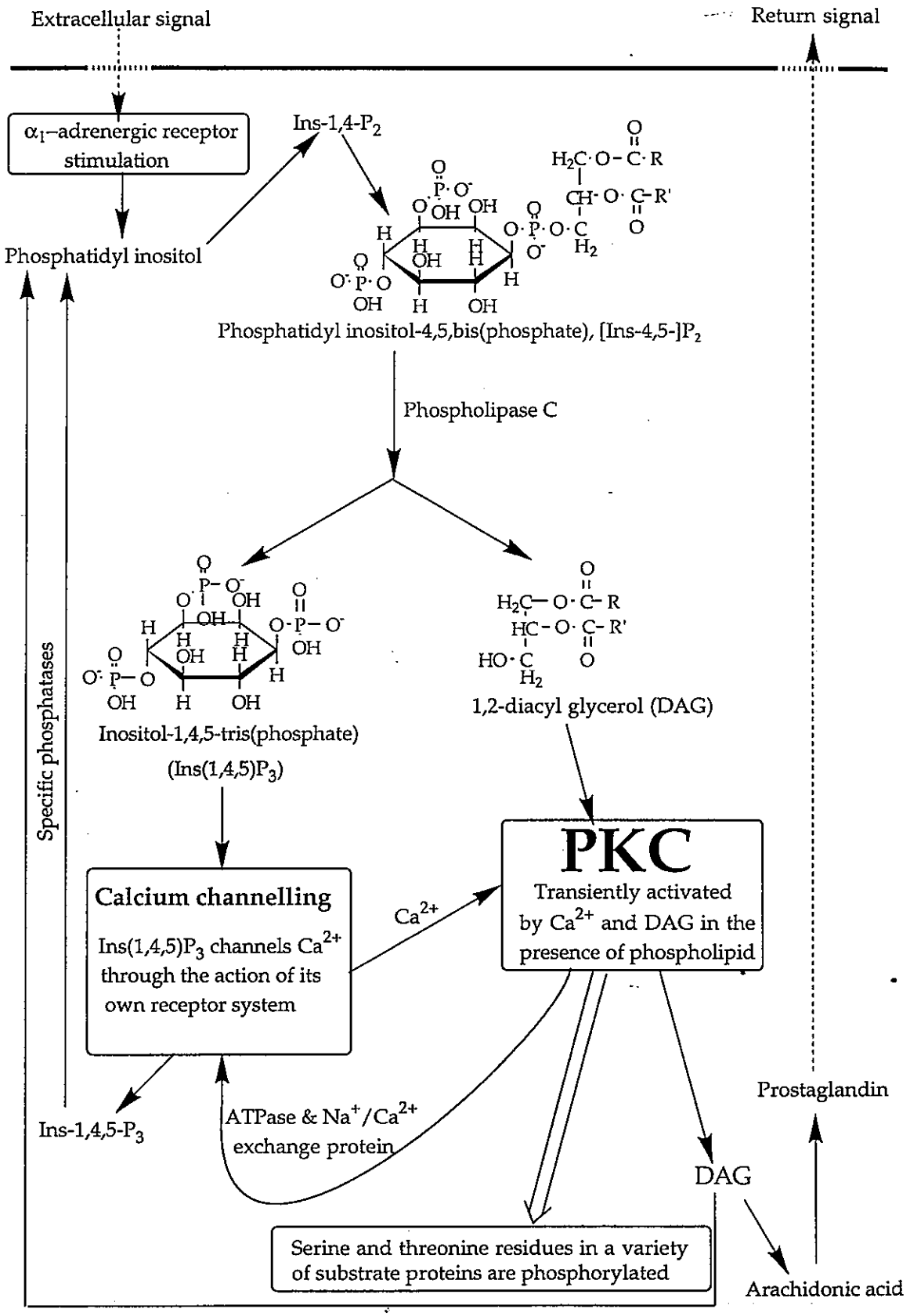


Figure 1.6 - The normal activation of Calcium dependent PKC in response to stimulation of cells by extracellular signals (adapted from[18,20,21,27,32-34,36-38]).

1.2 OUTLINE OF THE PRESENT STUDY

1.2.1 Background

Currently, no method exists for the control of *Pimelea* poisoning in cattle. At present when animals show signs of *Pimelea* poisoning the only management strategy is to move the herd to an area free of *Pimelea*, however for many property owners this is not possible. Various methods such as spraying and burning of the plant have been examined by the Queensland Department of Primary Industries (QDPI) to control *Pimelea* plant growth and germination, with limited success/cost effectiveness. *Pimelea* plants tend to germinate during autumn, flower in spring, and mature seed is dropped during early summer[2]. Anecdotal evidence suggests that the optimum conditions for germination of the *Pimelea* plant occur following late summer rain. Optimum growth conditions occur with little or no competition from pasture grasses following drought or overstocking[39].

Pimelea poisoning of cattle is continual and on-going. Losses in beef production in Queensland, New South Wales, the Northern Territory, and South Australia is increasing. Current agricultural technology has been unsuccessful, therefore control of the disease by animal health means is considered the best option. This approach has been received favourably by producers.

The highly specific nature of PKC activation by the daphnane orthoester toxins seems to indicate that alteration of key functionalities may result in a decreased toxic effect. If this hypothesis holds true, the conversion of the toxins to a form displaying decreased pharmacological activity may ultimately be achieved by micro flora bacteria present (either naturally, or introduced) in the rumen environment of cattle. This would possibly be a more favoured option, as rumen bacteria are readily transmitted within the grazing herd.

This approach requires investigation of the nature of action of the daphnane orthoester toxins regarding their interaction with PKC. Investigation of the pharmacology of simplexin and related compounds will therefore be performed to ascertain the key functionalities required for their physiological potency. The summation of this information will therefore provide a complete pathogenesis of *Pimelea* poisoning.

Management of *Pimelea* infested areas requires the knowledge of toxin levels in pasture and forage such that animals may be moved when toxin levels become close to, or above a critical threshold. This requires the development of an assay, and will be investigated initially for the use of routine laboratory equipment such as gas chromatography (GC) and high performance liquid chromatography (HPLC).

1.2.2 Immunogen Feasibility

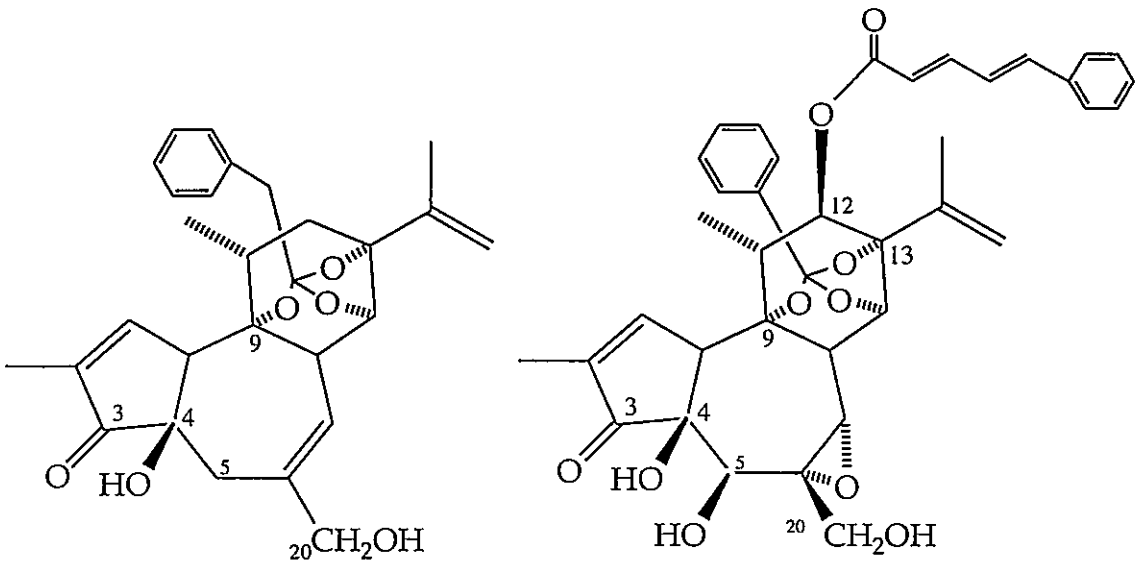
Using a vaccine or anti-dote is perceived by producers as being an efficient, and cost-effective method of control of poisoning. The production of anti-bodies against the phorbol nucleus was reported where rabbits were immunised with phorbol-13-monosuccinate which had been conjugated to bovine serum albumin (BSA)[40]. In

immuno assay experiments, the anti-sera was saturated with [^{3}H]-phorbol-12,13-dibutyrate, and its displacement by eighteen phorbol-related compounds was examined. The antibodies produced showed very high selectivity, with 4-a-phorbol and mezerein in particular, showing no serological activity[40].

In a previous study within our group, rabbits were immunised with extracts of *Pimelea* plant material which had been conjugated to Bovine Serum Albumin (BSA) via a succinylate linker. The presence of specific antibodies was confirmed by an Enzyme-Linked Immuno-sorbent Assay (ELISA) technique developed in our laboratory[41]. The purified rabbit antibodies produced were shown to prevent the contraction of bovine pulmonary venule preparations *in vitro*, caused by *Pimelea* toxins, in a dose-dependent manner. The cross-reactivity of antibodies raised against commercially available mezerein and resiniferinol orthophenyl acetate (Figure 1.7) was also investigated as a possible means of immunising cattle with conjugates produced from these commercially available orthoester toxins[41]. The results indicated that the mezerein antibodies gave higher over-all cross reactivity than *Pimelea* toxin or resiniferinol antibodies[41].

In the earlier studies, bovine anti-*Pimelea* toxin antibodies showed no neutralising effects against EC_{50} concentrations of *Pimelea* toxin on bovine pulmonary venule preparations *in vitro*[41]. The postulated reason for the lack of efficacy of the bovine anti-toxin antibodies *in vitro*, was that freeze drying the purified bovine IgG reduced its activity more than that of rabbit anti-toxin antibodies. It was also suggested that cross-linking of carrier protein during the conjugation process had an effect on the immune response of cattle, more so than in rabbits. During the current study, this hypothesis was to be examined by repeating the previous work and investigating some other conjugation approaches.

One of the prime objectives of the research described in this thesis was to refine the conjugation chemistry of plant extract to immunogenic carrier protein, and to attempt to develop a more systematic approach to the conjugation protocol. The anticipated result was that a series of conjugates would be produced, and the most efficacious would be chosen for field testing, based upon the toxin-specific antibody response (determined by ELISA), and the ability of the corresponding purified IgG antibody fraction to attenuate the effects of the *Pimelea* daphnane orthoesters *in vitro*.



Resiniferinol orthophenyl acetate Mezerein

Figure 1.7 - Structures of resiniferinol orthophenyl acetate and mezerein

1.2.3 Investigation of Daphnane Orthoester Pharmacology

An alternative to producing protective immunity in cattle may be the modification of the rumen environment, such that cattle may graze *Pimelea* plants without developing the symptoms of poisoning. This concept has been used previously in relation to beef production, for the introduction of the legume *Leucaena leucocephala*, which is an excellent source of protein for cattle[42]. *Leucaena* produces the non-protein amino acid mimosine (-[N-(3-hydroxy-4-oxopyridyl)]-a-aminopropionic acid), which can poison cattle fed large amounts of *Leucaena*. Enzymes present in the *Leucaena* plant convert mimosine to 3-hydroxy-4(1H)-pyridone (DHP) in the rumen environment, which causes goitre, and reduces thyroxine hormone production by the thyroid gland[42,43], thus reducing production capacity. The problem of *Leucaena* toxicity has been overcome in Australia by the introduction of the rumen micro flora bacteria *Synergistes Jonesii* which is found in animals in Indonesia and Hawaii, and neutralises DHP toxicity. Cattle inoculated with this bacteria can survive on a diet consisting of 100% *Leucaena* without developing DHP toxicity[43]. A similar approach to *Pimelea* poisoning may prove successful, therefore it was decided to further investigate this possibility.

The binding pharmacophore of PKC is an important place to begin the investigation, as the pivotal question is which functional groups of the simplexin molecule (and related toxins) are important for PKC binding and activation. This leads to the next key question, which is: can alteration of an essential functional group reduce the pharmacological activity of the toxin significantly enough to prevent the symptoms of *Pimelea* poisoning, and can a rumen bacterium perform the required operation? A focus of the current study has been structure-activity investigations of daphnane orthoesters in relation to the binding to, and activation of protein kinase C. The rumen is an anaerobic, reductive environment, therefore the investigation of reductive type chemical transformations were to be targeted initially.

Considerable interest in the PKC binding pharmacophore, due to a proposed link between PKC activation and tumour promotion, has resulted in a number of publications regarding structure-activity relationships concerning the activation of PKC by compounds possessing the phorbol skeleton[38,44,45]. These groups performed computer modelling of the PKC pharmacophore by comparing the phorbol esters with the bryostatins, aplysiatoxin, and diacylglycerol (the endogenous activator of PKC).

1.2.4 Analysis of *Pimelea* toxins

The development of an assay to determine levels of toxicity of live plant material and plant debris has been identified by producers and the QDPI as an area requiring further development. The current technology relies upon identification of compounds in concentrated plant extracts by high performance liquid chromatography (HPLC). However, simplexin (and related compounds) are not considered to possess high enough UV absorbing properties (log e 3.92)[46] for convenient measurement by conventional methods. Therefore, the addition of a stronger UV absorbing chromophore will be investigated in order to increase the sensitivity of the measurement of daphnane orthoester toxin levels by HPLC. Gas chromatography (GC) is also a rapid analytical process, and the derivitisation (to increase volatility) of the *Pimelea* toxins for potential GC analysis will also be examined. Knowledge of the toxicity of the *Pimelea* plant at various stages of maturity may be advantageous for use as a management tool, and may help explain observed variability in the extent of *Pimelea* poisoning between infested areas.

2.0 EXTRACTION, ISOLATION, CHARACTERISATION, AND DERIVATISATION OF TOXINS FROM *PimeleaTrichostachya*

2.1 INTRODUCTION

The objective of this series of experiments was to optimise toxin extraction from *Pimelea* plant, both in terms of yield and extraction time, as it was envisaged that large quantities of toxin would be required for conjugation reactions and further characterisation of components. Procedures for the extraction of daphnane orthoester toxins using chloroform[13], dichloromethane[47], ethanol[46,48] and methanol[15,17,49] have been published. Reported yields of these compounds varied from 0.00002% (w/w dry weight of plant material by methanol extraction at room temperature followed by silica chromatography) of huratoxin and a related compound from *Excoecaria agallocha*[15] to 0.003% (w/w dry weight) of a mixture of daphnanes from *Pimelea simplex*[49] (also using a methanol extraction followed by silica chromatography), and 0.0056% simplexin (w/w dry weight) from *Pimelea simplex*[46] (using ethanol extraction followed by Sephadex LH20 column chromatography and preparative TLC).

The extraction procedures outlined in all reports reviewed by this author involved several solvent partitioning steps as well as either gel permeation (Sephadex LH20), or silica chromatography. Therefore, improvement to the *Pimelea* toxin extraction, was to be achieved initially by endeavouring to reduce the number of steps in the extractions, through the adaptation of the method employed in a previous study from this laboratory[41]. The method involved the extraction of dried, milled *Pimelea* plant material with methanol or ethanol at room temperature, followed by partitioning with chloroform and water, where the chloroform layer retained toxic components. Vacuum assisted normal phase silica chromatography using a hexane:ethyl acetate gradient then followed, to yield *Pimelea* fractions A (50% ethyl acetate in hexane) and B (100% ethyl acetate). Finally, preparative scale C18 reverse phase high performance liquid chromatography (HPLC) using a methanol:water gradient was performed on the combined A and B fractions, yielding *Pimelea* fraction C, which contained the toxic components of the *Pimelea* plant. In general, these compounds were eluted when the solvent composition reached 90% methanol.

Examination of the extraction protocol showed that there were two main areas where improvements in the extraction procedure could be made. These were in the initial extraction and the silica chromatography. Improvement of the initial extraction procedure was to be achieved by attempting soxhlet extraction of the dried milled plant material, as it was envisaged that extraction might be more efficient at higher temperature. Soxhlet treatment of the seeds of *Diarthron vesiculosum* with hexane yielded 0.00041% w/w simplexin (dry weight plant material)[50], and it was unsure whether the heat treatment had facilitated degradation of the simplexin. Therefore, this possibility was to be investigated.

Improvement of the silica chromatography was to be achieved by using "flash" chromatography, a rapid normal phase separation technique[51].

The analysis of *Pimelea* toxins in extracts was performed using reverse phase high performance liquid chromatography (HPLC), and the feasibility of extending that technique into analysis of plant and forage samples was to be investigated. In order to develop a useful assay, the technique should be one that can be performed rapidly. For this reason, the feasiblity of analysing the derivatised *Pimelea* toxins by gas chromatography (GC) was also examined.

The work described in this chapter therefore details the methods employed for the improvement and optimisation of extraction efficiency, further characterisation of the toxic principles of the extract of *Pimelea* plant, scale-up of the extraction process, and the feasibility of derivatising *Pimelea* toxins for potential analysis of plant and forage samples by GC or HPLC.

2.2 EXPERIMENTAL

2.2.1 General

Soxhlet extractions were performed using Quick-fit EX5/75 (150g-1Litre scale parallel experiment), three EX5/63 (70g-1 Litre scale optimisation experiment), and EX11/7 (1000g-20 Litre scale subsequent large scale extractions) soxhlet apparatus. Analytical reagent (AR) grade methanol was used as the extraction solvent in all cases.

High performance liquid chromatography (HPLC) was performed using a Waters 600E multi-solvent delivery system with a Waters 486 variable wavelength detector and Maxima (Baseline) soft-ware. This system was later up-dated to a Waters 600 series system controller with 996 Photo Diode Array (PDA) detector and Millennium soft-ware. Stationary phases for all HPLC separations were Nova-Pak C-18 reverse phase supplied by Waters Australia. Analytical scale separations were performed using Waters 3.9 x 150mm cartridge columns, and preparative scale separations were performed using a Waters 25 x 100 mm Radial Compression Module with 25 x 100 mm C-18 cartridge. Fractions were collected from the preparative scale separations using a Gilson Model 203 Micro Fraction Collector. All solvents used were HPLC grade, filtered through Millipore 0.45mm membrane filters, and degassed by sparging with high purity helium gas prior to use.

Nuclear magnetic resonance spectra (^{1}H and ^{13}C) were recorded using a Bruker AMX 300, 300MHz nuclear magnetic resonance spectrometer in $CDCl_3$ unless otherwise stated. Low resolution GC-MS data were obtained using a Shimadzu GC-14-H, GCMS-QP2000A quadrupole gas chromatograph-mass spectrometer incorporating a direct injection probe. GLC analyses were performed using a Varian 3300 Gas Chromatograph with a non-polar SE30 stationary phase (15m x 0.53mm ID with 1.2µm film thickness) and a Varian 3400 Cc Gas Chromatograph with a polar carbowax stationary phase (30m x 0.32mm ID with 0.25µm film thickness).

Infrared spectra were recorded using a Perkin Elmer 1600 series Fourier Transform Infrared Spectrometer. Compounds were presented as KBr disks.

Compounds eluted with the main "toxic" fractions were given "PC" codes in the laboratory, which was an abbreviation for *Pimelea* C. The compounds corresponding to the peaks PC4, PC5, PC6, and PC7 were targeted for analysis (Refer to Figure 2.1).

Mezerein was chosen as a model daphnane due to its commercial availability (CalBiochem Australia Pty Ltd), and its structural similarity to simplexin.

2.2.2 Parallel Room Temperature and Soxhlet Extraction Techniques

Approximately equal quantities of milled, dried *Pimelea trichostachya* plant material were extracted with methanol by using the two different extraction techniques. The room temperature extraction involved the suspension of dried, milled plant material (150 grams) in 2.5 litres of AR-grade methanol stirring at room temperature for a total of twenty two hours, (the solids were filtered off after fourteen hours, and fresh solvent added). The soxhlet method involved refluxing 600mL of methanol at 65°C for twenty two hours in a 1L extraction flask connected to a Quickfit® EX5/75 soxhlet apparatus. The plant material (153 grams) was packed into the appropriate soxhlet thimble, with no attempt made to maximise plant material:solvent surface area. The solids were removed from the room temperature extraction by filtering, while the soxhlet extract was allowed to cool to room temperature prior to solvent removal under vacuo, followed by chloroform:water partitioning. The aqueous layer was discarded, as it had been shown previously that the toxic components were retained in the chloroform layer[41]. After concentrating the extracts, a dark coloured, tar-like residue resulted. The crude, partitioned extracts from both extractions were processed separately using the method previously employed[41], with the exception of the silica chromatography. "Flash" chromatography[51] rather than vacuum assisted chromatography was adopted as a time saving approach and this methodology was likely to give a greater separation. Table 2.1 shows the solvent profile used.

Fraction	Volume (mL)	Hexane:Ethyl acetate
1	250	90:10
2	250	75:25
A	250	50:50
B	250	0:100

Table 2.1 - Solvent profile used for purification of *Pimelea* extracts (This scale enabled the processing of approx 2g crude, partitioned extract).

Fractions A and B were shown previously[41] to contain the toxic components, and the fractions were combined as it was possible to resolve the components by HPLC. This approach was adopted as a time saving approach using gradient 1 (Table 2.2). Compounds producing peaks corresponding to those most toxic observed by Nayyar[41] were isolated, purified, and structurally evaluated by one and two dimensional ^{1}H and ^{13}C NMR, mass spectrometry, and infrared spectrophotometry where indicated.

Time (minutes)	Flow rate (mL/min)	%Methanol	%Water
0.00	1.00	60	40
13.00	1.00	65	35
21.00	1.00	70	30
29.00	1.00	75	25
45.00	1.00	85	15
53.00	1.00	90	10
90.00	1.00	90	10
100.00*	1.00	60	40
120.00*	1.00	60	40

* These gradient steps were used to re-equilibrate the column.

Table 2.2 - Gradient 1, Analytical HPLC gradient.

2.2.3 Identification of Toxins from *Pimelea trichostachya.*

The confirmation that simplexin and related daphnane toxins had survived the soxhlet treatment was required. This was achieved following comparison of the chromatograms of *Pimelea* extracts produced by Nayyar, 1994[41] by isolation and purification of a series of components found to be toxic using the fish bio-assay technique[41]. The compounds were separated using gradient 2 (Table 2.3), and collected by fraction collector. During purification, the target compounds were separated from co-eluting chlorophylls on the preparative scale by using an acetonitrile:water gradient (gradient 3, Table 2.4). The purified compounds were stored at -15°C, under a nitrogen atmosphere prior to NMR analysis in $CDCl_3$. These compounds were also analysed by low resolution direct injection mass spectrometry.

Time (minutes)	Flow rate (mL/min)	%Methanol	%Water
0.00	5.00	60	40
3.00	5.00	65	35
7.00	5.00	70	30
10.00	5.00	75	25
13.00	5.00	80	15
19.00	5.00	90	10
23.00	5.00	90	10
70.00*	5.00	60	40
80.00*	5.00	60	40

* These gradient steps were used to re-equilibrate the column.

Table 2.3 - Gradient 2, Preparative HPLC gradient.

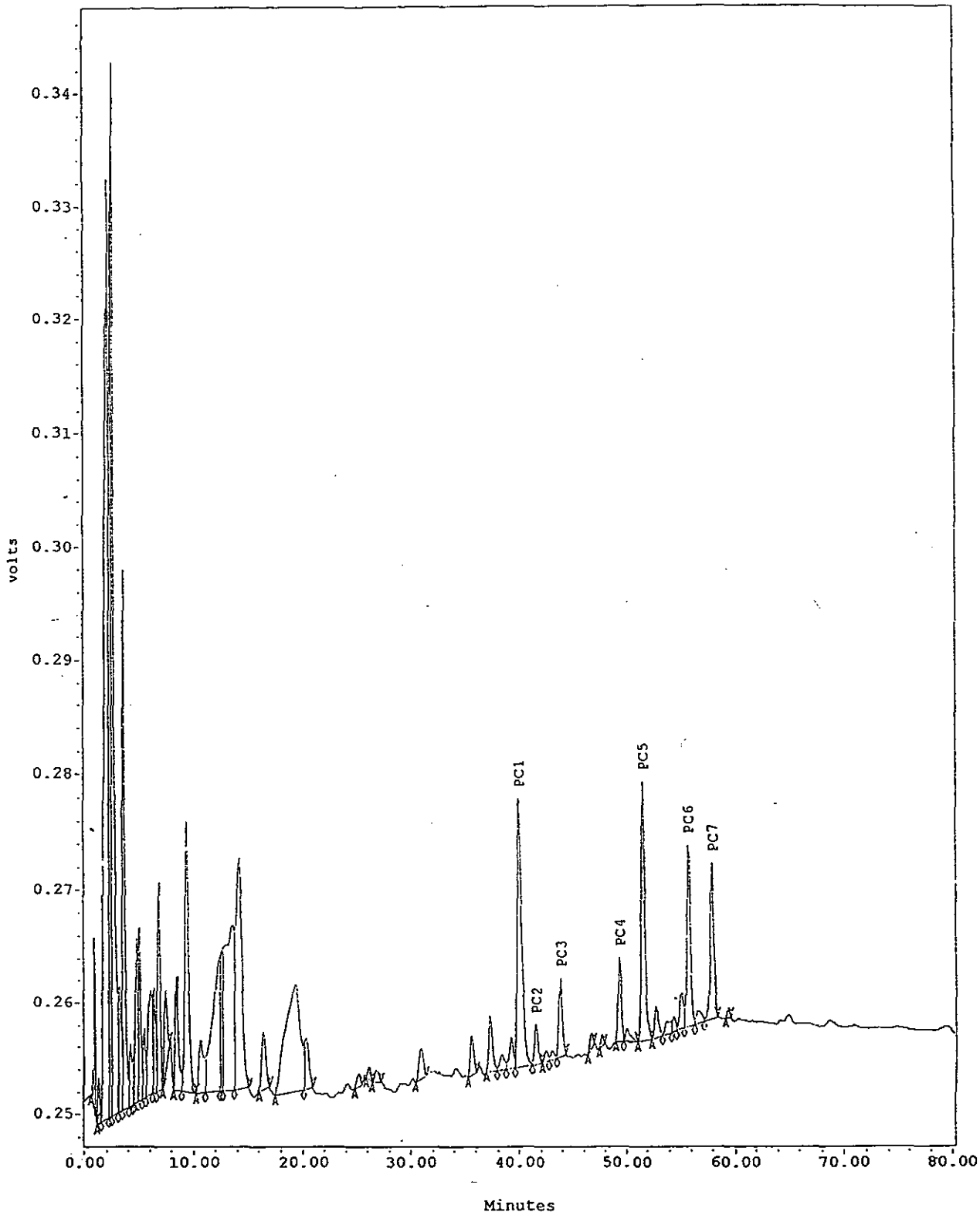


Figure 2.1 - HPLC trace of Pimelea AB fraction showing the toxic fraction.

Time (minutes)	Flow rate (mL/min)	%Acetonitrile	%Water
0.00	12.00	80	20
2.00	12.00	80	20
4.00	12.00	95	5
14.50	12.00	95	5
‡14.60	12.00	0	100
‡16.00	12.00	0	100
*17.00	12.00	20	80
*18.00	12.00	20	80

‡ Used for column flushing

* used to re-equilibrate the column.

Table 2.4 - Gradient 3, Preparative HPLC gradient.

2.2.4 Optimisation of the Extraction Process

The solvent:plant material ratio was increased by performing the soxhlet extraction using three Quickfit EX5/63 soxhlets assembled with a one litre extraction flask containing 800mL of methanol. Approximately 70 grams of plant could be extracted in one extraction using this equipment. The plant material was extracted for seventy two hours, with samples (20mL) being taken from the extraction flask at 4, 8, 16, 24, 48, and 72 hours. These samples were each concentrated, extracted into chloroform, and chromatographed as described above for the bulk extracts. Relative simplexin levels were determined by duplicate HPLC analyses, using pure simplexin as an external standard.

2.2.5 Scale-up of *Pimelea* plant extraction

The soxhlet extraction technique was scaled-up using Quickfit EX11/7 pilot scale soxhlet extraction equipment. The thimble was packed with approximately 900 grams of a fresh batch of dried, milled *Pimelea trichostachya* plant material. Glass wool-filter paper sandwiches were placed every 100g in order to increase the solvent-plant material surface area interface by allowing percolation of solvent. The plant material was extracted for 48 hours.

2.2.6 Preparation of Toxin Derivatives for GC Analysis

The preparation of potentially volatile trimethylsilyl (TMS) and phenyl boronate derivatives of mezerein was first investigated by derivatising the hydroxyl functions of cyclohexyl methanol, cyclohexanol (TMS derivatives)[52-54], and 1,3-propane diol (phenyl boronate derivative)[55,56] as model compounds. All derivatisation reactions were performed in clean, dry Wheaton reaction vials using dry pyridine as the solvent.

Either cyclohexyl methanol (0.0088 mmol) or cyclohexanol (0.01mmol) was treated with either N-methyl(N-trimethylsilyl)-trifluoroacetic acid (MSTFA) (0.8 mmol) in pyridine (1mL), or 1,1,1,3,3,3-hexamethyl disilazane (HMDS) (0.1 mmol) with trimethyl silyl chloride (TMS) (0.1 mmol). The reaction vessels were warmed to

approximately 50°C for 5 minutes to ensure complete reaction. The reaction products were then analysed by GC-MS, which confirmed 100% conversion of cyclohexyl methanol and cyclohexanol to their respective TMS ethers. For the production of their respective phenyl boronate derivatives, 1,3-propane diol (0.066 mmol) and mezerein (0.0015 mmol) were each dissolved in pyridine (1 mL) and treated with phenyboronic acid (0.1 mmol and 0.002 mmol respectively).

The success of the derivitisation chemistry for the model compounds was confirmed firstly by GC, followed by GC-mass spectrometric analysis. Elution of model compounds and their TMS derivatives was achieved iso-thermally at 100°C. However, elution of 1,3-propane diol and its phenyl boronate derivative was achieved isothermally at 120°C. Elution of the TMS derivatives of cyclohexyl methanol and cyclohexyl methanol was attempted iso-thermally at 120°C using a polar carbowax column, however better separation was achieved using the non-polar SE30 column.

Positive results for the TMS and phenyl boronate derivatisation reactions of mezerein were confirmed by HPLC. A temperature ramp from 220°C up to 250°C (using the SE30 column) was employed (Figure2.2) in an attempt to elute the derivatives of mezerein by GC.

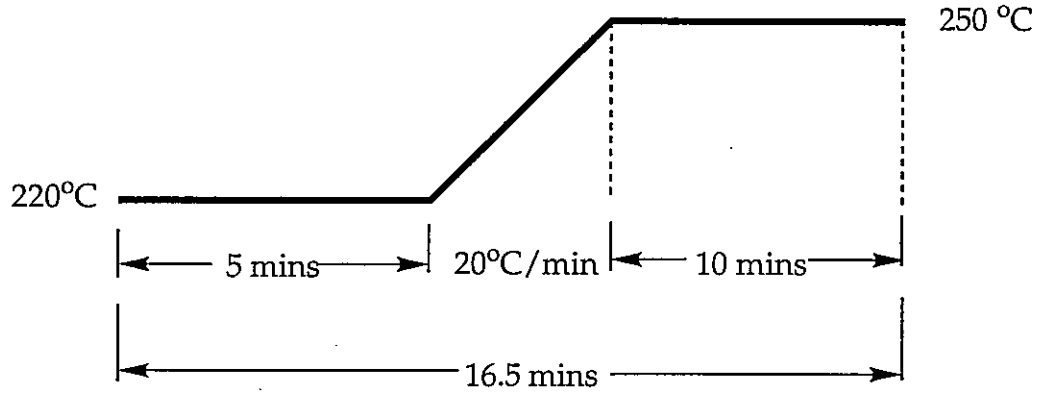


Figure 2.2 - GC ramp employed for attempted elution of mezerein and derivatives by GC, (injector temp, 250°C, detector temp, 260°C).

2.2.7 Derivatisation of Toxins for HPLC Assay

The addition of a UV. chromophore to the toxins was attempted initially by protection of the C4, 5, and 20 hydroxyl groups of mezerein, and simplexin as either benzoate, or 4-nitrobenzoate esters. In the first instance, mezerein (0.0076 mmol) was dissolved in dry THF (1mL), and stirred at room temperature. Triethylamine (0.03mmol) was added to the stirred mixture, followed by benzoyl chloride (0.03 mmol). The progression of the reaction was monitored by TLC eluting with 50:50 ethyl acetate:hexane with mezerein as the reference (R_f 0.49). The reaction product was indicated by a higher R_f (0.90) spot, and the reaction was complete when the spot corresponding to mezerein had disappeared, which occured after approximately 14 hrs. The solvent was removed from the reaction mixture by rotary evaporation, and the remaining residue was dissolved in ethyl acetate (2mL) and quenched with water (2mL). The aqueous layer was discarded, and the remaining organic layer was washed with 0.1N NaOH (5 mL), followed by 5%

w/v sodium bicarbonate solution (3 x 5mL). The resulting product was purified by silica chromatography, yielding 4.9mg of product. The analytical HPLC gradient was modified to reduce retention of the compounds (gradient 4, Table 2.5) in order to increase the analysis rate. For example, the retention time of mezerein was 38.6 minutes using gradient 1, while it was reduced to 12.1 minutes using gradient 4.

Time (minutes)	Flow rate (mL/min)	%Methanol	%Water
0.00	1.00	80	20
7.00	1.00	95	5
30.00	1.00	95	5
35.00	1.00	80	20

* These gradient steps were used to re-equilibrate the column.

Table 2.5 - Gradient 4, Analytical HPLC gradient.

The derivatisation procedure was repeated by treating mezerein (0.0076mmol) and simplexin (0.0094 mmol) with molar excesses of 4-nitrobenzoyl chloride, in an analogous manner.

2.3 RESULTS AND DISCUSSION

2.3.1 Isolation and Characterisation of *Pimelea* Toxins

The dried, milled *Pimelea* plant material was extracted using the room temperature and the soxhlet extraction methods in a parallel experiment to ascertain whether the plant toxins survived soxhlet extraction conditions. The yields of crude partitioned extract from the room temperature extraction was slightly higher than that of the soxhlet extraction. However, the AB fraction yield from the soxhlet extraction technique was almost double that of the room temperature extraction. HPLC analysis of the AB fractions indicated that the relative intensities of the peaks corresponding to the desired compounds were higher for the soxhlet extraction. Using preparative scale HPLC, the AB fractions were processed to give *Pimelea* fraction C, and it was shown that the fraction C yield by soxhlet was almost ten times that of the room temperature extraction. Table 2.6 summarises these findings.

Extract	Room temperature extraction	Soxhlet extraction
Milled, dried plant material	150g	153g
Crude, partitioned extract	3.91g	3.45g
Fractions A and B	161mg	256mg
Fraction C	≈4mg	≈38mg

Table 2.6 - Comparative extraction yields.

The corresponding yields of the toxic *Pimelea* fraction were 0.0027% w/w dry plant material by room temperature extraction, and 0.025% w/w dry plant material by soxhlet extraction. These data confirm that the soxhlet technique was more efficient for extraction of toxins from the *Pimelea* plant material, in that firstly, less than one quarter of the solvent used for the room temperature extraction was required for the soxhlet extraction technique. Secondly, an increase in yield of almost one order of magnitude was achieved using the soxhlet technique without optimisation, which was a significant improvement in *Pimelea* toxin yield.

The compounds corresponding to the peaks PC5 and PC6 were equivalent to the peaks corresponding to the most toxic compounds by fish bio-assay[41]. The compound PC5 (from both the room temperature, and the soxhlet extractions) was isolated, purified, and characterised by nuclear magnetic resonance spectroscopy. The ^{1}H spectrum of PC5 from both the room temperature and soxhlet extraction techniques were identical, and gave excellent agreement with those published for simplexin[46,57] (Figures 2.3 and 2.4).

The compound corresponding to PC6 was found to also possess a parent daphnane type structure and was in good agreement with that of huratoxin, which is also known to be present in *Pimelea trichostachya*[46]. The proton assignments of this compound were

confirmed by ^{1}H-^{1}H homnuclear correlation (Figure 2.5 and 2.6 summarises the ^{1}H NMR shift assignments for this compound).

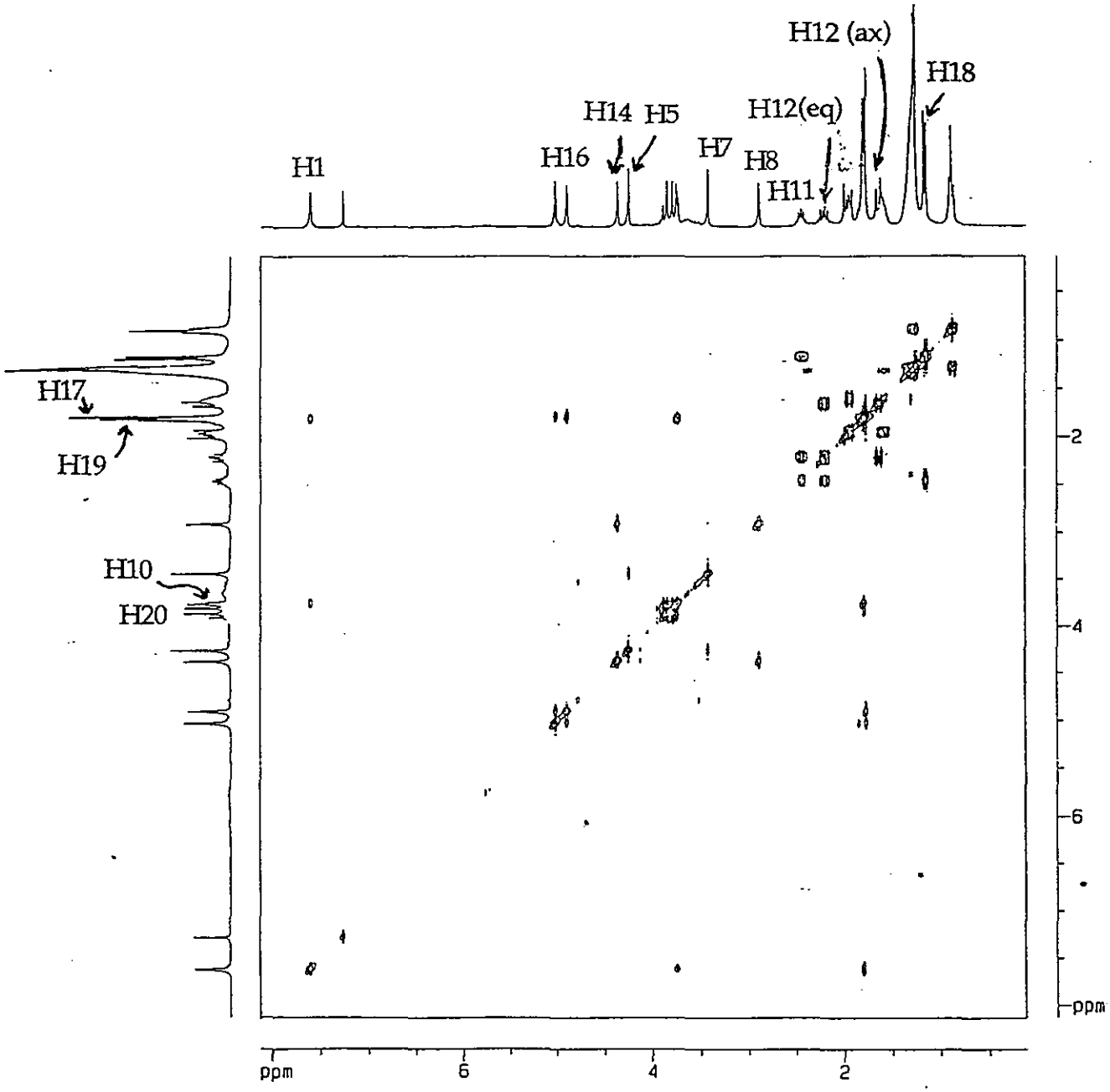


Figure 2.3 - ^{1}H-^{1}H Homonuclear Correlation (COSY) NMR spectrum for PC5 - (Simplexin)

Chemical Shift, δ ,(ppm)	Coupling (m, J(Hz))	Environment	Observed Coupling to
7.60	m, 7.0	H1	H10 and H19
5.01	methylene d, 36.5	H16	H17
4.37	d, 2.4	H14	H8
4.26	d, 0.0	H5	H7
3.96, 3.90, 3.86, 3.80	AB quartet, 29.4	H20	
3.75	d, 2.6	H10	H1 and H19
3.44	d, 0.0	H7	H5
2.91	d, 2.2	H8	H14
2.47	p, 15.7	H11	H18 and H12 (eq)
1.96	dd, 14.1	H12 (eq)	H12 (ax) and H11
1.65	d, 13.7	H12 (ax)	H12 (eq)
1.81	dd, 2.7	H19	H10 and H1
1.78	dd, 0.6	H17	H16
1.17	d, 7.1	H18	H11
0.89	t	CH_3- side chain	
1.96	m	-CH_2- side chain	
3.97, 3.95, & 3.60	s (exchangeable)	-OH	

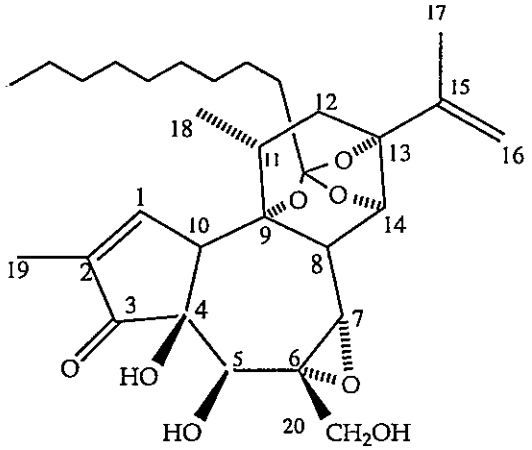


Figure 2.4 - ^{1}H NMR shift assignments for PC5 (simplexin)

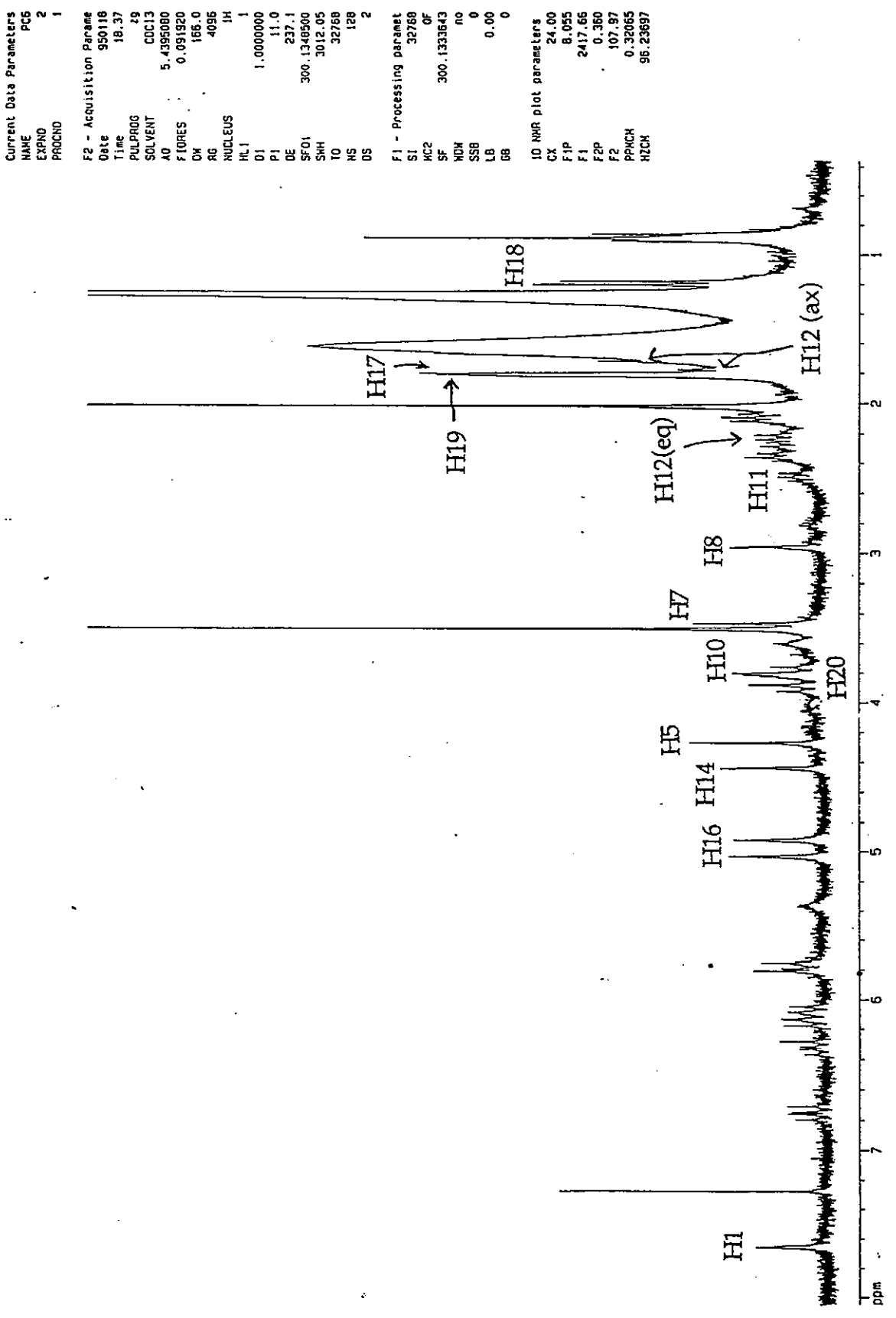


Figure 2.5 - ^{1}H- NMR spectrum of PC6 - (huratoxin)

Chemical Shift, δ ,(ppm)	Coupling (m, J(Hz))	Environment	Observed Coupling to**
7.60	m, 6.9	H1	H10 and H19
6.75	dd, 12.2	H_c	H_b and H_d
6.35	dd, 11.1	H_d	H_b and H_c
6.10	ddd, 11.0	H_b	H_a H_c and H_d
5.88	ddd, 13.4	H_a	$-CH_2-$ and H_b and H_c
5.01	methylene d,36.0	H16	H17
4.37	d, 2.5	H14	H8
4.26	d, 0.0	H5	H7
3.93, 3.89, 3.80, 3.76	AB quartet, 35.5	H20	
3.75	d, 2.3	H10	H1 and H19
3.44	d, 0.0	H7	H5
2.91	d, 2.3	H8	H14
2.47	p, 14.4	H11	H18 and H12 (eq)
1.96	dd, 14.0	H12 (eq)	H12 (ax) and H11
1.65	d, 13.9	H12 (ax)	H12 (eq)
1.81	dd, 2.7	H19	H10 and H1
1.78	dd, 0.6	H17	H16
1.17	d, 7.4	H18	H11
0.89	t	CH_3- side chain	
1.96	m	$-CH_2-$ side chain	
3.97, 3.95, & 3.60	s (exchangeable)	-OH	

** Confirmed by ^1H COSY

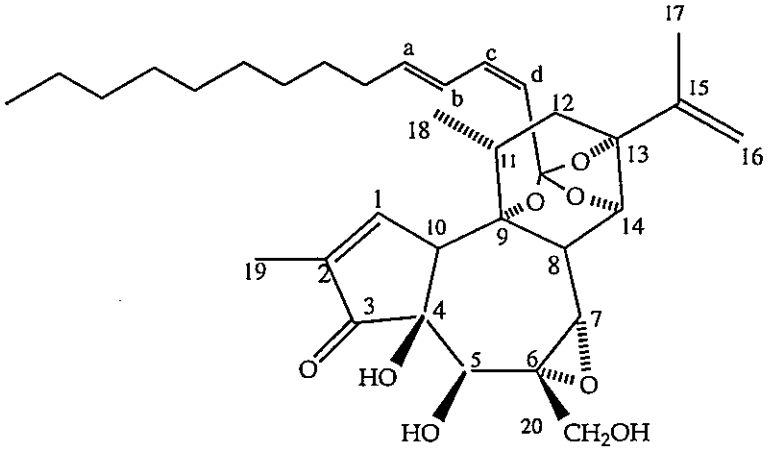


Figure 2.6 - ^1H NMR shift assignments for PC6 (huratoxin)

The compounds PC4 and PC7 (found to be toxic by fish bio-assay however not as potent as PC5 and PC6)[41] were also purified for characterisation, and these compounds were found not to be of the daphnane type. The ^{1}H NMR spectrum of PC4 showed striking similarity to that of arachidonic acid. Using direct injection mass spectrometry, it was shown that this compound possessed elements of the 11,14,17-eicosatrienoic acid structure (no molecular ion observed), and the fragmentation pattern of this compound gave exceptional agreement (Figure 2.7), ($C_{20}H_{34}O_2$), (m/e:- 163 $C_{12}H_{19}$; 149 $C_{11}H_{17}$; 135 $C_{10}H_{15}$; 109 C_8H_{13}; 108 C_8H_{12}; 95 C_7H_{11}; 69 C_5H_8; 55 C_4H_7), (^{1}H :- d 6.63 s (broad) 1H; d5.38 m 6H; d2.81 m 4H; d2.62 m 2H; d2.10 m 4H; d1.63 m 2H; d1.29 m 11H; d0.98 t 3H).

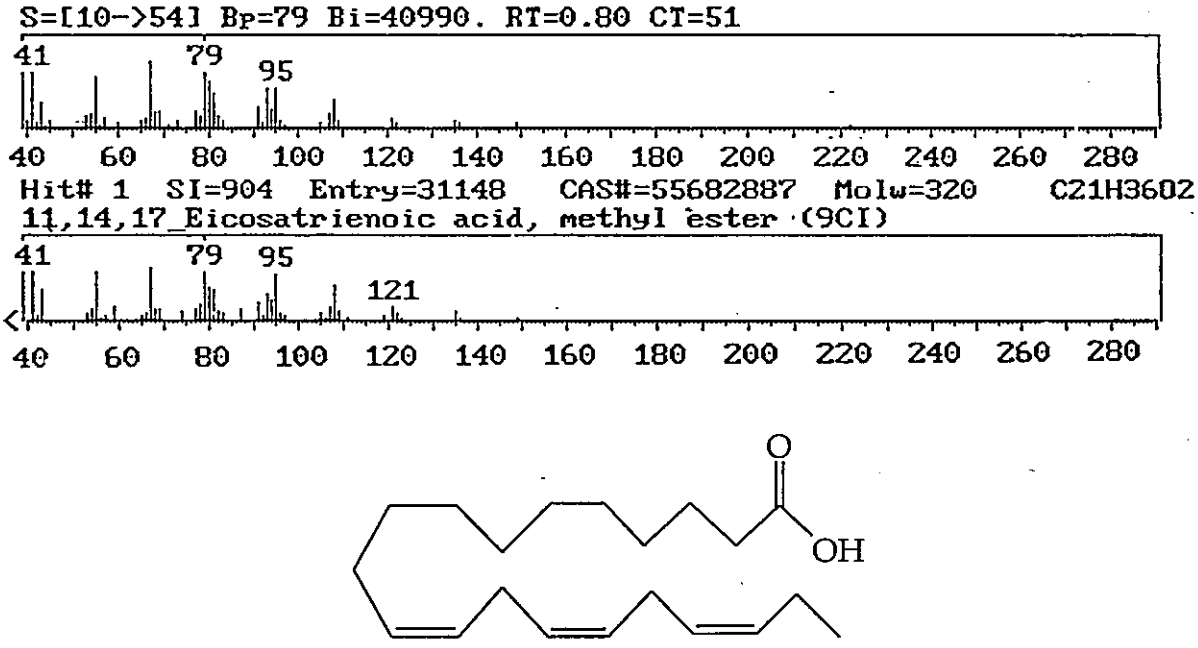


Figure 2.7 - *Pimelea* constituent PC4 - 11,14,17-eicosatrienoic acid.

Using the same techniques, the compound corresponding to the peak labelled PC7 was identified. The molecular ion also was not seen in the mass spectrum, however the fragmentation pattern closely resembled that of *trans*-7-tetradecene ($C_{14}H_{28}$), (m/e:- 139 $C_{10}H_{19}$; 125 C_9H_{17}; 111C_8H_{15}; 97 C_7H_{13}; 83 C_6H_{11}; 69 C_5H_9; 55 C_4H_7; 41 C_3H_5), (^{1}H :- d5.36 ddd 1H; d2.35 ddd 2H; d2.01 ddd 2H; d1.68 ddd 2H; d1.28 m 4H; d0.89 t 3H), (IR:- 3050cm^{-1}, C-H str alkene; 2925, 2854cm^{-1}, C-H str aliphatic; 1709cm^{-1}, C=C str alkene, 1458cm^{-1}, C-H def alkane; 968cm^{-1}, H-C=C-H str *trans*-alkene; 722cm^{-1}, C-H rock of -CH_2-); as also shown in Figure 2.8.

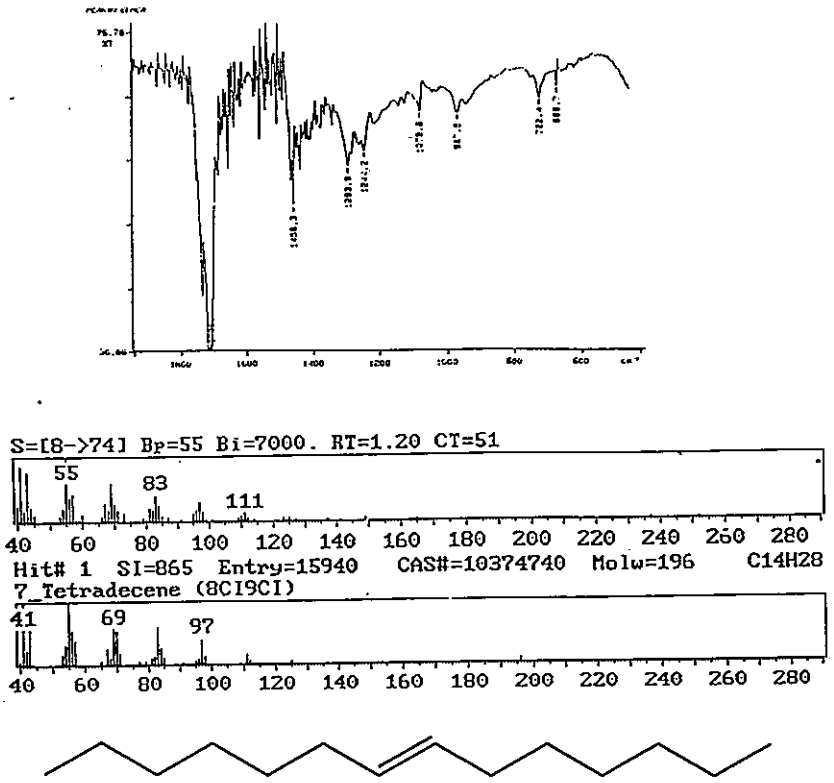


Figure 2.8 - *Pimelea* constituent PC7 - *trans*-7-tetradecene.

The appearance of 11,14,17-eicosatrienoic acid raises an interesting point in this work, in that compounds possessing this type of structure are precursors to prostaglandins in animal systems. For example, $\Delta^8,\Delta^{11},\Delta^{14}$-eicosatrienoate is the pre-cursor to the prostaglandin PGE_1[58]. Although 11,14,17-eicosatrienoic acid and *trans*-7-tetradecene were found to be toxic by fish bio-assay[41], the possibility exists that these compounds were found to be toxic due to residual simplexin and huratoxin present as contaminants in the corressponding fractions, which may have given rise to the observed toxicity.

2.3.2 Optimisation and Scale-up of Soxhlet Extraction

Once it had been established that the daphnane orthoester toxins from *Pimelea trichostachya* survived soxhlet treatment, the process was optimised prior to scaling up. Three Quickfit EX5/63 soxhlets were loaded with approximately twenty grams of ground *Pimelea trichostachya* plant material per thimble, and connected to a one litre flask, thus attempting to maximise the plant material:solvent surface area. The plant material was extracted into 800mL of AR methanol over a period of seventy two hours. Samples (20mL) were taken from the flask at specified time intervals, and worked-up to give the AB fraction products as previously described. These fractions were analysed using reverse phase HPLC and pure simplexin as an external standard. The results are expressed as the amount of simplexin for a fixed volume aliquot, calculated relative to peak area at 240nm. The results are presented as the average of duplicate analyses (Table 2.7).

Figure 2.9 illustrates the crude and AB fraction yields (in milligrams) as a function of time. It is noted here that the AB fraction yield peaks after twenty four hours, then slowly decreases, while the crude yield peaks at forty eight hours, and remains reasonably static thereafter.

Time, (hrs)	Weight of Crude Extract (mg)	Weight of AB fractions (mg)	Simplexin on column (µg)
0	0	0	0
4	23.9	5.0	2.7
8	47.4	14.6	4.0
16	56.9	25.8	5.2
24	68.1	34.8	6.0
48	85.7	28.1	5.9
72	89.3	22.4	4.7

Table 2.7 - Extraction time-course data

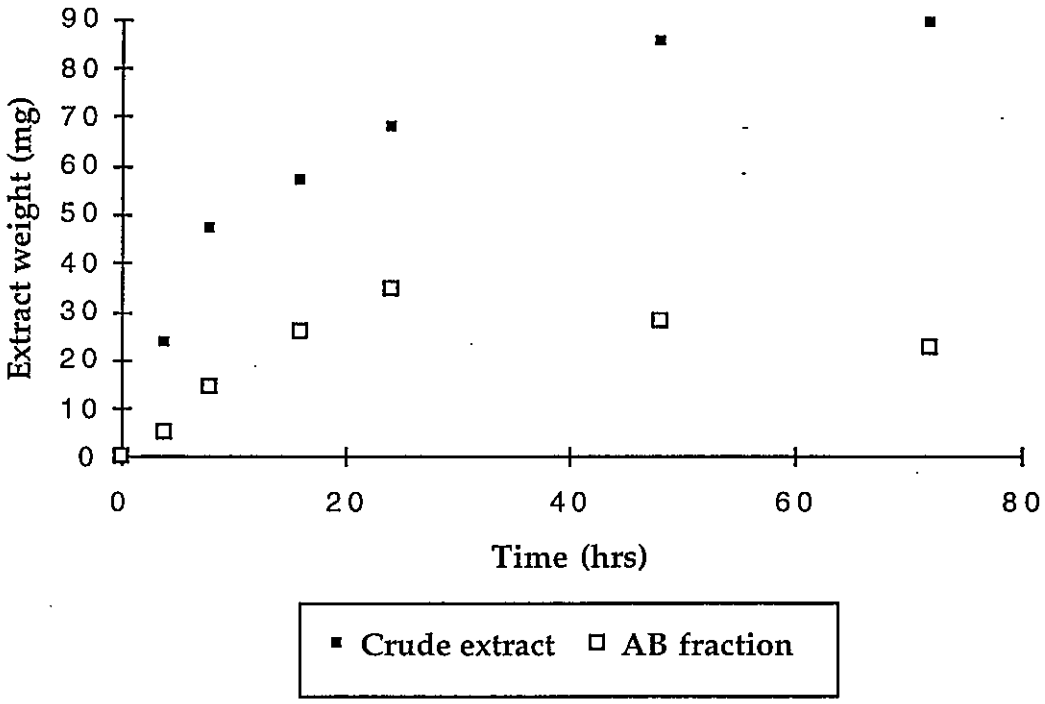


Figure 2.9 - Extract weight as a function of time for *Pimelea* extract optimisation

The relative levels of simplexin as a function of time are illustrated (Figure 2.10), and this profile demonstrates that the yield peaks after 24 hours, remains relatively constant, then declines after 48 hours. At this stage, the rate of decomposition exceeds the rate of extraction. The correlation of Figure 2.9 with Figure 2.10, enabled the prediction of the maximum simplexin yield, while minimising entrainment of extraneous material. Most of the more polar compounds (such as carbohydrates) would be removed from the crude initial extract during partitioning with chloroform and water, while most of the less polar substances would be removed from the extract during silica chromatography. Therefore, maximum simplexin recovery coupled with a minimum mass yield of Fraction AB indicated an optimisation of the yield and purity of recovered simplexin under these conditions. Therefore, all subsequent extractions were performed for 48 hours.

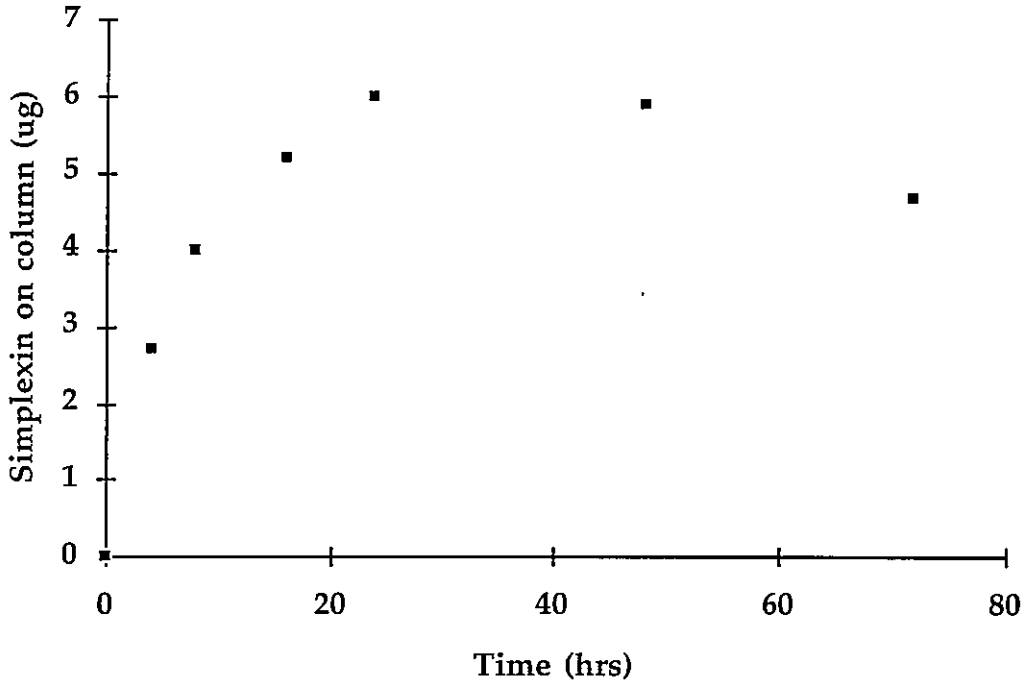


Figure 2.10 Simplexin levels relative to time for *Pimelea* extraction optimisation

Compared with previous extractions, the AB fraction composition upon scale-up showed a vast improvement in simplexin and huratoxin yields . A typical chromatogram of the pilot scale extraction is shown in Figure 2.11, and comparison with Figure 2.1 emphasises the extent of the improvements made. The *Pimelea* AB fraction yields (predominantly simplexin with huratoxin) were approximately 0.6% w/w of the dry plant material using this scale, which represents an increase in yield of more than two orders of magnitude compared with previous studies (0.003% w/w[49] and 0.0056% w/w[11]).

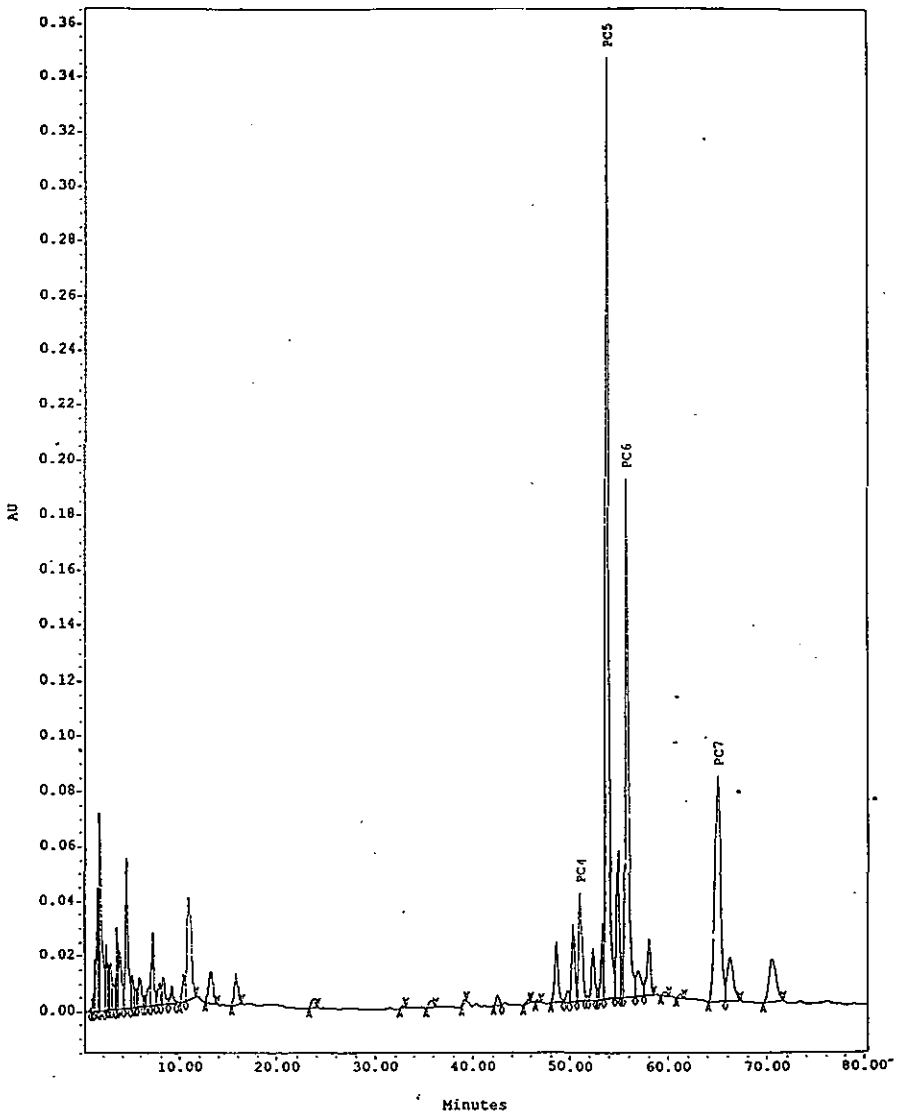


Figure 2.11 - Typical chromatogram of the AB fraction (pilot scale extraction).

2.3.3 Derivatisation of Toxins

Examination of the parent structure of simplexin (Figure 2.12) shows that the only UV absorbing chromophore is the enone function present in the five membered ring moiety. The molar absorptivity coefficient (e) of simplexin was experimentally found to be 5911 at 240 nm (log e 3.77, 0.094 mM in methanol (literature $\log_{e(244\ nm)}$ 3.91 ethanol[46,47]), which is relatively low, indicating detection by HPLC using conventional UV detection may not be sensitive enough for determination of simplexin levels in plant or forage samples.

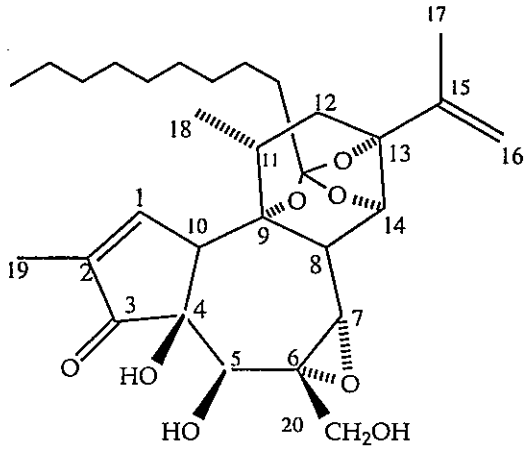


Figure 2.12 - simplexin

The concentration - response curve for simplexin was found to be linear over the range of 40 ppb to 800 ppb based upon a 5µL injection, or an analytical column load of 0.200 µg to 4.00 µg (Figure 2.13), indicating adherence to the Beer-Lambert law over the specified range. Detection of simplexin in samples at 40ppb may be achieved, however in order to obtain samples of approximately this concentration, substantial sample size (approximately 7g of dried plant sample) would need to be processed to obtain levels within the detection range of the HPLC system. Therefore, analysis of samples of low simplexin concentration by HPLC may not be achieved.

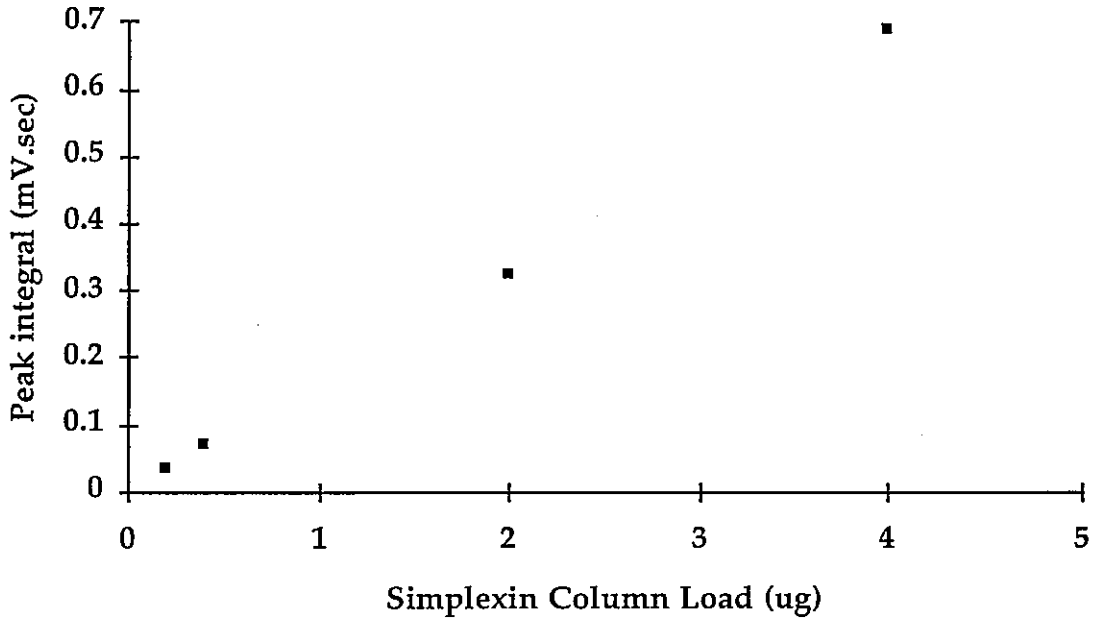


Figure 2.13 - Peak Integral as a function of Simplexin Column Load (µg).

The *Pimelea* toxins were found not to be eluted using GC, and this property was assumed to be due to lack of volatility. Direct injection-low resolution mass spectrometry of simplexin and huratoxin proved not to produce fragmentation patterns characteristic of the daphnane compounds, confirming lack of volatility. Therefore the

feasibility of producing volatile trimethyl silyl (TMS), and phenyl boronate derivatives of the daphnane orthoester compounds was examined.

The feasibility of producing TMS derivatives of the daphnane orthoesters was first examined by derivatisation of the model compounds cyclohexyl methanol, and cyclohexanol in order to simulate the primary and secondary hydroxyl functions of the daphnane orthoesters. Two different silylation methods were investigated in order to determine the preferred derivatisation method[52,54]. The preferred method was that of Sweeley *et al*, 1963[52], which involved the treatment of the model alcohol with 1,1,1,3,3,3-hexamethyldisilazane and trimethyl silyl chloride in dry pyridine. This method appeared to be the cleanest approach, and the results showed 100% conversion of both cyclohexyl methanol (retention time (R_t) 1.15 minutes) and cyclohexanol (R_t 0.71) to the respective TMS ethers (cyclohexyl methanol-TMS-ether, R_t 2.08 minutes, m/e 171, $C_9H_{19}OSi$ - loss of CH_3 radical, and cyclohexanol-TMS-ether (R_t 1.21, m/e 172, $C_9H_{20}OSi$ - molecular ion)). Mezerein (the model daphnane orthoester) (R_t 12.10 minutes using HPLC gradient 3) was treated in the same manner as the model compounds resulting in 100% conversion to a mixture of mono- (12.4%, R_t 19.39 mins, HPLC), di- (82.9%, R_t 28.17 mins, HPLC), and tri- (4.7%, R_t 30.46 mins, HPLC) TMS ethers. No signals were obtained when this mixture was injected into the GC, which indicated that the derivatives were not volatile enough for GC or GC-MS analysis.

The feasibility of further derivatisation of mezerein was investigated by producing the 4,20-phenyl boronate derivative[55]. The compound 1,3-propane diol was chosen as a model compound in order to validate the method. Using an SE30 column, elution of 1,3-propane diol by GC was achieved isothermally at 80°C with a retention time of 1.02 minutes, while the corresponding phenyl boronate derivative was eluted isothermally at 120°C with a retention time of 3.61 minutes. The identity of this compound was confirmed by GC-MS (m/e: 162, $C_9H_{11}O_2B$; 104, C_6H_5OB). The corresponding C4, C20 phenyl boronate derivative of mezerein was also not volatile enough to be eluted by GC. The HPLC trace of the reaction mixture showed that the phenyl boronate derivative of mezerein had a retention time of 15.50 minutes, which was slightly longer than that of mezerein (12.10 minutes). The lack of volatility of this derivative also confirms that it was not feasible to produce volatile derivatives of the daphnane orthoesters. Therefore potential analysis of these compounds in plant or forage samples was not achievable by gas chromatography.

The treatment of mezerein with benzoyl chloride was performed in order to potentially derivatise the daphnane orthoester by the addition of a UV chromophore to the hydroxyl functions at position 4, 5 and 20 for improved analysis by HPLC. The reaction produced a mixture of di- and tri- benzoate esters of mezerein, which indicated that addition of a strongly absorbing UV chromophore (such as cinnamate, or retinoate) may be achieved (appreciably increasing the sensitivity of assay by HPLC). This rationale was however not pursued, as the reaction produced a mixture of di- and tri-derivatised products. The investigation of a PKC activation assay and a radio-ligand binding assay to measure daphnane orthoester concentrations in *Pimelea* extracts is currently being investigated in this laboratory[59].

2.4 CONCLUSIONS

The efficiency of extraction of simplexin and huratoxin from *Pimelea* plants has been increased by more than two orders of magnitude compared with yields obtained in previous studies. Soxhlet extraction of *Pimelea trichostachya* using methanol followed by rapid normal phase silica chromatography, was found to yield a simplexin-huratoxin mixture (predominantly simplexin) equivalent to 0.60% w/w of dried plant material compared with 0.056% w/w (the highest yield obtained in previous studies). This has enabled the accumulation of sufficient quantities of simplexin and huratoxin for subsequent studies, and this approach may prove econmically viable for future simplexin production.

The compounds corresponding to the most toxic by fish bio-assay were found to be the daphnane orthoesters simplexin and huratoxin. Elements of the structures of 11,14,17-eicosatrienoic acid and *trans*-7-tetradecene were also identified in extracts of *Pimelea trichostachya*. The derivatisation of the daphnane orthoesters for potential toxin assay by GC and HPLC was found not to be feasible.

3.0 SYNTHESIS AND CHARACTERISATION OF *Pimelea* TOXIN - PROTEIN CONJUGATES FOR THE PRODUCTION OF ANTIBODIES IN CATTLE

3.1 INTRODUCTION

The generation of specific antibodies for use in screening assays for low molecular weight toxins has been a fairly widely accepted practice (for example, refer to Baden *et al*[60]). However, obtaining protective immunity for grazing animals against various toxins through immunisation with toxin-protein conjugates has more recently been attempted with limited success[61,62].

The molecular weights of *Pimelea* daphnane orthoester toxins range from approximately 500 to 600 $gmol^{-1}$ (simplexin 532$gmol^{-1}$ and huratoxin 584$gmol^{-1}$). The low molecular weight limit of a substance upon which the immune system will generate an antibody response is approximately 1,500 Da; therefore the *Pimelea* toxins are not naturally immunogenic. The strategy employed in this study was to covalently link the daphnane orthoester toxins to an immunogenic carrier protein with a four carbon succinylate linker. In a previous study it was shown that highly specific antibodies could be raised against the phorbol nucleus in rabbits by employing a similar strategy[40]. The results of a previous study in our laboratory[41] indicated that variations in the conjugate preparation, such as the extent of toxin incorporation and the degree of protein cross-linking occuring during conjugate preparation, affected the ability of the conjugate to produce specific anti-toxin antibodies.

In the current project, the aim was to improve the conjugation protocol to control the degree of toxin incorporation, and limit the extent of protein cross-linking. The original conjugation protocol[41] involved the succinylation of *Pimelea* toxins (mainly at the C20 hydroxyl function), followed by the production of a mixed anhydride by reaction of the free succinylate carboxyl grouping with *i*-butyl chloroformate (*i*-BCF). The anhydride bond was then displaced through nucleophilic attack by the primary amine nitrogen of the lysine residues of the protein, thereby releasing carbon dioxide and *i*-butanol. The main problem with this procedure is that any excess *i*-BCF present in the reaction mixture facilitates protein cross-linking through the activation of the free carboxyl groups of aspartate and glutamate residues present in the carrier protein. The elimination of this problem (while maintaining use of a succinylate linker) may be achieved by the activation of the succinylated toxin as an N-hydroxysuccinimide (NHS) ester (adapted from[63-65]). Displacement of NHS from the activated toxin could then be achieved by nucleophilic attack on the imide carbonyl by free lysine amine nitrogen groups on the carrier protein (Figure 3.1). This approach was to be examined initially by performing the reaction sequence using the carbonyl function of benzoic acid as a model. The carrier protein chosen for preparing conjugates for the cattle vaccination experiments during this study was ovalbumin, which has a molecular weight of 45,000 Da and 20 lysine residues which can be utilised for conjugation[66].

The conjugates produced using the *i*-BCF and NHS activation methodologies were prepared using the *Pimelea* AB fraction. The rationale for this was that the mixture of

toxin components present in the extract mixture with appropriate functionality would be attached to the carrier protein, thereby generating a broad spectrum antigen. A third conjugate was to be produced from a highly purified mixture of simplexin and huratoxin using *i*-BCF activation, to determine if a more selective antibody response resulted. The conjugate which produced the most efficacious antibodies (based on toxin specific antibody response (ELISA), and attenuating effect on the contraction of bovine pulmonary venule by *Pimelea* toxins *in vitro*) would then be used in the field evaluation trials.

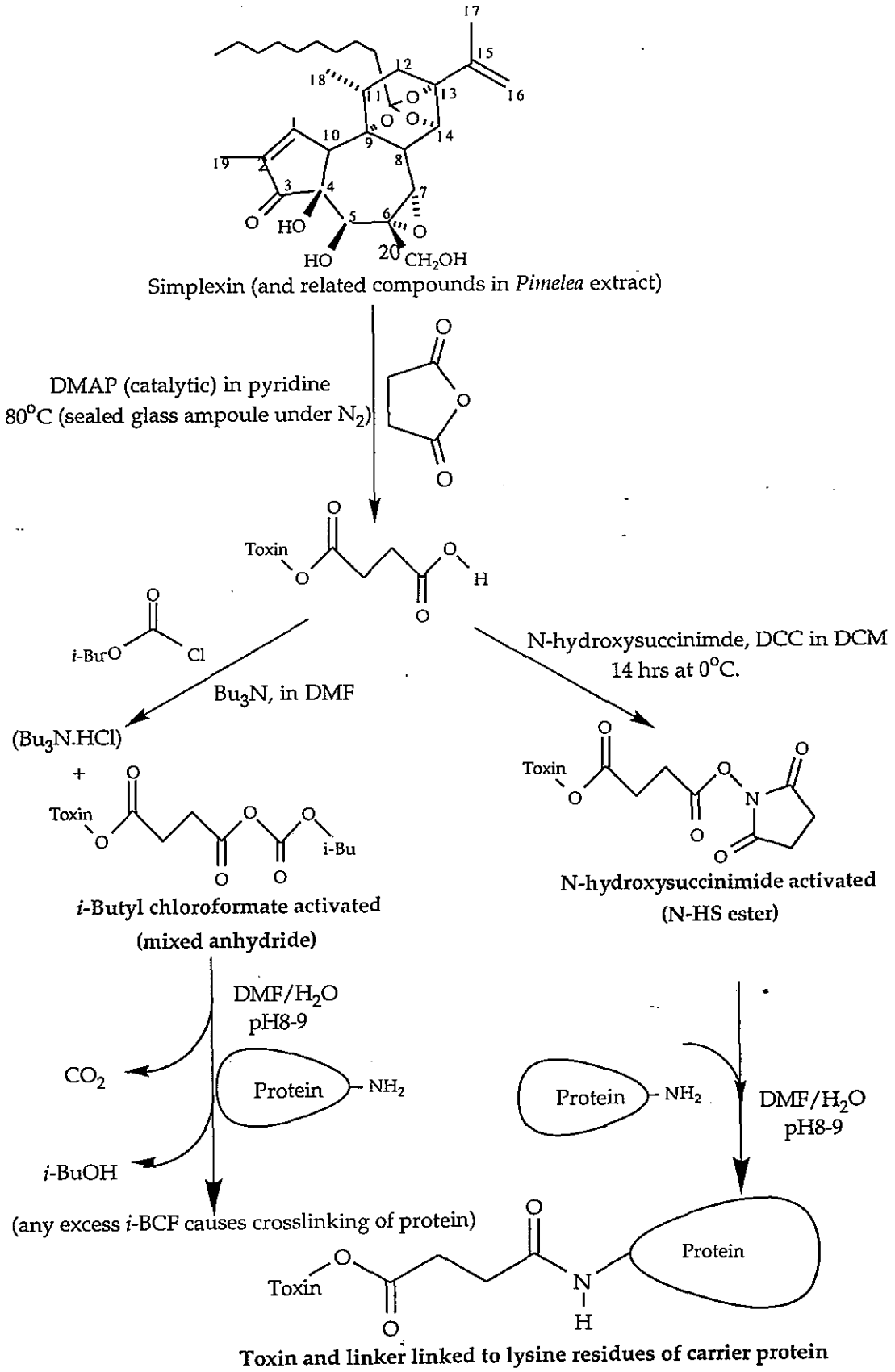


Figure 3.1 - Summary of succinylate conjugation protocols used in this study

The effect of linking the *Pimelea* toxins to cysteine residues of ovalbumin via a *p*-maleimidophenylcarbamate linker[67] on resulting antibody response was also to be examined. This method involves the reaction of a hydroxyl function with *p*-maleimidophenyl isocyanate (PMPI), forming a carbamate. This then reacts with carrier protein, forcing a Michael type attack on the methylene carbon of the maleimido moiety by activated sulphydryl groups of cysteine residues present (Figure 3.2). Ovalbumin possesses 6 cysteine residues for coupling[66].

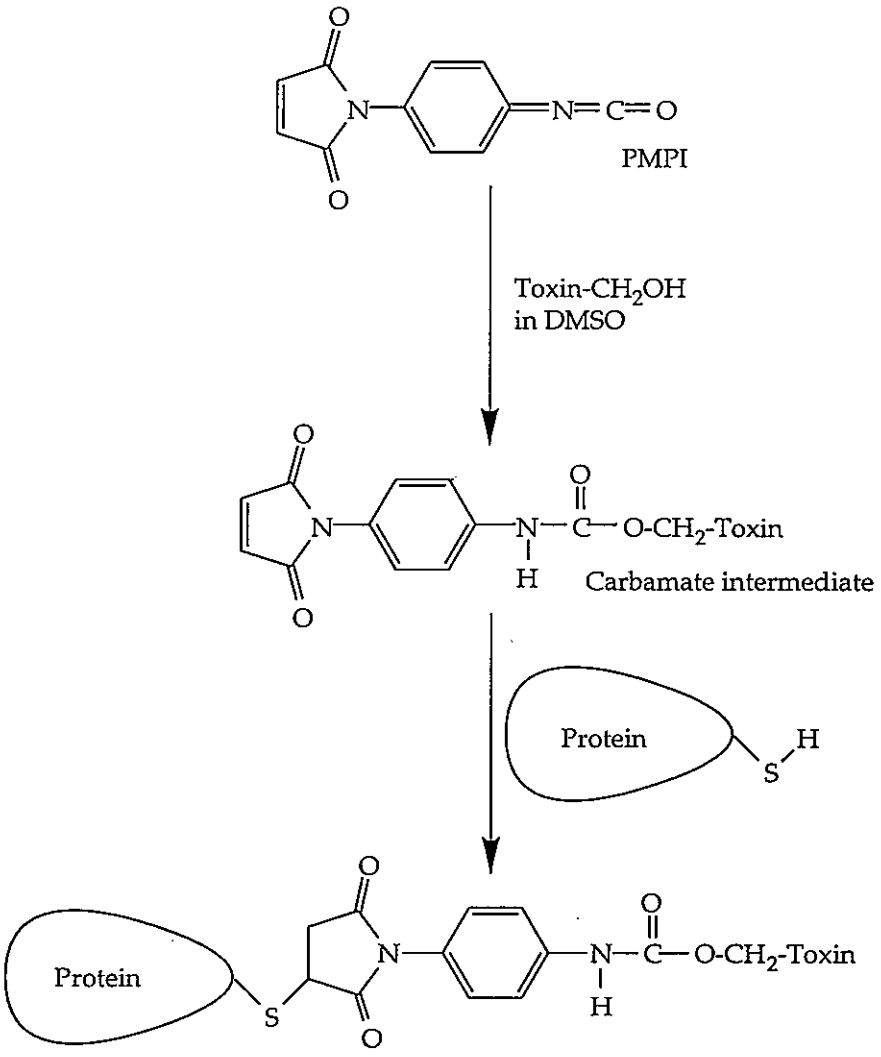


Figure 3.2 - Linking of toxin to cysteine residues via a PMPI linker.

3.2 EXPERIMENTAL

3.2.1 Derivatisation of Model Compounds

3.2.1.1 N-Hydroxysuccinimide ester of benzoic acid

Benzoic acid (0.82 mmol) was dissolved in dry dichloromethane (DCM) (5mL) and cooled to 0°C. N-hydroxysuccinimide (0.90 mmol) was added to the stirring solution, followed by N,N'-dicyclohexyl carbodiimide (DCC) (0.90 mmol), and dimethylaminopyridine (DMAP) (0.041 mmol - catalytic). The reaction mixture was stirred at 0°C. Thin layer chromatography (TLC) (25% ethyl acetate in hexane) of the reaction mixture after 14 hours revealed a spot of R_f 0.17 compared with benzoic acid (R_f 0.38). The N,N'-dicyclohexyl urea produced by the reaction was removed by vacuum filtration, and the resulting product was purified using "Flash" silica chromatography[51] with 25% ethylacetate in hexane as the eluent. The compound (0.34 mmol) was analysed by NMR and shown to be the N-hydroxysuccinimide ester of benzoic acid (Figure 3.3). (^{1}H NMR:- d8.14 d 2H; d7.68 d 1H; d7.51 dd 2H; d2.89 s 4H; ^{13}C:- d205; 169; 135; 131; 129; 125; 26).

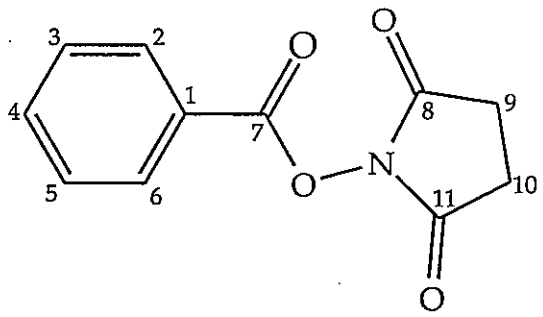


Figure 3.3 - N-Hydroxysuccinimide ester of benzoic acid

3.2.1.2 Displacement of N-hydroxysuccinimide

t-Butylamine (0.91 mmol) was dissolved in a 50:50 mixture of dimethyl formamide (DMF) and water. To this, N-hydroxysuccinimide ester of benzoic acid ((0.046 mmol) was added. The reaction was monitored by TLC (as above), and the reaction was judged to be complete when the spot corresponding to the N-hydroxysuccinimide ester of benzoic acid (R_f 0.18) had disappeared, and two new spots had appeared (R_f 0.0 corresponding to N-hydroxysuccinimide, and a new compound (R_f 0.46)). The solvent was removed from the product, and it was purified using "Flash" chromatography (25% ethylacetate in hexane as the eluent). The product was found to be N-*t*-butylbenzamide (^{1}H NMR:- d7.73 d 2H; d7.44 m 3H; d5.93 s (broad) 1H; d1.84 s, 9H; ^{13}C:- d170; d132; d129; d127; d30) (Figure 3.4).

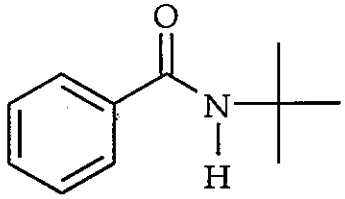


Figure 3.4 - N-*t*-Butylbenzamide.

3.2.2 *Pimelea* toxin conjugation

3.2.2.1 *i*-Butylchloroformate "activation" (CR1A)

Pimelea fraction AB (1.82mmol, calculation based on an average toxin molecular weight of 550gmol^{-1}) was dissolved in dry pyridine (10mL) containing succinic anhydride (2.2mmol) and DMAP (0.04mmol - catalytic). The reaction mixture was sealed in a glass ampoule under a nitrogen atmosphere and heated overnight at 80°C. The reaction mixture was allowed to cool to room temperature prior to removal of the solvent *in vacuo*. The succinylated toxin was activated by producing a mixed anhydride by treatment with *i*-butylchloroformate (*i*-BCF). Conditions were carefully controlled such that excess *i*-BCF was avoided to minimise cross linking of carrier protein (succinylated *Pimelea* toxin (1.8mmol in dry DMF (20mL)); tributylamine (2.5mmol); *i*-BCF (2.2mmol)). A 3.4% (v/v) aliquot of the reaction mixture was taken for conjugation to human serum albumin (HSA) (1.45x10^{-3}mmol) for enzyme linked immunosorbent assay (ELISA) experiments (CR1A-HSA). The remaining activated toxin mixture was added dropwise to a stirring solution of ovalbumin (0.044 mmol in 50mL of 50% DMF in 0.1M phosphate buffer; pH 8.5-9.0) while monitoring and maintaining pH. The reaction mixture was left stirring for 14 hours (0°C), and the resulting conjugate (CR1A-OV) was dialysed with 50mM sodium bicarbonate solution (60 hrs). The resulting conjugate solution was freeze dried prior to formulation into vaccines. Toxin inclusion ratios were determined by UV difference at 240nm and Poly acrylamide gel electrophoresis (PAGE).

3.2.2.2 N-Hydroxysuccinimide activated conjugation (CR1B)

Pimelea AB fraction (1.8mmol) was dissolved in dry pyridine (10mL) and the *Pimelea* fraction was succinylated as described above. The product was then dissolved in dry DCM for reaction with N-hydroxysuccinimide (HPLC analysis showed the peaks corresponding to simplexin and huratoxin had reacted). The succinylated *Pimelea* toxin extract was activated with N-hydroxysuccinimide (2.3mmol), DCC (1.82mmol), and DMAP (0.09mmol) in DCM (as described above) prior to conjugation to carrier protein. A 3.4% aliquot (v/v) of the activated toxin mixture was taken for conjugation to HSA (1.45x10^{-3}mmol) for future ELISA experiments on an equivalent scale as described above. The remaining NHS ester of the succinylated *Pimelea* AB fraction was dissolved in DMF (30mL) and added dropwise to a stirred solution of ovalbumin (0.044 mmol in 50mL of 50% DMF in 0.1M phosphate buffer; pH 8.5-9.0) while monitoring and maintaining pH. The reaction mixture was left stirring overnight (0°C), and the resulting conjugate (CR1B-OV) was dialysed with 50mM sodium bicarbonate solution (60 hrs). The resulting conjugate solution was also freeze-dried prior to formulation into vaccines. Inclusion ratios were determined by PAGE.

3.2.2.3 Conjugation via a *p*-Maleimidophenyl Isocyanate linker (CR2A)

Mezerein (0.015 mmol) was dissolved in dimethylsulphoxide (DMSO) (1mL) and reacted with PMPI (0.023 mmol) for 14 hrs under a nitrogen atmosphere. The reaction mixture was then diluted with water and extracted into ethyl acetate. The product was purified by preparative HPLC and yielded mezerein-20-*p*-maleimidophenylcarbamate

(0.007 mmol), (retention time 4.78 minutes relative to mezerein, 12.4 minutes; ^1H NMR: d7.75; 7.60; 7.43, m 10H (benzene ring and vinylic proton of C12 substituent of mezerein, and *p*-disubstituted benzene of PMPI moiety - overlapping); 7.37; 6.9; 6.8; 6.0; 5.42 s, 2H (maleimido protons); 5.1; 5.05 ; 4.95; 4.35; 3.83; 3.80; 3.65; 2.52; 1.96; 1.75; 1.40).

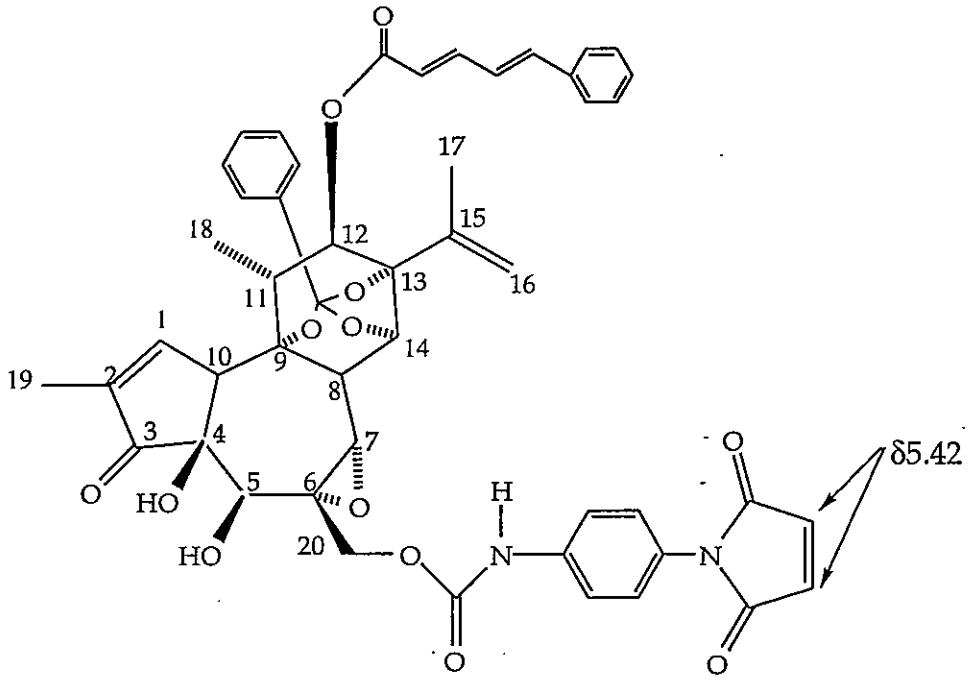


Figure 3.5 - The C-20 PMPI derivative of mezerein

This reaction was also repeated using *Pimelea* AB fraction for eventual conjugation to ovalbumin, however only partial reaction completion was achieved. Due to time constraints, this sequence was not repeated.

3.2.2.4 Simplexin-huratoxin conjugation (CR1C)

A purified mixture of simplexin and huratoxin (0.18mmol) was conjugated to ovalbumin ($8.18x10^{-3}$ mmol) using the *i*-BCF activation method (CR1C-QV). As with the previous two conjugates, a 10% aliquot of the activated toxin was taken for conjugation to HSA ($9.13x10^{-4}$ mmol) for use in ELISA testing (CR1C-HSA). Inclusion ratios were determined by UV difference at 240nm.

3.2.3 Toxin inclusion by UV difference

Solutions of conjugates equivalent to $8.89x10^{-4}$mM ovalbumin were prepared, and their UV spectra recorded from 220-300nm. The difference in absorbance at 240nm compared with an equivalent $8.89x10^{-4}$mM ovalbumin solution was assumed to be due to the enone function of simplexin and huratoxin molecules which had been conjugated. The concentration of these compounds was calculated by applying Beer's law (Abs = ebc), and the inclusion ratio determined by the ratio of simplexin/huratoxin concentration compared to ovalbumin. The molar toxin inclusion ratios of the *i*-BCF activated conjugates could be determined using this method, however due to the absorbance of NHS in this spectral region, interference by residual NHS prevented the

use of UV absorbance difference for the NHS-activated conjugates. The UV difference methods were also employed for the calculation of toxin inclusion ratios for the *i*-BCF activated HSA conjugates, while PAGE was used for the NHS-activated HSA conjugate.

3.2.4 Toxin inclusion by polyacrylamide gel electrophoresis (PAGE)

Sodium dodecylsulphate (SDS) polyacrylamide gels were prepared fresh prior to use (BIO-RAD Mini-Protean II PAGE manual), and samples were prepared by diluting 1:4 with fresh sample buffer and heating at 95°C for four minutes. Ovalbumin (Mwt. 45,000 Da), bovine serum albumin (BSA) (Mwt. 67,000Da), and human serum albumin (HSA) (Mwt. 69,000 Da) were used as standards. A plot of R_f as a function of molecular weight was used to generate a standard curve, therefore allowing molecular weights of the ovalbumin and HSA conjugates to be determined by interpolation.

3.2.5 Immunisation of Cattle with Experimental Vaccines

Vaccines (5mL per animal) were batched as oil-in-water emulsions containing a suitable adjuvant, and corresponding conjugate doses (1mg per animal). The aqueous phase (300mL per litre of vaccine) consisted of Quil A (400mg), saline solution (150mL), and DEAE dextran solution (150mL). The oil phase (700mL per liter of vaccine) consisted of Arlecil adjuvant (70mL) (a saponin) in ondina oil (630mL). The emulsions were homogenised using an Ultra-Turax T25 at 13,500 rpm for 3 minutes, followed by 20,500 rpm for 3 minutes. A drop-test was performed to confirm the homogeneity of the emulsions.

Three groups of eight animals (heifers) (two vaccinated, one control) were immunised subcutaneously (control animals were bled only) with the experimental vaccines (CR1A-OV, group 1; CR1B-OV, group 2; control, group 3). The conjugate CR1C-OV was tested at a later date in a trial consisting of two groups of five vaccinated, and five control animals. Vaccinated heifers were given booster vaccinations after 4 weeks (CR1A-OV and CR1B-OV) and 3 weeks (CR1C-OV). Blood samples (20 mL) were taken from the tail at these times for the collection of plasma for ELISA antibody assay, while bulk samples (150 mL) were taken from the jugular vein 2 weeks post secondary vaccination (CR1A-OV and CR1B-OV) and 10 days post secondary (CR1C-OV) for serum IgG purification. Table 3.1 summarises the bleeding schedule. Most immunised animals developed swelling (3-5cm diameter) around the subcutaneous injection site. This condition was less predominant in the CR1B-OV vaccinate group.

Group	Time (days)			
CR1A-OV	0†	28‡	35	42*
CR1B-OV	0†	28‡	35	42*
CR1C-OV	0†	21‡	31*	

† Primary vaccination; ‡ Secondary vaccination;* Bulk bleed

Table 3.1 - Bleeding schedule for trial animals

3.2.6 Enzyme Linked Immunosorbent Assay (ELISA) Antibody analysis

Plasma samples (obtained by centrifugation of non-clotted blood) were diluted 1 in 500 for antibody testing. This was achieved by utilising an ELISA technique developed in our laboratories. The corresponding HSA conjugates (CR1A-HSA, CR1B-HSA, and CR1C-HSA) were coated on to Immulon 4 microtitre plates by dissolving the appropriate conjugate (0.5μg mL^{-1}) in coating buffer (0.05M carbonate buffer pH9.6), and incubating for 14 hrs at 4°C(50μL per well). The plates were washed 5 times (wash solution: 0.01M Na_2HPO_4, 0.001M EDTA, 0.14M NaCl, and 0.05% v/v Tween 20; pH 7.5), and blocked with 1% w/v skim milk powder in assay buffer (300μL per well) for 1 hour at room temperature (assay buffer: 0.01M Na_2HPO_4, 0.001M EDTA, 0.14 M NaCl, and 0.03 mM BSA). Following blocking, the plates were washed and serum (50μL per well of 1:500 diluted serum) from vaccinated, positive, and negative control animals for each of the vaccination conjugates was incubated away from light for 2 hours at room temperature. The plates were then washed 5 times, and 50μL per

well of anti-IgG-horseradish peroxidase (second antibody-HRP) conjugate was incubated in the dark at room tempature for 1 hour. Following the final wash (5 times), the wells were incubated with standard ABTS substrate until adequate colour had developed for positive control samples (typically 30 minutes in the dark). Further colour development was stopped using 100µL per well of 1% SDS. Absorbances were read using a BIO-RAD 3550 microplate reader (at 405nm)

The relative ovalbumin response was determined by repeating the procedure with each of the vaccinate serums while using an ovalbumin plate coating. This was performed to tentatively test the specificity of the antibodies. By comparing the values obtained by HSA-conjugate and ovalbumin plate coatings, an indication of the relative toxin specific antibody response could be obtained.

3.3 RESULTS AND DISCUSSION

3.3.1 Conjugate characterisation

The estimation of toxin:protein inclusion for the *i*-BCF activated *Pimelea* AB conjugate (CR1A-OV) was performed using UV difference, and PAGE to allow comparison between the two methods.

The *Pimelea* toxin inclusion ratio for CR1A-OV was found to be 17:1 by UV difference at 240nm (Figures 3.6 and 3.7), while it was found to be 16:1 by PAGE (Figure 3.8). These results indicate close agreement, therefore it was concluded that PAGE was a valid method for determination of the inclusion ratio of *Pimelea* toxin to ovalbumin for the CR1B-OV conjugate. A plot of R_f (distance bromophenol blue marker moved, relative to the distance the analyte moved on the gel) as a function of molecular weight of the molecular weight standards produced a straight line ($R^2 \geq 0.998$) (Figure 3.9), therefore the molecular weights of the conjugates could be interpolated.

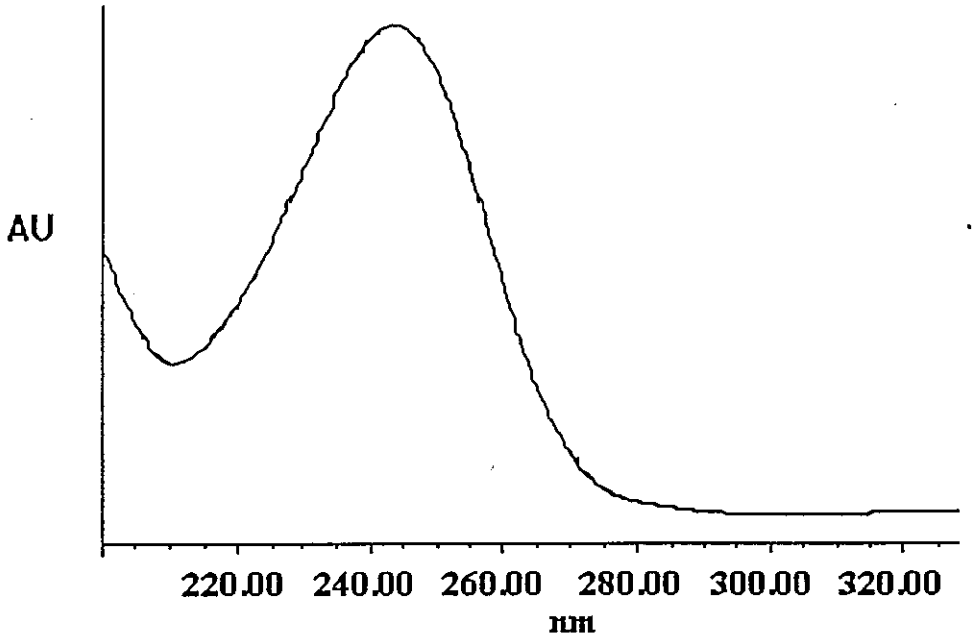


Figure 3.6 - UV spectrum of simplexin.

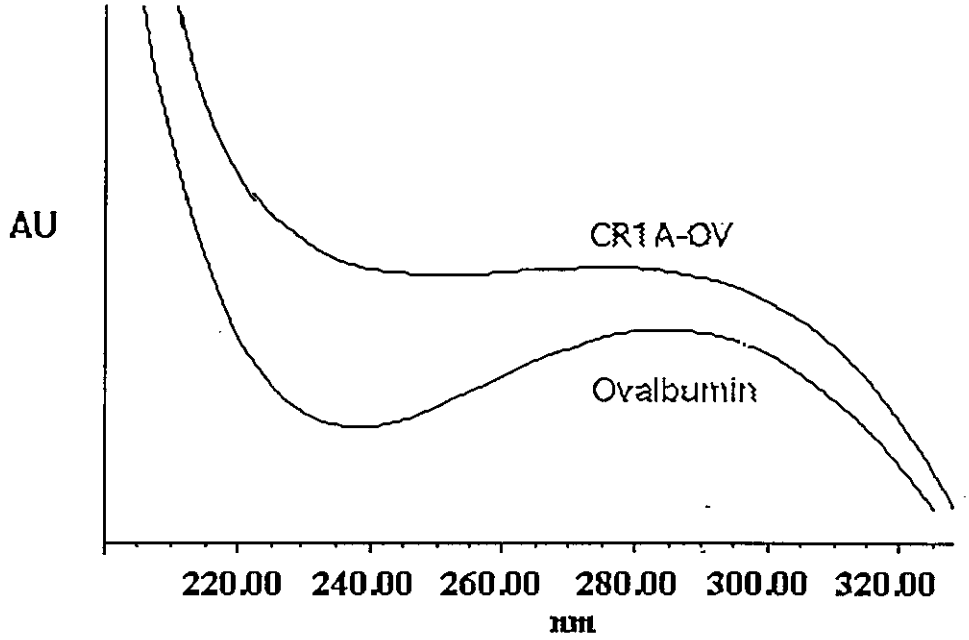


Figure 3.7 - UV spectra of ovalbumin and CR1A-OV

The toxin inclusion ratio calculated for the other conjugates prepared for this study are given in Table 3.2.

Conjugate	Inclusion ratio	Characterised by
CR1A-OV	17 16	UV difference PAGE
CR1A-HSA	6	PAGE
CR1B-OV	13	PAGE
CR1B-HSA	7	PAGE
CR1C-OV	13	UV difference
CR1C-HSA	8	PAGE

Table 3.2 - Summary of inclusion ratios of *Pimelea* toxins:carrier protein.

Figure 3.8 - PAGE Gels confirming the attachment of the *Pimelea* toxins to ovalbumin (Gel A: CR1A-OV and CR1B-OV) and HSA (Gel B: CR1A-HSA and CR1B-HSA), (BSA = bovine serum albumin; Oval = ovalbumin; and HSA = human serum albumin)

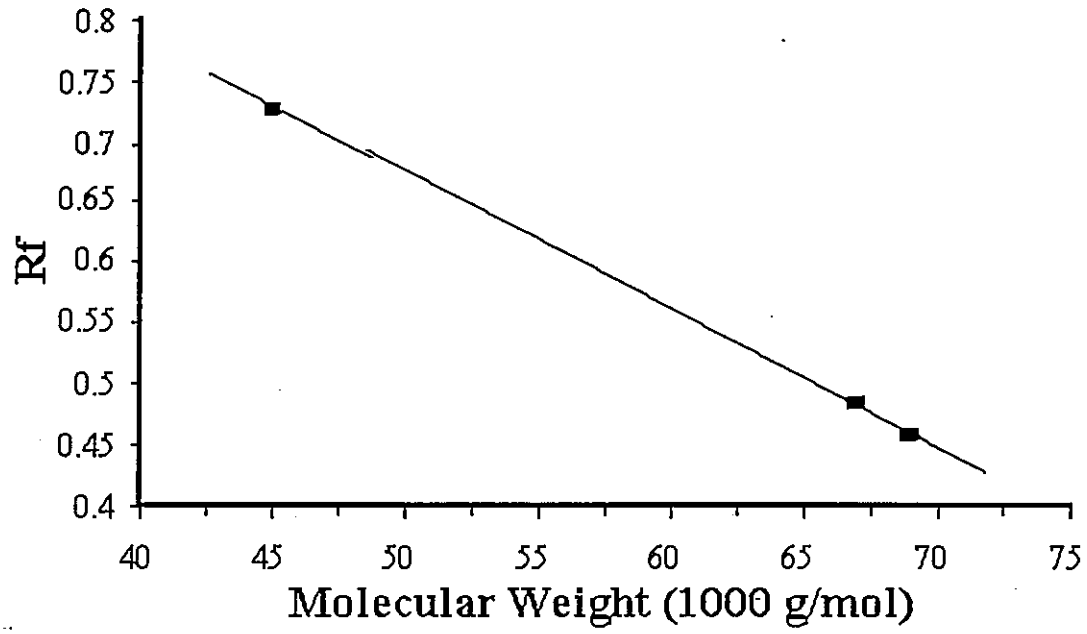
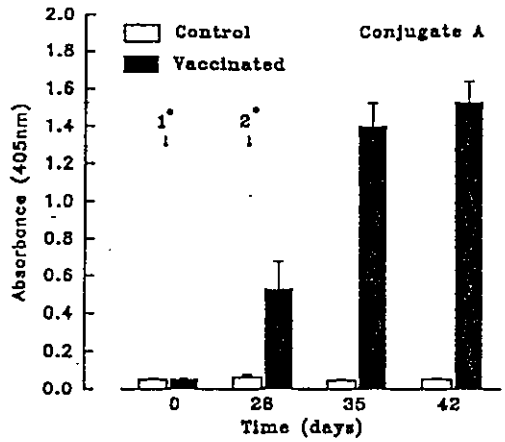
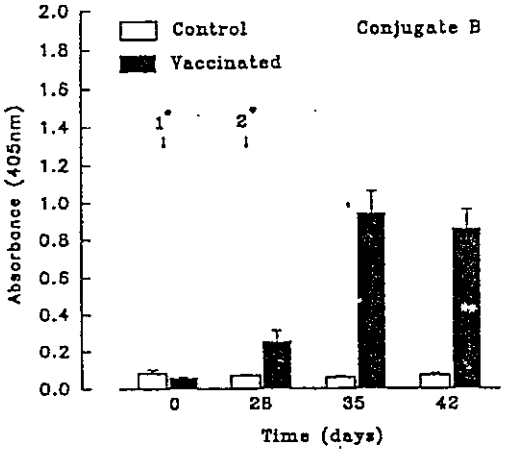


Figure 3.9 - Molecular weight calibration curve (PAGE)

3.3.2 Assessment of Antibody Responses by ELISA

The results of the previous study in our laboratories[41] showed that antibody titres peaked two weeks after secondary immunisation. However, due to time and animal access constraints, the trial for the CR1C-OV conjugate was reduced to secondary immunisation at 21 days (rather than 28 days), and blood samples were taken at 10 days post secondary (rather than at 7 and 14 days). This should not significantly effect the interpretation of results, as the antibody titres were still able to be compared with control levels. Plasma samples were diluted 1:500 in ELISA assay buffer for determining the time-course of antibody response for each experiment. The plasma samples from each vaccination experiment were assayed either with the corresponding HSA conjugates or ovalbumin as plate coatings. In this way it was possible to determine the toxin-specific antibody response in relation to the response generated to ovalbumin itself.





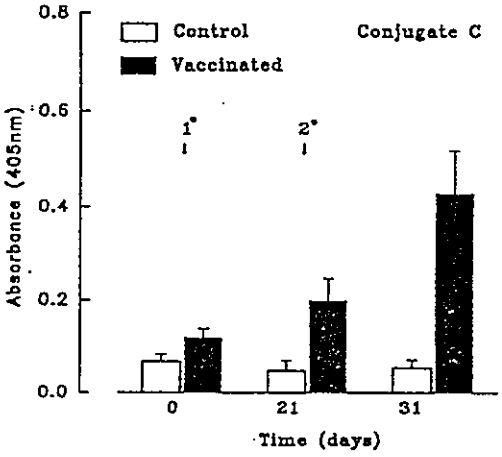


Figure 3.10 - ELISA Time-course plots for CR1A-OV (a); CR1B-OV (b); and CR1C-OV (c)

The animals immunised with the CR1A-OV conjugate developed a strong specific response, giving an ELISA absorbance (1:500 serum dilution, at 405nm) of 1.52±0.10 at 2 weeks post-secondary vaccination (Figure 3.10a). The corresponding value for the CR1B-OV group was 0.84±0.10 which, within error margins, was the same as the 1 week post-secondary vaccination (Figure 3.10b). The corresponding ovalbumin response (Figure 3.11) for CR1B-OV (0.96 ± 0.1) was higher than that of CR1A-OV (0.43 ± 0.10), which indicates that immunisation with CR1A-OV gave a higher toxin-specific response than CR1B-OV.

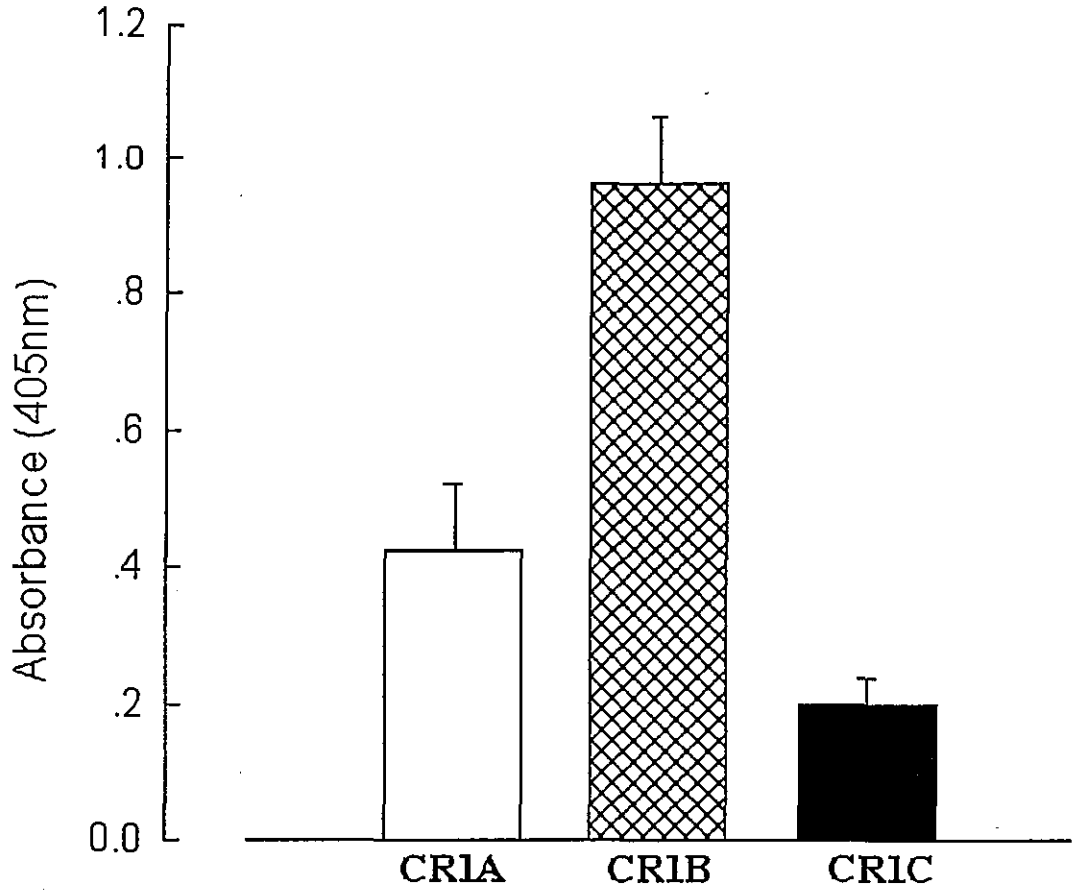


Figure 3.11 - Relative ovalbumin response for CR1A; CR1B; and CR1C groups.

PAGE analysis of the conjugates (Figure 3.8) showed no evidence of significant protein cross-linking, indicating that either the *i*-BCF toxin-activation chemistry is superior to the NHS activation chemistry, or the toxin inclusion ratio plays a significant role in the specificity of resulting antibodies. To investigate this possibility further, a third conjugate was synthesised. This time however, the toxin used for conjugation was a mixture of highly purified simplexin and huratoxin rather than the *Pimelea* fraction AB. The toxin-inclusion ratio was determined to be 13:1 (by UV difference).

The 10-day post secondary toxin-specific antibody response for the CR1C-OV conjugate (Figure 3.10c) was 0.43±0.09, which was significantly lower than that of the corresponding CR1A-OV conjugate. The toxin-specific antibody response as well as the relative ovalbumin response was generally lower than that of the other *i*-BCF activated toxin. The reason for the lower overall antibody titre is attributed to the reduced times between primary and secondary immunisations, and post-secondary bleed time. This meant that animals immunised with CR1C-OV had less time time to respond than those immunised with CR1A-OV and CR1B-OV. A simple method for comparison of the efficacy of the three conjugates is to divide the toxin-specific response by the corresponding ovalbumin response (this was termed the relative toxin specific antibody response (RTSAR)) (Table 3.3). This data shows that the relative toxin-specific response for the CR1C-OV conjugate is reasonably high (compared to CR1B-OV), supporting the notion that the toxin-specific response is dependant on the toxin-inclusion ratio.

Conjugate	Toxin specific response	Relative ovalbumin response	RTSAR
CR1A-OV	1.52 ± 0.10	0.43 ± 0.10	3.53
CR1B-OV	0.84 ± 0.10	0.96 ± 0.11	0.88
CR1C-OV	0.43 ± 0.09	0.20 ± 0.03	2.15

Table 3.3 - Relative toxin and ovalbumin responses (by ELISA).

3.4 CONCLUSIONS

The feasibility of activating succinylated *Pimelea* toxin as an N-hydroxysuccinimide ester was examined firstly by conducting the experiment with benzoic acid as a model compound. The carboxyl group of benzoic acid was coupled to N-hydroxysuccinimide in high yield, and the resulting compound was stable enough to be purified by chromatographic methods. It was also shown that the N-Hydroxysuccinimide moiety could be displaced by a primary amine nitrogen under mild conditions, also in relatively high yield. Application of this conjugation method was achieved in parallel with the established *i*-BCF activation route in experiments designed to compare the efficacy of the conjugates. The feasibility of conjugating *Pimelea* toxin to the cysteine residues of ovalbumin via a PMPI linker was also examined, however while this chemistry could be achieved with the pure model compound mezerein, it was not successful with the *Pimelea* toxin mixture, presumably due to interference by more reactive components than the daphnane toxins.

From the cattle vaccination experiments, the conjugates produced via *i*-BCF activation gave relative toxin-specific antibody responses of 3.53 (CR1A-OV) and 2.15 (CR1C-OV) which was significantly higher than the conjugate produced via NHS activation (CR1B-OV - 0.88). The efficacy of the corresponding purified antibodies derived from each of the cattle vaccination experiments was to be evaluated *in vitro* to select the best conjugate for formulation into a vaccine for testing under field conditions (Chapter 4).

4.0 ASSESSMENT OF BOVINE ANTI-TOXIN ANTIBODIES *In Vitro* & FIELD EVALUATION OF AN EXPERIMENTAL VACCINE

4.1 INTRODUCTION

The selection of a vaccine for examination under field conditions was to be based on the toxin-specific antibody response generated by immunisation with experimental conjugates, and the ability of purified bovine IgG antibody fraction generated by immunisation with these conjugates, to attenuate the effects of *Pimelea* toxins on bovine pulmonary venule preparations *in vitro*.

The techniques used in this study were based on the observation that the *Pimelea* toxins caused irreversible contraction of bovine pulmonary venule preparations *in vitro*[10]. In a recent study in our laboratories, it was shown that purified rabbit anti-toxin serum IgG attenuated the effects of the *Pimelea* toxin *in vitro* in a dose dependant manner[41]. In the present study, the EC_{50} for the *Pimelea* toxin extract needed to be established before testing purified antibodies for anti-toxin efficacy. The EC_{50} concentration is the concentration required to give a response equal to 50% of the maximum contractile response generated by 1μM 5-HT (5-hydroxytryptamine).

The *Pimelea* poisoning season in Southern Queensland occurs mainly from August to December when adequate seasonal rainfall has not been received in the previous summer. Ideally, animals should be immunised with the experimental vaccine prior to them developing symptoms of *Pimelea* poisoning. In this study, vaccine trials were conducted in Roma (Queensland) and Marree (South Australia).

4.2 EXPERIMENTAL

4.2.1 General

For the *in vitro* studies, eight 25mL glass organ baths and Grass FT03 force transducers (Grass Instruments) were interfaced to a MacLab 8 analogue-digital converter using Chart software and two Transbridge TBM4 amplifiers (World Precision Instruments). Data was recorded using a Macintosh II CX microcomputer. Carbogen gas (5% CO_2 in O_2) was bubbled through a sinter in the base of the organ bath to oxygenate the tissue. Bovine pulmonary venule preparations were dissected from fresh bovine lung and tensioned between the force transducers and a support post. Each preparation was immersed in physiological Tyrodes solution and maintained at 35±1°C by a heated water jacket (Figure 4.1).

4.2.2 Physiological Tyrodes Solution

Physiological Tyrodes solution was prepared using AR grade reagents and diluted with demineralised water. The Tyrodes solution (2L) was prepared fresh prior to each *in vitro* assay and consisted of: Stock A (100 mL), Stock B (100 mL), Stock C (100 mL), and glucose (2.0 grams). All stock solutions were prepared in 2 litre batches with AR grade reagents and demineralised water. Stock A consisted of NaCl (2.74M), KCl

(109mM), $MgCl_2$ (33mM), and NaH_2PO_4 (17mM). Stock B solution was 0.45M $NaHCO_3$ and stock C solution was 36mM $CaCl_2$.

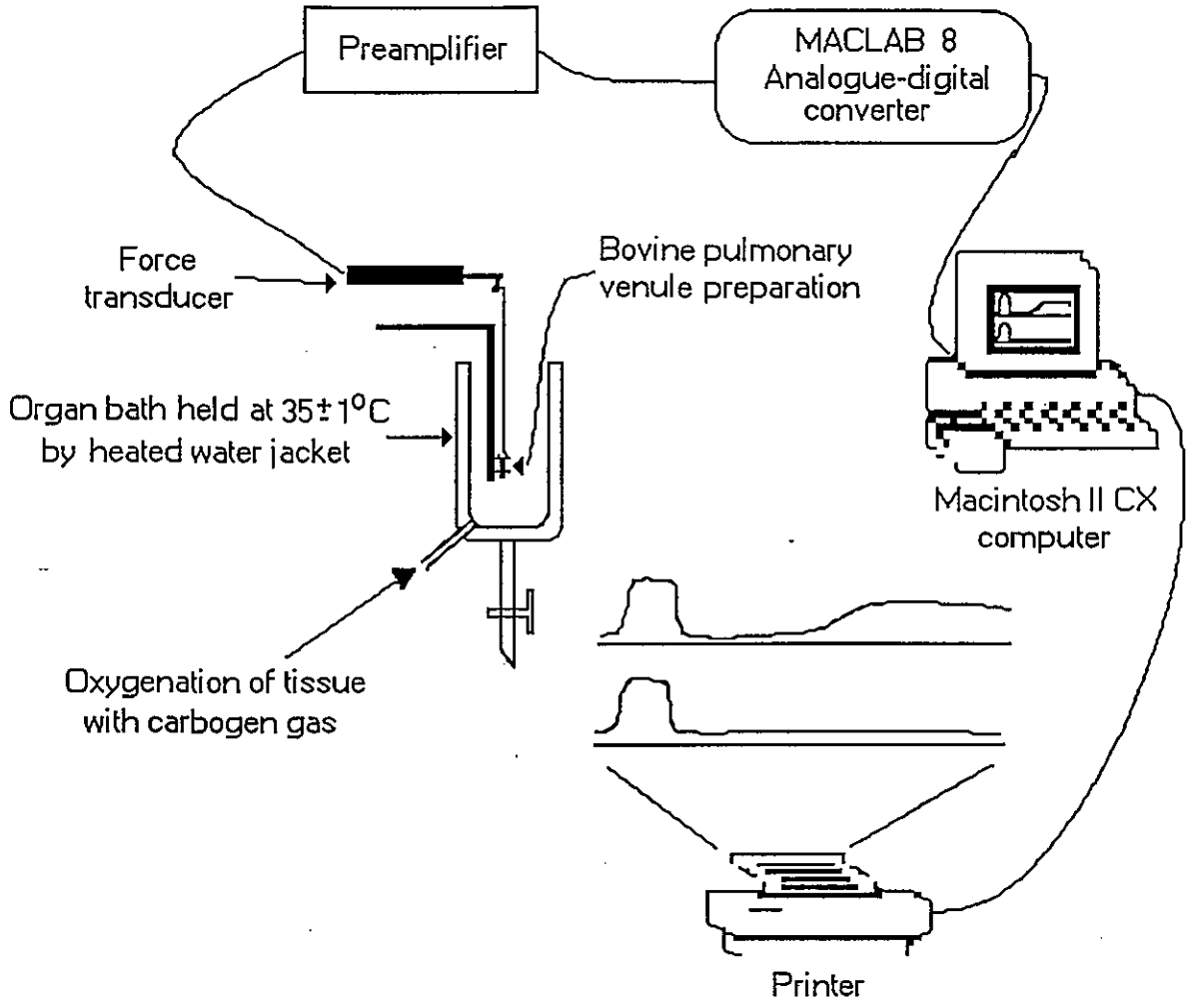


Figure 4.1 - Organ bath system used for *in vitro* assessment of bovine anti-toxin antibodies

4.2.3 Affinity Purification of Bovine Serum IgG

Blood (150 mL) was collected from the jugular vein of animals displaying the highest toxin-specific antibody response (determined by ELISA) from each of the vaccinate groups. The blood was allowed to clot prior to the removal of the serum fraction following centrifugation. Serum (30mL) was de-salted and the IgG fraction was separated using Econo-Pac® serum IgG purification kits (Bio-Rad, USA). The resulting IgG fractions were freeze dried (yielding: A, 80 mg; B, 65 mg; and C, 85 mg), and absorbance of a sample (1mg dissolved in 5 mL distilled water) of each (at 280 nm) confirmed the presence of the eluted protein fraction. The resulting antibodies were used in the *in vitro* assay without further de-salting.

4.2.4 Effective curve for *Pimelea* fraction AB

Sections of fresh bovine lung (approx. 150g) were obtained daily from the killing floor of local meat works within 30 minutes of slaughter. The lung sample was placed in

carbogenated Tyrodes solution (4°C) for transportation back to the laboratory. Sections of pulmonary venule were dissected from the lung and connective tissue was removed. The preparation was then cut into 2-4mm lengths (ringlets). The sections of pulmonary vein were connected to force transducers while immersed in physiological Tyrodes (35±1°C) in the organ baths. The tissue samples were equilibrated and tensioned to the optimum preload (25mN)[41]. Typical contractile responses were observed by contracting the tissue with 5-hydroxytryptamine (5-HT) (1μM). After maximum contraction was observed (30 minutes), the tissue was washed with fresh Tyrodes solution (3 times) and the tension allowed to equilibrate back to the optimum pre-load. The tissue was exposed to the desired concentration of *Pimelea* AB fraction (in ethanol), and the force of contraction monitored over 10 hours. Molar concentrations of *Pimelea* toxin were calculated based on an average molecular weight of 550 $gmol^{-1}$. The effective curve (EC) was generated by plotting the force of contraction induced by *Pimelea* toxin as a percentage of the 5-HT contraction with respect to toxin concentration. Each experiment was repeated four to six times in order to generate data for valid statistical analysis.

4.2.5 Attenuating Effects of Bovine Serum IgG *in vitro*

These experiments were conducted in the presence of purified antibodies to examine their attenuating effects. Control tissues (2 repeats per assay) were treated with *Pimelea* toxin without antibodies at EC_{50} and EC_{70} doses. Purified bovine serum IgG fraction (1mg and 5mg quantities) produced by the respective animals immunised with CR1A-OV, CR1B-OV, and CR1C-OV conjugates was incubated with the tissue preparations for 30 minutes prior to the addition of EC_{50} or EC_{70} doses of toxin. The attenuating effects of the antibodies were also expressed as a %5-HT contraction. At the conclusion of these experiments, tissues were contracted with 5-HT to confirm the viability of tissue preparations.

4.2.6 Preparation of Vaccines, and Immunisation of Trial Animals for Evaluation of Experimental Vaccine

For the field trials a 50:50 mixture of CR1A-OV and CR1C-OV conjugates was used. Vaccines were batched in the same manner as described in Chapter 3, and shipped to the Roma or Marree properties in refrigerated packaging.

4.2.6.1 Queensland trial (Roma)

Animals were given primary immunisations in early June, followed by secondary immunisations 4 weeks post primary. Animals (3 x 50 vaccinate, and 3 x 50 control) were immunised with the experimental vaccine on properties in the Maranoa district (Borah, Lillypool, and Woodlands). Blood (20mL) was collected from the tail at the time of secondary immunisation and two weeks post secondary immunisation for analysis of plasma antibodies by ELISA. Recent rains had been received in this district.

4.2.6.2 South Australian trial (Marree)

Animals on properties in the Marree district were immunised with experimental vaccines with the same active constituent as the Queensland trial (Clayton - 33

vaccinate and 32 control, Wilpoorinna - 50 vaccinate and 50 control, Etadunna - 62 vaccinate and 63 control, and Dulkaninna - 45 vaccinate and 45 control). Animals at Etadunna and Clayton received secondary immunisation 4 weeks post-primary, while animals at Wilpoorina and Dulkaninna were administered secondary vaccination at 2 weeks post primary. Animals were weighed and given condition scores based on their visual appearance (between 1.0 (poor) and 4.0 (good)). This region was experiencing drought conditions, and as a consequence *Pimelea* was a major constituent of the biomass. Many animals on the properties were suffering *Pimelea* poisoning to some extent at the time of primary immunisation. Blood (20mL) was taken for ELISA antibody testing. Plasma samples were diluted 1:500 for ELISA analysis.

4.3 RESULTS AND DISCUSSION

4.3.1 Analysis of Anti-*Pimelea* Toxin Antibodies *in vitro*

The *Pimelea* AB fraction (a mixture of predominantly simplexin and huratoxin) was found to induce contraction of bovine pulmonary venule preparations over a concentration range of 1nM to 1 mM *in vitro* (Figure 4.2). The EC_{50} and EC_{70} doses for *Pimelea* AB fraction were 30nM and 50nM respectively, which was within the range of the previous study performed in this laboratory. The control tissues produced EC_{50} contraction of 32±12% 5-HT, and EC_{70} contractions of 42±20% 5-HT, which was in agreement with the effective curve (the maximum contraction induced by toxin was 50±10%) (Figure 4.2). The attenuating effects of the antibody were expressed as %5-HT contraction, and the results of this study are expressed in Tables 4.1 and 4.2.

Conjugate	Toxin Inclusion Ratio	%5-HT (EC_{50} toxin, 5 mg antibody)	EC_{50} toxin, 1mg antibody
CR1A-OV	17	2.9±5.1%	13.0±4.2%
CR1B-OV	13	20.8±19.4%	16.1±19.3%
CR1C-OV	13	No contraction	No contraction

Table 4.1 - The attenuating effects of anti-*Pimelea* fraction AB *in vitro* to EC_{50} doses of *Pimelea* fraction AB

Conjugate	Toxin Inclusion Ratio	%5-HT (EC_{70} toxin, 5 mg antibody)	EC_{70} toxin, 1mg antibody
CR1A-OV	17	N.D.	N.D.
CR1B-OV	13	17.0±9.8%	18.0±8.8%
CR1C-OV	13	No contraction	No contraction

Table 4.2 - The attenuating effects of anti-*Pimelea* fraction AB *in vitro* to EC_{70} doses of *Pimelea* fraction AB

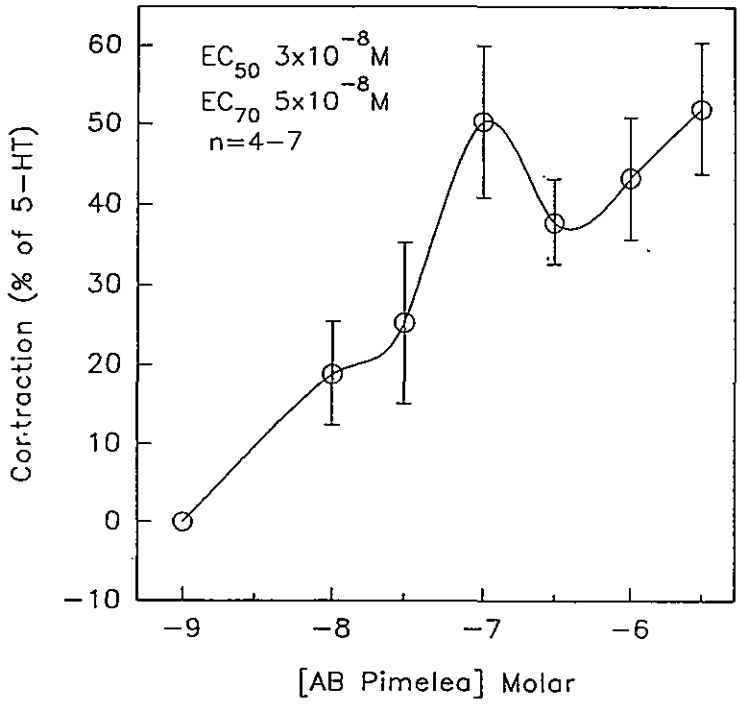


Figure 4.2 - Effective curve for the *Pimelea* AB fraction

The bovine antibodies induced by vaccination with each of the three conjugates displayed attenuating effects against *Pimelea* toxins *in vitro*. The antibodies produced by the CR1A-OV conjugate showed attenuating effects against *Pimelea* toxin in an approximate dose-dependent manner. However, the antibodies induced by immunisation with the CR1B-OV conjugate displayed variable efficacy. At 1mg per 25mL, the antibodies induced by immunisation with the CR1C-OV conjugate displayed total attenuating effects against EC_{50} and EC_{70} doses of *Pimelea* AB fraction *in vitro* (Figure 4.3). The data presented in Tables 4.1 and 4.2 suggests that the efficacy of toxin-specific antibody responses in cattle are influenced by the inclusion ratio and the conjugation chemistry employed. The toxin inclusion ratios of the CR1B-OV and CR1C-OV conjugates were equivalent, however the chemical pathways leading to the final conjugation to carrier protein were different, supporting the notion that the linking chemistry has an effect on the antibody recognition. A possible explanation for this is that the addition of activated toxin to carrier protein causes some form of conformational change to the carrier protein (depending on the form of activated toxin) effecting antibody recognition.

Based on the toxin-specific antibody responses determined by ELISA (Chapter 3), and the ability of the purified serum IgG to attenuate the effects of *Pimelea* toxin *in vitro*, the CR1A-OV and CR1C-OV conjugates gave superior responses than the CR1B-OV conjugate. While the CR1A-OV conjugate induced a higher toxin-specific antibody response than CR1C-OV (measured by ELISA), the latter displayed superior attenuating effects *in vitro*. An experimental vaccine for the field trial studies was therefore formulated from a 50:50 mixture of the CR1A-OV and CR1C-OV conjugates. As in all previous studies, each vaccination contained 1 mg of active constituent (conjugate).

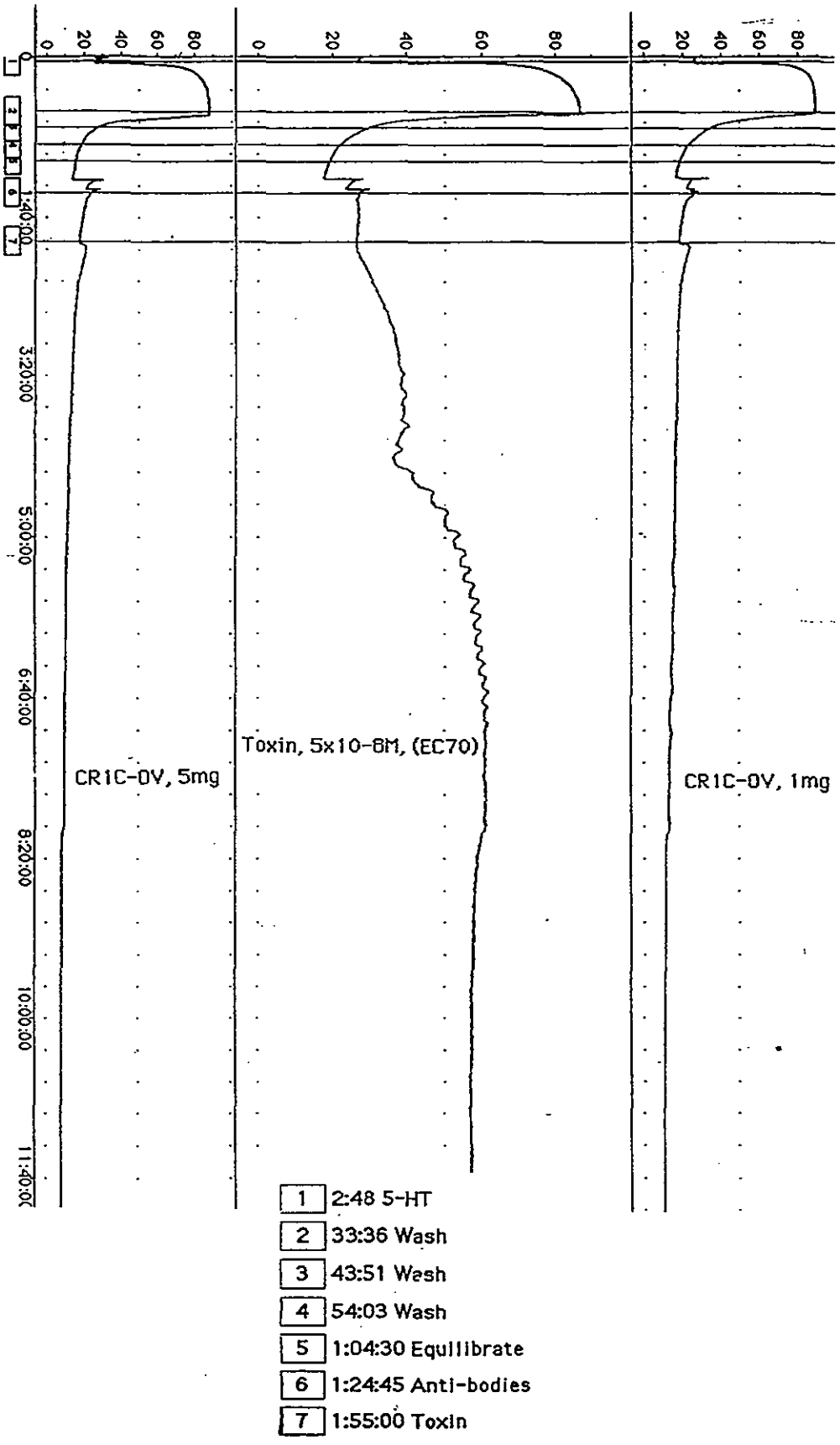


Figure 4.3 - Typical attenuating effect of the CR1C-OV induced bovine serum IgG antibodies against EC_{70} doses of *Pimelea* toxin *in vitro* (mg per 25mL)

4.3.2 Field Evaluation of the Experimental Vaccine

4.3.2.1 Roma trial

Animals were immunised on 3 properties in the Maranoa (Roma) district in early June to allow subsequent toxin-specific antibody responses to peak prior to the forthcoming *Pimelea* poisoning season. The two week post-secondary toxin-specific antibody responses of animals immunised with the experimental vaccine on Borah, Lillypool, and Woodlands displayed strong toxin-specific ELISA absorbances (Figure 4.4). This region received good winter rains and follow-up rain was also received. Summer rainfall in this region has also been favourable for pasture growth, therefore *Pimelea* poisoning has not been considered a threat this year in the Roma region, thus not providing a result regarding the protective ability of the vaccine under these field conditions.

4.3.2.2 Marree Trial

Animals immunised with the experimental vaccine in the Marree district developed strong post secondary toxin-specific antibody responses (Figure 4.5). Although most animals were suffering *Pimelea* poisoning to some extent at the time of primary immunisation, this did not effect the two week post-secondary toxin-specific responses. The toxin-specific responses for animals administered secondary immunisation after 2 weeks post-primary were approximately half that of the animals immunised 4 weeks post primary (Figure 4.6), however this did not effect the 2 week post-secondary toxin-specific responses (Figure 4.5).

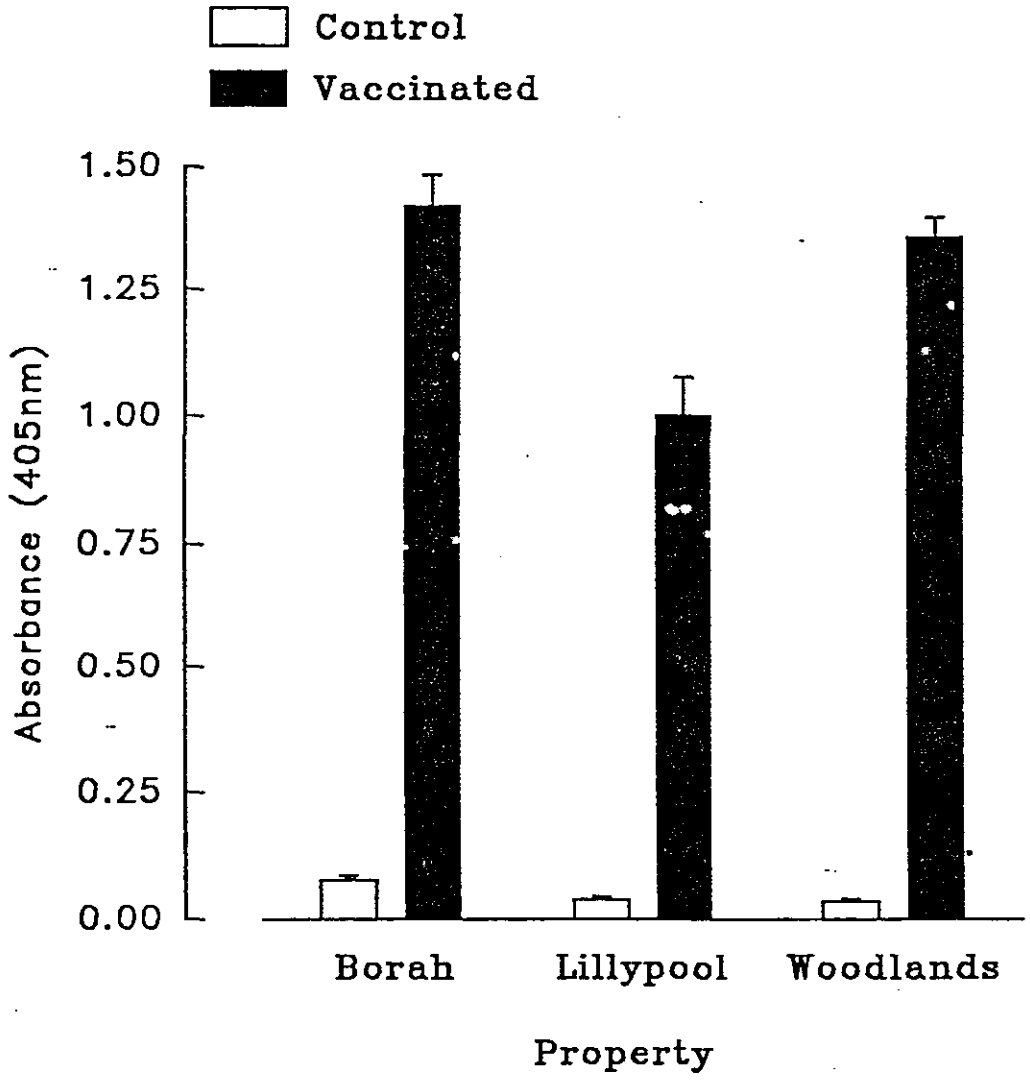


Figure 4.4 - Two week post secondary toxin-specific antibody responses of trial animals (Queensland) as measured by ELISA (plasma diluted 1:500)

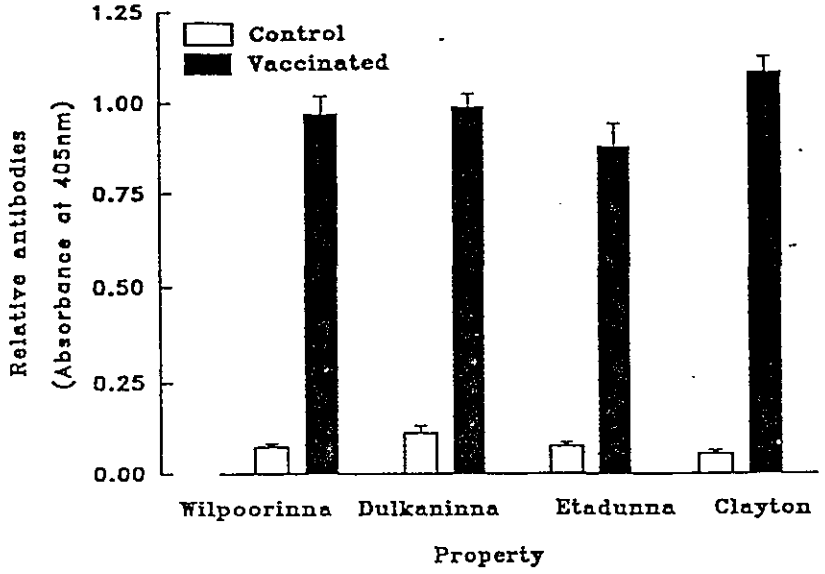


Figure 4.5 - Toxin-specific antibody responses (ELISA) for Marree Trial Animals (post secondary immunisation)

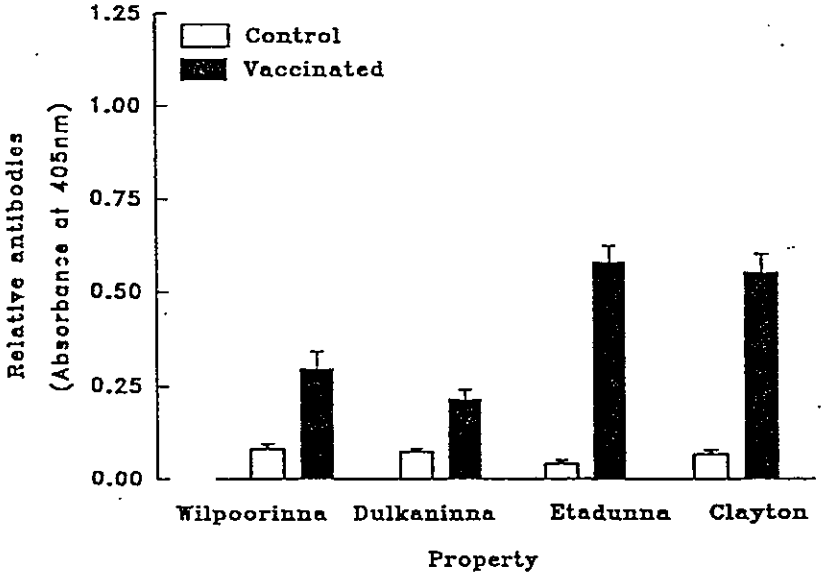


Figure 4.6 - Toxin-specific antibody responses (ELISA) for Marree trial animals (showing post primary responses)

For the South Australian properties, the number of cattle deaths in 1995 (prior to the beginning of the current trial) were 200 out of 200 affected animals at Wilpoorina, 40 out of 60 at Etadunna, 90 out of 100 at Dulkaninna, and 30 out of 100 affected animals at Clayton. Condition of the animals following immunisation was monitored and documented using a visual index on Wilpoorina, Etadunna, and Dulkaninna. The Clayton animals were not graded. There was a non-significant trend for increased liveweight of vaccinate compared with control animals on Wilpoorina, Etadunna, and Dulkaninna after secondary immunisation (Figure 4.7).

The average condition scores of vaccinated and control animals on Wilpoorina, Etadunna, and Dulkaninna show that there was a slight improvement in the condition of vaccinated animals on these properties (Table 4.3) after secondary immunisation. Animals were not given a condition score where there was uncertainty regarding the extent of the oedema.

Property	Time (days)	Number of animals per group (Vaccinate, Control)	Condition score (Control mean)	Condition score (Vaccinate mean)
Wilpoorina	47	34, 39	1.7	1.8
Etadunna	45	30, 36	2.8	2.9
Dulkaninna	31	39, 37	1.9	2.1

Table 4.3 - Average condition scores of Marree trial animals

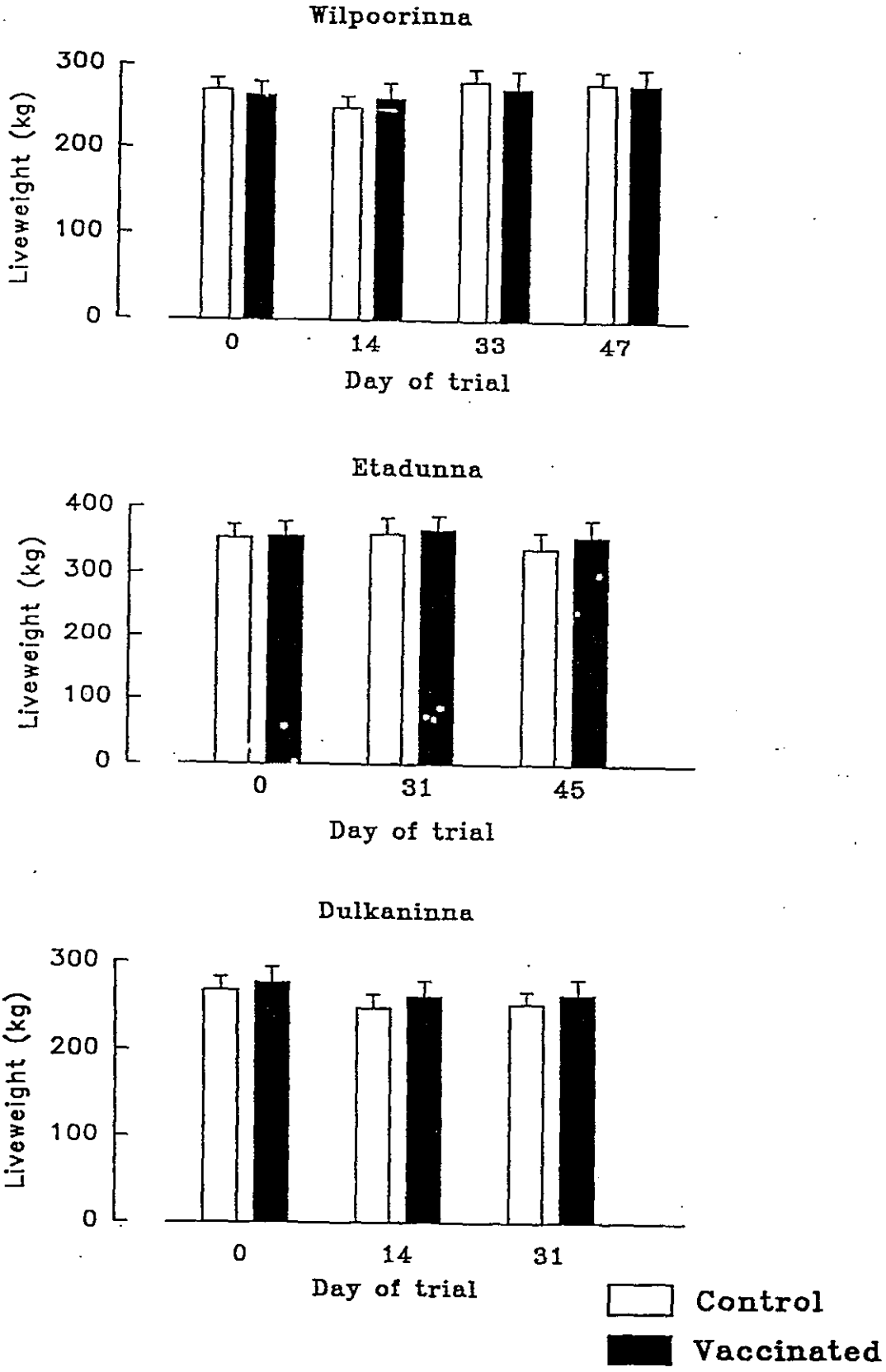


Figure 4.7 - Post secondary immunisation liveweights of Marree trial animals

4.4 CONCLUSIONS

The results of the *in vitro* experiments indicate that the freeze-dried bovine anti-toxin atibodies were efficacious against the *Pimelea* toxins. The lack of any attenuating effect of bovine anti-toxin antibodies observed in the previous study[41] was most likely due to the highly cross-linked protein-toxin conjugates used for immunisation. In the present study, care was taken to prevent protein cross-linking while preparing the conjugates. The *i*-BCF activation method seemed to produce more efficacious antibodies than those produced by NHS ester activation, presumably due to more advantageous toxin epitope presentation on the protein using this chemistry.

These data indicate that immunisation with a vaccine produced from a simplexin-huratoxin-ovalbumin conjugate of varying toxin inclusion ratio might have the potential to offer protection against the effect of *Pimelea* poisoning under field conditions. Cattle developed strong toxin-specific antibody responses (2 weeks post secondary immunisation) regardless of whether or not they were suffering *Pimelea* poisoning at the time of primary immunisation.

In a future study, trial animals from *Pimelea* free properties should be immunised to maximise antibody response, then introduced to *Pimelea* infested areas for evaluation of whether these antibodies offer any protective properties to cattle.

5.0 STRUCTURE - ACTIVITY STUDIES OF MEZEREIN AND SIMPLEXIN ANALOGUES : PKC BINDING & ACTIVATION

5.1 INTRODUCTION

5.1.1 The Structural Requirements for PKC Activation

Compounds such as phorbol-12-tetradecanoate-13-acetate (TPA), teleocidin B and aplysiatoxin, as well as the bryostatins, are structurally unrelated to 1,2-DAG, yet activate PKC and are known tumour promoters. This has prompted structure-activity studies in order to establish a model, for the essential structural requirements for PKC activation[36,38,44,68-73].

Computer-assisted modelling was used for the analysis of tumour promoters to propose a rationale for the tumour promoting activity of phorbol esters, teleocidin B, and aplysiatoxin. These studies were performed by correlating the published bio-actvities of naturally occuring compounds, leading to the proposal of a stereo-chemical model in which: the oxygens in TPA at C-3, C-4, C-9, and C-20 correspond to O-11, N-13, N-1, and O-24 of teleocidin, and O-27, O-3, O-11, and O-30 of aplysiatoxin[44]. These functional groups, as well as similarly orientated hydrophobic groups, were suggested to be essential for bio-activity. The correlating structural features are illustrated in Figure 5.1.

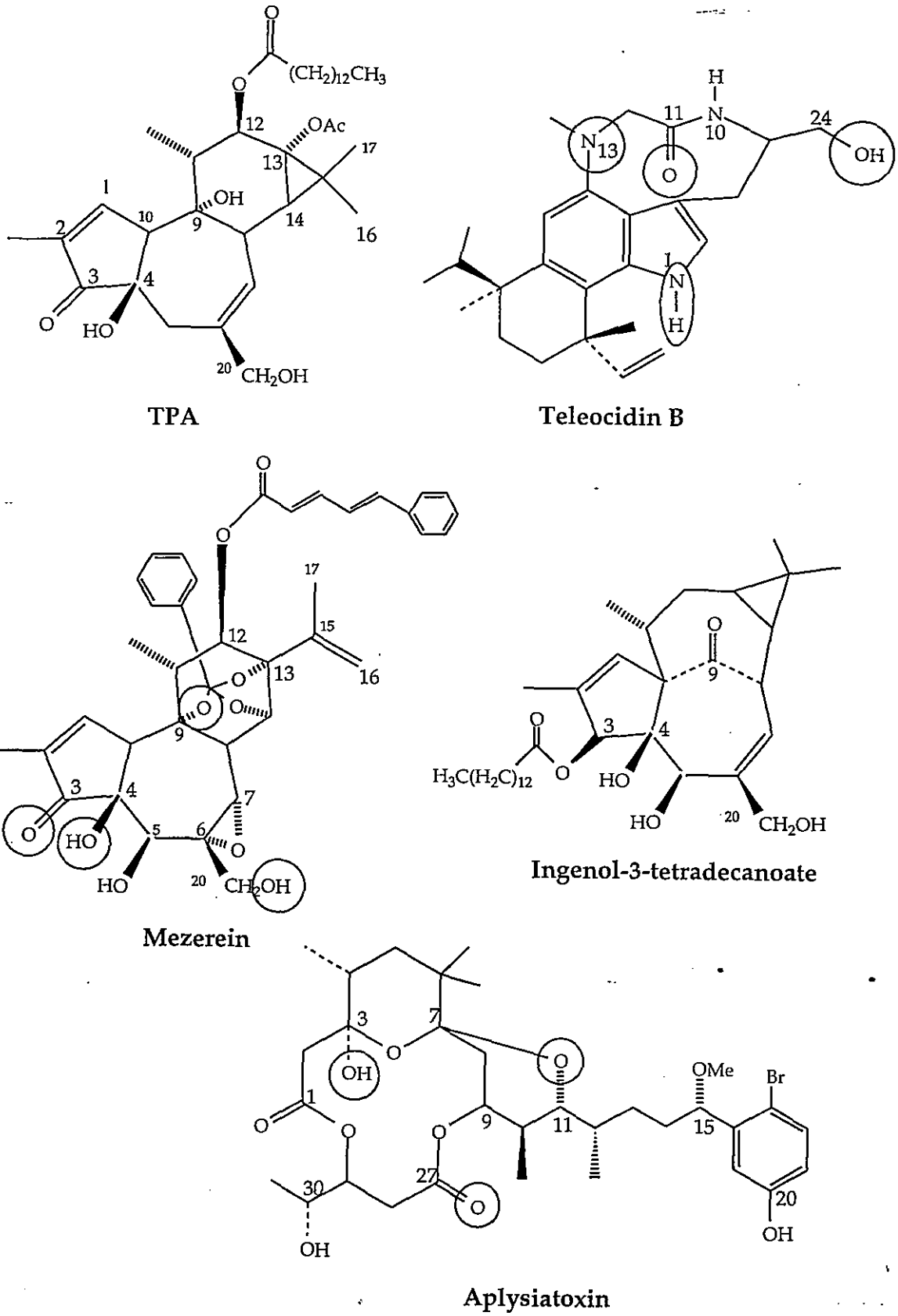
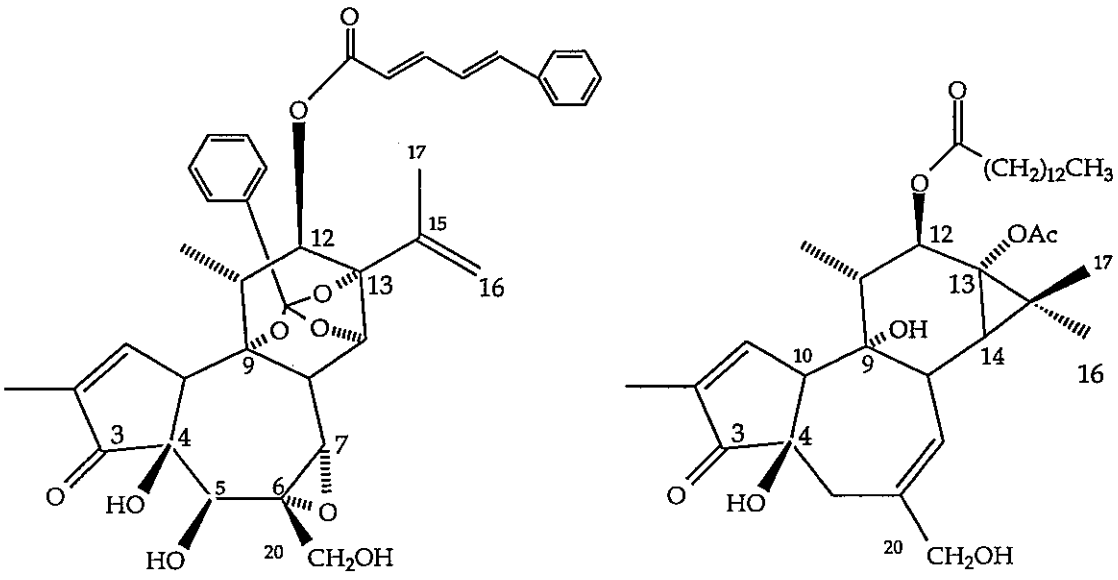


Figure 5.1 - Illustration of the correlating structural features of PKC-activating tumour promoting agents[44].

It was also concluded from these studies that the C12 substituent of phorbol esters must be hydrophobic, however the structure of this group may vary considerably without loss of bio-activity[44]. Steric hindrance generated by the change in orientation of the C2-methyl group upon hydrogenation of the 1,2-double bond of the cyclopentene ring, resulted in a considerable loss of bio-activity[68,69]. The C-3 carbonyl function of TPA was thought to be important for bio-activity, as compounds possessing a b-hydroxyl group, and compounds without an oxygen at this position possessed diminished activity. The addition of a b-hydroxyl group at the C-5 of TPA resulted in a slight reduction of bio-activity, which was more pronounced in compounds with a 5,6-double bond, or a carbonyl group at C7. This suggests that the stereochemistry of the carbons in the five and seven membered ring moieties is important for acitivity.

The minimum energy conformations of mezerein compared with TPA (Figure 5.2) showed excellent overlap, however it was not possible to explain why TPA acts as a complete tumour promoter, while mezerein functions only as an irritant, although almost as potent in the activation of PKC[44] (Concentration required to give 50% PKC activation: TPA - 2.5 nM; HHPA - 1nM; mezerein - 3nM; teleocidin - 18 nM; and aplysiatoxin -12nM). The addition of a 5-hydroxyl group to TPA, and the epoxidation of the 6,7-double bond of HHPA resulted in reduced bio-activity, however, both of these functions are present in mezerein. Furthermore, it was suggested that if the C-12 substituent of TPA was replaced with an unsaturated substituent, as in mezerein, the compound functions only as an irritant[44].



Mezerein **TPA**

Figure 5.2 - The structures of mezerein and TPA

Synthesis of a series of simplified PKC activators (possessing C-4, C-9, and C-20 hydroxyl groups, and the C-12 or C13- hydrophobic moieties of phorbol esters) produced results which showed competitive inhibition of phorbol ester binding to PKC[70], indicating agreement with the Jeffrey model proposed earlier.

In a later study, spatial arrangement of physical and chemical properties (rather than atomic position) was used to superimpose TPA, teleocidin B, 3-*O*-tetradecanoylingenol, (-)-indolactam-V, and aplysiatoxin for receptor mapping[71], and this approach suggested a hydrogen bonding mechanism for the relevant groups interacting in PKC binding. Based upon the superposition of teleocidins with TPA, a model was also proposed for the receptor cavity. Based on these results, it was proposed that -CH_2OH groups of C-9 in teleocidin, the C-6 of TPA, the amide N-H of teleocidin, and the C-4 hydroxyl group of TPA act as hydrogen donors, while the carbonyl groups at C-11 of teleocidin, and C-3 of TPA are hydrogen acceptors (Refer to Figure 5.1). The Itai model proposed that amide (position 10) of teleocidin was involved in the PKC receptor binding rather than N-13, as suggested in the Jeffrey model. Investigation of pK_a values of model tertiary aromatic amines[74] reveals values for N,N-dimethylaniline, and N,N-diethylaniline of 5.15 and 6.61 respectively, at 25°C. Thus, it may still be feasible that the N-13 of teleocidin is protonated under physiological conditions, and could thus act as a hydrogen donor when hydrogen bonding. Therefore, the ability of the N-13 of teleocidin to act as a hydrogen donor is pH dependent.

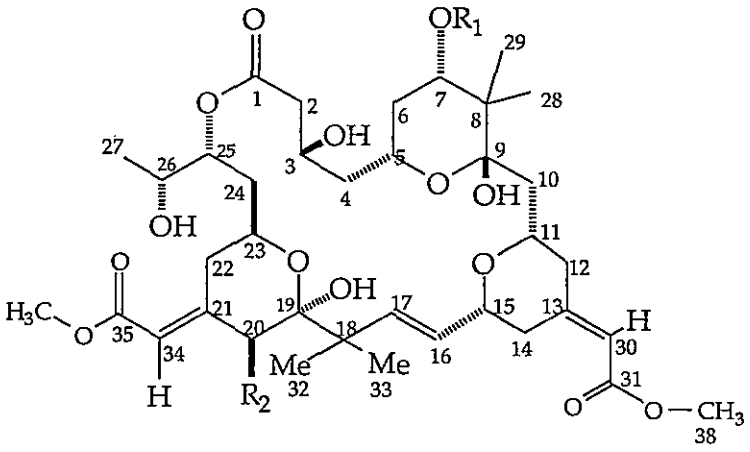
It has also been suggested that the indole N-H of teleocidin stereospacially corresponds to the C-9 hydroxyl group of TPA[70], and acts as a hydrogen bond donor to the receptor. The deficiency of this model is however revealed when the C-9 oxygen is bound in an O-9, O-13, O-14 ortho-ester function, as is the case with simplexin, mezerein, and related daphnane ortho esters. However, it has since been suggested that the C-9 oxygen acts as a hydrogen acceptor[71], which seems appropriate when considering compounds with the orthoester function.

The implications are that the indole N-H of teleocidins may not be essential for PKC binding (this nitrogen can not act as a hydrogen acceptor), and it follows that the C-9 oxygen of TPA also may not be essential for PKC binding.

5.1.2 The Effect of Bryostatins on PKC Activity

The structure-activity model was extended to the comparison of the phorbol esters to the bryostatins (PKC activating macrocyclic lactones isolated from marine bryozoans)[45]. This model correlated the C-26, C-1, and C-19 oxygens of the bryostatins (Figure 5.3) with close agreement to the C-20, C-9, and C-4 oxygens of the phorbol esters. The effect of bryostatins on arachidonic acid metabolite release, and epidermal growth factor binding in Friend erythroleukemia (C3 10T 1/2) cells was later studied[75], and it was concluded that the bryostatins inhibited phorbol ester action non-competitively.

Evidence showed that the C-19 oxygen of bryostatins acts as a hydrogen acceptor and examination of bryostatin 3 shows this oxygen is bound as a lactone oxygen (other bryostatins have a free hydroxyl here). Comparison showed close agreement with the C-9 oxygen of TPA.



Bryostatin	R_1	R_2
1	$COCH_3$	OCO
2	H	OCO
3	$COCH_3$	OCO
4	$COCH_2CH(CH_3)_2$	$OCOCH_2CH_2CH_3$
5	$COCH_2CH(CH_3)_2$	$OCOCH_3$
6	$COCH_2CH_2CH_3$	$OCOCH_3$
7	$COCH_3$	$OCOCH_3$
8	$COCH_2CH_2CH_3$	$OCOCH_2CH_2CH_3$
9	$COCH_3$	$OCOCH_2CH_2CH_3$
10	$COCH_2CH(CH_3)_2$	H

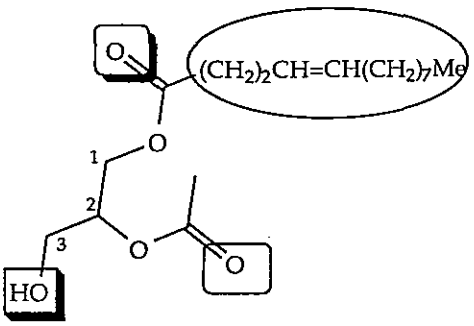
Figure 5.3- Structure of the bryostatins (adapted from[45]).

5.1.3 Further Refinements to the Structure-Activity Model for PKC Activation

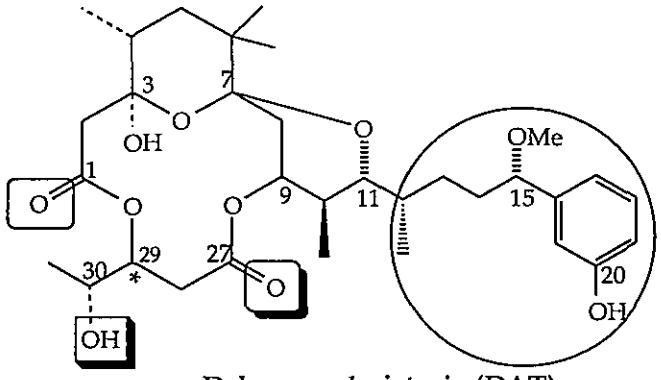
Further investigation of the structural basis for PKC activation by comparison of debromoaplysiatoxins (DAT's) with diacyl glycerols, the endogenous activators of PKC was reported[38]. This group argued that the C-29 stereo-centre (rather than the C-30 stereo-centre of DAT) was crucial for activity (Figure 5.4). It was shown that 3-deoxy-DAT was equi-potent with DAT regarding PKC activation, therefore concluding that the C-3 hydroxyl function of DAT was not essential for activity. Structural considerations showed that the C-30 hydroxyl group of DAT matched the C-3 hydroxyl group of diacylglyceride, the C-29 stereo-centre of DAT matched the C-2 stereo-centre of (*S*)-diacylglycerides, and the C-1 carbonyl group of DAT matched the C-2 ester moiety of diglyceride. The structure correlation of (*S*)-diacylglycerides with DAT, TPA, ingenol-3-tetradecanoate, bryostatin 1, and teleocidin B-1 as proposed[38] is summarised (Figure 5.4).

The Nakamura model shows the C-3 carbonyl oxygen of TPA matching the carbonyl oxygen of the C-2 ester moiety of diglyceride in contrast to other models[44,70,71]. This also suggests that the C-4 hydroxyl function of TPA is not required for PKC activation, as 4-deoxyphorbols are PKC activators[38]. However, it was previously suggested that the 4-b-hydroxyl group is required for optimal PKC activation[44].

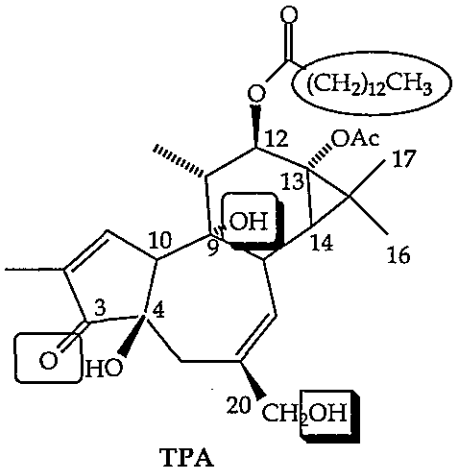
The interaction of the C-12 and C-13 ester groups of TPA and related compounds was investigated by the synthesis of sterically hindered analogues related to DAG, and the ability of these compounds to displace membrane-bound ^{3}H-labelled phorbol-12,13-dibutyrate ([^{3}H]-PDBu) was examined[72] (Figure 5.5).



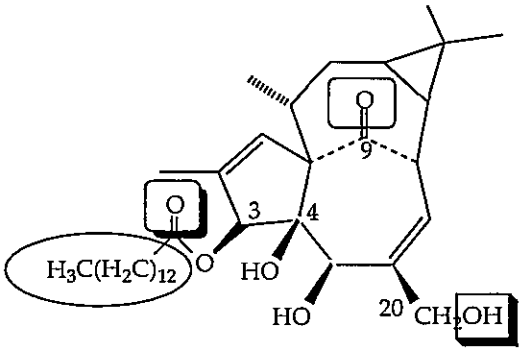
(S)-1-Oleoyl-2-acetylglycerol



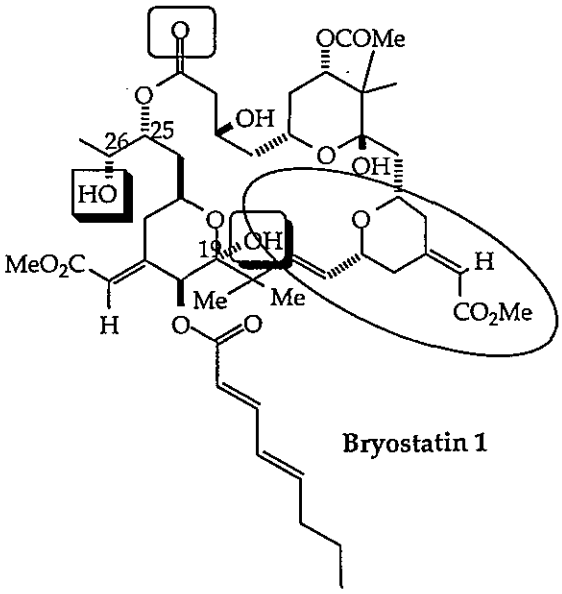
Debromoaplysiatoxin (DAT)



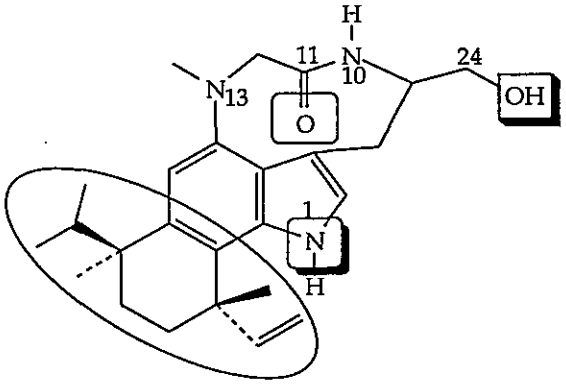
TPA



Ingenol-3-O-tetradecanoate

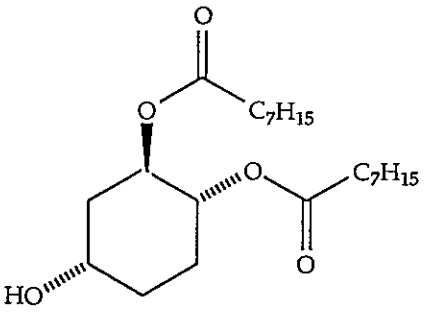


Bryostatin 1

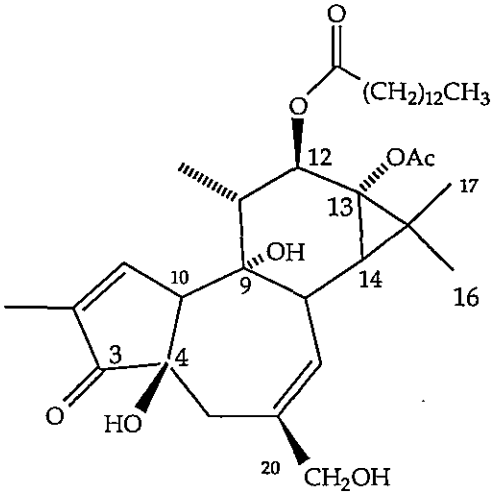


Teleocidin B-1

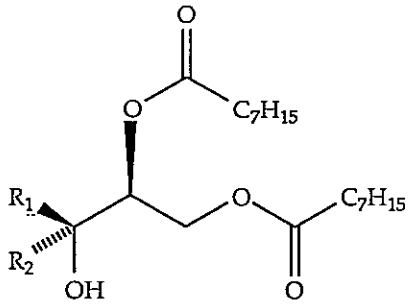
Figure 5.4 - Proposed structure-activity correlation[38]



DiC8



(TPA)



(1) R_1=H; R_2 =Me :
(2) R_1=Me; R_2=H :
(3) R_1=R_2=Me :
(4) R_1=R_2=$(CH_2)_5$

Figure 5.5 - Synthetic analogues of DAG[72]

The binding of these analogues to PKC was shown to be significantly weaker than that of [^{3}H]-PDBu (μM range compared with nM range of [^{3}H]-PDBu) and it was argued that the cause of this was that diacylglycerols have to be given substantial energy to adopt the optimum stereo-chemistry for PKC activation[73]. This showed that the 1,2-acetyl-*sn*-diacylglyceride conformer with molecular geometry similar to the ester region of b-phorbol was 3.2kcal/mol above that of the lowest energy conformer of diacylglyceride. It was also concluded that the C-9 hydroxyl group, as well as the C-12 and C-13 ester functions of TPA correlated closely with diacylglyceride. However, compounds which do not have the diacylglyceride-like region, such as teleocidins, aplysiatoxins, and bryostatins are potent PKC activators.

Much of the recent structure-activity research regarding the activation of PKC by phorbol esters has still been focussed on this region of the phorbol ester related molecules[76].

5.1.4 The PKC Receptor Interaction

A model for the binding of TPA to PKC was therefore proposed[36], in which the C-3 carbonyl oxygen was favoured over the C-4 hydroxyl group of TPA as being responsible for PKC binding. It was suggested that the second of 2 cysteine-rich regions of PKC containing an asparagine residue is essential for strong TPA binding, since an additional hydrogen bond to the C-20 hydroxyl group of TPA would be

allowed (Figure 5.6). PKC-z contains only one cysteine-rich region and is reported not to respond to TPA significantly[27].

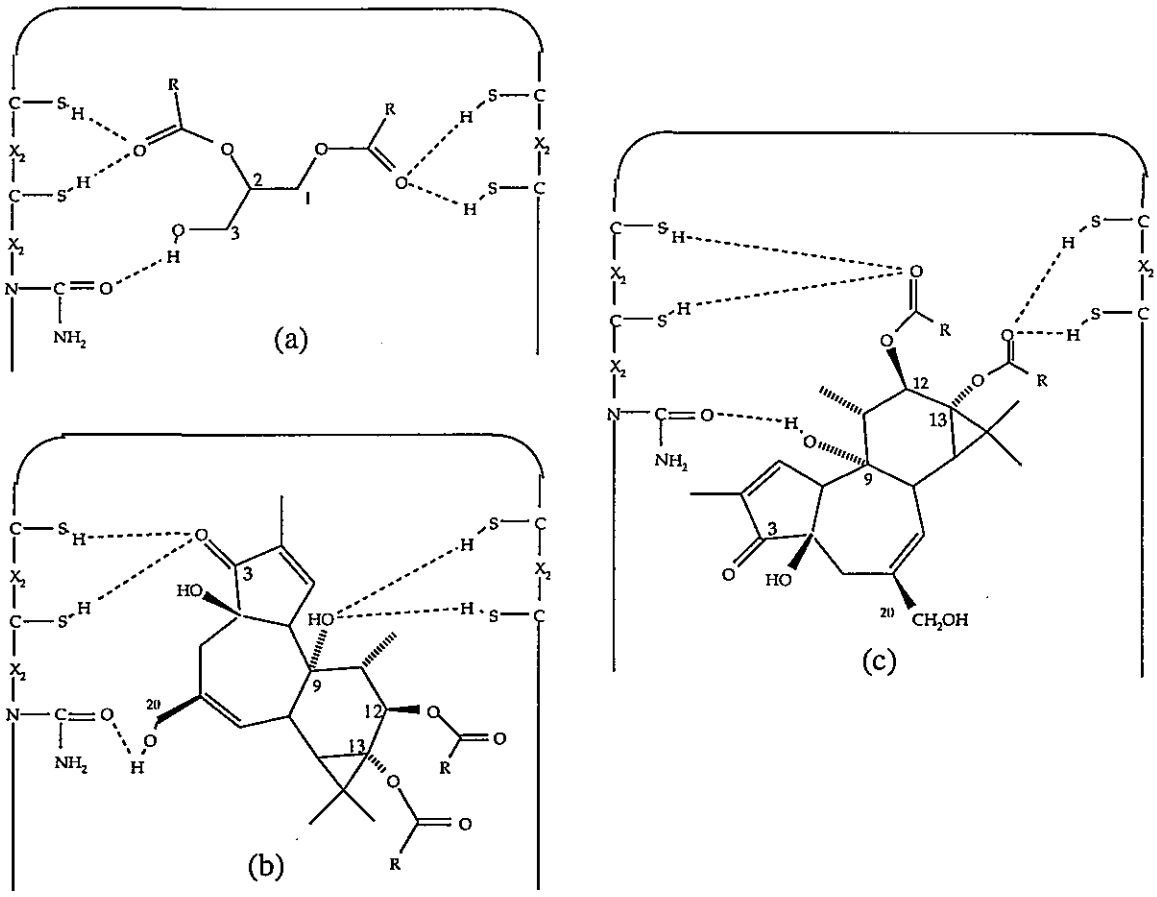


Figure 5.6 - Putative binding of diacylglycerol (a) and TPA (b) and (c) to the second cysteine-rich region of PKC. C-SH, cysteine; N-CO-NH2, asparagine; R, fatty acid residue[36]

This model suggests that the C-9 hydroxyl group of TPA might act as a hydrogen donor (Figure 5.6(c)), or a hydrogen acceptor (Figure 5.6(b)) in interacting with the PKC receptor. The model presented in Figure 5.6 (c) is inappropriate when considering the activation of PKC by simplexin and related daphnane orthoesters, as the C-9 oxygen is bound in a C-9, C-13, C-14 orthoester function. The Gschwentdt binding model (Figure 5.6(c)) also shows the ester carbonyl oxygens of the C-12 and C-13 ester moieties of TPA interacting with the cysteine residues of PKC which are not present in simplexin or huratoxin. Therefore it would be expected that the EC_{50} of simplexin would be significantly higher than that of TPA.

5.1.5 Relation to the Present Study

The literature models for PKC binding seem not to adequately explain why simplexin and related daphnanes, which have the C-9 oxygen incorporated into an orthoester function, and which lack the C-12 and C-13 ester moieties of TPA, are such potent PKC activators.

The aim of this structure-activity study was two-fold: firstly we wished to identify a derivative of simplexin with significantly reduced PKC activity, with the long-term view of achieving detoxicification by rumen microflora; secondly, investigation of new simplexin and mezerein analogues would provide new information regarding the essential functional groups for binding and activation of PKC by daphnane compounds. Mezerein was chosen as a model reference daphnane orthoester for this study due to its close structural resemblance to simplexin (Figure 5.7), and the advantage of being available commercially.

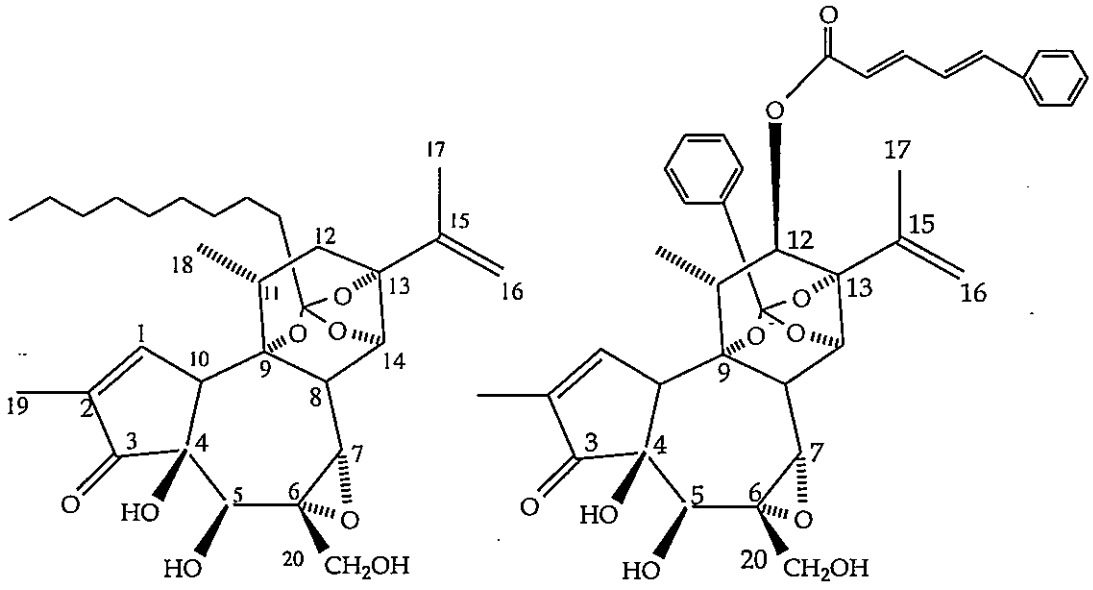


Figure 5.7 - simplexin and mezerein

To determine the functional role of the various oxygens with respect to PKC binding and activation, selective chemical alterations were performed to the structures of the respective compounds. Changes in PKC binding were measured using a radioligand binding assay[59], while PKC efficacy was determined using the *in vitro* techniques previously described (Chapter 4). Chemically, the rationale was to selectively protect the respective hydroxyl functions as either silyl ethers, benzoate, or 4-nitrobenzoate esters. The role of the C-3 carbonyl grouping of mezerein and simplexin was investigated by selective reduction of the C-3 carbonyl function to the respective hydroxyl functions.

5.2 EXPERIMENTAL

5.2.1 General

Geometry optimisation AN1 level calculations (gas phase, solvent free) were calculated for simplexin using a Silicon Graphics work station with Spartan III soft-ware.

Simplexin was purified from dried *Pimelea* plant material using the methods previously described (Chapter 2), while mezerein, was obtained from CalBiochem Australia Pty Ltd.

5.2.2 Preparation of derivatives of Simplexin and Mezerein

5.2.2.1 3-Hydroxymezerein

The procedure for selective reduction of the carbonyl group of enone moieties was adapted from the Luche reaction[77]. Mezerein (10 mg, 0.015 mmol) was dissolved in dry methanol (1mL), to which cerium chloride (6.8 mg, 0.018 mmol), then sodium borohydride (0.7 mg, 0.01 mmol) was added. The reaction was monitored by TLC (50% ethyl acetate in hexane) and when complete (approximately 2 hrs), the reaction mixture was diluted into ethyl acetate (2 mL). The resulting mixture was washed with 0.1N HCl (3 x 5 mL), followed by 5% sodium bicarbonate solution (3 x 5 mL), and water (3 x 5 mL). The product was then dried over anhydrous sodium sulphate. After removal of the solvent *in vacuo*, purification was achieved by preparative reverse phase HPLC (R_t (analytical) - 5.60 minutes; mezerein, 12.10 minutes, yielding 7.9 mg, 0.012 mmol, 80%). Identification was performed by ^1H, and ^1H-^1H homonuclear correlated NMR spectroscopy.

5.2.2.2 3-Hydroxysimplexin

Simplexin (9.6 mg, 0.018 mmol) was treated with cerium chloride (8 mg, 0.022 mmol) and sodium borohydride (0.7, 0.01 mmol) as described above for the reduction of mezerein. The reaction product was identified as 3-hydroxy simplexin (R_t (analytical), 3.85 minutes; simplexin, 13.45 minutes).

5.2.2.3 *t*-Butyldiphenylsilyl Ether of Mezerein

The *t*-butyldiphenylsilyl ether of mezerein was prepared using standard methods[78,79]. Mezerein (5 mg, 0.0076 mmol) was dissolved in dimethylformamide (2mL) and cooled to -10°C under a nitrogen atmosphere. Imidazole (1.3 mg, 0.019 mmol) was added to the mixture, followed by *t*-butyldiphenylsilyl chloride (2.3 µL, 0.0091 mmol). The reaction mixture was then warmed to room temperature and left stirring for 14 hrs prior to work-up. TLC of the product (40 % ethyl acetate in hexane) revealed a new spot corresponding to the product (R_f 0.22). The reaction mixture was then extracted into ethyl acetate (5 mL) and washed with saturated sodium bicarbonate solution (3 x 5 mL) followed by water (3 x 5 mL). The product was then dried over anhydrous sodium sulphate in chloroform. The product was purified using preparative reverse phase HPLC (yielding 6.1 mg, 0.0068 mmol, 75%), and identified as the 20-*t*-butyldiphenylsilyl ether of mezerein (Figure 5.8): (retention time - reverse phase HPLC (analytical) 6.38 minutes; mezerein, 12.10 minutes; ^1H NMR:- d7.75, m, phenyl group of orthoester and silyl ether protecting group (overlapping) (9H); 7.60, m, H1 (1H); 7.40, m, benzene ring & vinylic proton of C12 substituent, and silyl ether protecting group (overlapping) (12H); (6.92, m (2H); 5.98, d, (1H) - C12 substituent); 5.17, d, H16 (2H); 5.14, s, H12 (1H); 4.95, d, H14 (1H); 4.25, s, H5 (1H); (3.95, 3.75), AB quartet, H20, (2H); 3.84, d, H10 (1H); 3.71, d, H7 (1H); 3.50, s, H8 (1H); 2.55, q, H11 (1H); 1.92, s, H17 (3H); 1.80, s, H19 (3H); 1.42, d, H18 (3H); 1.05, s, *t*-butyl (9H)).

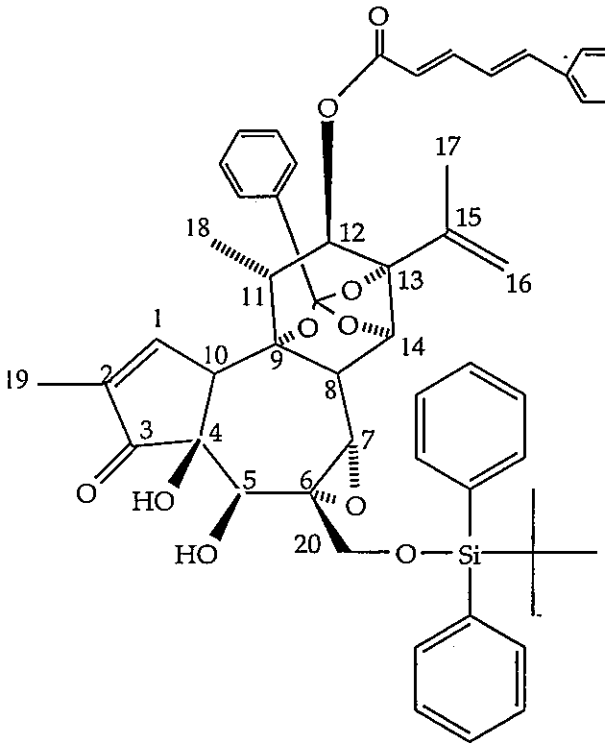
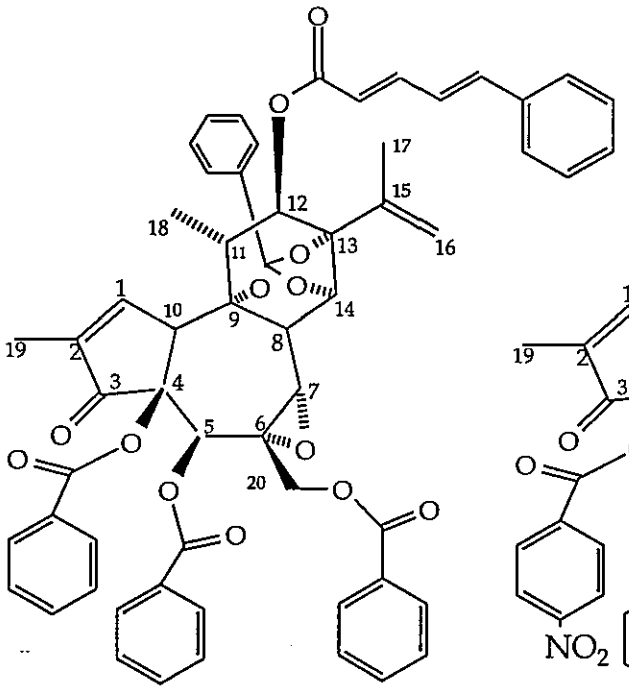


Figure 5.8 - 20-*t*-Butyldiphenylsilyl ether of mezerein.

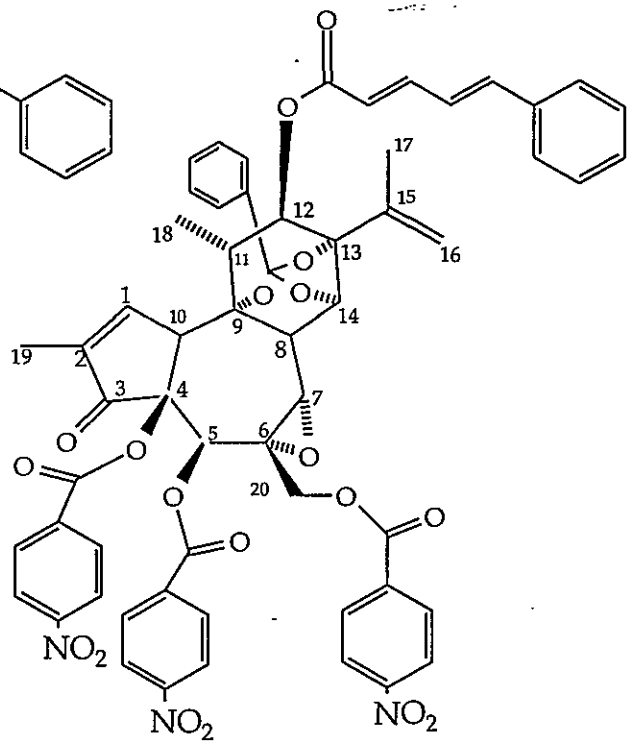
5.2.2.4 Benzoate and 4-nitrobenzoate Esters of Mezerein and Simplexin

Mezerein (5 mg, 0.0076 mmol) was treated with benzoyl chloride, while mezerein (5 mg, 0.0076 mmol) and simplexin (5 mg, 0.0094 mmol) were also treated with 4-nitrobenzoyl chloride as described previously (Chapter 2). The di- and tri- esters produced by the reactions were separated chromatographically by reverse phase HPLC. Identification of the esters was tentatively performed by correlating retention times with respective UV spectra produced by these compounds under the chromatographic conditions (PDA detection). The purified compounds isolated were: mezerein-4,5,20-tribenzoate, mezerein-4,5,20-tri-(4-nitro)benzoate, simplexin-5,20-di-(4-nitro) benzoate, and simplexin-4,5,20-tri-(4-nitro)benzoate: [mezerein-4,5,20-tribenzoate (1.8 mg, 0.0019 mmol; R_t 50.38; mezerein R_t 38.00 mins, gradient 1. ^{1}H NMR:- d8.04, m, phenyl protons from benzoate ester substituent (15H); 7.75, m, phenyl group of orthoester (5H); 7.60, m, H1 (1H); 7.40, m, benzene ring & vinylic proton of C12 substituent (6H); (6.92, m (2H); 5.98, d, (1H) - C12 substituent); 5.21, s, H5 (1H); 5.17, d, H16 (2H); 5.14, s, H12 (1H); 4.95, d, H14 (1H); (4.06, 4.02, 3.80, 3.76), AB quartet, H20, (2H); 3.84, d, H10 (1H); 3.71, d, H7 (1H); 3.50, s, H8 (1H); 2.55, q, H11 (1H); 1.92, s, H17 (3H); 1.80, s, H19 (3H); 1.42, d, H18 (3H)); mezerein-4,5,20-tri-(4-nitro)benzoate (3.1 mg, 0.0028 mmol; R_t 18.54 mins; mezerein R_t 12.10 mins, gradient 4. ^{1}H NMR:- d8.27, m, phenyl protons of 4-nitro benzyl ester groupings, typical of para disubstituted benzene (12H); 7.75, m, phenyl protons of orthoester function (5H); 7.60, m, H1 (1H); 7.40, m, benzene ring & vinylic proton of C12 substituent (6H); (6.92, m (2H); 5.98, d, (1H) - C12 substituent); 5.17, d, H16 (2H); 5.14, s, H12 (1H); 4.95, d, H14 (1H); 4.85, s, H5 (1H); 4.18, d, H7 (1H); (4.07, 4.03, 3.81, 3.77), AB

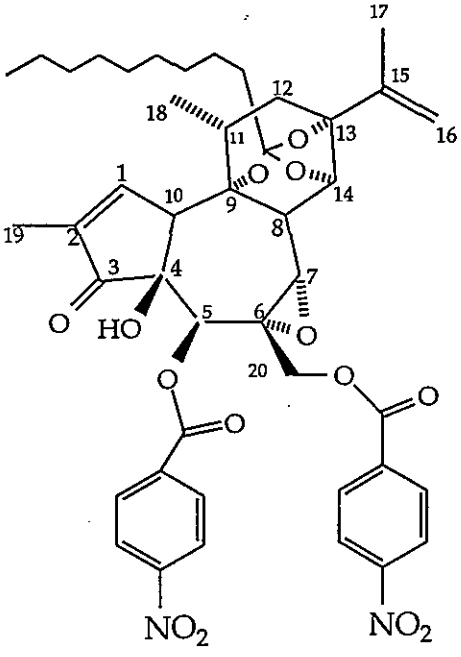
quartet, H20, (2H); 3.84, d, H10 (1H); 3.50, s, H8 (1H); 2.55, q, H11 (1H); 1.92, s, H17 (3H); 1.80, s, H19 (3H); 1.42, d, H18 (3H)); simplexin-5,20-di-(4-nitro)benzoate (3.9 mg, 0.0048 mmol; R_t 25.35 minutes; simplexin 20.29 minutes (gradient 4), ^1H NMR:- d8.27, m, phenyl protons of 4-nitro benzyl ester groupings, typical of para disubstituted benzene (8H); 7.60, m, H1 (1H); 5.01, d, H16 (2H); 4.42, d, H5 (1H); 4.39, d, H14 (1H); (4.05, 3.96, 3.94, 3.85), AB quartet, H20, (2H); 3.75, d, H10 (1H); 3.43, d, H7 (1H); 2.95, d, H8 (1H); 2.46, p, H11 (1H); 1.97, dd, H12eq (1H); 1.64, d, H12ax (1H); 1.81, dd, H19 (3H); 1.78, dd, H17 (3H); 1.17, d, H18 (3H); 3.60 (exchanges with D_2O), OH); simplexin-4,5,20-tri-(4-nitro)benzoate (3.7 mg, 0.0038 mmol; R_t 28.21 minutes, ^1H NMR:- d8.27, m, phenyl protons of 4-nitro benzyl ester groupings, typical of para disubstituted benzene (12H); 7.60, m, H1 (1H); 5.01, d, H16 (2H); 4.42, d, H5 (1H); 4.39, d, H14 (1H); (4.05, 3.96, 3.94, 3.85), AB quartet, H20, (2H); 3.75, d, H10 (1H); 3.43, d, H7 (1H); 2.95, d, H8 (1H); 2.46, p, H11 (1H); 1.97, dd, H12eq (1H); 1.64, d, H12ax (1H); 1.81, dd, H19 (3H); 1.78, dd, H17 (3H); 1.17, d, H18 (3H))] (Figure 5.9).



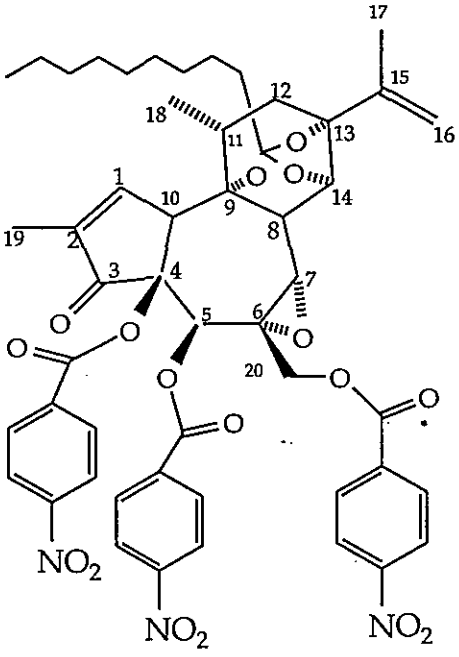
Mezerein-4,5,20-tribenzoate



Mezerein-4,5,20-(4-nitro)benzoate



Simplexin-5,20-di-(4-nitro)benzoate



Simplexin-4,5,20-tri-(4-nitro)benzoate

Figure 5.9 - Benzoate and 4-nitrobenzoate esters of mezerein and simplexin

5.2.3 Activation of PKC by Daphnane orthoesters and derivatives *in vitro*

The effect of the test compounds on bovine pulmonary venule preparations *in vitro* was determined in the same manner as described previously for the *Pimelea* toxins (Section 4.2.4). Tissue samples were collected and prepared as previously described. The effective curves for force of contraction induced by mezerein, 3-hydroxymezerein, simplexin, and 3-hydroxysimplexin as a percentage of the 1μM 5-HT contraction were determined.

5.2.4 Binding of Daphnane orthoesters to PKC

The binding properties of the test compounds were determined by competitive binding with 20-[^{3}H]-phorbol dibutyrate ([^{3}H]-PDBu) (599 Gbq mmol^{-1}, Amersham Life Sciences) to a PKC-rich homogeneous murine brain preparation[59]. Incubations were performed for 2 hours (30°C) and consisted of: [^{3}H]-PDBu (100μL of 20,000 dpm); brain preparation (100μL); graded concentrations of the competing agent (50μL); and binding buffer (50mM Tris-HCl (pH 7.4), 0.03 mM BSA) (750μL). Incubations were terminated by vacuum filtration through pre-wetted (with 2% polyethylenimine) glass filters. Bound radioactivity was determined by liquid scintillation counting following leaching with scintillant (4 mL, Optiphase "HiSafe2"). Analyses were performed in triplicate, and the inhibition curves (IC) were generated by plotting the percentage of bound radioactivity (less non-specific binding) (as opposed to total bound [^{3}H]-PDBu) with respect to concentration of competing agent. IC_{50} values represent the concentration of reagent required to give a 50% displacement of [^{3}H]-PDBu from the tissue preparation.

5.3 RESULTS AND DISCUSSION

5.2.1 Preparation of 3-hydroxymezerein and 3-hydroxysimplexin Derivatives

The main concern when applying the Luche reaction to mezerein and simplexin was that the C6-C7 epoxide function would also be reduced by excess sodium borohydride present in the reaction mixture. Analysis of the ^{1}H NMR spectrum of the product derived from the reduction of mezerein was similar to that of mezerein, except the signal for H1 was shifted up-field to d5.60, and the H19 signal was coupling to a new signal at d3.60, corresponding to a proton at the C-3 position (Figure 5.10) (^{1}H NMR:- d7.75, 7.55, 7.40, 6.92, 5.98, 5.60, 5.17, 5.14, 4.95, 4.38, 4.05, 3.95, 3.84, 3.60, 3.58, 2.55, 1.92, 1.65, 1.42). As expected, the product was a diastereomeric mixture, and was not further purified.

The resulting ^{1}H COSY spectrum of 3-hydroxymezerein (Figure 5.10) confirmed that the parent structure of mezerein had been maintained, and that no other finctional groups had been reduced. Accordingly, the reaction was repeated with simplexin. The coresspondinng ^{1}H NMR spectrum also showed that the parent structure of simplexin had been maintained (^{1}H NMR:- d5.61, m, H1 (1H); 4.96, methylene d, H16 (2H); 4.35, d, H14 (1H); 4.30, d, H5 (1H); 3.82, (3.96, 3.90, 3.86, 3.80), AB quartet, H20 (2H); 3.76, d, H10 (1H); 3.65, d, H3 (1H); 3.43, d H7 (1H); 2.91, d, H8 (1H); 2.42, p, H11 (1H); 2.20, dd, H12eq (1H); 2.01, m, -CH_2- orthoester moiety) 1.67, d, H12ax (1H); 1.80, dd, H19 (3H); 1.74, dd, H17 (3H); 1.21, d, H18 (3H); 0.88, t (CH_3-orthoester); and 4.10, 4.05, 3.97, & 3.93, exchange with D_2O).

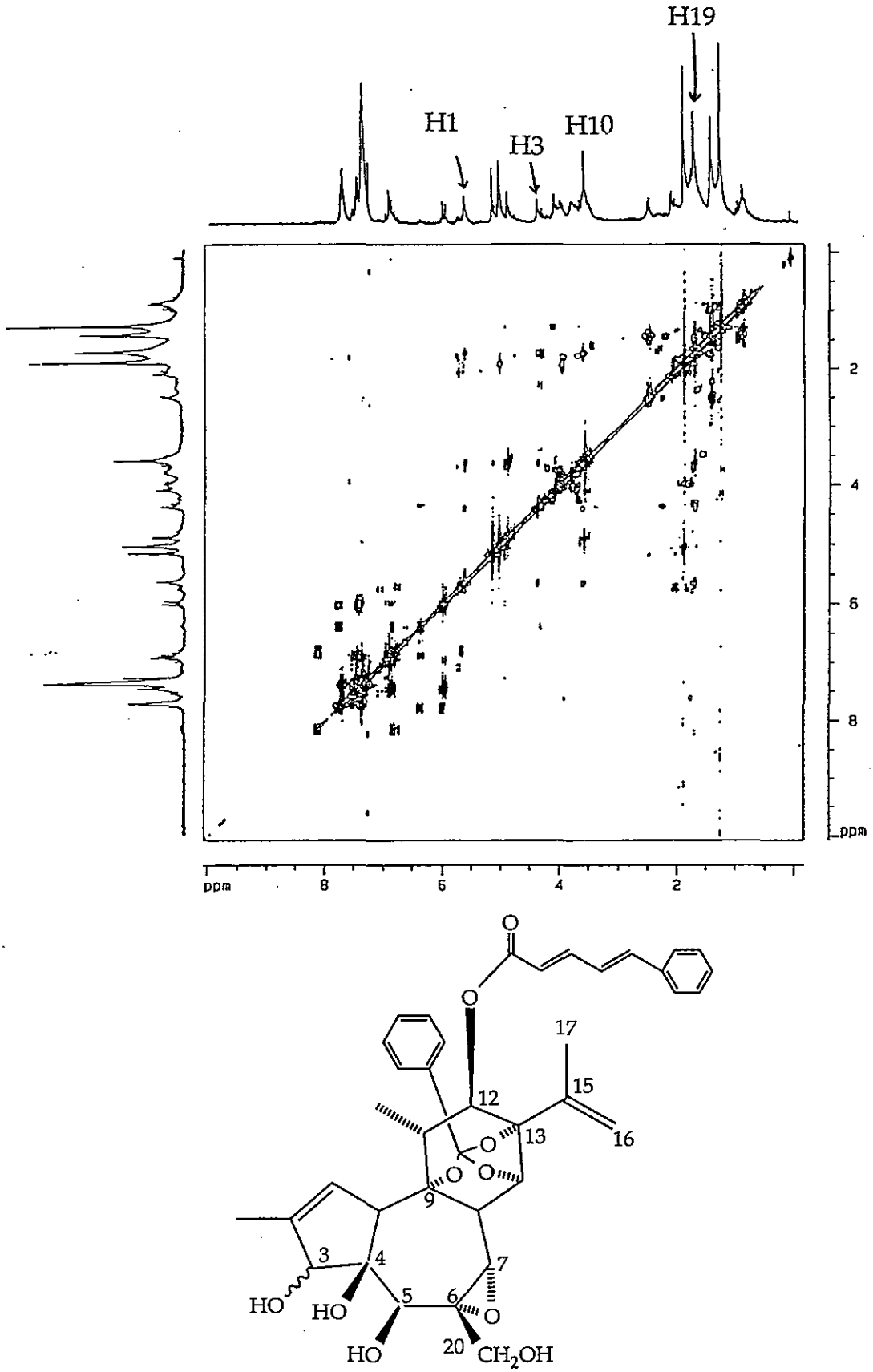


Figure 5.10 - ^{1}H Homonuclear correlation spectrum of 3-hydroxymezerein

5.3.2 Mezerein (Structure, Binding, and PKC Activation)

The selective protection of the C-20 hydroxyl group of mezerein was performed to determine whether the C-20 hydroxyl group was essential for binding to PKC, as it was previously suggested that the C-20 hydroxyl group of phorbol esters and daphnane orthoesters was required for PKC activation[44]. The IC_{50} of mezerein was found to be 16.6 nM, while the IC_{50} for the corresponding C-20-*t*-butyldiphenylsilyl ether was found to be 30.2 nM. Although this value is approximately twice that of mezerein, the compound still binds strongly to PKC (nM), and it can be concluded that the C-20 hydroxyl group of mezerein is not essential for binding to the PKC pharmacophore. However, it may still be required to achieve optimum activation of PKC *in vitro*, or *in vivo*.

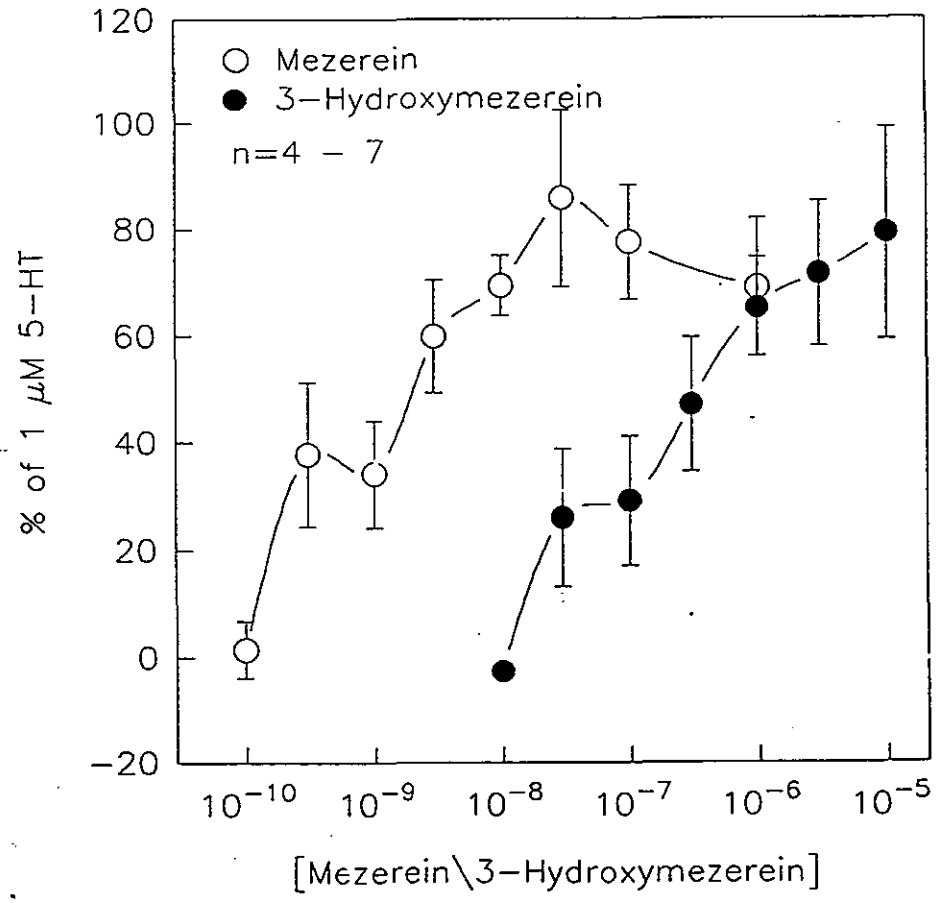
The protection of the C-4, C-5, and C-20 hydroxyl groups of mezerein as benzyl and 4-nitrobenzyl esters was performed to confirm whether a change in the protecting groups had any effect on the PKC binding of the structural analogues of mezerein. In both cases, the retention times of the protected analogues of mezerein were longer than mezerein under the reverse phase HPLC conditions (4-nitrobenzyl esters had longer retention that the corresponding benzyl esters of mezerein), indicating that the protected variants possessed higher lipophilic character than mezerein. The IC_{50} of mezerein-4,5,20-tribenzoate and mezerein-4,5,20-tri-(4-nitro)benzoate were 1445 nM and 144.5 nM respectively.

The protection of the C-4 and C-5 hydroxyl groups of mezerein results in an increase in IC_{50} of 87.0 times (as benzyl esters) and 8.70 times (as 4-nitrobenzyl esters). These results indicate that the lipophilic and steric changes induced by the added substituents affect PKC binding. Mezerein-4,5,20-tri-(4-nitro)benzoate had a longer elution time using reverse phase HPLC, therefore was more lipophilic than mezerein-4,5,20-tribenzoate, which as anticipated resulted in a lower IC_{50} (mezerein-4,5,20-tri-(4-nitro)benzoate would have a greater interaction with the membrane tissue due to its higher lipophilicity than mezerein-4,5,20-tribenzoate). Therefore it is concluded that the C-4 and C-5 functionalities of mezerein are important for PKC binding, and the difference of IC_{50} due to varying the protecting ester groups may be attributable to the differences in lipophilic character induced by the substituents.

The selective reduction of the C-3 carbonyl group of the cyclopentenone moiety of the daphnane orthoesters was achieved using the Luche reaction[77]. The selective reduction of the C-3 carbonyl group of mezerein to a hydroxyl group (a- or b-) resulted in an increase of IC_{50} to 178 nM (a 10.7 fold increase). Therefore, conversion of the C-3 carbonyl group to a hydroxyl group resulted in a substantial reduction of the PKC binding capacity of mezerein. The factors affecting this property may be linked to the change in functional group interation with the binding site, and the change in lipophilic character of the mezerein derivative. Using the *in vitro* assay system described in the previous chapter, the EC_{50} of mezerein was found to be 1.5 nM, which compares favourably with a value of 3 nM obtained in a previous study[44]. The effective curve of mezerein (relative to 5-HT) and 3-hydroxymezerein show that reduction of the C-3 carbonyl results in an increase of EC_{50} from 1.5 nM to 220 nM (a 147-fold increase)

(Figure 5.11). Therefore, the functional group interaction (carbonyl compared with hydroxyl functionality) at the C-3 position is also important for PKC activation.

The difference between the EC_{50} (1.5 nM, derived from organ bath experiments) and IC_{50} (17 nM, derived from radioligand binding assay) for the parent compound may attributed to a receptor reserve for PKC activation (ie. not all receptor sites are required for maximum PKC activation).



Mezerein EC_{50} 1.5 nM
3-Hydroxymezerein EC_{50} 220 nM
Concentration Ratio 147

Figure 5.11 - Effective curve for mezerein and 3-hydroxymezerein.

5.3.3 Simplexin (Structure, Binding, and PKC Activation)

The C-4, C-5, and C-20 hydroxyl groups of simplexin were protected as 4-nitrobenzyl esters. The IC_{50} concentration of simplexin was found to be 10.9 nM, while simplexin-5,20-di-(4-nitro)benzoate was found to have an IC_{50} of 1075 nM. This represents a reduction in binding capacity of a factor of almost two orders of magnitude (98.6-fold). This provides good evidence that the hydroxyl function at the 5 position contributes significantly for the binding of simplexin to PKC. The contribution of the C-20 hydroxyl group to the binding of simplexin to PKC cannot be estimated from this data, however the C-20 *t*-Butyldiphenylsilyl ether of mezerein had an IC_{50} of 30.2 nM, therefore it may be assumed that the large increase in IC_{50} observed for the 5,20-dibenzoate analogue of simplexin is mainly contributed by derivatisation of the C-5 hydroxyl group. Simplexin-4,5,20-tri-(4-nitro)benzoate was found to have an IC_{50} of 4068 nM (a 373-fold increase), also providing solid evidence that the C-4 hydroxyl group of simplexin is required for binding of simplexin to PKC.

Simplexin was found to have a very large effective range *in vitro* (from sub pM to μM concentrations). The EC_{50} of simplexin was found to be 0.24nM (Figure 5.12), which indicates that it is a more potent PKC activator than mezerein. The IC_{50} value for 3-hydroxysimplexin was found to be 45 nM (which is a 4.1-fold increase compared with the corresponding mezerein transformation of a 10.7-fold increase). However 3-hydroxysimplexin was found to be inactive *in vitro* in the μM concentration range (Figure 5.12), which indicates that the C-3 carbonyl of simplexin is not essential for PKC binding, yet it is essential for PKC activation. These data suggest that 3-hydroxysimplexin might be useful as a selective protein kinase C inhibitor. The difference in behaviour of 3-hydroxysimplexin and simplexin *in vitro* may be due to differences in the PKC subtypes between species (ie. the binding assay used murine brain preparation whereas the *in vitro* assay used bovine pulmonary venule), however it may be argued that the mode of action of the daphnane orthoesters is through mimicing the role of the endogenous activator, DAG. The receptor site for DAG binding (for DAG dependent PKC) is expectected to be the similar across species. The reference compound mezerein, was also exposed to the same structural alteration and assay treatments, resulting in different bioactivity findings.

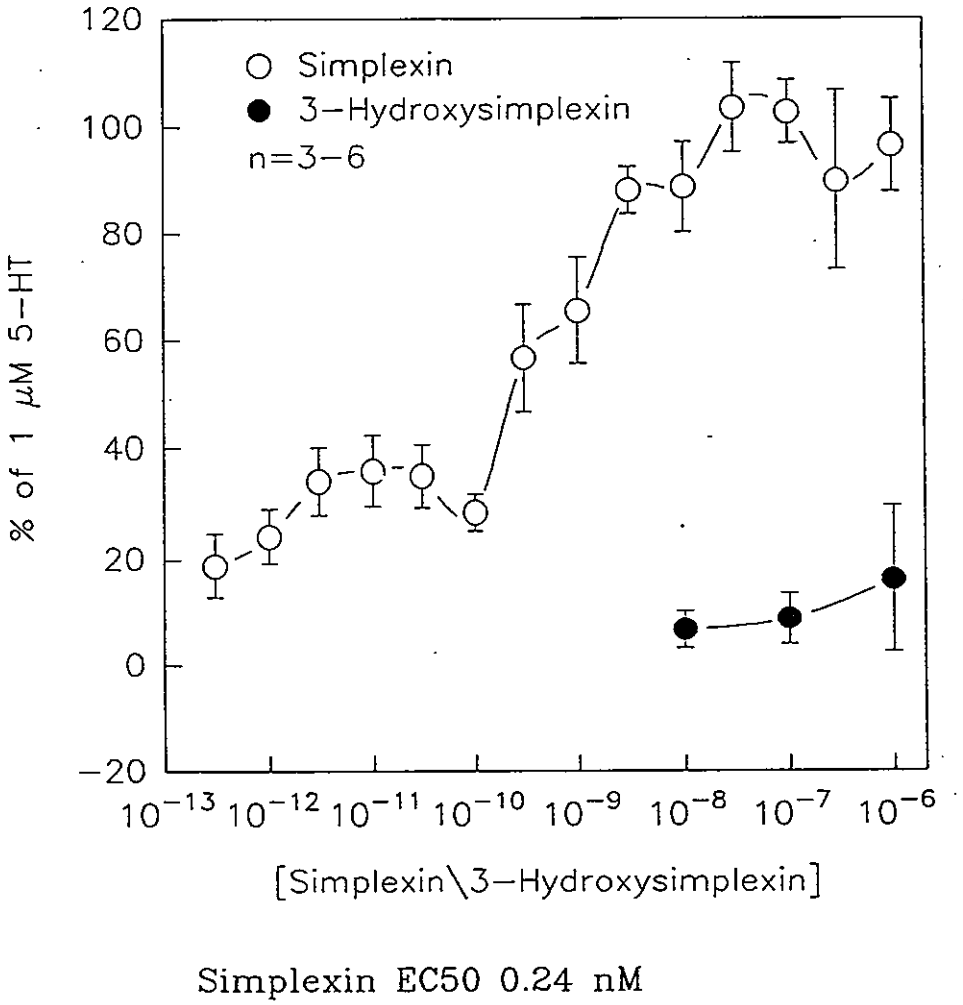


Figure 5.12 - Effective curve for simplexin and 3-hydroxysimplexin

5.3.4 Conformational Analysis of Simplexin

The structure-activity investigation of simplexin analogues conducted for this study has yielded new information regarding crucial functional groups required for the activation of PKC. When examining the structural features of simplexin, it becomes apparent that the molecule has descrete regions which could dictate its membrane, cytoplasm, and PKC interactions. It is postulated that the orthoester function provides a novel interaction with membrane lipids, as it is conceivable that the orthoester oxygens could interact with the polar portions of membrane lipids, thus providing additional binding forces which assist the PKC-active portion of simplexin and related daphnane orthoesters to bind to PKC (Figure 5.13 and Figure 5.14). Therefore, this strong membrane interaction may be one of the underlying causes of prolonged PKC activation by these compounds.

The active region of simplexin is restricted to a relatively small area spanning from the C-3 carbonyl group of the 5-membered ring moiety to the C-20 hydroxyl group of the 7-membered ring system, however the above structure-activity evidence seems to suggest that the position of the C-4 and C-5 groups are essential for bindng to PKC. The resulting activity of PKC therefore depends on the conformation adopted by the receptor which is governed by the nature and position of the oxygen at the C-3 position and possibly the at the C-20 position (Figure 5.15).

It is postulted that the structural alteration of mezerein to 3-hydroxymezerein results in different comparable bio-activity than the corresponding alteration of simplexin because the predominant lipid interaction for mezerein is through the C12 ester substituent (thus the position of the PKC active region of mezerein is in a different 3-dimensional conformation in the cell cytoplasm than simplexin and 3-hydroxysimplexin).

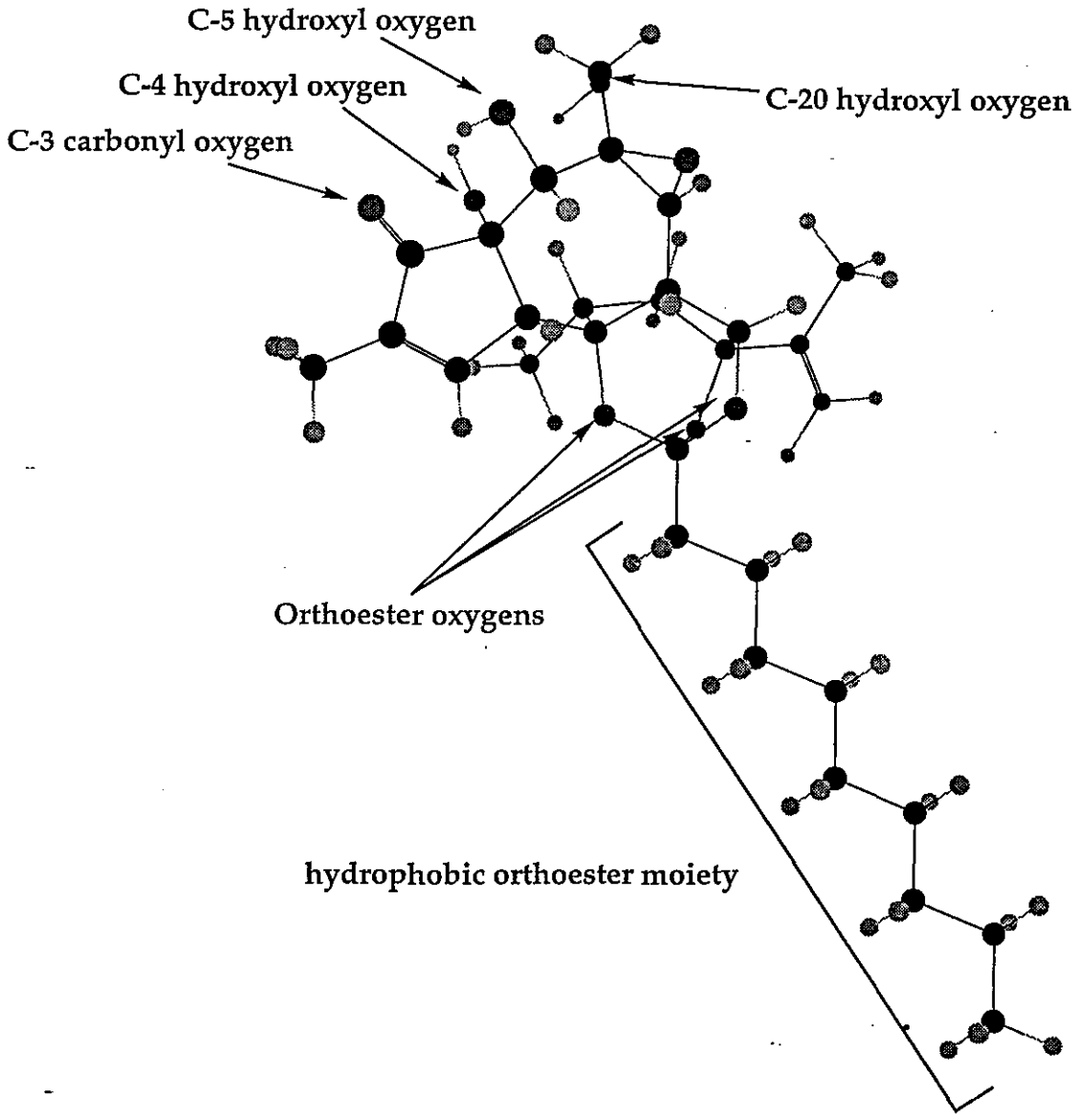


Figure 5.13 - The 3-Dimensional structure of simplexin

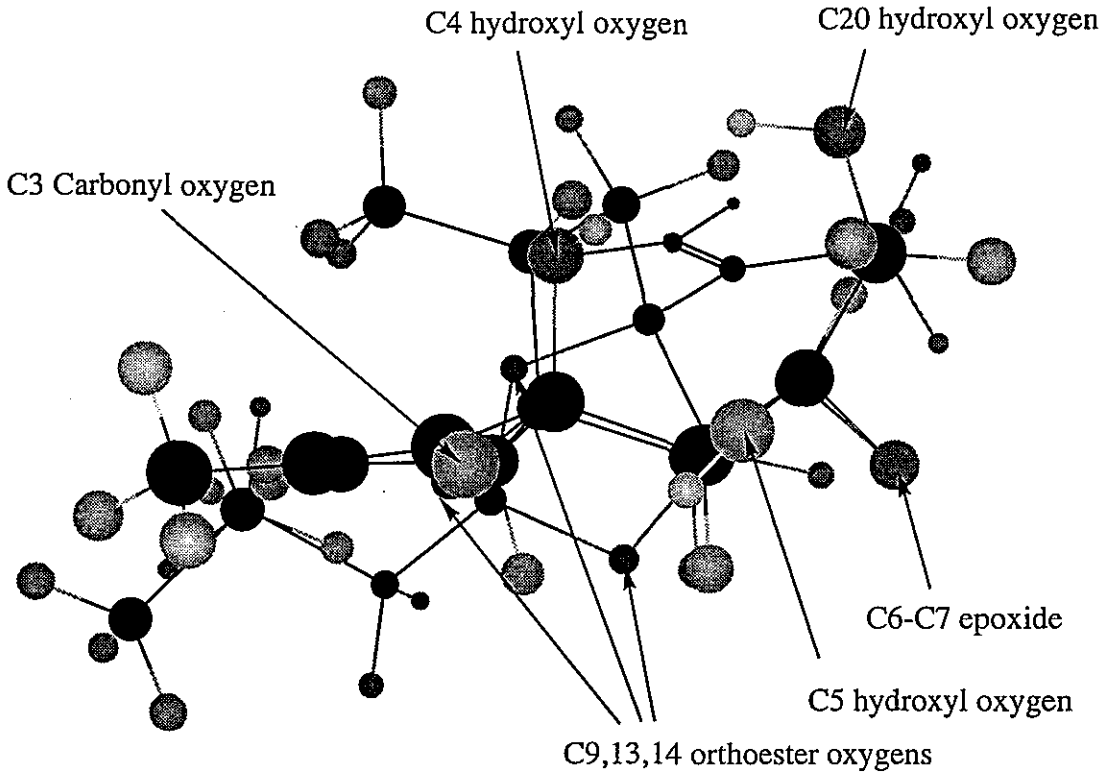


Figure 5.14 - PKC-active portion of simplexin

5.4 CONCLUSIONS

It was shown that selective reduction of the C-3 carbonyl group of the enone moiety of both mezerein and simplexin significantly reduced the *in vitro* activity of these compounds on bovine pulmonary venule preparations. The practical implications of this finding are that reductive conversion to this (or similar) inactive versions of the *Pimelea* toxins in the anaerobic rumen environment may provide a means of reducing the susceptibility of cattle to the effects of *Pimelea* poisoning. This alteration significantly reduced the PKC binding ability of mezerein and simplexin to a PKC-rich murine brain preparation (although the change was not as marked for simplexin).

It was shown by the above data that the selective reduction of the C-3 carbonyl group of simplexin resulted in a more pronounced effect on the EC_{50} concentration *in vitro* than the corresponding transformation of mezerein. The protection of the C-4, C-5, and C-20 hydroxyl groups of simplexin as 4-nitrobenzyl esters also resulted in a more pronounced change in the binding affinity compared with the corresponding 4,5,20-tri-(4-nitro)benzyl ester of mezerein. This evidence proves that daphnane orthoesters such as mezerein (and possibly the phorbol esters) may bind to the PKC receptor in more than one way in a similar manner to that demonstrated (Figure 5.6)[36]. However, limitations in this model arise when considering the C-9 oxygen of the daphnane orthoesters. The difference in observed properties of the simplexin structural analogues suggest that simplexin may only interact with the PKC receptor in one way. Hence simplexin possesses unique PKC binding and activating properties.

The IC_{50} and EC_{50} values obtained in this work demonstrated a distinct difference between PKC binding and activation, and it is postulated that the PKC isoenzyme adopts its activating (or otherwise) conformation once the activator is bound. It is has been shown in this work that the essential requirements for compounds of the daphnane orthoester type for binding to PKC are the C-4 and C-5 hydroxyl functions. The role of the C-20 hydroxyl group in PKC activation requires further analysis. The C-3 carbonyl group of simplexin is required for optimum binding, however its role appears to be more significantly related to the activating properties once bound to the PKC isoenzyme *in vitro*.

6.0 CONCLUSIONS & DIRECTIONS FOR FUTURE WORK

6.1 CONCLUSIONS

The primary aims of this study were to improve toxin yields by extraction from milled, dried *Pimelea* plant material, develop toxin-specific antibody producing conjugates for immunisation of cattle, field test an experimental vaccine, and investigate a structure-activity relationship of the daphnane orthoesters. This required the, optimisation of soxhlet extraction techniques for the extraction process, and confirmation of the structure of the daphnane orthoester toxins in extracts by NMR spectroscopy. The identification of other compounds in the extract was also achieved. As a result, it was then possible to obtain relatively large quantities (greater than 100-fold improvement over previous studies) of *Pimelea* toxin in a relatively short period of time which facilitated preparation of conjugates. Sufficient quantities of conjugate were produced for experimental and field animal trials to examine the feasibility of inducing protective immunity against *Pimelea* toxicity in cattle. This also enabled the purification of enough quantities of simplexin to conduct the structure-activity study of the daphnane orthoester toxins.

The investigation of conjugation methods for the attachment of the *Pimelea* toxins to immunogenic carrier proteins has given some insight into the factors which dictate the specific immune response in cattle. It was shown that a high toxin:protein inclusion ratio (17-fold inclusion) gave the highest toxin-specific antibody response by ELISA technique, however the ability of these purified antibodies to attenuate the effects of *Pimelea* toxins *in vitro* was less than for a conjugate of lower inclusion ratio, but higher toxin purity (13-fold, using the same toxin activation chemistry). The antibodies produced from the N-hydroxysuccinimide activated conjugate were shown not to produce a strong toxin-specific antibody response or be as efficacious at attenuating the effect of *Pimelea* toxins *in vitro*.

Vaccination of trial animals with an experimental vaccine batched as a 50:50 mixture of conjugates (attempting to exploit the high toxin-specific antibody response produced by one conjugate, with the high antibody efficacy produced by the other conjugate) gave good antibody responses both in healthy animals, and those already affected by *Pimelea* poisoning. Unfortunately, favourable seasonal conditions prevented the vaccinated animals in the Roma trial from being challenged with *Pimelea* during 1995. The Marree field trial was inconclusive, although the trend (non-significant) was that the vaccinated animals performed slightly better in condition scores and liveweight.

The investigation of a structure-activity relationship of mezerein and simplexin has also provided some new data regarding the requirements of these molecules for PKC binding and activation. The approach used in this study was to perform selective functional group manipulations on a carefully chosen reference compound, followed by the target compound. The activity of each of these derivatives was examined using a PKC radioligand binding assay, and an *in vitro* organ bath assay using bovine pulmonary venules.

Selective reduction of the C-3 carbonyl group of simplexin reduced PKC receptor affinity slightly, but caused a very marked reduction in *in vitro* efficacy. The C-4 and C-5 hydroxyl groups were shown to contribute PKC receptor binding.

6.2 DIRECTIONS FOR FUTURE WORK

The field evaluation of the experimental vaccine must be repeated under ideal conditions in order to gain a clear insight into the feasibility of immunising cattle against the effects of *Pimelea* poisoning. The ideal situation would be one where cattle where immunised with the experimental vaccine, and antibody responses were allowed to peak, while grazing non-*Pimelea* infested pastures, followed by relocation of these animals to areas containing *Pimelea* plants. If a trial under these conditions proves to be successful, the evaluation of parameters affecting toxin-specific immune responses in cattle immunised with *Pimelea* toxin-protein conjugates needs to be further investigated, and optimised. The toxin levels in the plants at the time of trial should be measured in order to also gain an effective range of the potential vaccine treatment.

With reference to the management of *Pimelea* poisoning, the toxin levels throughout the growth cycle of the plant should be determined in order to ascertain information regarding when the *Pimelea* plant is most toxic, and what soil and environmental conditions govern this factor. As a result, conditions which favour or disfavour the production of the daphnane toxins in *Pimelea* plants would be determined, and this factor will aid in the management of the condition.

High priority should be placed on the further investigation of the potential of 3-hydroxysimplexin as a selective PKC inhibitor, due to the clinical and therapeutic implications. PKC inhibitors such as rottlerin[22] and sphingosine[80] have been investigated, however the effective concentrations of these compounds is typically in the μM range, and their mode of action is typically non-specific, or specific on particular protein kinase sub-types[81]. Other approaches for PKC inhibition has been through the synthesis of substrates and substrate analogues to block the action of PKC[24].

The effect of selective hydrogenation of the 1,2-double bond on the PKC activity of simplexin should be determined (Figure 6.1). Methods for selective hydrogenation of an enone moiety have been published[82-84]. The saturation of the enone moiety should also be performed to examine its effect on PKC activity (Figure 6.1). The activity of the enantiomers produced upon the selective reduction of the C-3 carbonyl should also be performed following diastereomeric separation.

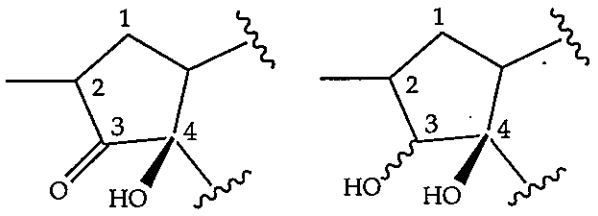


Figure 6.1 - Suggested hydrogenated analogues for future examination

Application of the knowledge gained regarding the structure-activity of simplexin and mezerein may lead to a new series of synthetic PKC active compounds. These may be developed and examined for PKC activity and include the basic structural requirements. (For example, refer to Figure 6.2)

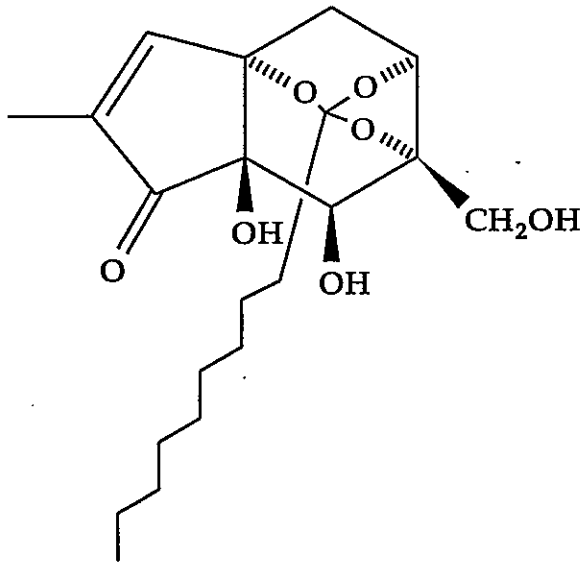


Figure 6.2 - Suggested new synthetic PKC active analogue

Furthermore, the phorbol ester prostratin (Figure 6.3) has been shown to prevent cell killing by human immunodeffiency virus (HIV)[85], however due its perceived tumour promoting properties (through PKC activation), further investigation of this compound was halted initially[16]. Prostratin was later found not to be a tumour promoter, therefore its anti-HIV properties were examined[85].

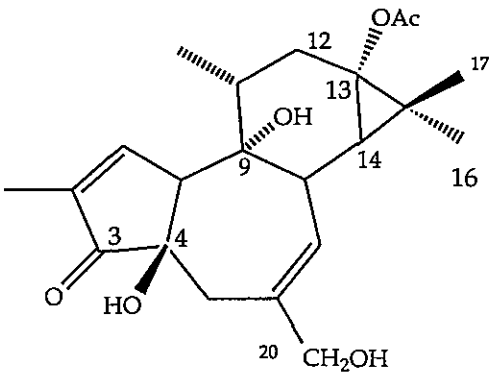


Figure 6.3 - Prostratin

The parent daphnane orthoester structure is similar to the parent tigliane structure, and prostratin was found to be non- tumour promoting. These molecules share common functional groups, therefore the anti-viral and anti-microbial properties of 3-hydroxysimplexin (non-PKC activating) and simplexin-4,5,20-tri-(4-nitro)benzoate (non-PKC binding) should be investigated.

Finally, the diminished PKC activity of 3-hydroxysimplexin indicates that conversion of simplexin to this compound by rumen micro flora could be a feasible solution to reduction of the effects of *Pimelea* poisoning of cattle. A search for anaerobic microorganisms which can perform this reduction should be given high priority in future work.

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APPENDIX C

DEVELOPMENT OF PROTEIN KINASE C ASSAY METHODOLOGIES FOR THE QUANTIFICATION OF DAPHNANE TOXINS IN *PIMELEA TRICHOSTACHYA.*

1. INTRODUCTION

1.1 *Pimelea* poisoning of cattle

Pimelea poisoning is an increasing problem to Australian cattle producers, representing an estimated loss of $14-20 million p.a. to the Australian cattle industry (Pressland and Dadswell, 1992). Areas affected by *Pimelea* poisoning include: (i) Maranoa district in southern Queensland; (ii) Marree district in northern South Australia; (iii) Cobar district of northern New South Wales; and (iv) southern Northern Territory. Economic losses are incurred from cattle deaths by ingestion of *Pimelea* plants or inhalation of the dried plant material, and also from costs associated with treatment of survivors, reduced pasture area due to *Pimelea* infestation, and the costs of controlling the infestation.

Management of *Pimelea* poisoning is difficult. It occurs in extensive environments, making physical or chemical control of the plant impractical. Since *Pimelea* is native to Australia, there are no apparent natural biological control agents. Some producers have experimented with burning affected areas; however experiments studying the effects of burning have given inconclusive results. A study carried out by the Queensland Department of Primary Industries (QDPI) examining the effects of burning on *Pimelea* infestations, showed that two properties out of the five studied had an increased amount of *Pimelea* plants after burning, while one showed no effect, and two showed a decrease in the *Pimelea* population (Pressland and Dadswell, 1992). The plants are hard-seeded, and hence the seeds possibly remain viable in the soil for long periods.

Pimelea spp. are low-growing herbaceous annuals of the family Euphorbiaceae (Figure 1.1). Three species in particular have been reported to cause *Pimelea* poisoning in cattle (Pressland and Dadswell, 1992):

(i) *Pimelea simplex* which comprises two sub-species: *simplex* and *continua;* these are commonly named the desert rice flower;
(ii) *Pimelea trichostachya*, commonly known as the flax or poverty weed; and
(iii) *Pimelea elongata.*

Pimelea plants are native to Australia and tend to grow best in light, sandy, relatively infertile soils. The QDPI has shown that *Pimelea* germination occurs with day temperatures of 20-25°C and night temperatures of 15-20°C. Outbreaks tend to occur in the spring-early summer (August-December), generally when a dry summer is followed by early, light winter rains (Pressland and Dadswell, 1992).

The first incidence of *Pimelea* poisoning was recorded in 1938 at St. George in south-western Queensland; hence *Pimelea* poisoning is often termed St. George disease. It is also commonly known as "Marree disease", "Bighead Disease" and "Flax Weed Poisoning". More recent reports have documented *Pimelea* poisoning in the Roma and Maranoa areas of south-western Queensland, and the more central regions of Charleville, Cunnamulla, Quilpie, Thargomindah and Mitchell. Severe incidences of *Pimelea* poisoning have also been recorded in the Marree area of South Australia (Table 1.1).

The first incidence in South Australia was reported in 1958. In 1960 in South Australia, there were significant losses of introduced cattle, and major losses occurred again in 1980.

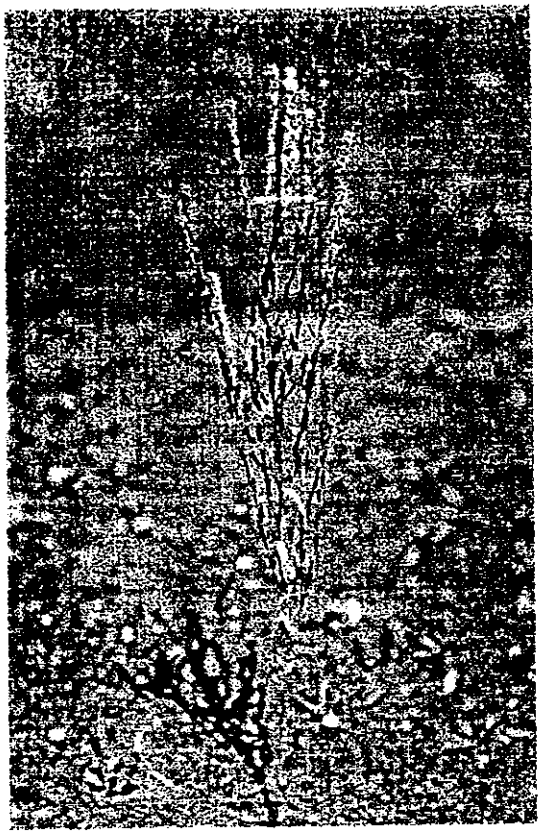
Severe poisoning was reported in the Marree area in 1990, with one property alone losing almost 600 head of cattle, with an additional 1500 animals diagnosed with St. George disease. A further outbreak has occurred in 1995. On one property, there have already been well over 200 cattle deaths, with at least 200 sick animals (Table 1.1).

Table 1.1: History of *Pimelea* Poisoning in the Marree Area of South Australia. (adapted from: J. Cawthorn, pers. comm.)

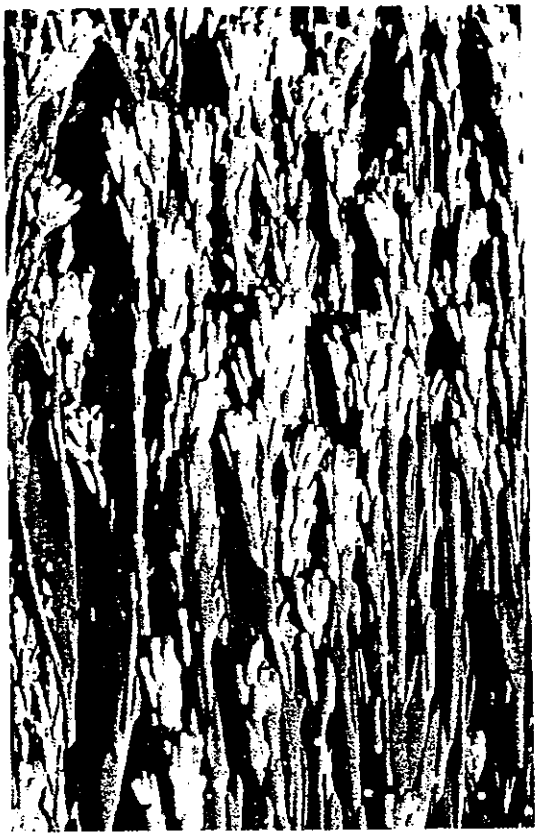
1980	STATION	SICK ANIMALS	DEATHS
	Finnis Springs	30	40
	Clayton	88	7
	Mundowdna	300	200
	Muloorina	100	50
	Dulkaninna	40	40
	TOTAL	558	337

1990	STATION	SICK ANIMALS	DEATHS
	Mulka	60	100
	Moolawatana	50	50
	Murnpeowie	1500	597
	Mundowdna/ Wilpoorina	200	110
	Muloorina	60	30
	Dulkaninna	300	300
	Etadunna	20	2
	Mungeranie	30	30
	TOTAL	2220	1219

1995	STATION	SICK ANIMALS	DEATHS
	Mulka	10	-
	Moolawatana	2	-
	Murnpeowie	200	100
	Mundowdna/ Wilpoorina	200	200
	Muloorina	1	-
	Clayton	100	30
	Dulkaninna	100	90
	Etadunna	60	40
	TOTAL	673	460



Pimelea elongata (whole plant)



Pimelea elongata (seed heads)

Pimelea trichostachya (whole plant)

Pimelea trichostachya (seed heads)

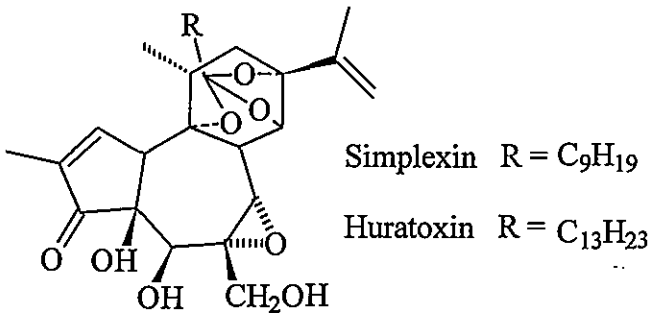
Figure 1.1: *Pimelea* species.

1.2 *Pimelea* toxins

The clinical effects of *Pimelea* poisoning in cattle are directly attributable to the biological actions of diterpenoid toxins present in the plants. The major toxic component of *Pimelea simplex* was isolated and characterised by Freeman and co-workers in 1975 (Freeman *et al.*, 1975). This compound was named simplexin and it has structural similarities to huratoxin, a piscicidal constituent of *Hura crepitans* from the Euphorbiaceae family (Figure 1.2) (Freeman *et al.*, 1975). Simplexin has an LD_{50} in mice of 1mg/kg. Simplexin is a potent skin irritant and is moderately active as a co-carcinogen (Freeman *et al.*, 1975). Freeman *et al.* (1975) describes one experiment in which 9mg of simplexin injected intravenously into a calf (100kg), caused death within 30 minutes.

Freeman *et al.* (1979) reported that the family Euphorbiaceae is well known for containing toxic, irritant and co-carcinogenic diterpene esters of the tigliane, daphnane and ingenane types (Figure 1.3). Mezerein, a toxic and irritant substance isolated from *Daphne mezereum* (family Thymelaceae), is a daphnane toxin that is structurally similar to simplexin and huratoxins, also daphnanes (Figure 1.4). Sub-toxin B is a tigliane ester (Figure 1.2).

The *Pimelea* diterpenoid toxins are similar in structure to the phorbol esters (Figure 1.4), which are known co-carcinogens that activate various isoenzymes of protein kinase C (PKC) (Brasseur *et al.*, 1985). The lipophilic *Pimelea* toxins are also believed to bind to PKC. The toxins are believed to be slowly metabolised and as a result they cause prolonged activation of PKC, which in turn leads to adverse pathological responses in target tissues.



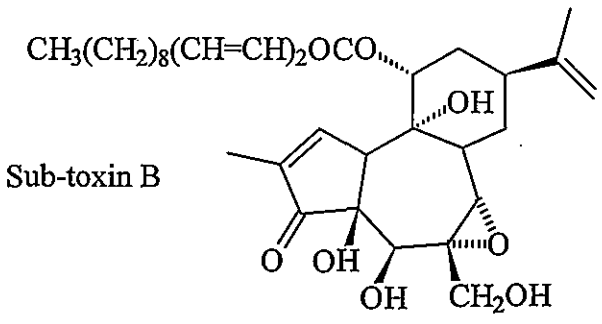


Figure 1.2: *Pimelea* toxins

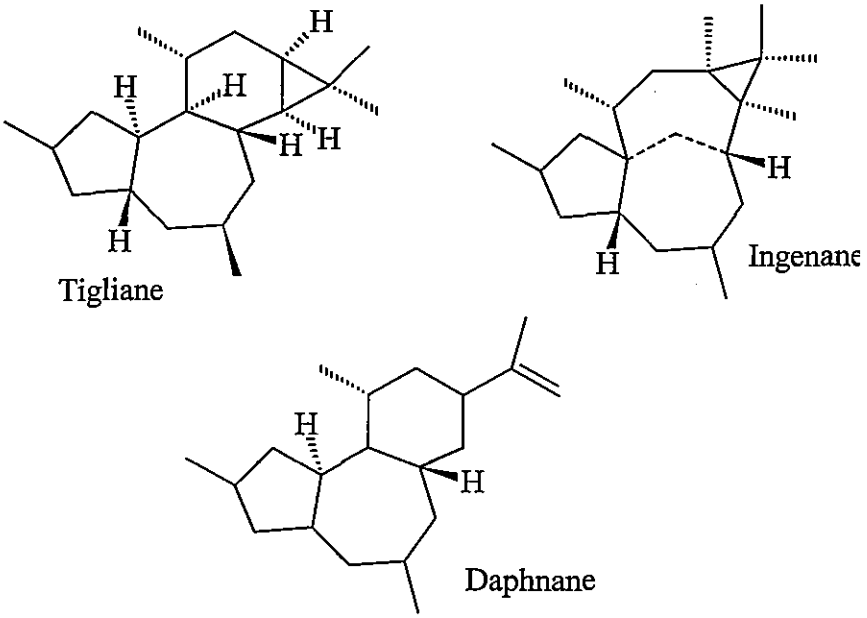


Figure 1.3: Parent structures of the diterpenoid toxins from Euphorbiaceae and Thylmelaceae.

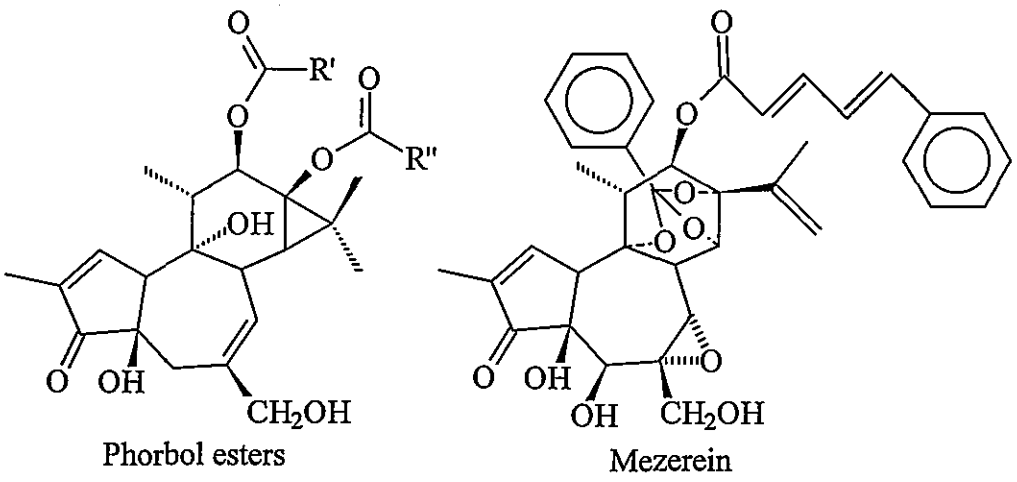


Figure 1.4 Tumour promoting phorbol and daphnane esters.

1.3 Physiological symptoms of *Pimelea* poisoning

Alexander and Jensen (1963) reported that the bovine pulmonary vasculature is unusual when compared with analogous tissues in horses and sheep, in that both the arteries and veins of cattle have a heavy muscular coat, with distinct disruptions in continuity in vessels of 300-400μm or less in diameter. These disruptions were classified as sphincter-like structures (Alexander and Jensen, 1963). Constriction of these sphincters, induced by *Pimelea* toxicity, leads to an increase in the pressure of the pulmonary capillary bed, the

pulmonary arterial system and the right ventricle, accompanied by variable pulmonary oedema (Alexander and Jensen, 1963). If constriction continues, dilation of the right ventricle causes the right atria ventricular valve to close incompletely, allowing regurgitation during ventricular systole (Alexander and Jensen, 1963). This leads to distension and pulsation of the jugular veins as a result of the increase in systemic venous pressure, followed by oedema, right-sided heart failure and eventually death (Figure 1.5). In most field cases, the animals show signs of wasting, there is a roughening of the coat, and they have an awkward stance, giving the impression of thoracic or abdominal pain (Pressland and Dadswell, 1992).

These distinct muscular sphincters present in cattle, are absent in horses and sheep (Alexander and Jensen, 1963) and the latter species can graze areas where *P. trichostachya* grows, without developing dependent oedema.

Clark (1973) studied the pathogenesis of the disease, by drenching cattle daily with dried, milled *Pimelea* plant material. Animals receiving more than 50mg/kg body weight/day developed jugular distension, diarrhoea and a mucous discharge from the eyes and nostrils within three days. Continuation at this dosage led to oedema of the neck and brisket, usually followed by death within seven days.

The extent and occurrence of diarrhoea depends on whether inhalation or ingestion of the plant predominates. Clark (1973) reported an inhalation experiment in which animals did not ingest *Pimelea* plant material. These animals developed oedema without diarrhoea. Clark (1973) also showed that these results could be achieved by intravenous injection of a crude extract of the plant. These findings suggest that diarrhoea (common in field cases) results from ingestion of plant material.

Figure 1.5 Cow showing clinical signs of *Pimelea* poisoning: severe oedema of the head, neck and brisket, and distinct jugular distension.

1.4 Protein kinase C (PKC)

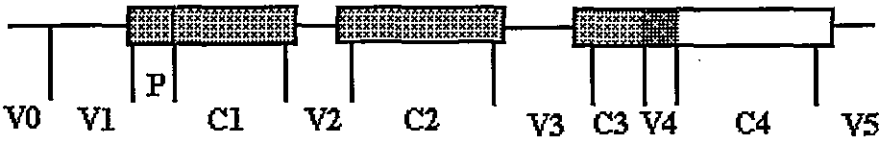
1.4.1 *Structure and properties of PKC*

The *Pimelea* toxins are thought to cause constriction of bovine pulmonary venules through activation of various isoenzymes of protein kinase C (PKC).

Protein phosphorylation plays a key role in regulating cellular functions. The kinase and phosphatase enzymes that are responsible for regulating protein phosphorylation and dephosphorylation are targets for various growth factors, hormones, neurotransmitters and other extrinsic agents (Parker *et al.* 1986).

Protein kinase C (Figure 1.6) represents a family of at least 11 isoenzymes that exhibit phospholipid-dependent protein-serine/threonine kinase activity (Nishizuka, 1992; Hug and Sarre, 1993). The enzyme plays a critical regulatory role in many important biological processes, including development, memory, proliferation and carcinogenesis (Lester and Epand, 1992). Protein kinase C (MW 77 000 g/mol), identified in 1977 by Nishizuka and co-workers, was initially described in rat liver, as a protease-activated protein kinase which phosphorylated histone H1.

PKC is comprised of two functionally different domains: a regulatory domain (amino terminal) and a catalytic or protein kinase domain (carboxyl terminal).



C1-4 conserved region
V0-5 variable region
P pseudosubstrate site

Figure 1.6 Structure of PKC (adapted from: Lester and Epand, 1992)

In general, the PKC isoenzymes can be divided into three classes (Nishizuka, 1992):

(i) Conventional PKC (cPKC): require both Ca^{2+} and diacylglycerol (DAG) for maximal kinase activity. These include the α, $\beta 1$, $\beta 2$ and γ isoforms.
(ii) Novel PKC (nPKC): require DAG, but are Ca^{2+}-independent. These include the δ, ε, (ε'), η, θ and possibly μ isoforms.
(iii) Atypical PKC (aPKC): require neither Ca^{2+} nor DAG for activity. These include the ζ and ι (known as λ in the murine system) isoforms.

The regulatory domains of all PKC isoenzymes are composed of a pseudosubstrate motif (Figure 1.6) and a tandem repeat of a cysteine-rich sequence (Gschwendt *et al.*, 1991). The pseudosubstrate motifs are polypeptide sequences that resemble a substrate phosphorylation site which interacts with the active site, rendering the enzyme inactive.

Binding of the natural activator of PKC, 1,2*sn*-diacylglycerol (DAG - Figure 1.7), causes a conformational change so that the pseudosubstrate motif no longer binds to the catalytic domain of PKC. Binding of calcium to a region located between the cysteine-rich region and the ATP-binding site of the conventional isoenzymes is believed to accentuate the conformational change induced by DAG. Isoforms which lack the calcium binding site (C2 in Figure 1.6) are probably activated by DAG alone.

It has been shown that the cysteine-rich sequences of PKC play a key role in the binding of DAG, phorbol esters and other structurally related compounds to the enzyme (Figure 1.8 - Gschwendt *et* al., 1991). In Figure 1.8, the phorbol esters are shown to bind to PKC in two possible conformations, b and c. The binding shown in c permits an additional hydrogen bond and thus may be a more favourable binding conformation. Phorbol esters, and presumably the *Pimelea* toxins mimic the actions of DAG due to a number of structural similarities, particularly the ester bonds. However, modifications of DAG and phorbol esters, or analogues synthesized on the basis of pharmacophores predicted from molecular modeling, have often yielded compounds devoid of most or all biological activity (Leli *et al.*, 1990). This suggests that a particular stereochemistry is essential for binding.

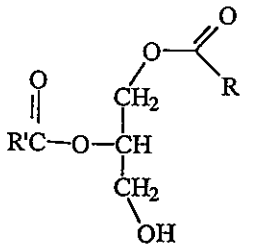


Figure 1.7: 1,2*sn*-diacylglycerol
(adapted from: Bohinski, 1987)

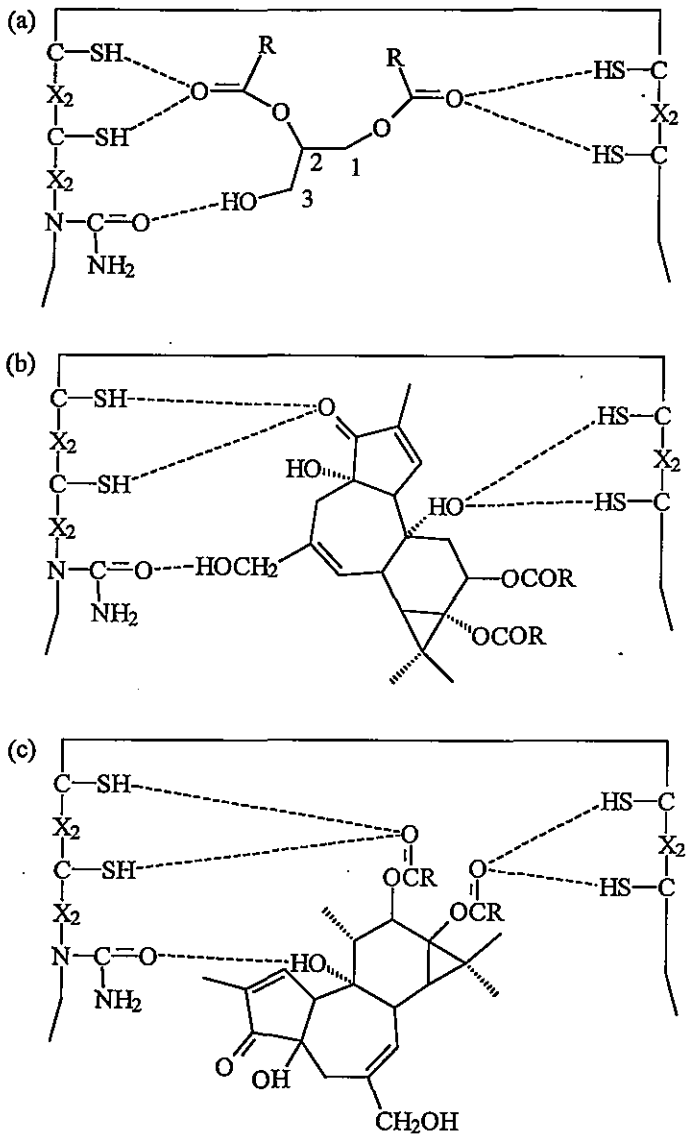


Figure 1.8 Proposed binding of (a) DAG and (b,c) phorbol esters to PKC (adapted from: Gschwendt *et al.*, 1991)

1.4.2 *Activation of PKC*

Under resting conditions, PKC appears to be loosely bound to membranes, and it is likely that the enzyme is dissociated from the membranes by the removal of calcium (Kikkawa and Nishizuka, 1986). PKC is then transiently activated by DAG, which is produced via an agonist-induced path involving the phospholipase C hydrolysis of inositol phospholipids. In the membrane, inositol phospholipids are broken down to phosphatidylinositol-4-phosphate (PIP) and phosphatidylinositol-4,5-biphosphate (PIP_2) by stimulation of α_1-adrenoceptors. PIP_2 is then further hydrolysed to inositol-1,4,5-triphosphate (IP_3) and 1,2*sn*-diacylglycerol (DAG) which is the only diacylglycerol that will activate PKC (Kikkawa and Nishizuka, 1986). Other stereo- and regio-isomers are

inactive, which suggests a highly specific lipid-protein interaction is needed for PKC activation by DAG (Kikkawa and Nishizuka, 1986). IP_3 serves as a mediator of Ca^{2+}-mobilisation from internal stores.

Free DAG is virtually absent from membranes under normal conditions. As soon as it is formed and activates PKC, it is either phosphorylated by DAG-kinase to phosphatidic acid, or it is hydrolysed by DAG-lipase to monoacylglycerol which is further hydrolysed to release arachidonic acid (Berridge, 1987). Arachidonic acids are the precursors to prostaglandins, thromboxanes and leukotrienes (Berridge, 1987). Hence, when cells are stimulated under normal conditions, PKC is only transiently activated by DAG, which has a half-life of 60 seconds. Active PKC then phosphorylates a wide range of cellular proteins for various physiological functions.

Tumour-promoting phorbol esters and the *Pimelea* toxins, are likely to cause prolonged activation of PKC since phorbol esters have a half-life of around 23 hours. This results in abnormal activation of various cellular functions controlled by PKC. Fish *et al.* (1988) have reported that phorbol esters cause a slowly developing contraction and an associated transmembrane calcium flux, in vascular smooth muscle. Phorbol myristate acetate (a phorbol ester) has been shown to cause severe lung injury (Kuroda *et al.*, 1988) closely associated with toxic oxidants released from activated granulocytes.

1.5 PKC radioligand binding assays

Mezerein has effects similar to those of the phorbol esters (Sastrodihardjo *et al.*, 1987) and has been shown to activate PKC both *in vitro* and *in vivo* (Miyake *et al.*, 1984). A PKC radioligand binding assay was used with rat brain as the PKC-rich tissue and [^{3}H]-phorbol-12,13-dibutyrate (PDBu) as the radiolabel (Miyake *et al.*, 1984). Mezerein was found to bind to PKC when added directly to the reaction mixture. However, phospholipid was essential for activation, as mezerein alone showed no effect (Miyake *et al.*, 1984). In another experiment, mezerein was also shown to activate PKC in intact cells, using human platelets labeled with ^{32}Pi (Miyake *et al.*, 1984).

Brasseur *et al.* (1985) showed, using calculations of physical constants, that the orientation of the CH_2OH group at the C3 position of DAG is similar to that of the same group as the C20 position of the phorbol esters (Figure 1.9). Using computer-assisted molecular modeling of the spatial relationships of various functional groups of tumour promoters and PKC activators, Jeffrey and Liskamp (1986) concluded that the oxygens at C3, C4, C9, and C20 of phorbol esters are important for binding to PKC.

It has been shown previously that a [^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]-PDBu) binding assay can be used to detect phorbol esters in crude extracts of Euphorbiaceae plants in a sensitive and specific manner (Beutler *et al.*, from Dr de Vries, pers. comm.). In a further study, a similar PKC [^{3}H]-PDBu binding assay was used to show that bryostatins have a sub-nanomolar affinity for the "phorbol ester receptor" in rat brain (de Vries *et al.*, 1988).

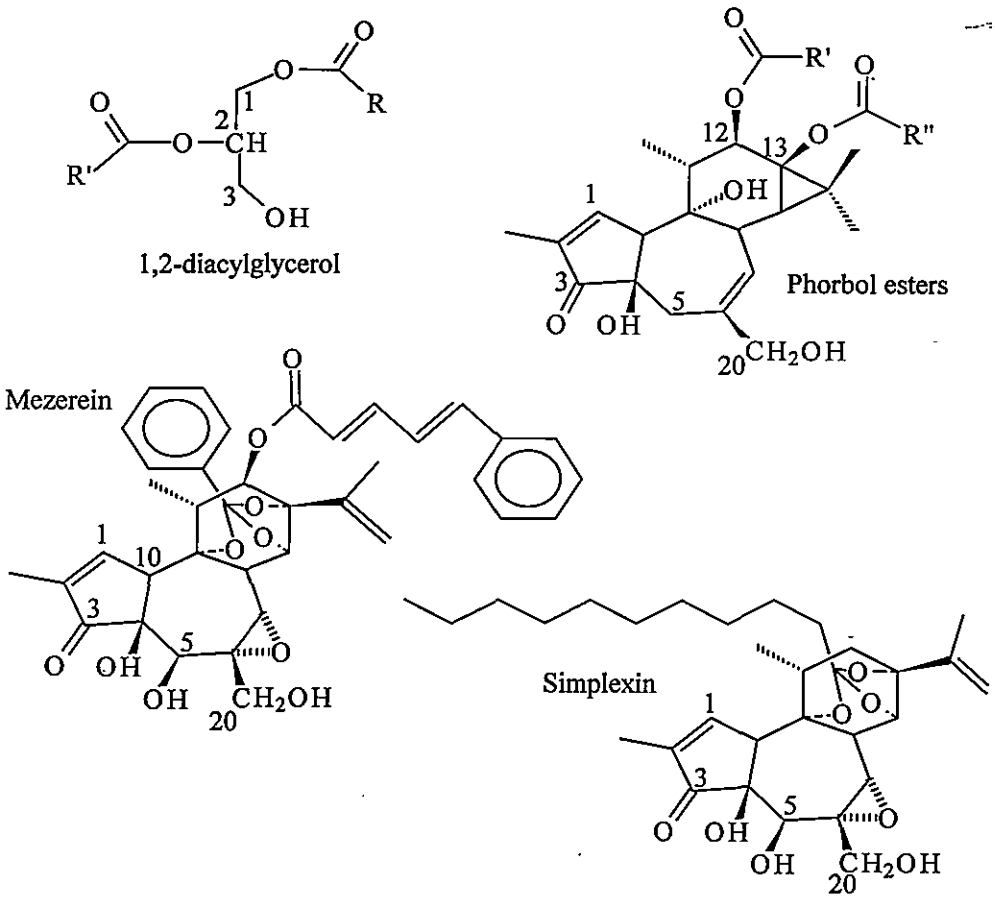


Figure 1.9 Structural analogies between DAG, phorbol esters, mezerein, and simplexin.

1.6 Objectives of the current study

The major aim of this study was to establish a reliable PKC radioligand binding assay and to then use this methodology to quantify daphnane toxins in *Pimelea* plants. The plants are reported to contain less than 0.005% simplexin (Freeman *et al.*, 1975). The sensitivity of direct high performance liquid chromatography (HPLC) UV detection of these levels is low, and these relatively non-volatile compounds are also incompatible with gas-liquid chromatography (GLC) analysis. A PKC radioligand binding assay would overcome these problems, as it has the advantages of being a sensitive, efficient, robust and reproducible technique.

To the author's knowledge, there are no reports on the distribution of toxins in *Pimelea* plants, or changes in toxin concentrations during the development and growth of the plants. A second major aim of the present study then was to use the PKC radioligand binding assay methodology to carry out a preliminary characterisation of the distribution of toxins in *Pimelea trichostachya.*

The third aim of this project was to investigate the viability of a commercially available colorimetric PKC assay kit of the SpinZyme format (Pierce) for the detection of daphnane toxin activity in *Pimelea* extracts. The kit is a non-radioactive kit which utilises a dye-labelled substrate. Using this kit, PKC catalyses the transfer of phosphate groups to the substrate. Phosphorylated peptide is separated from non-phosphorylated peptide using the specific affinity membranes supplied with the kit. Measurement of phosphorylated product is then achieved using absorbance or fluorescence spectrophotometry, and the absorbances are directly proportional to the amount of phosphorylated product, and hence the level of PKC activity.

2.0 A COMPETITIVE PKC RADIOLIGAND BINDING ASSAY FOR THE QUANTITATION OF *PIMELEA* TOXINS

2.1 Aims

The aims of the work described in this section were:

(i) to isolate and purify toxins from *Pimelea* plants;
(ii) to develop and validate a competitive PKC radioligand binding assay; and
(iii) to use crude plant extracts and purified toxins as competitors in the PKC radioligand binding assay to quantify toxin levels in plants.

2.2 Introduction

Previous reports have described the isolation of daphnane and tigliane orthoesters from *Pimelea* species (Freeman *et al.*, 1975; Tyler and Howden, 1985). Briefly, dried milled *Pimelea simplex* plants were extracted at least five times in ethanol. The crude extracts were combined and evaporated to a "thick, dark gum". The water-soluble, non-toxic component was removed by washing with distilled water. The water-insoluble toxic residue was passed through a Sephadex LH 20 column. The separate fractions were analysed for toxicity using guinea pig and mouse bioassays. Simplexin was purified from the toxic fractions by thin layer chromatography

Recently, the extraction and isolation procedures for *Pimelea* toxins were optimised using column chromatography and analytical and preparative HPLC (Nayyar, 1994; B. Lambert, unpublished). The latter procedures were used in the present study.

As described in Chapter 1, a [^{3}H]-phorbol-12,13-dibutyrate ([^{3}H]-PDBu) binding assay has been used previously to detect phorbol bioactivity in crude extracts of Euphorbiaceae plants (Beutler *et al.*, from pers. comm. D. de Vries, 1994). Also, this methodology has been adapted to demonstrate that bryostatins have a subnanomolar affinity for the "phorbol ester receptor" in rat brain (de Vries *et al.*, 1988). The PKC radioligand binding assay used in this project was developed from the methodologies described in these two papers. Basically, the competitive radioligand binding assay involves saturating a PKC-rich rat brain preparation with [^{3}H]-PDBu. Subsequent additions of graded doses of competing agent (ie. the toxin) displaces the [^{3}H]-PDBu from the receptor. The remaining membrane-bound [^{3}H]-PDBu can then be collected by vacuum filtration through glass fibre filters and measured by liquid scintillation counting.

2.3 Materials and Methods

2.3.1 *Extraction, isolation and purification of toxins*

Dried, milled *Pimelea trichostachya* plants were soxhlet extracted in methanol for 48 hours (Figure 2.1). The solvent was removed by rotary evaporation and the resulting dark green, thick, tarry residue was partitioned between chloroform and water in a separatory funnel. Using a fish bioassay, Nayyar (1994) found that the toxins were contained in the organic layer.

The combined chloroform extracts were evaporated to dryness using a rotary evaporator. The resulting crude extract was dissolved in a minimum volume of ethyl acetate, and applied to a 95 mm (id) flash chromatography column (Still *et al.*, 1978). The column was packed with silica gel (Merck Kieselgel G type 60) and eluted by the application of compressed air with two litres of each of the following solvent compositions: (i) 90:10 hexane:ethyl acetate; (ii) 75:25 hexane:ethyl acetate; (iii) 50:50 hexane:ethyl acetate; and (iv) 0:100 hexane:ethyl acetate.

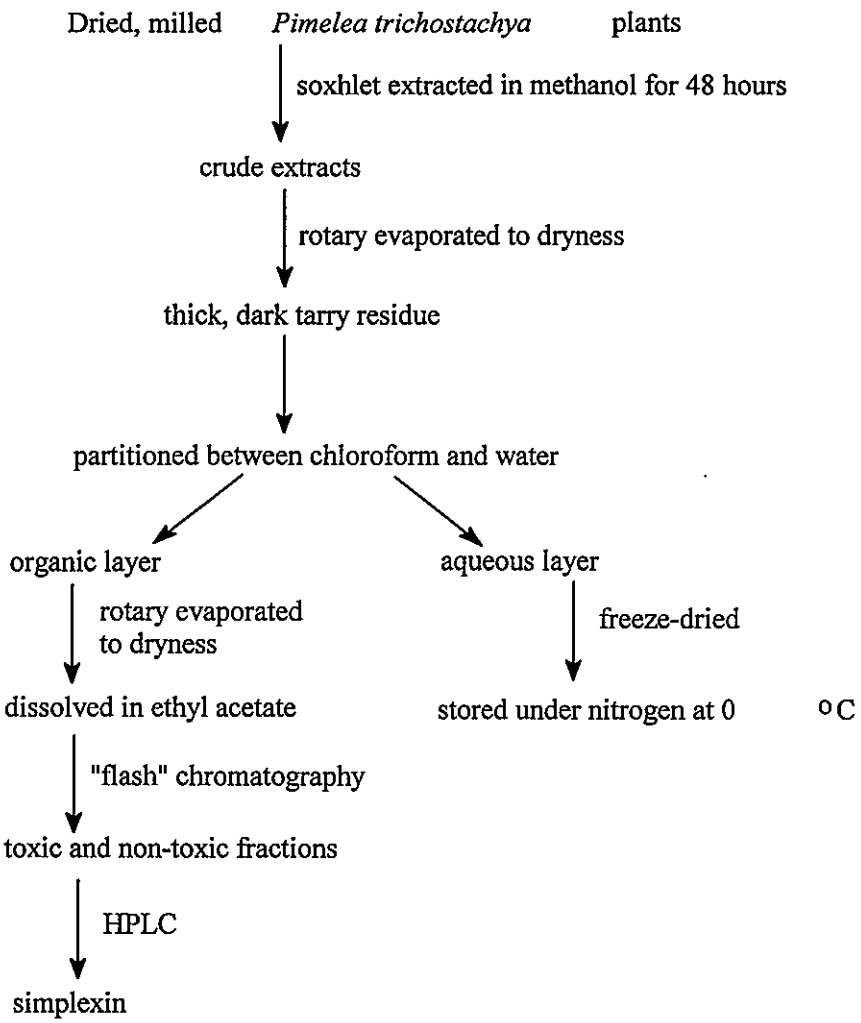


Figure 2.1 Extraction and isolation of simplexin
(adapted from: Nayyar, 1994; Lambert, unpublished)

The eluates were collected as four fractions, which have previously been labelled Fractions 1, 2, A, and B. These fractions were subjected to rotary evaporation and the residues stored under nitrogen at -20°C.

Samples of each fraction (2mg/mL in methanol) were analysed using reverse phase HPLC, using an optimised methanol/water gradient on a Waters Nova-Pak C18 cartridge column (3.9 x 150mm) with photodiode array detection at 240nm (Table 2.1). The HPLC system used was the Waters-Millenium 2010 Chromatography Manager, with a Waters 600 pump and controller and a Waters 996 PDA Detector (10mm flow cell). HPLC-grade methanol, filtered through a 0.45μm filter, and Millipore-filtered water were used for all separations. Solvents were degassed before use and then intermittently sparged with helium gas (10mL/min) throughout the analyses. For preparative separations, a Waters RCM Millipore column (25 x 10mm) was used with gradient elution (Table 2.2).

Table 2.1: Gradient elution for analytical separation.

#	Time (min)	Flow (mL/min)	% methanol	% water
1	0	1	60	40
2	13	1	65	35
3	21	1	70	30
4	29	1	75	25
5	45	1	85	15
6	53	1	90	10
7	90	1	90	10
8	100	1	60	40
9	120	1	60	40

Table 2.2: Gradient elution for preparative separation.

#	Time (min)	Flow (mL/min)	% methanol	% water
1	0	5	60	40
2	3	5	65	35
3	7	5	70	30
4	10	5	75	25
5	13	5	80	20
6	19	5	85	15
7	23	5	90	10
8	70	5	90	10
9	80	5	60	40

2.3.2 *PKC radioligand binding assay*

Rat heads (female Wistar rats) were snap-frozen in liquid nitrogen and dissected longitudinally to expose the brain. The brains (10.65g) were removed while solid and homogenised for two minutes in 10 volumes of homogenisation buffer (10mM Tris-Cl, pH 7.4 at 25°C) using an Ultra-Turrax T25 at 11 500 rpm.

The homogenate was centrifuged at 4°C for 10 minutes at 14 000 rpm on a Sorvall RC5C centrifuge using an SS34 rotor. The supernatant was discarded and the pellet resuspended (in homogenisation buffer) and recentrifuged three times. The final pellet was resuspended in homogenisation buffer and stored in aliquots at -70°C.

[20(n)-^{3}H]-PDBu (specific activity 599 Gbq mmol^{-1}, Amersham Life Sciences) was used at a constant concentration of 20 000 dpm/100µL in the assay. The total assay volume was 1mL.

Incubations were initiated by the addition of 100µL of rat brain preparation to triplicate tubes containing 100µL of [^{3}H]-PDBu and 50µL of graded doses of competing agent (unlabelled PDBu, mezerein, Fraction B, or simplexin) and binding buffer (50mM Tris-Cl, pH 7.4 at 25°C, 2mg/mL bovine serum albumin (BSA)). Incubations were carried out at 30°C for 120 minutes in a shaking water bath, and terminated by vacuum filtration through glass fibre filters (Whatman GF-C) pre-wetted with 2% polyethylenimine, which is used to assist retention of protein on the filter disk. The disks were then washed with 4mL of cold binding buffer and placed in scintillation vials. Optiphase 'HiSafe2' scintillation mixture (4mL) was added to each vial and bound radioactivity was measured by liquid scintillation counting, using an LKB Wallac counter.

It was found that the filter disks had to be leached overnight in the scintillant to obtain reproducible results. Initially, the bound radioactivity was measured immediately after the addition of scintillant, and the counts obtained were much less than expected. After leaching the filter disks in the scintillant overnight, consistent results were obtained.

The data obtained by liquid scintillation counting were represented as B/B_0 values: B is the bound radioactivity (in cpm) less the non-specific binding mean; B_0 is the total [^{3}H]-PDBu bound to the PKC in the absence of labeled competing agents, less the non-specific binding mean. Non-specific binding is a measure of the amount of [^{3}H]-PDBu that binds to the filter disks in the absence of rat brain preparation. Hence, the B/B_0 value represents the [^{3}H]-PDBu remaining bound to PKC after displacement with a particular concentration of competitor as a percentage of the total [^{3}H]-PDBu bound to PKC.

2.4 Results

2.4.1 *Extraction and isolation of toxins*

Initially, 740g of *Pimelea trichostachya* was soxhlet extracted to yield 128g of dark green, gummy extract. After the chloroform/water partition, 22g of crude extract was obtained and applied to the flash chromatography column. The masses of each of the four fractions collected are given in Table 2.3.

Table 2.3: Masses of Flash Chromatography Fractions

Fraction	Solvent Composition (Hexane : Ethyl acetate)	Mass Obtained (grams)
1	90 : 10	4.03
2	75 : 25	4.05
A	50 : 50	3.62
B	0 : 100	3.29

Each of the fractions were analysed by analytical HPLC as described earlier. It had been shown previously (Nayyar, 1994; B.Lambert, unpublished), that under the conditions outlined in Table 2.1, simplexin and similar *Pimelea* toxins have a retention time of 50 - 60 minutes, with characteristic UV absorption spectra. The chromatogram obtained for Fraction 1 is shown in Figure 2.2. The peaks have very low absorbances (< 0.005 units) with no major peaks between 50 and 60 minutes. The corresponding UV spectra of these peaks displayed none of the characteristic diterpenoid absorbances. As expected from previous work, Fraction 2 was also devoid of any of the characteristic toxin peaks (Figure 2.3).

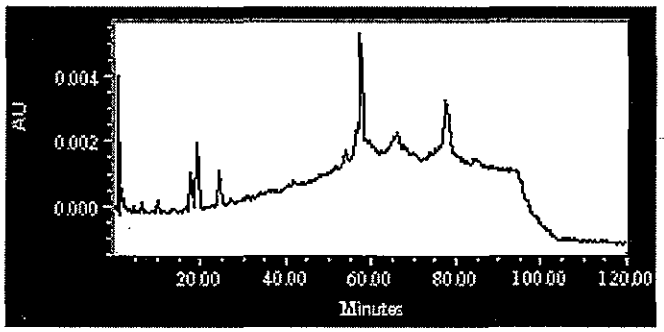


Figure 2.2: HPLC Chromatogram of Fraction 1.

Fraction A does contain a very small amount of toxins (Figure 2.4). The peaks between 50 and 60 minutes have very small absorbances, but the UV spectra are identical to those obtained for the *Pimelea* diterpenoids in previous studies (Nayyar, 1994; B. Lambert, unpublished).

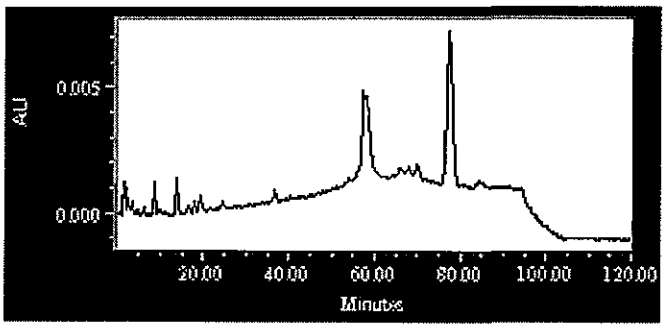


Figure 2.3: HPLC Chromatogram of Fraction 2.

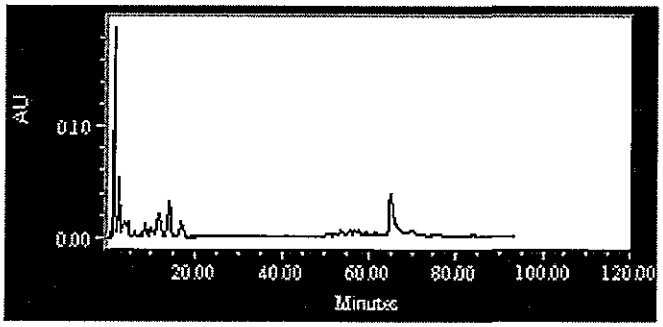


Figure 2.4: HPLC Chromatogram of Fraction A.

The peaks at 54 and 56 minutes have been identified in previous studies by proton nuclear magnetic resonance (nmr) studies as simplexin and huratoxin, respectively (B. Lambert, unpublished). The UV spectra obtained for these peaks suggest that other plant pigments may be co-eluting with the toxin peaks (Figure 2.5). When separated using preparative HPLC, the compounds are green in colour, confirming that pigments have co-eluted with the toxins on HPLC (Lambert, unpublished). These pigments have such high molar absorptivity co-efficients, that only a minute amount of co-eluting pigment is required for the UV spectra of the toxins to be obfuscated.

Fraction B, eluted from the flash chromatography column with 100% ethyl acetate, contained most of the toxins. This was expected, since the toxins are quite polar and likely to be eluted with a more polar solvent. The chromatogram (Figure 2.6) has characteristic peaks between 50 and 60 minutes, and UV absorbances of almost 0.5 units. Fraction B also has the unique UV spectra (Figure 2.7), with the toxin peaks co-eluting with plant pigments

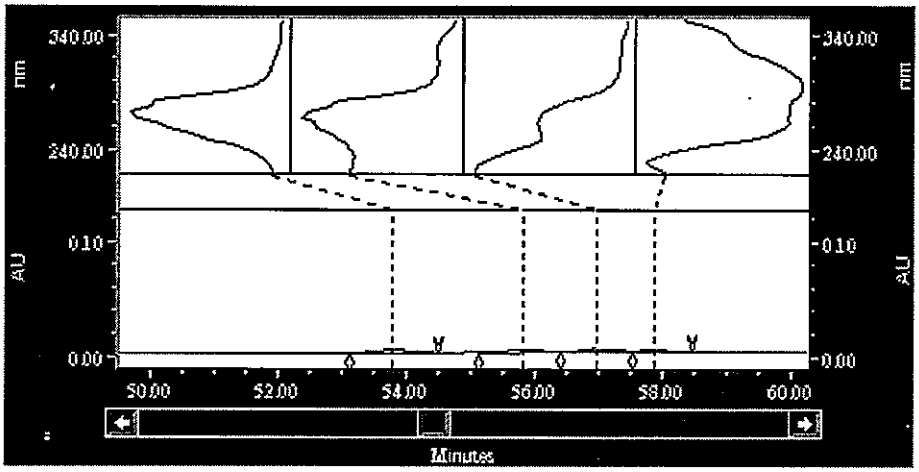


Figure 2.5: UV Spectra of the diterpenoid peaks of Fraction A.

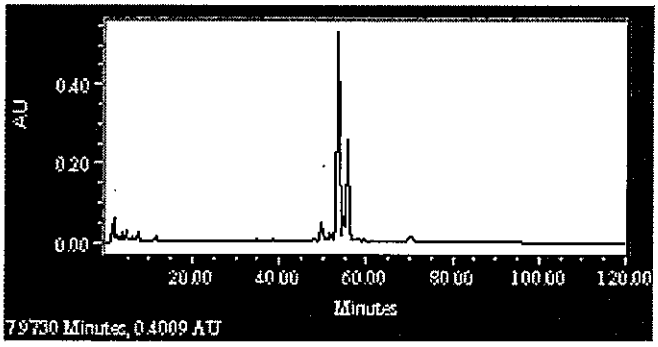


Figure 2.6: HPLC Chromatogram of Fraction B.

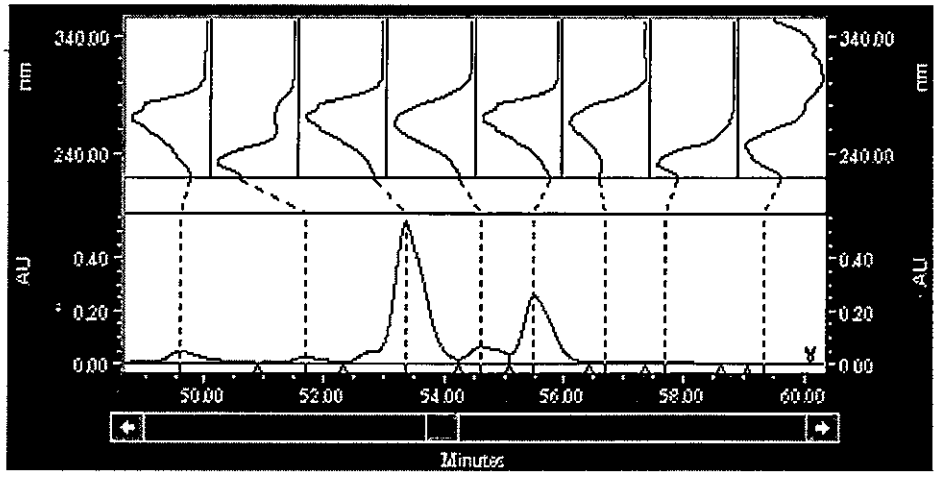


Figure 2.7: UV Spectra of the diterpenoid peaks of Fraction B.

2.4.2 *PKC radioligand binding assay*

In order to determine the quantity of rat brain preparation required for maximal [^{3}H]-PDBu in the assay, a series of tubes were set up with varying volumes of rat brain preparation, using the methodology described in Section 2.3.2 (Table 2.4).

The results indicate that the volume of rat brain required for maximal binding is 100μL in a 1mL assay (Table 2.5). This volume was used for all subsequent assays.

Table 2.4: Optimisation of Rat Brain Preparation

Tube Number	Volume of brain (μL)	Volume of [^{3}H]-PDBu (μL)	Volume of buffer (μL)
1 (Non-specific)	0	100	900
2 (Total [^{3}H]-PDBu in 100μL)	0	100	0
3	50	100	850
4	100	100	800
5	200	100	700

Table 2.5: Results for Rat Brain Optimisation Assay

non-specific binding: 648cpm

total counts ([^{3}H]-PDBu in 100μL): 18128cpm

Tube Number	Average cpm (less non-specific mean)
3	12 545
4	15 309
5	15 073

An assay was then set up as described in Section 2.3.3, using mezerein (Figure 1.4) as the competing agent. The data was analysed as described and plotted as B/B_o values against log [competing agent]. The dose response curve for mezerein is shown in Figure 2.8.

From the curve, the IC_{50} value for mezerein is 17nM. That is, a mezerein concentration of 17nM is required to displace 50% of the bound [^{3}H]-PDBu from the PKC receptor in rat brain.
Further assays were carried out with unlabelled phorbol-12,13-dibutyrate, Fraction B, and simplexin. These curves are shown in Figures 2.9, 2.10, and 2.11, respectively. The IC_{50} values for each are given in Table 2.6.

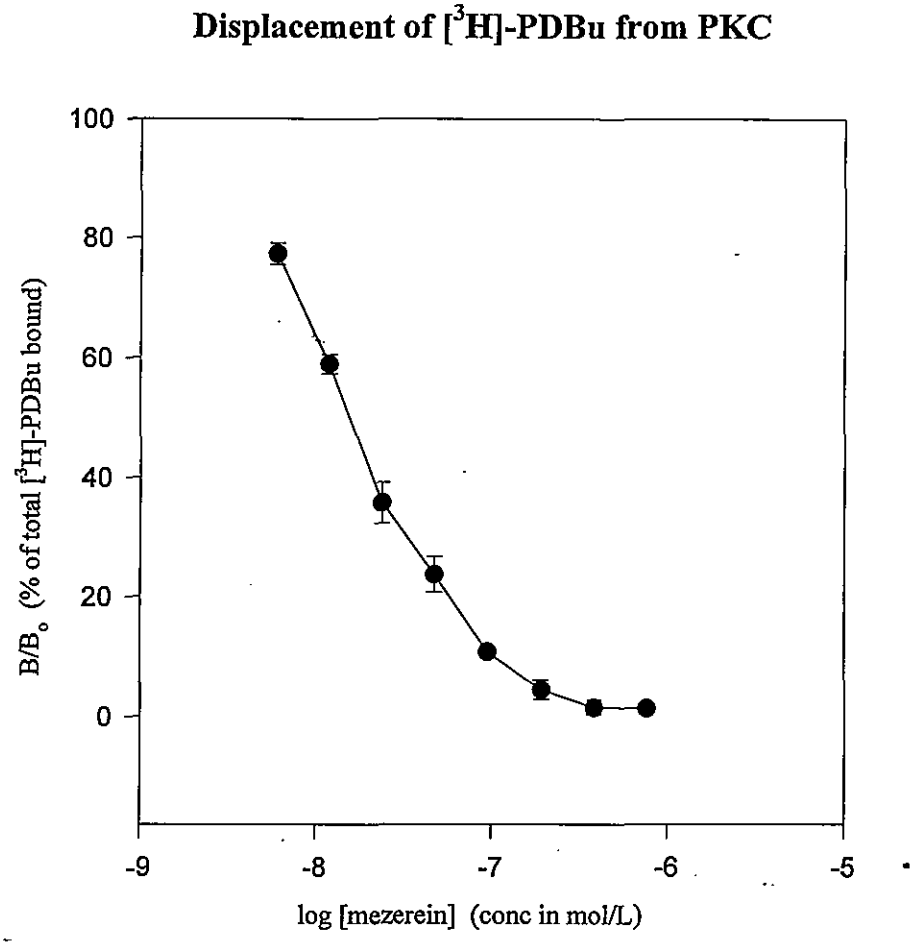


Figure 2.8: Dose Response Curve for Mezerein.

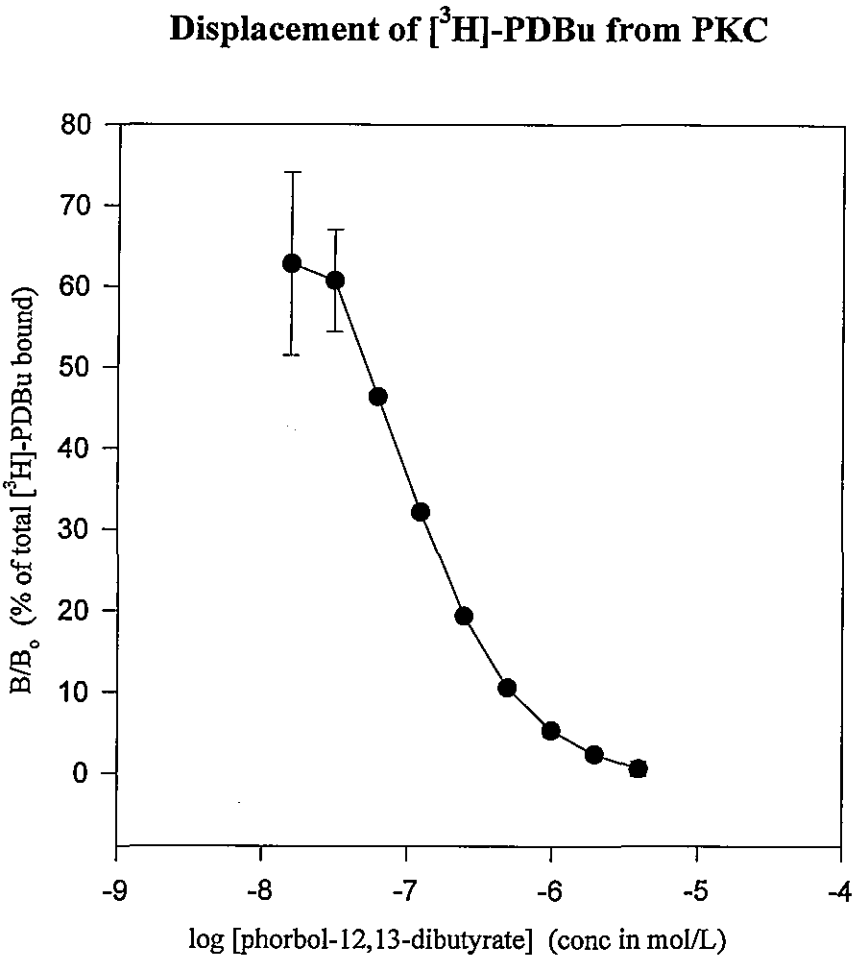


Figure 2.9: Dose Response Curve for Phorbol-12,13-dibutyrate.

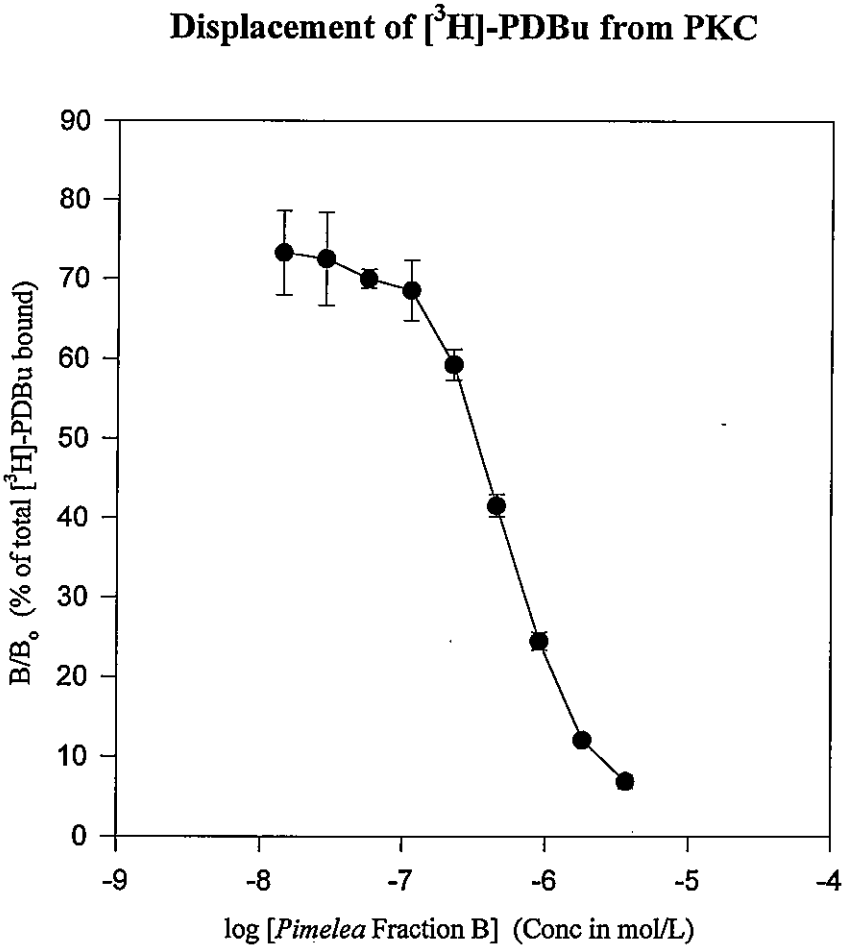


Figure 2.10: Dose Response Curve for Fraction B.

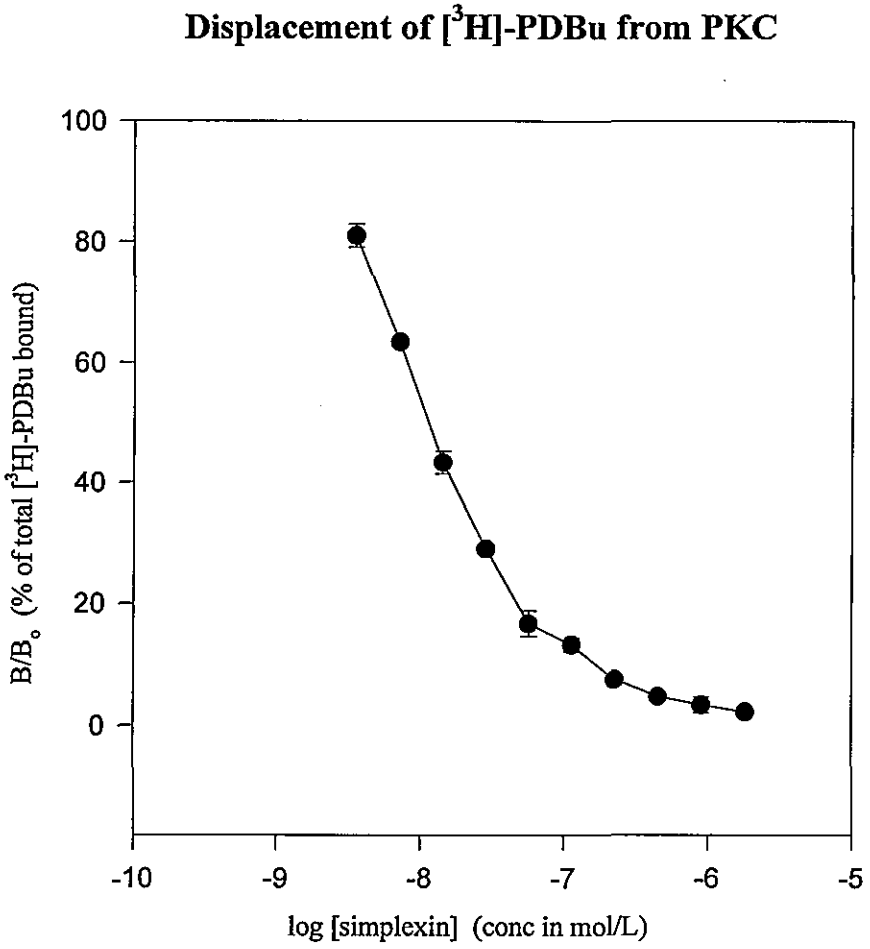


Figure 2.11: Dose Response Curve for Simplexin.

Table 2.6: IC_{50} Values

Compound	IC_{50} Value (nM)
Mezerein	17
Phorbol-12,13-dibutyrate	55
Fraction B	331
Simplexin	11

2.5 Discussion and conclusions

The extraction and isolation of toxins from *Pimelea trichostachya* gave results consistent with those obtained in previous studies (Nayyar, 1994; B. Lambert, unpublished). This indicates that the large scale procedure is reproducible and efficient, using soxhlet extraction and flash chromatography. The Waters-Millenium HPLC system provides convenient UV spectral analysis of each peak, facilitating identification and separation of the toxin peaks.

The diterpenoid toxins, primarily simplexin, isolated from *Pimelea trichostachya* were found to have an IC_{50} value of 11nM. This indicated that simplexin has a higher affinity for the PKC receptor in rat brain than both phorbol-12,13-dibutyrate (55nM) and mezerein (17nM). Mezerein and simplexin differ from phorbol esters in that they both contain the unique orthoester functionality. The IC_{50} values suggest that simplexin and mezerein have a higher affinity for the PKC receptor in rat brain, indicating that this moiety is likely to be intricately involved with receptor binding of these compounds (refer to Figures 1.2 and 1.4).

The PKC radioligand binding assay proved to be a reliable, robust, and reproducible assay, and non-specific binding was always less than 5% of the total [^{3}H]-PDBu bound. The only major drawback with the radioligand binding assay was the vacuum filtration step, since each tube had to be manually filtered and washed separately. This was a time consuming process, requiring almost four hours to filter 64 tubes.

If this assay protocol was to be adopted on a routine basis, a "multifiltration" apparatus or cell harvester would be required since it would allow simultaneous filtration of 48 or more tubes at once. In order to overcome the problem of leaching bound protein and [^{3}H]-PDBu in the scintillation cocktail, a procedure would be required to digest the filter disks immediately, so that liquid scintillation measurements could be carried out on the same day.
Overall, the PKC radioligand binding assay is able to quantify specific displacement of [^{3}H]-PDBu from PKC by *Pimelea* toxins, phorbol esters and other structurally related compounds in a sensitive and specific manner. The assay could be adopted for routine use, with only minor adjustments, to quantify toxin levels in *Pimelea* plants. The following chapter describes the use of the methodologies developed here to analyse the distribution of toxins in *Pimelea trichostachya* at different stages of the growth cycle.

A similar PKC assay should now be developed using bovine pulmonary venules in place of rat brain, since this tissue is the major site of action in *Pimelea* poisoning of cattle.

3.0 APPLICATIONS OF THE PKC RADIOLIGAND BINDING ASSAY

3.1 Aims

The aims of the work described in this chapter were to use the PKC radioligand binding assay to:

(i) carry out a preliminary characterisation of the toxin distribution in *Pimelea trichostachya*;
(ii) determine if antibodies raised against the *Pimelea* toxins bind simplexin using the PKC radioligand binding assay; and
(iii) carry out structure-binding studies of some structurally modified toxin-derived compounds.

3.2 Introduction

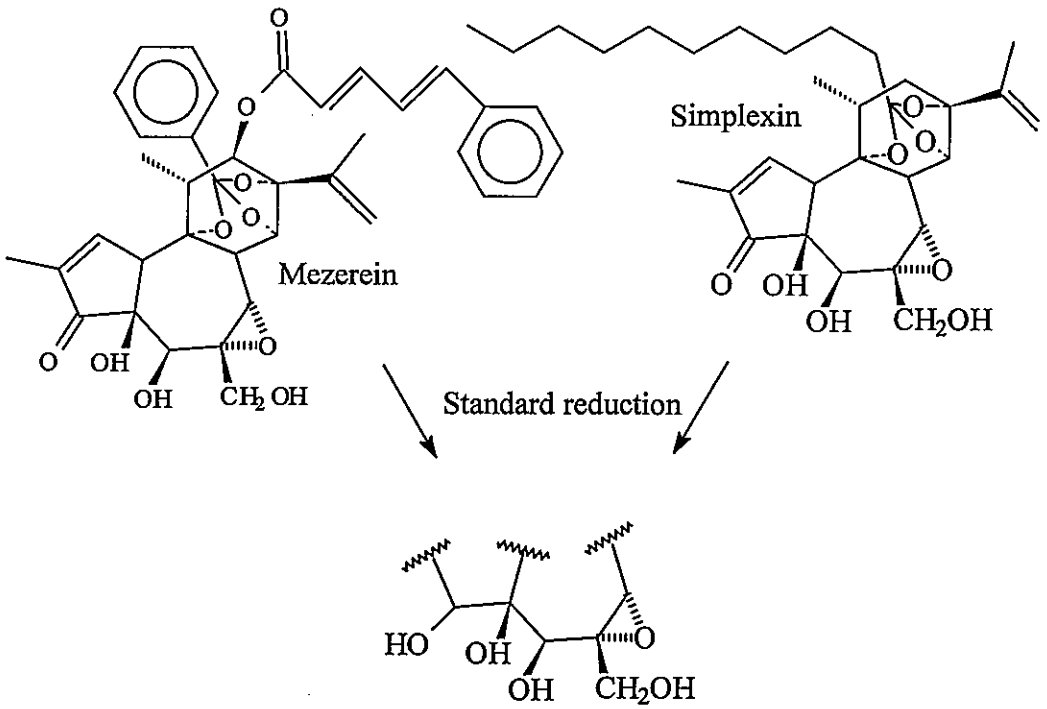
One of the specific objectives of this project was to quantify toxin levels in *Pimelea* plants. As mentioned previously, there is no information available on the distribution of toxins in *Pimelea* plants, or changes in toxin concentrations during the growth cycle. The PKC radioligand binding assay methodology was used to carry out a preliminary characterisation of the distribution of toxins in *Pimelea trichostachya*.

Plant samples (roots, stems, and flowers or seeds) were collected periodically by Ross Newman (QDPI Veterinary Officer, Roma), from properties on which cattle were being monitored for the development and progression of *Pimelea* poisoning. These samples were analysed for toxin concentrations using the PKC radioligand binding assay described in Chapter 2.

In a complementary study, antibodies were raised in cattle, against the *Pimelea* toxins (B. Lambert, unpublished). The specificity of these antibodies for the *Pimelea* toxins was to be established via *in vitro* organ bath studies. However, since these studies are extremely time-consuming, it was proposed that a result might be obtained more rapidly with the PKC radioligand binding assay. The assay procedure described in Section 2.3.2 was used, with the addition of the antibodies at concentrations equivalent to those used in the organ bath studies.

In addition to these studies, various modifications to the toxins and the structurally related compound mezerein, were being prepared as part of the *Pimelea* research program. Previous studies have shown that the C3 carbonyl and the C4, C5, and C20 hydroxyl groups are important for the binding of phorbol esters, mezerein and related compounds to PKC (Jeffrey and Liskamp, 1986). C3-reduced mezerein, and C3-reduced simplexin were prepared, in order to determine the importance of the C3 carbonyl functionality (Figure 3.1). Mezerein was also modified so that the C20 hydroxyl group was protected as a silyl ether (Figure 3.2) and the C4, C5 and C20 hydroxyls protected as benzyl esters (Figure 3.3) and p-nitrobenzylesters (Figure 3.4). Simplexin was then modified so that the C5 and C20 hydroxyl groups were protected as p-nitrobenzylesters (Figure 3.5) and the C4, C5 and C20 hydroxyls protected as p-nitrobenzylesters (Figure 3.6).

The affinity of these compounds for the PKC receptor was to be established, using the PKC radioligand binding assay described in Section 2.3.2. This allows for the determination of which functional groups are most essential for binding to the PKC receptor in rat brain.



C3-hydroxy- simplexin or -mezerein

Figure 3.1 Reduction of the C3-carbonyl of mezerein and simplexin.
(adapted from: Lambert, unpublished)

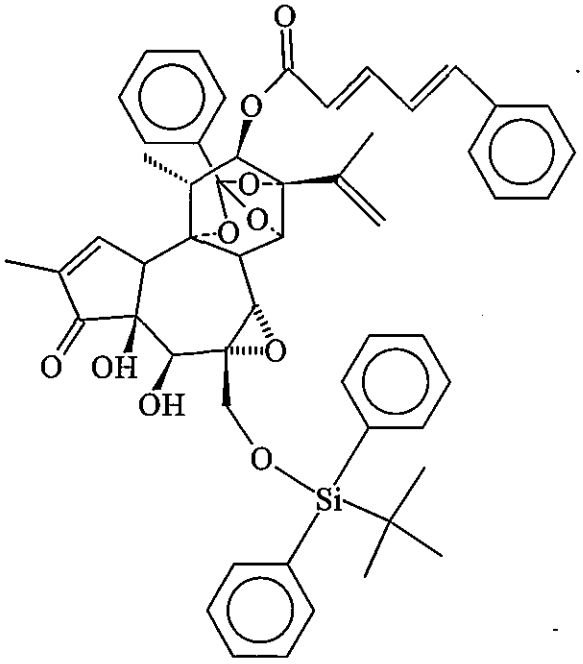


Figure 3.2 Protection of the C20 hydroxyl of mezerein as a silyl ether - R3F

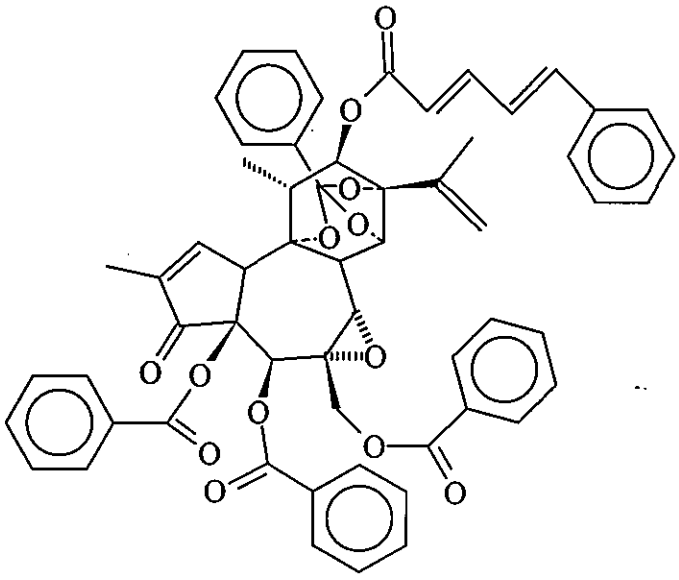


Figure 3.3 Protection of the C4, C5 and C20 hydroxyls of mezerein as benzyl esters - R3A

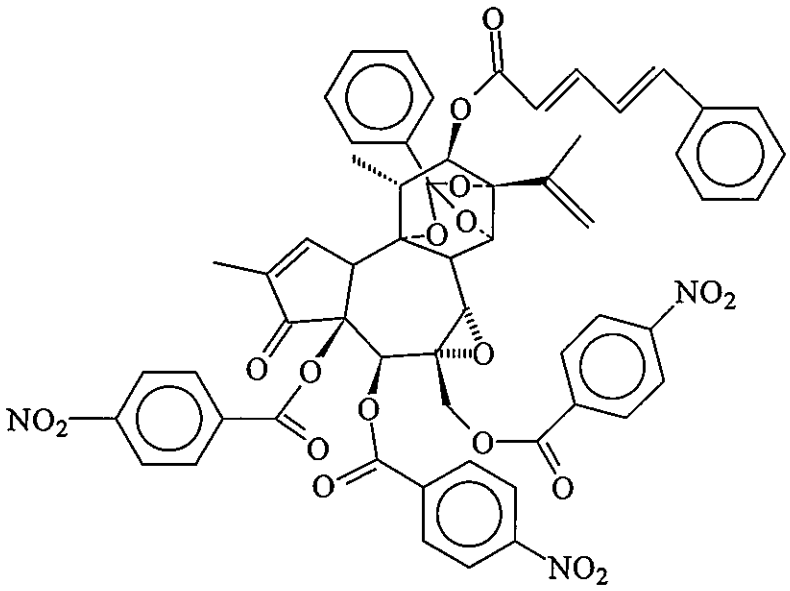


Figure 3.4 Protection of the C4, C5 and C20 hydroxyls of mezerein as p-nitrobenzylesters - R3C

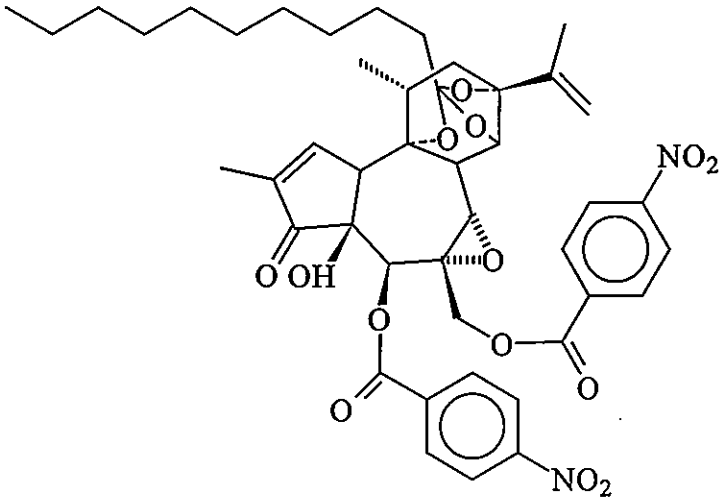


Figure 3.5 Protection of the C5 and C20 hydroxyl groups of simplexin as p-nitrobenzyl esters - AR3C2

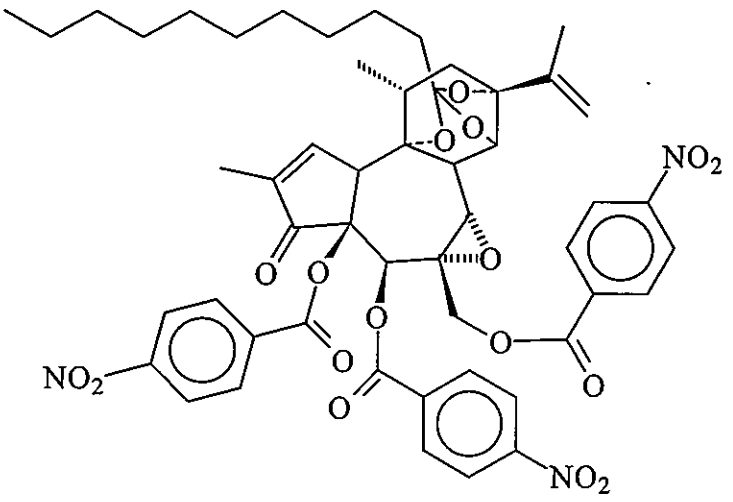


Figure 3.6 Protection of the C4, C5 and C20 groups of simplexin as p-nitrobenzylesters - AR3C3

3.3 Materials and Methods

3.3.1 *Toxin distribution study*

Each plant sample was milled using a rotary hammer mill. The samples were collected from the same property in the Maranoa district. Roots, stems and flowers were collected in winter and roots and stems were collected in summer. Samples (1g) were extracted in 1:1 v/v ethanol:dichloromethane, stirring overnight at room temperature. The extract was gravity filtered, rotary evaporated to dryness, and stored under nitrogen at -20°C.

Dilutions of the crude extracts were made in dimethyl sulphoxide (DMSO), with a maximal concentration of 200µg/mL. Aliquots (50µL) of each graded dose were added to triplicate tubes containing [^{3}H]-PDBu, rat brain preparation and binding buffer. The assay was carried out as described in Section 2.3.2.

3.3.2 *Immunogen study*

Antibodies raised in cattle against the *Pimelea* toxins were available as lyophilised antisera (B. Lambert, unpublished) and as serum from immunised cattle. The lyophilised powder could be added directly to the assay. However, the IgG was first precipitated from the serum using 35% ammonium sulphate ($(NH_4)_2SO_4$) precipitation. A saturated $(NH_4)_2SO_4$ solution (0.54 x serum volume) was added dropwise to the stirring serum, over a period of 15 minutes. This was left to equilibrate for 30 minutes, then centrifuged at 3000 rpm for 15 minutes at 4°C on a Beckman J2-21M centrifuge. The pellet was resuspended to its original volume in binding buffer (50mM Tris-Cl, pH 7.4 at 25°C, 2mg/mL BSA), and the ($(NH_4)_2SO_4$) precipitation repeated. For the final step, the pellet was redissolved in binding buffer (refer to Section 2.3.2) and desalted by dialysing in binding buffer at 4°C.

The level of protein in the dialysed product was then determined using a Bio-Rad Protein Assay (Bradford Assay) with BSA standards. In this procedure, 50mL of Bradford dye reagent was diluted with 4 volumes (200mL) of millipore filtered water and gravity filtered through Whatman No 1 filter paper. 100μL of the standards or samples were added to tubes containing 5mL of diluted Bradford dye, and the solutions were vortexed and left to react for 10 minutes. Absorbances at 595nm were measured on a UNICAM 8620 Series UV/VIS single beam spectrophotometer.

Varying concentrations (0.4, 2 mg/mL - equivalent to those used in corresponding organ bath studies) of the freeze-dried, affinity purified antibodies were added to the PKC radioligand binding assay. A second method for separating membrane-bound and free [^{3}H]-PDBu was also investigated, using adsorption onto activated charcoal. For this procedure, free [^{3}H]-PDBu is expected to be adsorbed onto the activated charcoal, while that bound to the receptor remains in solution. Incubations were carried out for 60 minutes at 30°C and terminated by immersion of the tubes in ice for 15 minutes. Activated charcoal (200μL) was then added and the tubes shaken and left in ice for a further 10 minutes. The tubes were then centrifuged for 15 minutes at 2000 rpm at 4°C on a Beckman J-6 Induction Drive Centrifuge. Scintillation cocktail was then added to the supernatant and the membrane-bound radioactivity was measured by liquid scintillation counting.

3.3.3 *Structure - binding studies*

Dilutions of the modified compounds were prepared in DMSO and the affinities of these compounds for the PKC receptor were determined using the PKC radioligand binding assay (Section 2.3.2).

3.4 Results

3.4.1 *Toxin distribution study*

The data obtained for the toxin distribution study were plotted as dose response curves as described in Chapter 2, and the IC_{50} values for each sample were determined. From the IC_{50} value, the mass of plant extracted and the mass of the crude extract, the % w/w equivalent simplexin was calculated (Table 3.1).

Table 3.1: Toxin Distribution Study

Sample	Mass of plant (g)	Mass of extract (mg)	IC_{50} value of extract (µg/mL)	% w/w equivalent simplexin
26/8/92 Roots	1.0089	29.8	1.2115	0.016
26/8/92 Stems	1.0317	49.4	1.4125	0.022
26/8/92 Flowers	1.0272	65.9	1.4275	0.096
9/12/92 Roots	1.0376	25.4	1.4036	0.040
9/12/92 Stems	1.0093	30.5	0.8004	0.024

3.4.2 *Immunogen study*

The 35% $(NH_4)_2SO_4$ precipitation procedure was carried out for serum from a control (non-immunised animal), serum from cattle immunised with 'Conjugate A' and serum from cattle immunised with 'Conjugate B' (B. Lambert, unpublished). Table 3.2 shows the volumes of sera and saturated $(NH_4)_2SO_4$ used for the precipitation.

The precipitation procedure and dialysis was carried out as described in section 3.2.1 and resulting IgG protein concentrations determined using a Bradford protein assay (Table 3.3). The calibration curve obtained for the BSA standards is shown in Figure 3.7.

Table 3.2: $((NH_4)_2SO_4)$ precipitation

Serum	Volume of serum (mL)	Volume of $((NH_4)_2SO_4)$ (mL)
Control	26	14
A	26	14
B	26	14

Table 3.3: Bradford Protein Assay

Serum Sample	Absorbance at 595nm	Concentration of protein (mg/mL)
blank	0.383	-
Control	0.400	0.50
A	0.456	1.70
B	0.445	1.46

Calibration Curve for the Determination of IgG in Serum

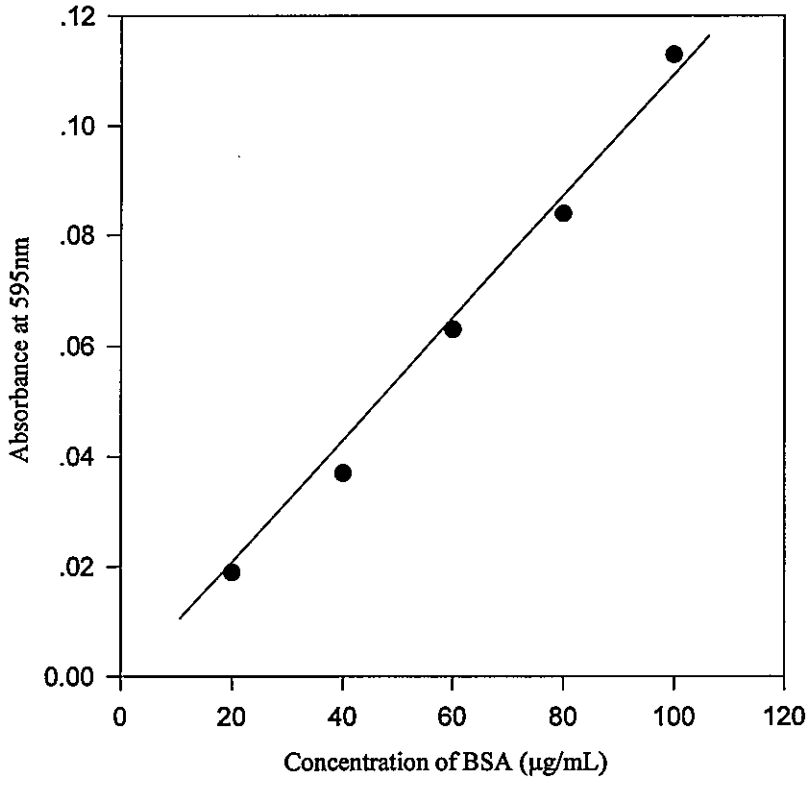


Figure 3.7: Standard Curve for Bradford Protein Assay

An initial PKC radioligand binding assay was carried out in the absence of rat brain preparation, using the freeze-dried, affinity purified IgG to determine if antibodies raised against the *Pimelea* toxins bind [^{3}H]-PDBu. In a previous study, it was demonstrated that there is no cross reactivity between simplexin and antibodies raised against [^{3}H]-PDBu, and this was believed to be a result of the unique tricyclic orthoester functionality present in simplexin, but lacking in phorbol esters (MJ D'Occhio, unpublished).

Antibodies raised against [^{3}H]-PDBu (supplied by Professor A. Tashjian of Harvard University), were used as a positive control in the assay, and a further series of tubes were prepared with Control, Conjugate A, and Conjugate B antibodies at concentrations equivalent to those use in *in vitro* studies (B. Lambert, unpublished). The counts obtained for each tube, including the positive control, were equivalent to non-specific binding, indicating that the antibodies had passed through the glass fibre filters.

Included in this assay were two sets of tubes using the charcoal method of separating free [^{3}H]-PDBu from bound for the positive control antibodies. The tubes consisted of: a tube for total [^{3}H]-PDBu bound and a tube containing a known concentration of antibodies raised against [^{3}H]-PDBu. These tubes gave the expected results, with the antibodies binding [^{3}H]-PDBu, thus resulting in a decrease in the number of counts.

From this data, it was proposed that the charcoal method be employed as the standard method of separation of free from bound radioactivity. To validate this proposal, a further experiment was undertaken as outlined in Table 3.4.

The results obtained were unexpected. The non-specific binding tube (Tube 4) showed the expected number of counts, while the other tubes showed reproducible, but ambiguous results. It appeared to us that in the presence of the rat brain preparation, the activated charcoal separation of free from bound radioactivity is adversely affected. The experiment was repeated to ensure that there were no operator errors and the same results were obtained.

Table 3.4: Validation of the charcoal method of separation

Tube 1	Tube 2	Tube 3	Tube 4
40µL rat brain prep	40µL rat brain prep	40µL rat brain prep	-
100µL [^{3}H]-PDBu	100µL [^{3}H]-PDBu	100µL [^{3}H]-PDBu	100µL [^{3}H]-PDBu
-	5µL PDBu	5µL Fraction AB	-
260µL buffer	255µL buffer	255µL buffer	300µL buffer

As a result, this method of separation was abandoned for the membrane assays, but could still be used to determine whether the antibodies raised against *Pimelea* toxins, bind [^{3}H]-PDBu. It was determined that the antibodies raised against the *Pimelea* toxins did not bind [^{3}H]-PDBu.

The *Pimelea* antibodies were then added to the PKC radioligand binding assay described in Section 2.3.2 (filtration method) to determine their efficacy in preventing the toxins from binding to the PKC receptor. The concentration of toxin required to displace 50% of the bound [^{3}H]-PDBu from PKC was used in the assay. The *Pimelea* antibodies were added in concentrations equivalent to those used in *in vitro* studies (B. Lambert, unpublished). Again, the antibodies raised against [^{3}H]-PDBu were used as a positive control, with labelled PDBu used as the competitor.

The results showed that the antibodies had no significant effect on toxin binding to PKC. It was thought that pre-incubation of the antibodies with the toxin may be required, before the addition of the rat brain preparation. The assay was repeated with a pre-incubation step of 30 minutes at 30°C. However, the results obtained were almost identical to those without the pre-incubation step. Due to time restraints, further experiments were abandoned since these antibodies had already proven to be efficacious in *in vitro* organ bath studies.

3.3.3 *Structure-binding studies*

The structurally modified compounds (Figures 3.1 - 3.6) were added in graded doses, as competing agents in the PKC radioligand binding assay outlined in section 2.3.2. The dose response curves for these compounds are shown in Figures 3.8 - 3.14. The IC_{50} values for these structurally modified compounds, compared with those for mezerein and simplexin, are given in Table 3.5 (refer to Figures 3.1 - 3.6 for the structures).

Table 3.5: IC_{50} Values

Competitor	IC_{50} Value (nM)
mezerein	17
3-hydroxymezerein	184
R3F	36
R3A	1501
R3C	144
simplexin	11
3-hydroxysimplexin	44
AR3C-2	1075
AR3C-3	4352

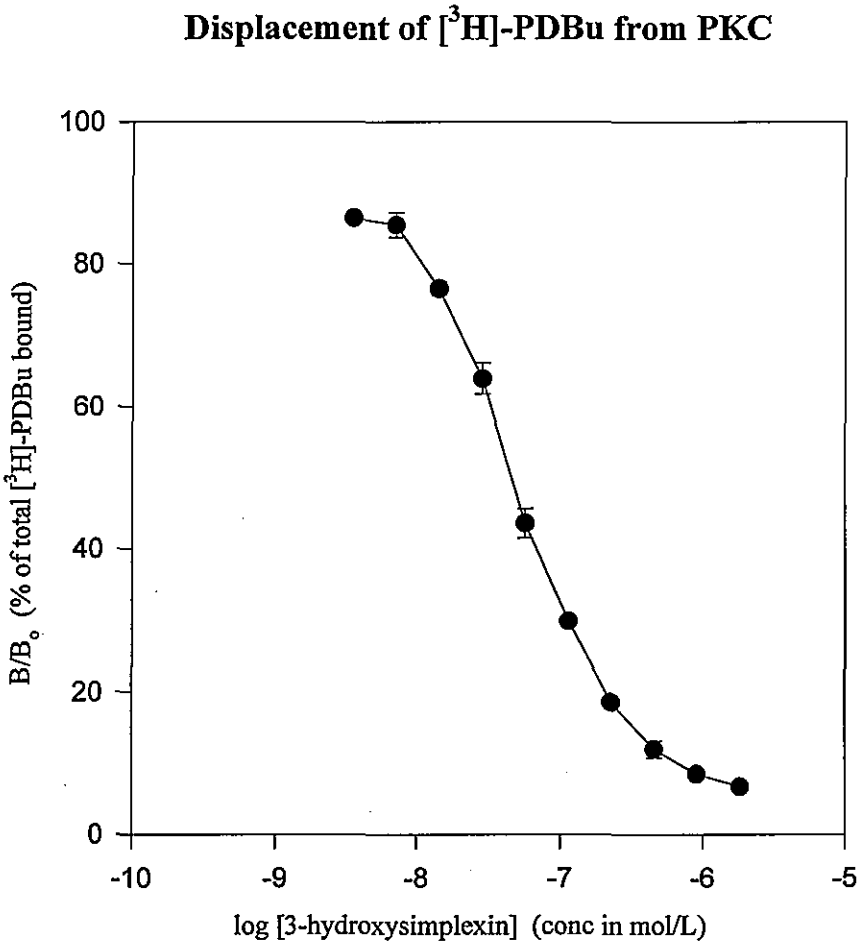


Figure 3.9: Dose response curve for 3-hydroxysimplexin.

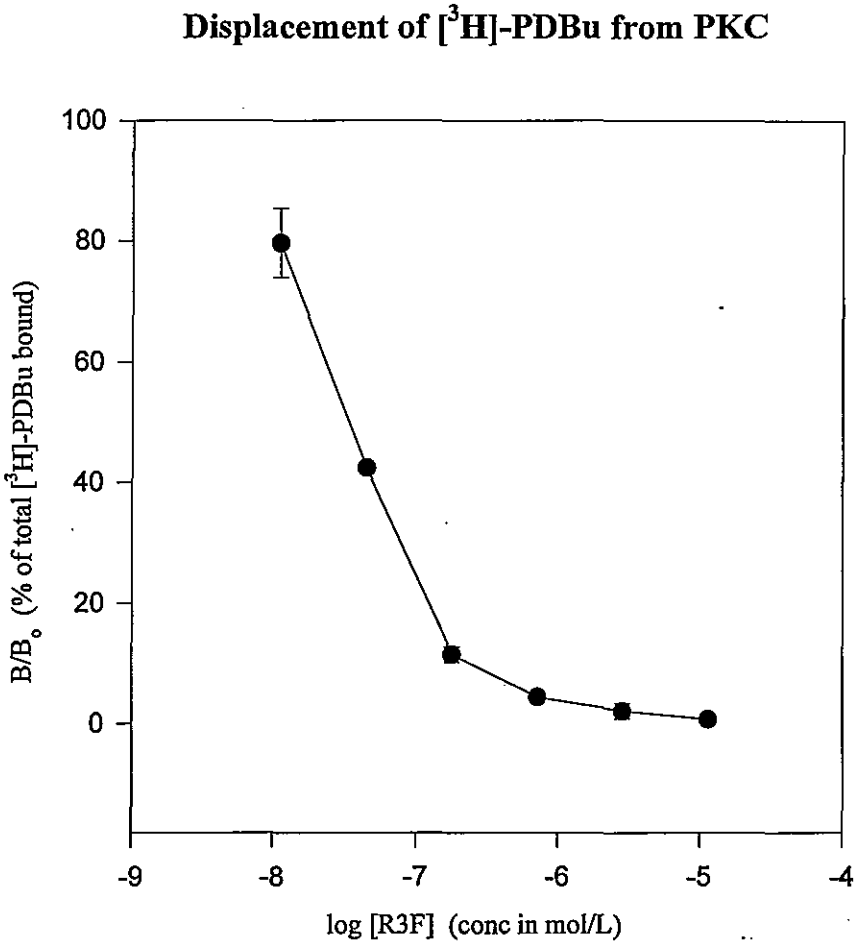


Figure 3.10: Dose response curve for R3F
(C20 OH of mezerein protected as a silyl ether).

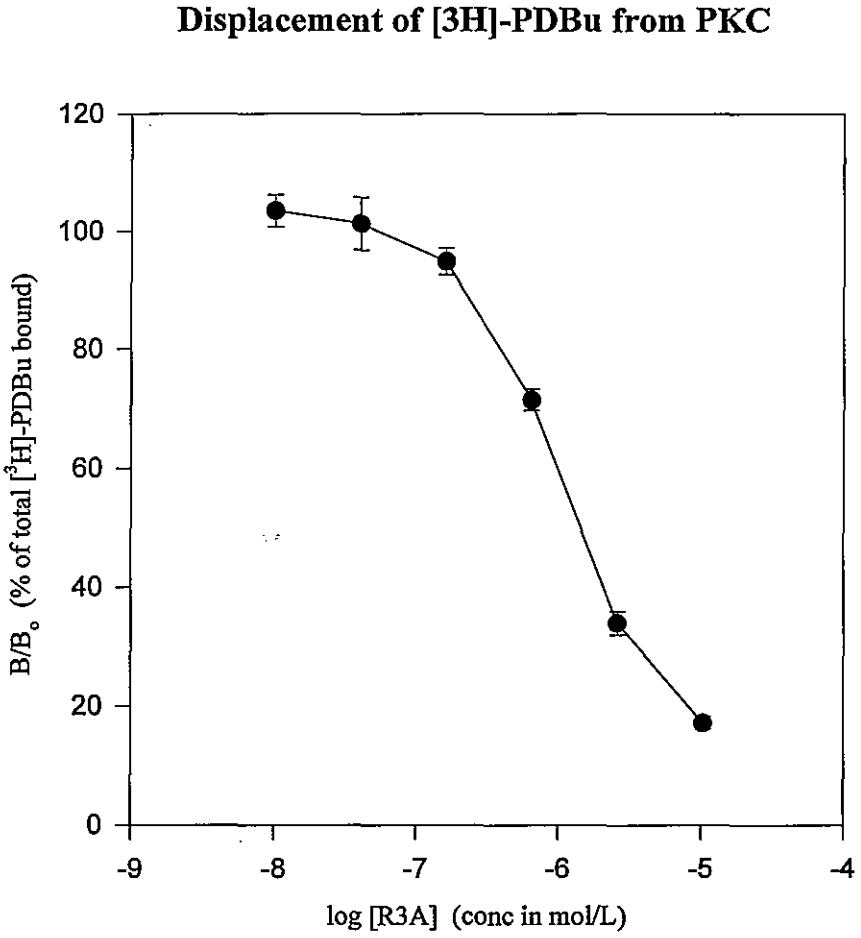


Figure 3.11: Dose response curve for R3A

(C4, C5 and C20 OHs of mezerein protected as benzyl esters)

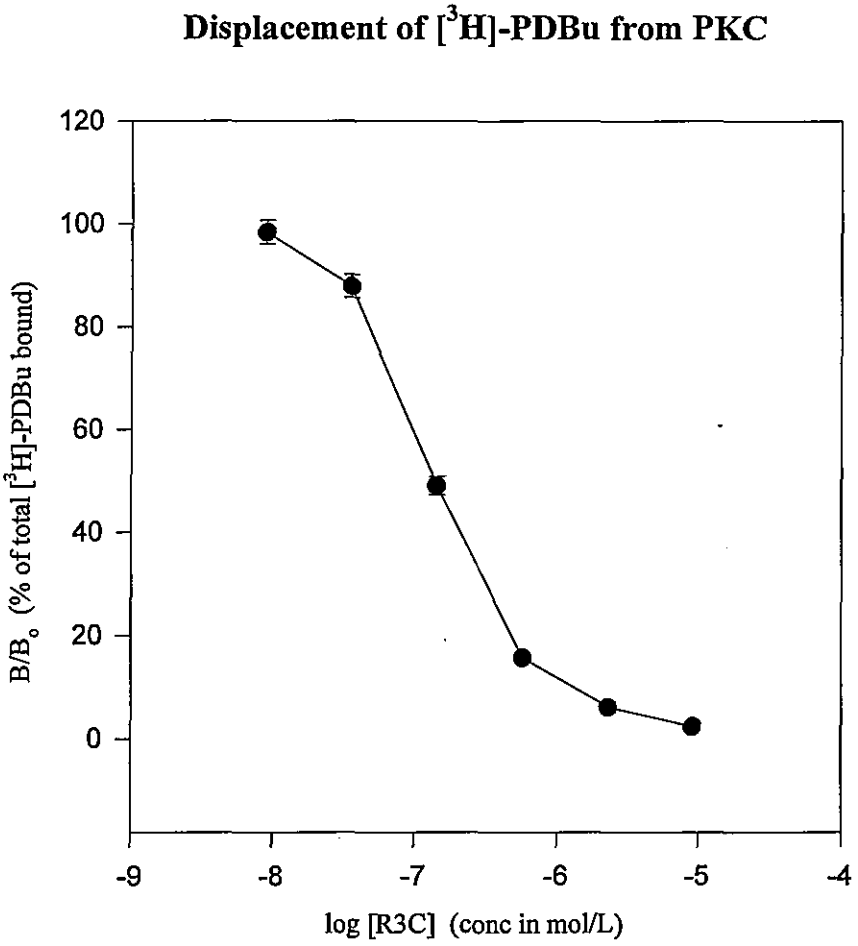


Figure 3.12: Dose response curve for R3C
(C4, C5, and C20 OHs of mezerein protected as p-nitrobenzylesters)

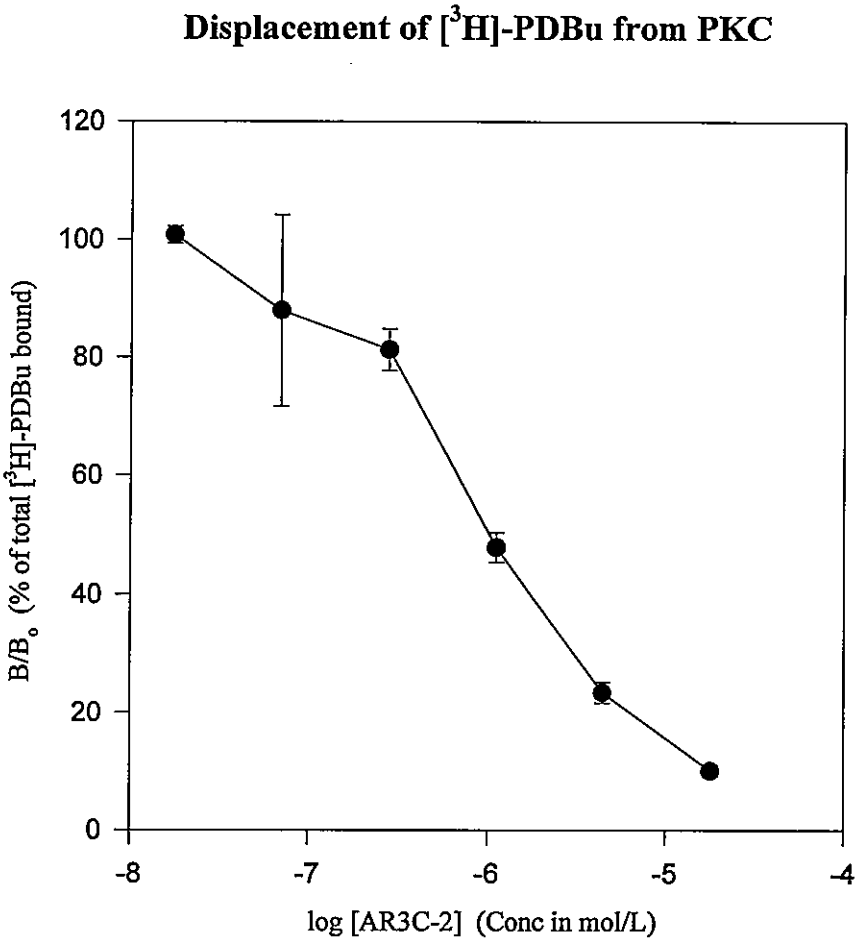


Figure 3.13: Dose response curve for AR3C-2
(C5 and C20 OHs of simplexin protected as p-nitrobenzylesters)

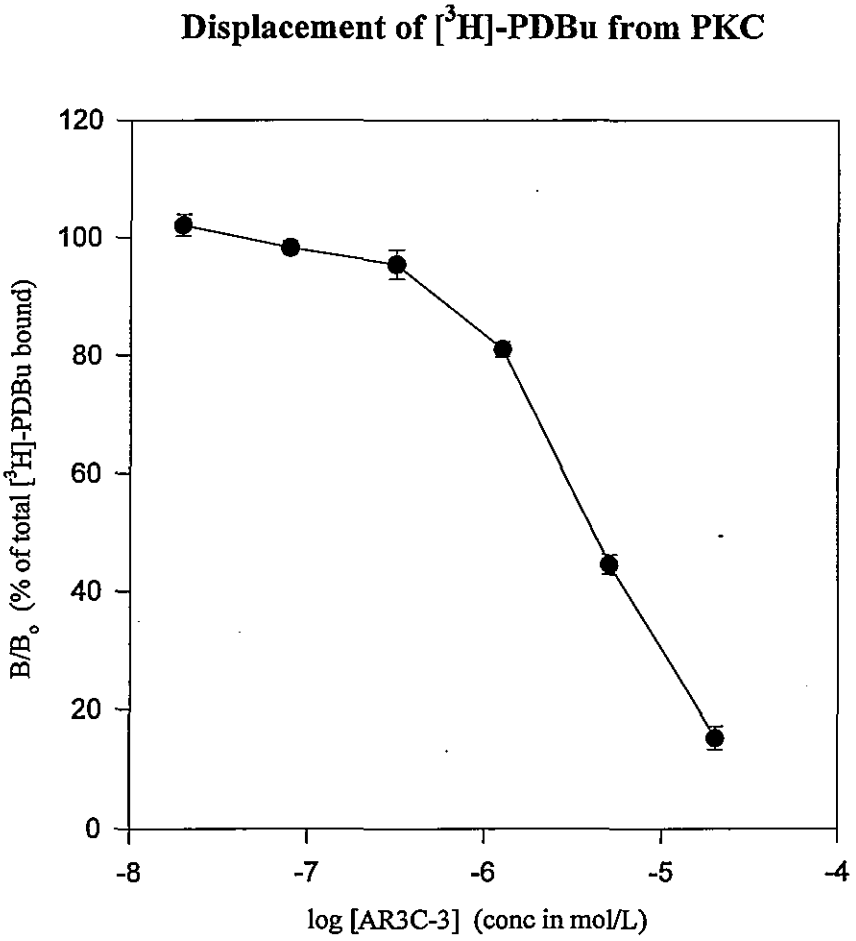


Figure 3.14: Dose response curve for AR3C-3

(C4, C5 and C20 OHs of simplexin protected as p-nitrobenzylesters)

3.5 Discussion and conclusions

The PKC radioligand binding assay developed in the previous chapter has been used successfully to analyse the distribution of toxins in *Pimelea* plants (Table 3.4). The flowers were found to contain a six-fold higher concentration of simplexin than the roots and a four-fold higher concentration than the stems. This may explain the higher incidence of poisoning during the flowering season. While the toxin concentrations in the stems did not appear to change significantly from winter to summer, the level in the roots more than doubled in this period.

Adaptation of the PKC radioligand binding assay to demonstrate efficacy of antibodies raised against *Pimelea* toxins was not successful. The antibodies did not bind [^{3}H]-PDBu, and they also failed to bind the *Pimelea* toxins in the binding assay, despite pre-incubation. The competing agents were prepared in DMSO and this solvent may interfere with antibody action (A. Hoey, pers. comm.). It is suggested that the competing agents be prepared in ethanol. It has also been reported that the presence of Ca^{2+} and/or Mg^{2+} salts (0.005M) in the binding buffer can increase the activity of some antisera (Chard, 1990). It may be worthwhile to prepare a binding buffer containing these salts for the assay. Further experiments need to be conducted in order to demonstrate antibody-toxin binding under the PKC radioligand binding assay conditions.

The PKC radioligand binding assay was adopted successfully for structure-binding studies to determine the functionalities most important for binding to the PKC receptor in rat brain. The results show that the C3 carbonyl is important for binding; C3-reduction of mezerein decreased the binding 11-fold and C3-reduction of simplexin decreased the binding 4-fold (Figure 3.1). Protection of the C20 hydroxyl group of mezerein resulted in a compound with only half the affinity of mezerein (Figure 3.2). Protection of the C5 and C20 hydroxyl groups of simplexin produced significantly decreased binding (by almost 100-fold) (Figure 3.5). Protection of the C4, C5 and C20 hydroxyl groups as p-nitrobenzyl esters decreased the binding affinity 400-fold, compared with simplexin.

It was concluded from these results that while the C3 carbonyl and C5 and C20 hydroxyl groups of simplexin are important for binding, the C4 hydroxyl group appears to be essential.

The competitive PKC radioligand binding assay developed during this study, has been used successfully to determine toxin levels in *Pimelea* plants and to analyse the affinity of various structurally modified derivatives of the toxins for the PKC receptor in rat brain. The assay is, however, a competitive displacement binding assay, and does not provide any information on the biological activity of these compounds. In the following chapter, a PKC Colourimetric SpinZyme Assay Kit, purchased from Pierce, was investigated in an attempt to quantify activation of PKC by *Pimelea* toxins.

4.0 INVESTIGATION OF A PKC ASSAY KIT TO MEASURE TOXIN LEVELS IN *PIMELEA TRICHOSTACHYA.*

4.1 Aims

The aim of the work described in this chapter was to investigate the feasibility of using a commercially available colorimetric PKC assay kit to quantify activation of PKC by *Pimelea* toxins.

4.2 Introduction

In previous studies, activation of PKC by various phorbol esters, DAG analogues and other structurally related compounds, has been measured primarily by *in vitro* studies (Deth and van Breeman, 1974) and enzyme assays (Castagna *et al.*, 1982). The *in vitro* studies involve organ bath studies, measuring tissue contraction following treatment with various activators (Deth and van Breeman, 1974; Fish *et al.*, 1988). However, these studies, while being sensitive, are time-consuming and difficult to prepare. In PKC assays using radioisotopes, the level of activity was determined by measuring the incorporation of ^{32}P into H1 histone from [γ-^{32}P]-ATP (Castagna *et al.*, 1982; Couturier *et al.*, 1984; Kuo *et al.*, 1991; Leibersperger *et al.*, 1990; Oishi *et al.*, 1988).

In the current study, a non-radioactive assay was investigated. The Pierce Colorimetric PKC Assay Kit, SpinZyme format employs a unique separation method to separate dye-labelled, phosphorylated peptide from non-phosphorylated peptide. The separation units contain membranes which have a specific affinity for the phosphate groups incorporated onto the peptide (Pierce, 1995). The detection system uses a fluorescent label attached to the substrate (peptide) molecules, which can be measured using absorbance or fluorescence spectroscopy.

4.3 Materials and Methods

4.3.1 *Preparation of PKC from rat brain*

Rat brains (female Wistar rats) were removed immediately after death and washed in ice cold homogenisation buffer (20mM Tris-Cl, pH 7.5 at 25°C, 2mM EDTA, 2mM DTT, 1mM PMSF). Brains were then homogenised for two minutes in four volumes of homogenisation buffer using an Ultra-Turrax T25 at 11500 rpm. The homogenate was centrifuged on a Beckman L8-70M Ultracentrifuge with a 50.2TI rotor at 11000 rpm for 60 minutes at 4°C. Aliquots of the supernatant were added to the assay immediately before incubation.

4.3.2 *Colorimetric PKC assay*

A summary of the reaction components is given in Table 4.1.

Pre-mixed reaction mixture - equal volumes of: Activator Solution (phosphatidyl-L-serine in water, 1mg/mL); Reaction Buffer (10mM ATP, 50mM $MgCl_2$, 0.5mM $CaCl_2$, 0.01% Triton X-100, 100mM Tris(hydroxymethyl)-amino methane, pH 7.4); and, PKC Substrate (dye-labelled glycogen synthase).

PKC dilution buffer - equal volumes of: tris-buffered saline (25mM Tris, 0.15M sodium chloride, pH 7.2) and glycerol.

A PKC standard was not supplied with the kit.

Table 4.1: Summary of Reaction Components

-	**Assay samples** (μL)	**Negative Control** (μL)	**Standard Curve** (μL)
Pre-mixed reaction buffer	15	15	15
Assay sample	10	-	-
PKC dilution buffer	-	10	-
Diluted PKC standard	-	-	10
Total volume	**25**	**25**	**25**

The assay was performed in microfuge tubes, with incubations initiated by the addition of 10μL of PKC (rat brain preparation) to 15μL of pre-mixed reaction mixture. The contents of each tube were vortexed, and the tubes capped and incubated at 30°C for 30 minutes. After incubation, 20μL of each sample was applied carefully to the centre of the corresponding affinity membranes of the SpinZyme separation units.

Non-phosphorylated peptide was washed from the membranes by the application of 250μL of phosphopeptide binding buffer (0.1M sodium acetate, 0.5M sodium chloride, 0.02% sodium azide, pH 5.0). The tubes were incubated, upright for three minutes at 30°C, then centrifuged on a Beckman Microfuge for 60 seconds. This procedure was repeated for a total wash volume of 500μL.

Following the final spin, the buckets (containing the membranes) were removed from the separation units and placed into the corresponding empty Eppendorf tubes. The phosphorylated peptide was then eluted from the affinity membranes by the application of 250μL of phosphopeptide elution buffer (0.1M ammonium bicarbonate, 0.02% sodium azide, pH 8.0). The tubes were incubated, upright for three minutes at 30°C, then centrifuged on a Beckman Microfuge for 60 seconds. This procedure was repeated for a total wash volume of 500μL.

4.3.3 *Detection of phosphorylated product*

Samples (250µL) were transferred to individual wells of a flat-bottom 96-well plate, and the absorbances at 570nm recorded on a BioRad Model 3550 Microplate Reader.

4.4 Results

4.4.1 *Preparation of PKC from rat brain*

Two grams of rat brains were homogenised in 8mL of homogenisation buffer and prepared as described in section 4.3.1. It has been reported that PKC catalyses the transfer of 5 - 6 nmol of [γ-^{32}P]-ATP to mixed histone/min/mg protein (Couturier *et al.*, 1984). In order to estimate the specific activity of PKC in the rat brain preparation, a Bradford protein assay was carried out as described in section 3.3.2. The results of the Bradford assay are plotted in Figure 4.1. Table 4.2 shows the results for the rat brain preparation.

From this data, the rat brain preparation was found to contain 1.56mg/mL protein. This might equate to approximately 0.80units of PKC activity/10µL of rat brain preparation. Consequently, dilutions were made on the rat brain preparation and added to the colorimetric PKC assay kit (Couturier *et al.*, 1984).

Table 4.2: Determination of protein in rat brain preparation

Sample (µg/mL BSA)	Absorbance at 595nm
Rat brain blank	0.018
Rat brain	0.935
Rat brain 1 in 2 dil	0.635
Rat brain 1 in 5 dil	0.330
Rat brain 1 in 10 dil	0.191
Rat brain 1 in 20 dil	0.137

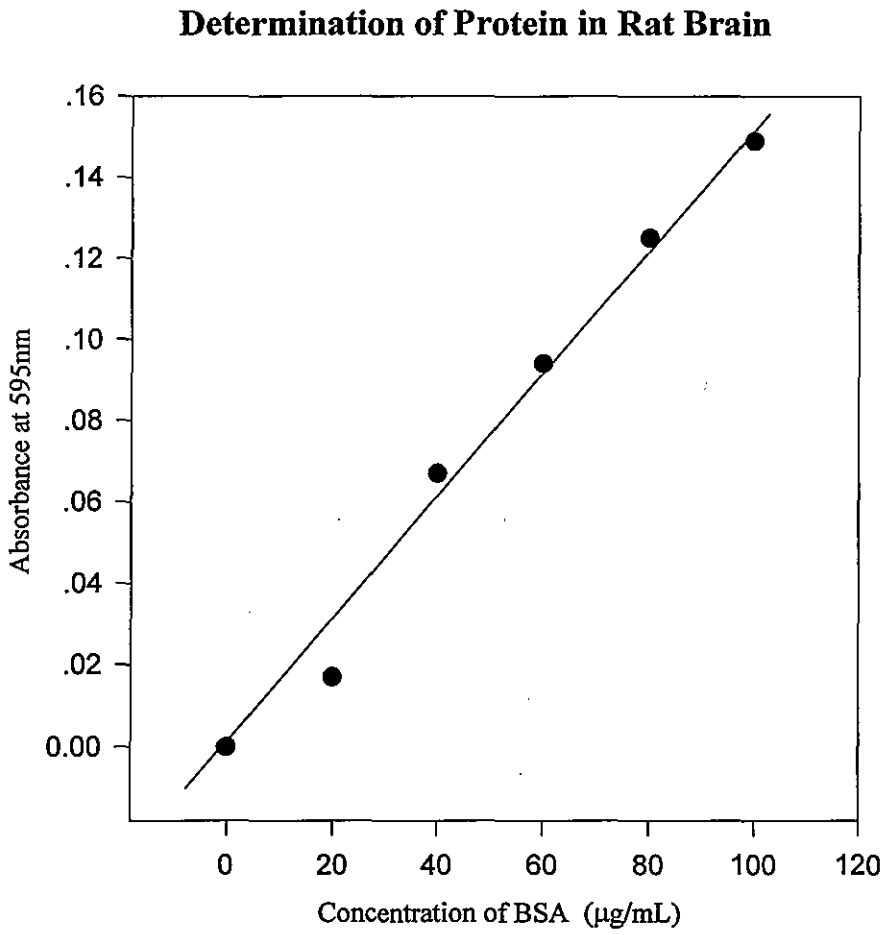


Figure 4.1 Determination of PKC in rat brain homogenate

4.4.2 *Colorimetric PKC assay*

The initial assay was carried out in triplicate with a negative control and dilutions on the rat brain preparation (Table 4.1). This assay was carried out in ELISA plates. The results are shown in Table 4.3.

The results obtained indicated problems with the assay system. The expected trend is that absorbance should increase with increasing PKC concentration. Since the assay was carried out in ELISA plates, it was thought that the protein had bound to the ELISA plates. In order to overcome this problem, the assay was repeated in microfuge tubes. The absorbances were then measured in an ELISA plate that had been 'blocked' with 1% skim milk powder in 0.01M phosphate-buffered saline, 0.2% BSA, pH 7.5. Almost identical results were obtained, suggesting other problems with the assay.

When the kit and reagents arrived from Progen Industries, the ice pack had melted and the contents were warm. They were then stored in the freezer for two months before use. The results obtained above seem to suggest that either: the reagents had degraded before

they were used; the rat brain preparation had lost its activity; or, the rat brain preparation was too dilute. In order to try to determine the cause of the problem, the experiment was repeated using a more concentrated rat brain preparation (homogenising in only two volumes of buffer, rather than four). The results are shown in Table 4.4.

These results confirmed that there was a problem with the kit. On consultation with Progen Industries (agents for Pierce), it was concluded that the reagents had degraded before use. Alex Baker at Progen offered to replace the activator solution and glycogen synthase and provide a small amount of PKC standard in order to determine whether there was a problem with the rat brain preparation.

Only enough PKC standard was supplied for one tube, so an assay was performed as shown in Table 4.5. Although the absorbance values were much lower than expected, PKC activity was detected. The Pierce instruction manual reports that at 0.2U PKC, an absorbance of approximately 0.3 should be observed. In an effort to increase the absorbances, the rat brain was further concentrated and the total buffer wash volumes (500µL) were decreased to 300µL.

Table 4.3: PKC assay results

Tube	Description	Average Absorbance at 595nm
1	Negative control	0.080
2	0 PKC	0.078
3	0.01U PKC	0.074
4	0.02U PKC	0.076
5	0.04U PKC	0.068
6	0.08U PKC	0.066

Table 4.4: PKC assay results (concentrated brain preparation)

Tube	Description	Average Absorbance at 595nm
1	Negative control	0.091
2	rat brain	0.096
3	rat brain 1 in 2 dil	0.096
4	twice the volume of rat brain	0.091

Due to time constraints, only one further assay could be performed. Hence, an experiment was designed to determine whether the Pierce colorimetric PKC assay kit gave positive results using the daphnane toxins as activator in place of the phosphatidylserine
(Table 4.6).

Phorbol esters and mezerein are reported to have a much higher potency than DAG in activating PKC. Disappointingly, the results obtained were all less than those for the negative control.

Table 4.5: PKC assay with PKC standard

Tube	Description	Average absorbance at 595nm
1	Negative control	0.102
2	Zero PKC	0.101
3	0.2U PKC (standard)	0.114
4	rat brain	0.117
5	rat brain 1 in 2 dil	0.109

Table 4.6: PKC assay with various activators

Tube	Description	Average Absorbance at 595nm
1	Negative control	0.112
2	Negative control (DMSO)	0.084
3	Positive control	0.115
4	Positive control (DMSO)	0.101
5	400mg/mL mezerein	0.101
6	15mg/mL mezerein	0.096
7	40mg/mL simplexin	0.097
8	5mg/mL simplexin	0.111
9	400nM C3-reduced simplexin	0.109
10	40nM C3-reduced simplexin	0.105

4.5 Discussion and conclusions

Investigation into the feasibility of using the Pierce Colorimetric PKC Assay Kit for routine analyses of PKC activation by daphnane toxins, has shown that the assay is not a robust one. The reagents had degraded before use, and even with fresh reagents the absorbances were very low ($\simeq$ 0.1).

Further discussion with Progen Industries led us to conclude that a non-radioactive peptag PKC assay kit from Promega should be trialed, as apparently, the kit is known to be robust, reliable and efficient.

For further continuation of this work, a PKC standard is essential for the accurate determination of levels of PKC in rat brain preparations.

5.0 GENERAL DISCUSSION AND FUTURE DIRECTIONS

The competitive PKC radioligand binding assay, described in section 2.3.2, proved to be a reliable, robust and reproducible assay. It can be adopted, with minor adjustments, for routine analyses of plant samples for *Pimelea* toxins.

A common request from producers is an assay to screen changes in toxin concentrations at different stages of the growth cycle of *Pimelea*. The radioligand binding assay allows for quantification of toxin levels in extracts through their displacement of [^{3}H]-PDBu from the PKC receptor in rat brain. Further work in this area could focus on determining toxin levels in plants from different geographical locations. Plant samples used in the present study were from one property in the Roma/Maranoa district. It has been demonstrated that toxin concentrations vary considerably in different parts in the plant obtained from one property.

Pimelea poisoning results from either ingestion of the plant or inhalation or the dried plant debris. Some farmers believe that the plant becomes more toxic as it dries. The plants used in the present study were old, dried plants and it would be of interest to investigate fresh plants to determine when the plant is most toxic.

A further development would be to utilise bovine pulmonary venule tissue as the PKC source in the competitive PKC radioligand binding assay as the pulmonary venule tissue is the primary target of the *Pimelea* toxins. This would involve replacing the rat brain preparation in the assay with a corresponding pulmonary venule preparation.

The structure-binding studies also proved to be informative. The results showed that while the C3 carbonyl and the C5 and C20 hydroxyl groups of mezerein and simplexin are important for binding, the C4 hydroxyl is a critical requirement. Further work in this area could involve identifying microflora that could perform this reductive transformation of daphnane toxins within the rumen. Interestingly, B. Lambert has found that reduction of the C3 carbonyl of simplexin very significantly reduces the efficacy of the compound to contract bovine pulmonary tissue, yet in this study, it was demonstrated that PKC affinity of the reduced compound is only decreased about four-fold.

Another potential future study direction could be the determination of toxin levels in biological tissues, particularly in the reproductive tissues, as this would provide an insight into how *Pimelea* poisoning affects the reproductive performance of surviving cattle. In previous work, it has been shown that synthetic phorbol esters (structurally similar to simplexin) inhibit the *in vitro* maturation (Bornslager *et al.*, 1986a,b) and fertilisation (Endo *et al.*, 1987a,b) of mammalian oocytes.

To the author's knowledge, there is no information in the literature concerning the storage of *Pimelea* toxins within cattle tissues, or on the threshold concentrations required to initiate physiological responses. An interesting study would be to investigate the storage and release of radiolabelled simplexin after ingestion/inhalation, as this could provide valuable information on the half life of elimination of the toxin from animal tissues.

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APPENDIX D

FIELD STUDY WITH AN EXPERIMENTAL VACCINE IN THE MARREE DISTRICT OF SOUTH AUSTRALIA

September - December 1995

1.0 SUMMARY

Plants of the *Pimelea spp* contain compounds that act as toxins in cattle and cause *Pimelea* poisoning. *Pimelea* poisoning in cattle is characterised by clinical diarrhoea, oedemas of the head and brisket, and increases in cardio-pulmonary and systemic blood pressure. Continued exposure of cattle to *Pimelea spp* plants leads to morbidity and/or mortality. An outbreak of *Pimelea* poisoning occurred in the Marree District of South Australia in late-winter (August) of 1995. On four stations, cattle showing varying degrees of *Pimelea* poisoning (diarrhoea, oedemas, poor body condition) were vaccinated with an experimental vaccine against toxins found in *Pimelea spp* plants. Vaccination induced significant anti-toxins antibodies in cattle on all properties, two weeks after secondary vaccination. There were no apparent differences in longitudinal changes in liveweight, general health status, or body condition, between control cattle and cattle vaccinated against *Pimelea* toxins. On one station, a commercial gastric stimulant was used to encourage feeding in a small group of cattle with relatively severe *Pimelea* poisoning. After 14 days of treatment with gastric stimulant, cattle showed increased general activity and, 20 days after the end of treatment, grazed across a wider area compared with a contemporary group of cattle that did not receive gastric stimulant. During the trial, toxin concentrations in *Pimelea trichostachya* were measured using a radioreceptor assay. It was found that significant amounts of toxins were present in flowers during both the growing and senescent stages of the plant life-cycle (September-March). The preliminary conclusion from the present trial is that the experimental vaccine does not facilitate the recovery of cattle already exhibiting *Pimelea* poisoning at the time of vaccination. Treatment with gastric stimulant may enhance recovery from *Pimelea* poisoning and this treatment should be further evaluated in larger groups of cattle.

2.0 INTRODUCTION

2.1 Background

Pimelea poisoning in cattle occurs when animals come in contact with *Pimelea spp* plants [1, 3]. These plants contain a range of toxins [3, 6, 15] which, after ingestion and/or inhalation, cause pathophysiological responses in cattle that include diarrhoea, increased blood pressure, and oedemas of the head and brisket [2]. Continued exposure to *Pimelea* toxins leads to heart, liver and kidney damage, resulting in morbidity and/or mortality [2].

A major site of action of *Pimelea* toxins in cattle are thought to be small veins within the lungs [2, 5, 12]. A unique feature of the smooth muscle layer of bovine pulmonary venules is the presence of specific receptors that bind *Pimelea* toxins. The binding of toxins to these receptors causes a constriction of sphincters in pulmonary venules, which places pressure on the right ventricle of the heart. Continued pressure leads to distension of the right ventricle

and also causes seepage of blood plasma from pulmonary venules into extracellular spaces; the latter contributes to oedema formation in the head and brisket [2]. A secondary increase in systemic blood pressure, together with elevated body temperature, can cause damage to the liver and kidneys [2].

The actions of *Pimelea spp* toxins on bovine pulmonary venules may require ingestion, absorption of toxin into blood, and transport to the lungs via systemic circulation. Alternatively, toxins my be absorbed directly through mucosal layers of pulmonary bronchioles and alveoli after inhalation, rather than absorptive processes that involve ingestion. In addition to actions on pulmonary venules, toxins present in *Pimelea spp* plants may also cause pathophysiological responses in cattle by other mechanisms. For example, clinical diarrhoea, which generally occurs at an early stage in *Pimelea* poisoning (TR Whyte, G Nayyar, G Pegg, MJ D'Occhio, unpublished), may result from ingestion of plant material and actions of *Pimelea* toxins directly on the intestinal mucosa.

2.2 Vaccination against toxins found in *Pimelea spp* plants

The toxins found in *Pimelea spp* plants are not considered to be immunogenic and under normal circumstances cattle do not develop antibodies to these toxins. This likely explains, in part, why cattle do not appear to acquire a resistance to *Pimelea* poisoning. Cattle can, however, be induced to produce antibodies to *Pimelea* toxins. This is achieved when toxins are purified and chemically linked to an immunogenic protein, and the protein-toxins conjugate is administered to cattle in a vaccine formulation [5, 8]. Vaccination of cattle with immunogenic toxins-protein conjugates leads to the production of antibodies against *Pimelea* toxins, and these antibodies are present in systemic circulation of vaccinated cattle. Furthermore, anti-*Pimelea* toxins antibodies purified from the blood of vaccinated cattle were shown recently to neutralise toxins, and prevent their constricting actions on isolated bovine pulmonary venules, *in vitro* [5, 6]. This demonstrated that, in an *in vitro* organ bath system, anti-toxins antibodies bound to toxins and prevented the binding of toxins to receptors on pulmonary venules. These laboratory observations led to the suggestion that anti-toxins antibodies may also bind toxins in circulation and prevent the development of *Pimelea* poisoning in vaccinated cattle [5, 8, 10, 11].

Accordingly, groups of cattle were vaccinated in field trials on stations that had a history of *Pimelea* poisoning in the Maranoa District of Queensland. The strategy in these field trials was to vaccinate cattle before the typical seasonal occurrence of *Pimelea* poisoning. The reason for this strategy was that vaccination was regarded as having more potential as a preventive measure against *Pimelea* poisoning, rather than a treatment for *Pimelea* poisoning. Vaccination resulted in immune responses and production of anti-toxins antibodies in cattle on all properties [MJ D'Occhio, GG Pegg, WJ Aspden, R Newman and D Burton, unpublished]. However, the occurrence of *Pimelea* poisoning was relatively low in these field trials, and it has not been possible to determine whether vaccination of cattle with the experimental vaccine against *Pimelea* toxins affords protection against poisoning.

2.3 Outbreak of *Pimelea* poisoning in the Marree District of South Australia

In late-winter (August) of 1995, *Pimelea* poisoning was reported on a number of stations in the Marree District of South Australia (Table 1.1). In the absence of any proven treatments for *Pimelea* poisoning in cattle [7], it was decided to vaccinate cattle on four stations where

animals were showing varying degrees of *Pimelea* poisoning. It was recognised that vaccination of cattle already showing signs of *Pimelea* poisoning was not the preferred strategy for an immunological approach which, ideally, would be used before poisoning occurred. However, it was considered that vaccination of cattle under the prevailing circumstances in the Marree District would provide information on whether vaccination prevented further progression of *Pimelea* poisoning, and/or facilitated recovery of animals showing signs of *Pimelea* poisoning.

A second strategy used on one station with cattle showing *Pimelea* poisoning was to treat a small number of animals with a gastric stimulant. Continued exposure to *Pimelea* toxins is thought to precipitate rumen stasis, and the gastric stimulant was tested in an effort to stimulate feeding in cattle with advanced *Pimelea* poisoning.

During the course of the trial, *Pimelea trichostachya* plants were collected at approximately monthly intervals on one station. These plants were used to study the distribution of toxins in roots, stems and flowers of *Pimelea spp* plants, and to monitor seasonal changes in toxin concentrations, using a recently developed toxins radioreceptor assay [13, 14].

Table 1.1 Occurrence of *Pimelea* poisoning in the Marree District of South Australia in mid- to late-winter (August-September) 1995.

	Number of affected cattle	Number of dead cattle
Station 1	10	0
Station 2	2	0
Station 3	200	100
Stations 4 and 5	200	200
Station 6	1	0
Station 7	100	30
Station 8	100	90
Station 9	60	40
Total	673	460
Total affected and dead	1,133	

3.0 METHODS

3.1 Immunogenic protein-toxins conjugate

Toxins were extracted from *Pimelea spp* plants using a series of solvent extraction procedures, and further purified by high pressure liquid chromatography (HPLC) [5, 8]. Purified toxins were chemically linked to an immunogenic carrier protein [4] using recently described conjugation chemistries [5, 8].

3.2 Vaccine formulation and vaccination protocol

The protein-toxins conjugate used for vaccination had previously been shown to induce anti-toxins antibodies in cattle that neutralised the actions of toxins on bovine pulmonary venules, *in vitro* [6]. For vaccination, the conjugate was prepared in a water-in-oil emulsion [4] and injected subcutaneous. Two vaccinations, typically 4 weeks apart, are required to induce significant circulating anti-toxins antibodies (WJ Aspden, MJ D'Occhio and GG Pegg, unpublished). On each property, cattle were assigned at random to equal numbers of control or vaccinated animals.

3.3 Vaccination trial

Cattle were vaccinated on 4 stations, designated Stations A to D. On Station A, cattle (n = 100) were primarily Shorthorn x Santa Gertrudis with a small proportion of Hereford x Santa Gertrudis. Entire and gonadectomised males and females were included and ages ranged from 6 months to 4 years. Cattle on Station B (n = 90) were an equal mixture of Hereford, Hereford x Shorthorn and Hereford x Brahman. Animals ranged in ages from 6 months to 4 years and included entire and gonadectomised males and females. The breeds on Station C (n= 124) were predominantly Hereford (60%) with some Hereford x Limousin (30%) and Hereford x Shorthorn (20%); they were calves, heifers and older cows. Cattle on Station D (n = 64) were mainly Hereford (90%) with a small number of Hereford x Brahman (10%); they included calves, heifers and cows.

On Station A and Station B, significant numbers of animals were showing signs of *Pimelea* poisoning, with a proportion of cattle at advanced stages of poisoning. It was decided, therefore, to use a 2-week interval between primary (September 1995) and secondary vaccinations on Stations A and B in an effort to induce significant circulating anti-toxins antibodies in these cattle relatively quickly. Only two animals on Station C had any apparent signs of *Pimelea* poisoning. Relatively few cattle on Station D had *Pimelea* poisoning and it was of reduced severity relative to Stations A and B. The conventional 4-week interval between primary (September 1995) and secondary vaccination was used on Stations C and D.

Blood samples were taken at the time of primary and secondary vaccinations and at various intervals thereafter to measure circulating anti-toxins antibodies.
Liveweight, general health status and body condition were recorded throughout the trial.

3.4 Gastric stimulant trial

A preliminary trial to examine responses to a gastric stimulant of cattle showing clinical signs of *Pimelea* poisoning was carried out on Station A. The gastric stimulant (Gastric Stimulant Powder [60 g sachets], Parnell Labs, Alexandria, NSW) contained strychnine (2.0 g/kg), pulv. zingiber (410 g/kg), pulv. gentian (210 g/kg) and pulv. aloes (25 g/kg). The product is marketed as a general rumenal and intestinal stimulant for use in cattle, horses and sheep.

Six animals on Station A that had similar clinical signs of *Pimelea* poisoning (diarrhoea, oedema, poor body condition) were divided into two equal groups: Group 1, 2 bulls (2 years old) and 1 steer(9 months old); Group 2, 3 bulls (2-3 years old). Animals in Group 1 served as controls and were fed hay together with a daily ration of 500 g stablemate, 250 g oat chaff and 180 ml molasses. Animals in Group 2 received the same feed and ration, and also approximately 80 g/day of gastric stimulant (160 mg strychnine, 33 g zingiber, 16 g gentian, 2 g aloes); this was dissolved in warm water and mixed with molasses to form a slurry that was added to the ration. General observations on the condition of the animals were made at the end of a 14-day treatment period, and again after approximately 20 days.

3.5 Content of toxins in *Pimelea trichostachya*

The distribution of toxins in roots, stems and flowers of *Pimelea trichostachya*, and longitudinal changes in content of toxins, were determined in plants collected from Station A, on Day 0 (September 1995), and at approximately monthly intervals thereafter. Levels of toxins were measured using a recently validated membrane protein kinase C (PKC) radioreceptor assay, that uses rat brain as a source of membrane PKC receptors [13, 14]. In brief, toxins were extracted from plant tissue and graded aliquots of purified toxins were used to displace binding of radiolabelled [20(n)-^{3}H]phorbol-12,13-dibutyrate to membrane PKC receptor [13, 14]. Displacement curves were used to determine IC_{50} values (concentration of toxin required to displace 50% of [^{3}H]phorbol-12,13-dibutyrate from rat membrane PKC receptors) for each sample. Results were calculated as percentage weight of toxins/weight of plant material (% w/w), and expressed as % w/w equivalent of the purified toxin, simplexin.

3.5 Statistical analyses

Data for levels of anti-toxins antibodies in circulation and liveweight were analysed by analysis of variance procedures using the General Linear Models (GLM) procedure of SAS/STAT [16]. Results are presented as means ± SEM, unless otherwise indicated.

4.0 RESULTS

4.1 Responses to vaccination

4.1.1 Circulating anti-toxins antibodies

Results for circulating anti-toxins antibodies after primary and secondary vaccinations are shown in Figure 3.1 and results summarised in Table 3.1. Cattle on Stations A and B had circulating anti-toxins antibodies 2 weeks after primary vaccination (time of secondary vaccination), and there were no differences between the two stations. After secondary vaccination at 2 weeks, cattle on Stations A and B showed a further increase in circulating antibodies and again there were no differences between the two stations (Table 3.1).

Cattle on Stations C and D had significant and similar levels of anti-toxins antibodies 4 weeks after primary vaccination (time of secondary vaccination) (Figure 3.1). Levels of circulating antibodies at the time of secondary vaccination were greater ($P < 0.05$) for cattle on Stations C and D compared with cattle on Stations A and B (Table 3.1). This may have been due to the interval of 4 weeks between primary and secondary vaccination for cattle on Stations C and D, compared with 2 weeks for cattle on Stations A and B. It had been shown in previous studies that levels of antibodies in circulation tend to increase gradually after primary vaccination (WJ Aspden, GG Pegg, MJ D'Occhio, unpublished). Differences in levels of antibodies between cattle on Stations A and B compared with cattle on Stations C and D at the time of secondary vaccination may also have been related to the general health status of cattle at the time of primary vaccination, as a greater proportion of cattle on Stations A and B showed significant signs of *Pimelea* poisoning at the time of primary vaccination. A reduced health status can compromise immunological responses to vaccination in cattle.

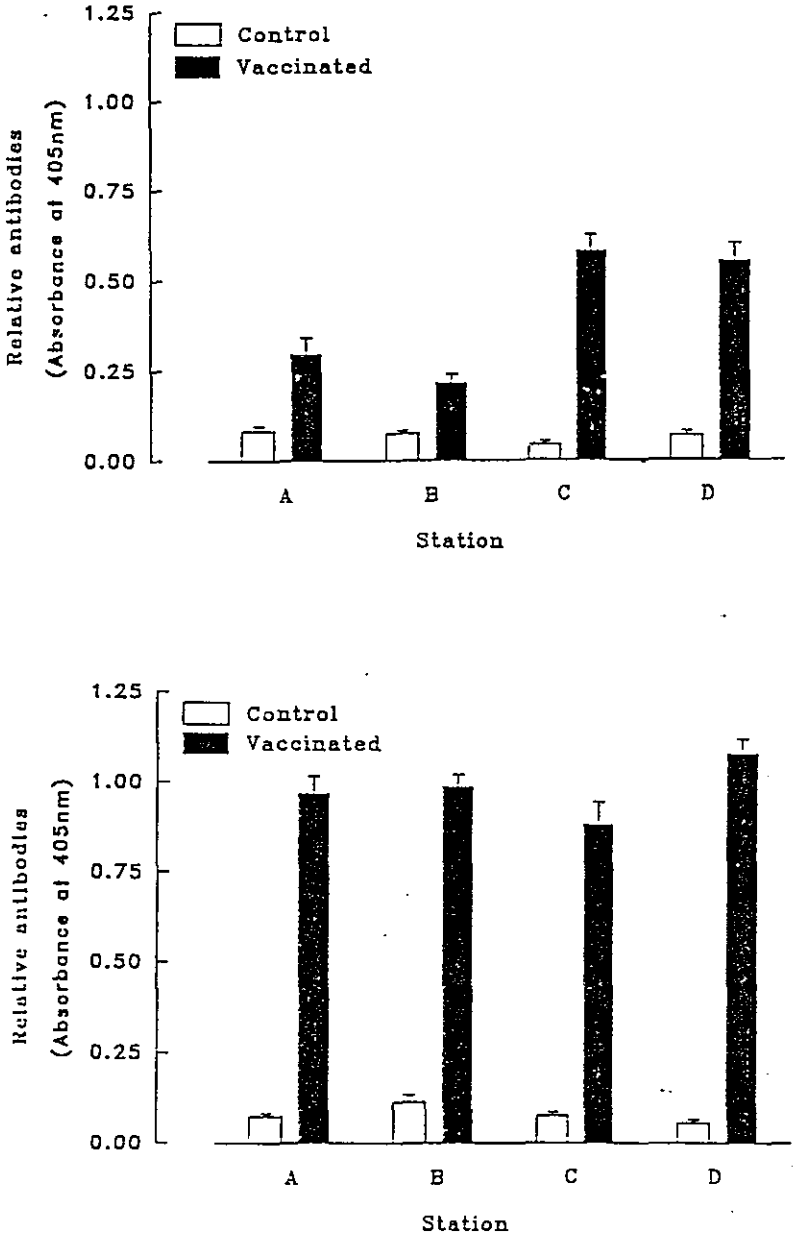

Figure 3.1 Circulating anti-toxins antibodies in cattle vaccinated against *Pimelea* toxins. Levels of antibodies are expressed as relative absorbances at 405 nm as determined by ELISA assay. Results are for levels of antibodies after primary vaccination (top panel) and secondary vaccination (bottom panel), and are presented as means ± SEM. Details on intervals between primary and secondary vaccinations are provided in Materials and Methods.

Table 3.1 Circulating anti-toxins antibodies after primary and secondary vaccinations. For cattle on Stations A and B, antibodies were determined 2 weeks after primary vaccination and for cattle on Stations C and D, 4 weeks after primary vaccination. Antibodies after secondary vaccination were determined at 2 weeks for all cattle. Results are presented as means ± SEM for relative absorbances at 405 nm.

	Absorbance at 405 nm	
	Post-primary vaccination	Post-secondary vaccination
Station A	$0.28 \pm 0.05^{a,y}$	$0.97 \pm 0.05^{b,y}$
Station B	$0.18 \pm 0.02^{a,y}$	$0.99 \pm 0.03^{b,y}$
Station C	$0.61 \pm 0.04^{a,z}$	$0.88 \pm 0.06^{b,y}$
Station D	$0.58 \pm 0.05^{a,z}$	$1.08 \pm 0.04^{b,y}$

^{a,b} means within rows without a common superscript differ ($P < 0.05$)
^{y,z} means within columns without a common superscript differ ($P < 0.05$)

Significant further increases in circulating anti-toxins antibodies occurred in cattle on Stations C and D after secondary vaccination at 4 weeks (Figure 3.1). There were no differences between cattle on Stations A and B (2-week vaccination interval) and cattle on Stations C and D (4-week vaccination interval) in levels of anti-toxins antibodies after secondary vaccination (Table 3.1).

On Station B, cattle showed a decline ($P < 0.05$) in circulating anti-toxins antibodies between 2 and 24 weeks after secondary vaccination (Figure 3.2). However, these cattle still had significant ($P < 0.05$) antibodies at 24 weeks, and it would be anticipated that further vaccination would return circulating antibodies to levels similar to those observed after secondary vaccination.

4.1.2 Changes in liveweight

Longitudinal profiles for changes in liveweight during the trial are shown in Figure 3.3. On Station A, both control and vaccinated cattle showed significant ($P = 0.001$) declines in liveweight between primary and secondary vaccination (- 20.3 ± 2.8 and - 16.0 ± 2.6 kg, respectively; $P = 0.26$). Liveweight then increased in both groups of cattle and remained relatively constant for the duration of the trial. There were no differences ($P = 0.41$) throughout the trial in changes in liveweight between control and vaccinated cattle on Station A. Cattle on Station B also showed a decline in liveweight between primary and secondary vaccinations (control, - 20.9 ± 4.9 kg; vaccinated, - 14.3 ± 3.8 kg) ($P = 0.26$). Liveweight for cattle on Station B increased from the time of secondary vaccination (Day 15) to Day 80 of the trial, and there were no differences ($P = 0.17$) in changes in liveweight between control (42.8 ± 5.7 kg) and vaccinated cattle (33.5 ± 3.6 kg). On Property C, control and vaccinated cattle did not differ in liveweight and, for both groups, liveweight remained relatively constant throughout the trial. Property C had only 2 recorded cases of *Pimelea* poisoning on Day 0. The apparent decline in liveweight between primary and secondary vaccination for cattle on Station D was due to the absence on Day 30 of relatively heavy control and vaccinated cattle. If these cattle are also excluded from data for Day 0, then the changes in liveweight between Day 0 and Day 30 were: control, - 3.8 ± 6.7 kg; vaccinated, - 7.0 ± 5.6 kg. Liveweights for cattle on Station D increased from Day 30 to Day 80 of the trial. Combined liveweight data for all stations for control and vaccinated cattle, respectively, are shown in Figure 3.3. The only outstanding feature of the combined data was a decline in liveweight between Day 0 and Day 15 of the trial. However, the data for Day 15 were contributed only by cattle on Stations A and B, which were the two stations with most cases of *Pimelea* poisoning. After Day 15, combined data for liveweight remained relatively constant up to Day 80 of the trial and there were no differences between control and vaccinated cattle (Figure 3.3).

4.1.3 Health status and body condition

The major indication of *Pimelea* poisoning on Day 0 for cattle on Station A (Table 3.2) and Station B (Table 3.3) was clinical diarrhoea. On Station B, for which there was the most complete data, the incidence of diarrhoea showed a significant ($P < 0.05$) decline between Day 0 and Day 15, for both control and vaccinated cattle (Table 3.3). From Day 15 to Day 50, the occurrence of clinical diarrhoea ranged from 5-10 % for control and vaccinated cattle on Stations A and B. There were only 2 observed cases of clinical diarrhoea on Day 0 for cattle on Station C (Table 3.4), and none for Station D (Table 3.5).

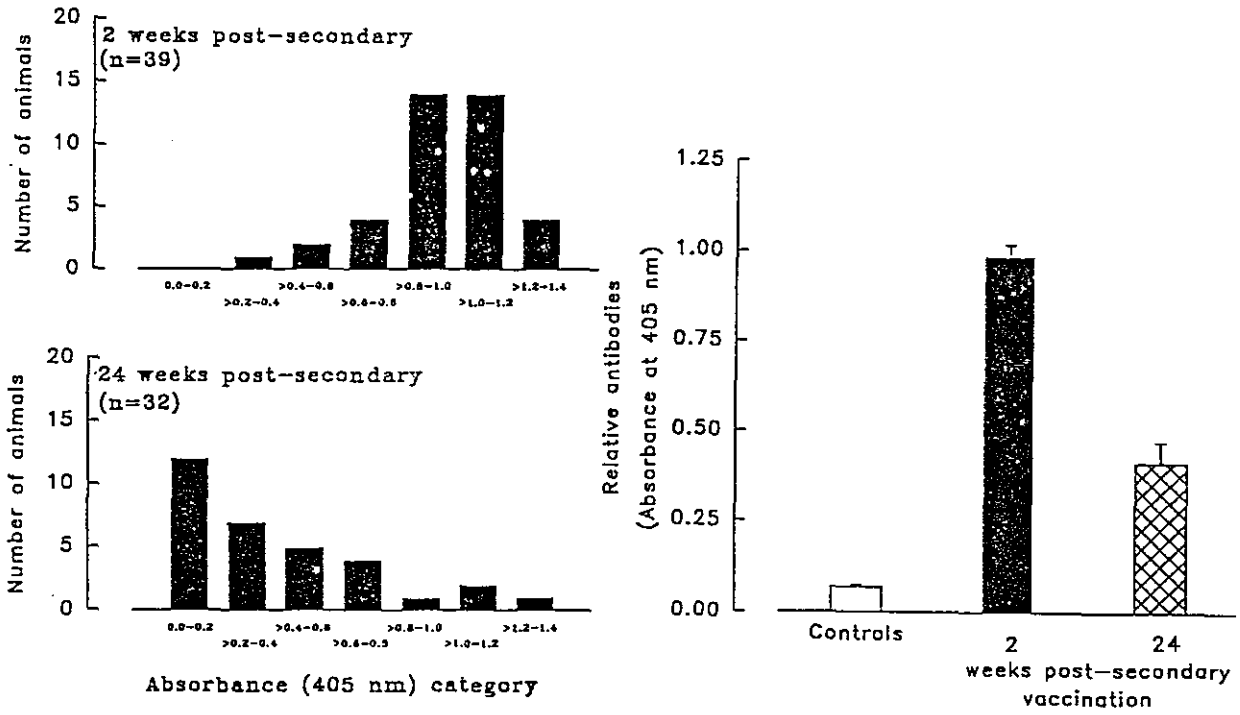

Figure 3.2 Relative distributions of circulating anti-toxins antibodies for cattle on Station B, 2 weeks and 24 weeks after secondary vaccination (left panel). Results are summarised in the right panel and are presented as means + SEM.

The proportion of cattle with an apparent oedema of the lower jaw and/or brisket was relatively low on all stations on Day 0 (Tables 3.2 to 3.5). However, cattle on Station A had signs of regressed oedemas. On Station A (Table 3.2) and Station B (Table 3.3) there was an increase ($P < 0.05$) between Day 0 and Day 15 in the number of cattle with an oedema, and there were no apparent differences between control and vaccinated cattle. An increase in the number of cattle with an oedema did not occur on Station C (Table 3.4) and there was only a relatively minor increase on Station D (Table 3.5).

There were no apparent differences between Day 0 and Day 80 in general health status between control and vaccinated cattle on all stations (Tables 3.2 to 3.5). On Station A, 6/50 control cattle and 10/50 vaccinated cattle ($P > 0.05$) died during the course of the trial, with most cattle dying between Day 0 and Day 15. On Day 0, cattle on Station A were transferred to a paddock that did not have significant growth of *Pimelea spp* plants.

On day 80, control and vaccinated cattle did not differ in the relative distributions of body condition scores (Tables 3.2 to 3.4).

Anecdotal information for Station B suggested that vaccinated cattle showed increased grazing and general activity earlier in the trial compared with control cattle. However, this was not reflected in the objective measurements of liveweight, general status and body condition discussed above (Table 3.3).

4.2 Responses to gastric stimulant

The general status of control cattle and cattle treated with gastric stimulant is summarised in Table 3.6. After 14 days of treatment, cattle that received gastric stimulant had regressed oedemas and showed increased general activity compared with control cattle. Oedemas had also regressed for control cattle on Day 14, but these cattle still had a "rough" coat. On Day 20 after treatment, cattle that had received gastric stimulant appeared more active and grazed across a wider area compared with cattle that had served as controls.

4.3 Toxin content in *Pimelea trichostachya*

Concentrations of toxins in *Pimelea trichostachya* are shown in Figure 3.4. In September 1995, the concentration of toxins was highest in flowers (0.096% w/w equivalent of pure simplexin) and lowest in roots (0.016% w/w simplexin). Toxins continued to be present in flowers from September 1995 to March 1996.

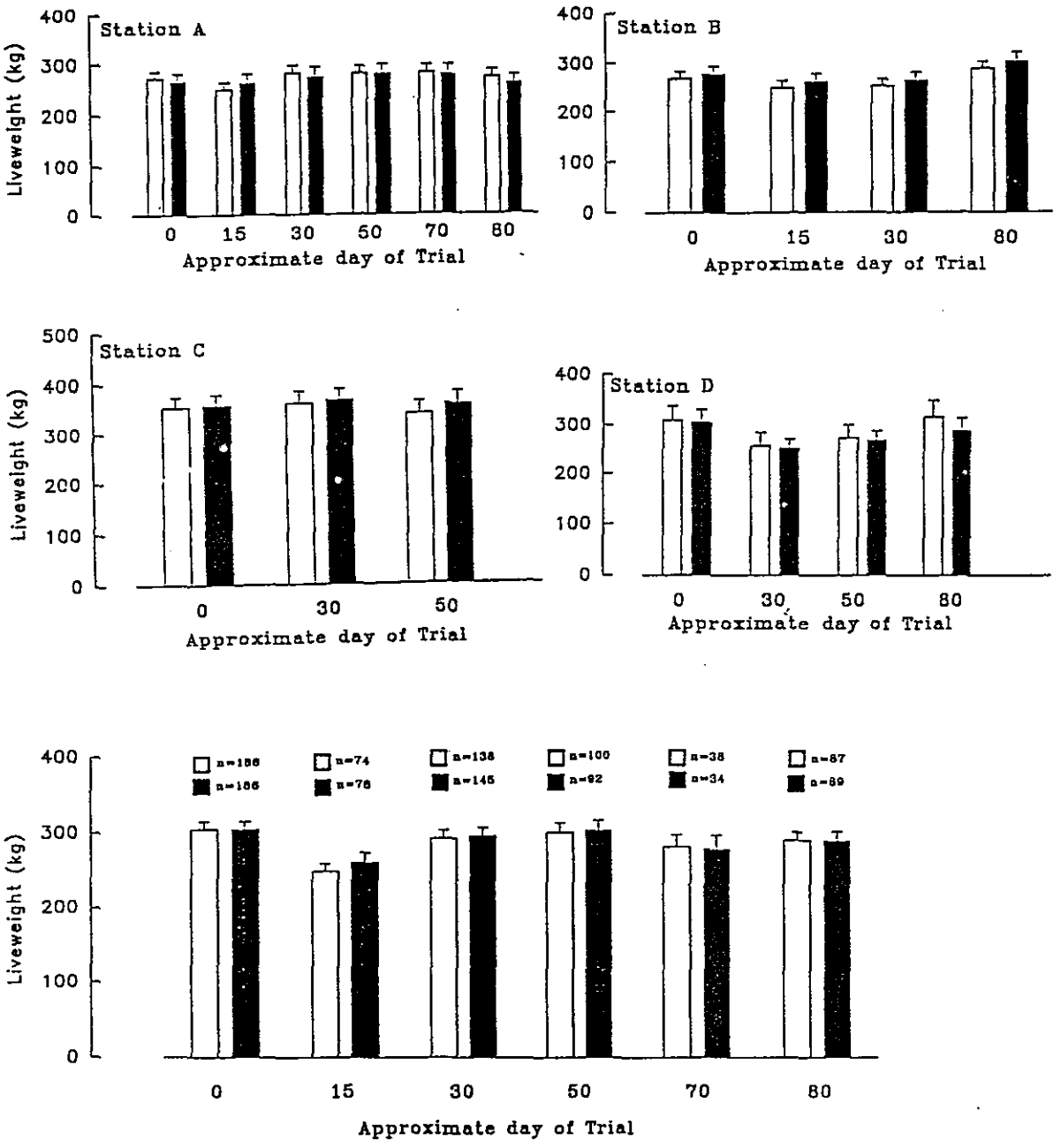

Figure 3.3 Longitudinal changes in liveweight for control cattle (open bars) and cattle vaccinated with the experimental *Pimelea* vaccine (closed bars) on Stations A to D. Combined data are shown in the bottom panel. Results are means + SEM; it should be noted that data for different days are contributed by different numbers of cattle, both within Stations and for combined data.

Table 3.2 Health status and body condition of control and vaccinated cattle on Station A.

	CONTROL			VACCINATED		
Clinical diarrhoea						
Day 0	not recorded for individual cattle					
Day 15	5/50			7/50		
Day 30	5/50			8/50		
Day 50	17/50			16/50		
Oedema[¶]	**SO**	**MO**	**HO**	**SO**	**MO**	**HO**
Day 0	0	2	0	0	2	0
Day 15	6	3	2	6	0	2
Day 30	7	3	3	4	3	3
Day 50	1	4	1	2	6	0

General status[§]	**VP**	**P**	**F**	**G**	**NC**	**VP**	**P**	**F**	**G**	**NC**
Day 0	0	0	1	0	47	2	0	0	0	45
Day 15	0	13	18	9	7	0	0	11	8	5
Day 30	0	23	10	4	11	0	20	10	3	16
Day 50	0	1	0	18	11	0	2	0	12	16

Body condition score on Day 50[‡]	**1**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**
Control (n = 39)	15	1	21	2	2	0	0
Vaccinated (n = 34)	9	1	23	0	1	0	0

[¶] SO, slight oedema; MO, medium oedema; HO, high oedema
[§] VP, very poor; P, poor; F, fair; G, good/clean; NC, no comment
[‡] Scale 1-9

Table 3.3 Health status and body condition of control and vaccinated cattle on Station B.

	CONTROL					VACCINATED				
Clinical diarrhoea										
Day 0	14/45					13/45				
Day 15	2/45					1/45				
Day 30	0/45					3/45				
Day 50	5/45					6/45				
Oedema[¶]	**SO**	**MO**	**HO**			**SO**	**MO**	**HO**		
Day 0	0	2	0			0	2	0		
Day 15	7	1	0			8	1	2		
Day 30	6	1	0			4	1	3		
Day 50	1	4	0			2	7	0		
General status[§]	**VP**	**P**	**F**	**G**	**NC**	**VP**	**P**	**F**	**G**	**NC**
Day 0	2	4	0	0	23	2	5	0	0	25
Day 15	0	4	0	26	4	0	5	0	29	4
Day 30	0	11	9	15	6	0	16	6	16	4
Day 50	0	1	0	26	6	0	1	0	23	6

Body condition score on Day 50[‡]	**1**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**	**4.0**
Control (n = 37)	7	1	13	2	12	1	1
Vaccinated (n = 39)	9	3	13	3	8	3	0

[¶] SO, slight oedema; MO, medium oedema; HO, high oedema
[§] VP, very poor; P, poor; F, fair; G, good/clean; NC, no comment
[‡] Scale 1-9

Table 3.4 Health status and body condition of control and vaccinated cattle on Station C.

	CONTROL							VACCINATED						
Clinical diarrhoea														
Day 0	1/63							1/62						
Day 30	0/63							0/63						
Day 50	2/63							1/62						
Oedema[¶]	**SO**		**MO**		**HO**			**SO**		**MO**		**HO**		
Day 0	0		0		0			0		1		0		
Day 30	0		0		0			0		0		0		
Day 50	1		1		0			0		2		0		
General status[§]	**VP**	**P**	**F**	**G**	**NC**			**VP**	**P**	**F**	**G**	**NC**		
Day 0	0	0	0	0	63			0	0	0	0	62		
Day 30	0	1	12	29	21			0	0	10	35	17		
Day 50	0	0	0	31	27			0	0	0	29	30		
Body condition score on Day 50[‡]	**1**		**1.5**		**2.0**	**2.5**	**3.0**	**3.5**		**4.0**				
Control (n = 36)	0		1		7	6	19	0		3				
Vaccinated (n = 30)	0		1		4	4	14	3		4				

[¶] SO, slight oedema; MO, medium oedema; HO, high oedema
[§] VP, very poor; P, poor; F, fair; G, good/clean; NC, no comment
[‡] Scale 1-9

Table 3.5 Health status and body condition of control and vaccinated cattle on Station D.

	CONTROL					VACCINATED				
Clinical diarrhoea										
	not recorded									
Oedema[¶]	**SO**		**MO**		**HO**	**SO**		**MO**		**HO**
Day 0	1		0		0	0		1		0
Day 30	2		0		0	5		0		0
Day 50	0		0		0	0		2		0
General status[§]	**VP**	**P**	**F**	**G**	**NC**	**VP**	**P**	**F**	**G**	**NC**
Day 0	0	3	0	0	22	2	3	0	0	24
Day 30	0	5	10	10	7	0	7	11	7	8
Day 50	0	0	13	13	6	0	3	11	11	7

[¶] SO, slight oedema; MO, medium oedema; HO, high oedema
[§] VP, very poor; P, poor; F, fair; G, good/clean; NC, no comment

Table 3.6 Characteristics of cattle with severe *Pimelea* poisoning that received supplementary feed for 14 days (Group 1, n = 3), or supplemetary feed + gastric stimulant for 14 days (Group 2, n = 3). Results are for condition at the start of the trial (Day 0), after 14 days of treatment (Day 14) and approximately 20 days after the end of treatment (Day 20).

	Group 1	Group 2
Day 0	**Animal 1**: PC[¶], diarrhoea, severe oedema **Animal 2**: PC, diarrhoea, oedema **Animal 3**: PC, diarrhoea, oedema	**Animal 1**: PC, diarrhoea, oedema **Animal 2**: PC, diarrhoea, severe oedema **Animal 3**: PC, diarrhoea, severe oedema
Day 14	**Animal 1**: residual oedema in brisket **Animal 2**: PC, no oedema, rough coat **Animal 3**: PC, no oedema, rough coat	**Animal 1**: no oedema, alert, "shiny" coat **Animal 2**: no oedema, alert **Animal 3**: no oedema, alert
Day 20	No oedemas; condition poor but improved; cattle not moving far from dam to graze	No oedemas; condition poor to fair; "shiny" coat; alert; moving away from dam to graze

[¶] PC, Poor condition

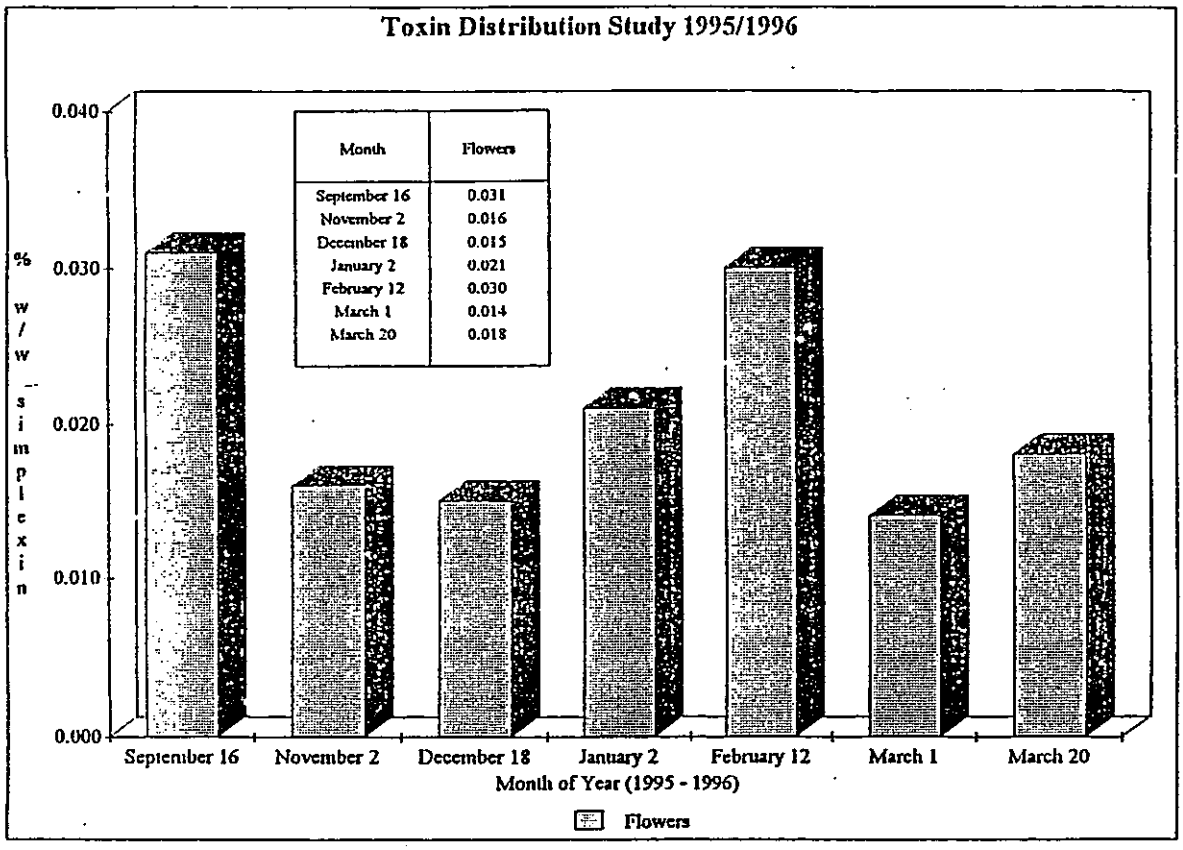

Figure 3.4 Longitudinal changes in concentrations of toxins in *Pimelea trichostachya* from September 1995 to March 1996 on Station A.

5.0 DISCUSSION

Vaccination of cattle against toxins found in *Pimelea spp* plants is being examined as a strategy for preventing the development of *Pimelea* poisoning in cattle that come in contact with these plants. The rationale for vaccination is that anti-toxins antibodies present in circulation should recognise and bind toxins, thereby preventing toxins from binding to target tissues and inducing pathophysiological consequences. The underlying assumption of the vaccination approach is that a major requirement for the occurrence of *Pimelea* poisoning in cattle is the absorption of toxins into blood and transport of toxins to target tissues by systemic circulation. This requirement has not been conclusively demonstrated as obligatory for all forms of *Pimelea* poisoning. Hence, the vaccination strategy is still conceptual and experimental, and it also remains untested under conditions of field exposure to *Pimelea* toxins.

The outbreak of *Pimelea* poisoning in the Marree District of South Australia in late-winter (August) of 1995 provided the opportunity to test an experimental *Pimelea* vaccine under field conditions. However, cattle were already showing signs of *Pimelea* poisoning at the time of vaccination, and these were not the preferred circumstances for testing the experimental vaccine. As discussed above, vaccination is being examined as a preventive strategy, rather than treatment, against *Pimelea* poisoning. In the absence of any treatment for *Pimelea* poisoning, it was determined that vaccination would nevertheless provide important information on whether this approach may prevent progression of the condition and/or facilitate the recovery of cattle with poisoning.

Vaccination against *Pimelea* toxins induced significant and consistent responses in anti-toxins antibodies in cattle on all stations, and maximal circulating antibodies after secondary vaccination were similar after a 2-week and 4-week interval between primary and secondary vaccinations. It should be noted, however, that antibody titres in the present study were determined at a plasma dilution of 1:500. It is possible that differences in maximal antibody titres may be observed at higher plasma dilutions.

There were no apparent longitudinal differences in liveweight, general health status or body condition between control cattle and cattle vaccinated against *Pimelea* toxins. A preliminary conclusion, therefore, is that vaccination of cattle showing *Pimelea* poisoning does not facilitate the rate of recovery from poisoning. It has not been established whether circulating anti-toxins antibodies actually bind toxins in circulation. This will require extensive studies on bound-to-free ratios of toxins in circulation of control and vaccinated cattle. It is possible that antibodies may bind toxins in circulation, but toxins are subsequently returned to the circulation during the processing of antibody-toxins complexes by macrophage cells and/or the liver. If this process occurs, it could establish a re-cycling of toxins in affected cattle.

Treatment of cattle showing *Pimelea* poisoning with a commercial gastric stimulant appeared to improve the general health status of cattle and the rate of recovery from poisoning. In a more recent trial (April 1996), gastric stimulant was reported to stimulate feeding in cattle with severe *Pimelea* poisoning in the Cobar District of NSW (N Dunn and L Dunn, personal communication). The findings in these two trials indicated that stimulation of feeding is important in cattle with *Pimelea* poisoning. Overcoming rumen stasis may facilitate the clearance of toxins either present in the rumen from where they may

continue to be absorbed, or toxins present in systemic circulation and other body tissues. However, both of the above trials involved relatively small numbers of cattle and the observations were anecdotal. Gastric stimulation, whilst providing positive findings on two stations, should therefore be tested in larger numbers of cattle with *Pimelea* poisoning.

The major indication of *Pimelea* poisoning at the start of this trial (Day 0) was clinical diarrhoea, and oedemas were first observed in significant numbers in the same cattle on Day 15. These observations were consistent with findings in laboratory studies where diarrhoea was also the first clinical sign of poisoning after exposure to *Pimelea spp* plant material, and this was followed by development of oedemas (TR Whyte, G Nayyar, GG Pegg and MJ D'Occhio, unpublished). It is possible that the relatively early occurrence of clinical diarrhoea results from a direct action(s) of *Pimelea* toxins on the intestinal mucosa, whilst the development of visual oedemas, which result from progressive seepage of blood plasma from vessels of the cardio-pulmonary circulation, occurs over some time.

Whilst the development of visual oedemas may occur over a period of time, it appears that oedemas can regress relatively quickly after the discontinuation of exposure to *Pimelea* plants. This was demonstrated in the present study by the significant regression of oedemas over 14 days in the gastric stimulant trial. In the gastric stimulant trial, supplementary feeding and re-alimentation may, however, have facilitated the clearance of *Pimelea* toxins from affected cattle relative to the rate of clearance of toxins under typical pasture conditions. The role of gastric stimulation in clearance of toxins from cattle and aiding in recovery from *Pimelea* poisoning is an important area that warrants further study. Removal of cattle from areas infested with *Pimelea spp* plants and transfer to areas with significant pasture (with and without supplementary feeding) has previously been shown to facilitate a relatively quick initial reversal of *Pimelea* poisoning (G Kruger, personal communication). In cases of severe oedemas of the head and jaw which prevent proper feeding, re-alimentation with gastric stimulation and supplementary feeding may be critical in achieving rapid regression of the oedema to allow normal feeding.

Susceptibility to *Pimelea* poisoning appears not to be related to breed, since in the present trial poisoning occurred in Hereford (*Bos indicus*), Shorthorn, Santa Gertrudis (*Bos taurus* x *Bos indicus*) and Hereford x Brahman (*Bos indicus*) cattle. Environmental conditions seem to be the major determinant of whether *Pimelea* poisoning will occur. In particular, rain in mid- to late-winter which is sufficient to stimulate significant growth of *Pimelea spp* plants, but not other pasture species, creates the circumstances for outbreaks of *Pimelea* poisoning. In this regard, management of pastures to ensure a continuation of an appropriate pasture density, may need to be considered in areas prone to outbreaks of *Pimelea* poisoning.

Generally, only a proportion of cattle in a herd are affected by *Pimelea* poisoning. It will be important to determine whether: (1) some cattle can discriminate *Pimelea spp* plant material and avoid ingestion/inhalation; (2) cattle differ in their capacity to metabolise *Pimelea* toxins to inactive end products either in the rumen, systemically, or other body tissues; and (3) cattle differ in their sensitivity to *Pimelea* toxins. The identification of individual cattle that efficiently inactivate *Pimelea* toxins, and/or have decreased sensitivity to toxins, would have important implications for breeding strategies in areas prone to *Pimelea* poisoning.

The first measurements of toxins in *Pimelea spp* plants was achieved in this trial. The highest concentrations of toxins were present in flowers, and toxins persisted during the

growing and senescent stages of the life-cycle of *Pimelea trichostachya*. It is likely that toxins are also present in flowers when the flowers fall off plants. Hence, *Pimelea* poisoning may occur by inadvertent ingestion/inhalation of residual dry *Pimelea* plant material present on the ground.

In summary, vaccination of cattle with an experimental vaccine against toxins present in *Pimelea spp* plants did not facilitate the recovery of cattle showing signs of *Pimelea* poisoning at the time of vaccination. Treatment of cattle showing advanced signs of *Pimelea* poisoning with a gastric stimulant appeared to improve the general health status of cattle. Encouraging feeding may be important in aiding the general clearance of toxins from the body of cattle with *Pimelea* poisoning, and the use of gastric stimulants and supplementary feeding should be further investigated. Other treatments that neutralise toxins, displace toxins from PKC receptors on bovine pulmonary venules [9, 12], or generally facilitate the clearance of toxins from cattle, should also be studied. The development of efficient protocols for treating cattle with *Pimelea* poisoning, rather than protocols that prevent poisoning, might be the preferred longer-term strategy since outbreaks of *Pimelea* poisoning appear not to be predictable, and significant poisoning generally only occurs in a proportion of cattle in a herd.

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APPENDIX E

FURTHER FIELD STUDIES ON PIMELEA POISONING IN CATTLE: TESTING OF AN EXPERIMENTAL PIMELEA VACCINE, GASTRIC STIMULANT AND EXPERIMENTAL ANTIDOTE

March - August 1996

1.0 SUMMARY

Pimelea poisoning occurs in cattle when animals come in contact with *Pimelea spp* plants. At present, there are no veterinary treatments that either prevent *Pimelea* poisoning, or aid in the recovery of cattle from *Pimelea* poisoning.

A trial was conducted in the Marree District of South Australia to evaluate both an experimental vaccine aimed at preventing *Pimelea* poisoning, and two potential treatments for cattle suffering from *Pimelea* poisoning. The trial comprised two Phases:

(i) Phase I: Cattle not previously exposed to *Pimelea spp* plants were vaccinated with the experimental vaccinated and were then exposed to pastures containing *Pimelea spp* plants; and

(ii) Phase II: Cattle with *Pimelea* poisoning were treated with Gastric Stimulant Powder or an antidote against Annual Ryegrass Toxicity (ARGT-Antidote) to determine if either treatment facilitated recovery from *Pimelea* poisoning.

Cattle vaccinated with the experimental vaccine developed significant anti-*Pimelea* toxins antibodies in circulation. Six weeks after exposure to *Pimelea spp* plants, significantly fewer vaccinated cattle had ongoing clinical diarrhoea compared with unvaccinated contemporary cattle. However, vaccinated cattle has similar occurrences of oedemas of the head and brisket as unvaccinated cattle. It was concluded, therefore, that vaccination with the experimental *Pimelea* vaccine did not reduce the susceptibility to *Pimelea* poisoning in cattle.

Treatment of cattle showing *Pimelea* poisoning with Gastric Stimulant Powder was associated with a greater acute positive change in liveweight compared with untreated cattle showing *Pimelea* poisoning. The significance of this observation was that encouragment of re-alimentation is important in aiding the recovery from *Pimelea* poisoning of emaciated cattle with possible rumen stasis. Treatment with the ARGT-Antidote has no apparent effects on acute liveweight change in cattle with *Pimelea* poisoning.

In summary, vaccination with the experimental *Pimelea* vaccine did not reduce susceptibility to *Pimelea* poisoning in cattle. Gastric Stimulant Powder would appear to have utility in aiding the recovery of cattle from *Pimelea* poisoning and should be further evaluated.

2.0 INTRODUCTION

2.1 Background

Pimelea poisoning of cattle occurs when animals come in contact with *Pimelea spp* plants [1, 4]. These plants contain a range of toxins [4, 7, 15, 16, 18] which, after ingestion and/or inhalation, cause pathophysiological responses in cattle that include diarrhoea, increased cadio-pulmonary blood pressure, and oedemas of the head and brisket [2]. Continued exposure to *Pimelea* toxins leads to an increase in systemic blood pressure,

potentially resulting in heart, liver and kidney damage. Cattle can become emaciated with resulting morbidity and/or mortality [2, 8].

A major target tissue for *Pimelea* toxins in cattle are small veins within the lungs [2, 6, 10, 14]. A feature of these pulmonary venules that is unique to cattle is the presence of specific receptors to which *Pimelea* toxins bind. The binding of toxins to these receptors induces a constriction of sphincters in the pulmonary venules, which places pressure on the right ventricle of the heart [2]. Over time, this pressure leads to a distension of the right ventricle and also causes seepage of blood plasma from pulmonary venules into surrounding tissue. The latter contributes to the development of oedemas in the head and brisket of cattle with *Pimelea* poisoning [2]. A secondary increase in systemic blood pressure, together with elevated body temperature, can cause permanent damage to the liver and kidneys [2].

The various pathophysiological responses in cattle to *Pimelea* toxins may occur as a result of different mechanisms of action of the toxins. For example, clinical diarrhoea, which tends to be an early symptom of *Pimelea* poisoning [TR Whyte, G Nayyar, G Pegg and MJ D'Occhio, unpublished results], may result from direct effects of toxins on epithelial cells of the intestinal mucosa. The effects of toxins on pulmonary venules could result either from direct absorption of toxins through the mucosal tissues of the lungs, or may require uptake of toxins into the blood, and subsequent transport of toxins to pulmonary venules in the lungs by systemic circulation.

The absorption of toxins into the systemic circulation is thought to be a common mechanism of poisoning associated with a variety of plant and other toxins [11, 17, 20], and is considered to also occur in *Pimelea* poisoning. This led to the suggestion that cattle might be vaccinated against toxins found in *Pimelea spp* plants [6, 7, 9]. Vaccinated cattle would produce anti-*Pimelea* toxins antibodies that would be present in circulation, and which would bind absorbed toxins and thereby prevent toxins from acting on target tissues (e.g. pulmonary venules). Vaccinated cattle may, therefore, have a reduced susceptibility to *Pimelea* poisoning. To develop an experimental *Pimelea* vaccine, toxins were purified from plant material, conjugated to a protein that was immunogenic in cattle [5, 6], and the toxins-protein conjugate was then formulated into a vaccine [5].

Cattle vaccinated with the *Pimelea* toxins-protein conjugate produced anti-*Pimelea* toxins antibodies which were present in systemic circulation [6, 7, 9, 13]. Additionally, anti-*Pimelea* toxins antibodies purified from the blood of vaccinated cattle attenuated the typical constricting effects of purified toxins on isolated bovine pulmonary venules in organ bath studies in the laboratory [6, 7]. This demonstrated that anti-*Pimelea* toxins antibodies recognised and bound toxins, and also had a neutralising action *in vitro* [6, 7].

The first opportunity to evaluate the experimental *Pimelea* vaccine was in cattle in the Marree District of South Australia that were showing *Pimelea* poisoning. Groups of cattle on four stations were vaccinated and monitored for recovery from poisoning. It was found that vaccination had no apparent beneficial effect on the rate of recovery of cattle from *Pimelea* poisoning [3].

However, vaccination against toxins found in *Pimelea spp* plants is intended as a strategy to prevent the occurrence of *Pimelea* poisoning, rather than as a treatment for cattle

already showing poisoning. Hence, the vaccine was not appropriately evaluated in the above trial.

2.2 Objectives in the present study

The first objective in the present study was to determine whether vaccination of cattle not previously exposed to *Pimelea* toxins with the experimental *Pimelea* vaccine would reduce the susceptibility of cattle to *Pimelea* poisoning.

A second objective was to examine potential treatments that might aid in the recovery of cattle showing *Pimelea* poisoning. A common feature of cattle with *Pimelea* poisoning appears to be a loss of appetite. A commercial product, Gastric Stimulant Powder (Parnell Laboratories (Aust) Pty Ltd, Alexandria, NSW), acts as a general rumenal and intestinal stimulant and, in preliminary trials, appeared to increase appetite and the rate of recovery of cattle with *Pimelea* poisoning [3; L Litchfield, L Dunn, R Newman and MJ D'Occhio, unpublished results]. In the present study, Gastric Stimulant Powder was further tested in cattle showing *Pimelea* poisoning from Phase I of the study. A second technology to be tested was the use of an experimental antidote. In recent studies, treatment with an antidote was found to enhance the likelihood of recovery in cattle suffering from Annual Ryegrass Toxicity [J. Edgar, unpublished results]. The toxins that cause Annual Ryegrass Toxicity have chemical features that are similar to the chemical properties of toxins found in *Pimelea spp* plants. Both groups of toxins are essentially water-insoluble and have other features that enable them to bind to membranes in various tissues. The antidote used in Annual Ryegrass Toxicity (ARGT-Antidote) is thought to act by binding to toxins and displacing them from membranes; additionally, the antidote-toxin complex is rendered water soluble which enhances the clearance of toxin through the kidneys. The ARGT-Antidote was, therefore, tested for its ability to also facilitate the recovery of cattle from *Pimelea* poisoning.

The present study comprised two phases:

(i) Phase I: Vaccination of cattle followed by exposure to pastures with *Pimelea spp* plants; and

(ii) Phase II: Testing of whether Gastric Stimulant Powder and the ARGT-Antidote facilitated the recovery of cattle from *Pimelea* poisoning.

Cattle not previously exposed to *Pimelea* toxins were vaccinated with the *Pimelea* toxins-protein conjugate, and when cattle has developed typical circulating anti-*Pimelea* toxins antibodies, they were transferred to a station that had a history of *Pimelea* poisoning. The vaccinated cattle were then monitored for signs of *Pimelea* poisoning and compared with a group of contemporary cattle that had not been vaccinated. Cattle that showed *Pimelea* poisoning were then assigned to different groups to evaluate the rate of recovery following treatment with Gastric Stimulant Powder or the ARGT-Antidote.

3.0 METHODS

3.1 Cattle

The cattle used in the trial were sourced from properties in southern South Australia (properties were free of *Pimelea spp* plants) and were provided by Kidman Holdings Pty Ltd, Managing Partner of S.Kidman and Company. The cattle ranged from approximately 12-24 months of age and included Angus, Charolais, Hereford, Murray Grey, Shorthorn, and various crosses of these breeds. These cattle comprised 63 heifers and 12 steers. An additional group of 20 Santa Gertrudis cattle (19 heifers; 1 steer) were also included in the study.

3.2 Experimental *Pimelea* vaccine

Toxins were extracted from *Pimelea spp* plants and purified [6], before conjugation to a protein that had previously been shown to be immunogenic in cattle [5, 6,]. The *Pimelea* toxins-protein conjugate was prepared into a water-in-oil vaccine formulation [5] and injected subcutaneous. Cattle received two vaccinations, 2-weeks apart. Circulating anti-*Pimelea* toxins antibodies were determined using an enzyme-linked immunosorbent assay (ELISA) [W.J. Aspden and M.J. D'Occhio, unpublished].

3.3 Gastric Stimulant Powder

The Gastric Stimulant Powder was supplied by Parnell Laboratories (Aust) Pty Ltd (Alexandria, NSW). The main constituents are strychnine, pulverised zingiber, pulverised gentian and pulverised aloes. For drenching, the powder was suspended in 1 litre of water and administered using a typical drenching tube. The daily dose of Gastric Stimulant Powder consisted of 60 g of powder that contained 120 mg strychnine, 24.6 g zingiber, 12.6 g gentian and 1.5 g aloes.

3.4 Annual Ryegrass Toxicity Antidote (ARGT-Antidote)

The ARGT-Antidote was developed by the CSIRO Division of Animal Health in the laboratory of Dr J. Edgar. The antidote binds strongly to toxins that cause ARGT and aids in the clearance of these toxins from poisoned animals. The antidote is administered in a gel that is injected intraperitoneal. In the present study, the volume of gel injected was 150 ml per treatment.

3.5 Experimental design

3.5.1 Phase I: Vaccination and monitoring for *Pimelea* poisoning

Experimental cattle were sourced in southern South Australia and transported to "Carrieton" Station, Hawker. At "Carrieton", the cattle were assigned at random to a Control Group (n = 37) that were not vaccinated, and a Vaccinated Group (n = 38) that were vaccinated with the experimental *Pimelea* vaccine. The interval between primary and secondary vaccination was 2 weeks. Three weeks after secondary vaccination (when vaccinated cattle has significant circulating anti-*Pimelea* toxins antibodies), the cattle were

transferred to a station in the Marree District that had a history of occurrence of *Pimelea* poisoning.

On arrival at the station in the Marree District, the cattle were joined with 20 local station cattle (Santa Gertrudis), 10 of which had been vaccinated with the experimental *Pimelea* vaccine. Therefore, the total number of Control and Vaccinated cattle initially used in the study were 47 and 48, respectively.

The combined group of cattle were moved to a paddock that had areas of *Pimelea spp* plants (*Pimelea trychostachia*) interspersed among Black Bluebush (*Maireana pyramidata*), Pearl Bluebush (*Maireana sedifolia*) and Cotton Bush (*Maireana aphylla*), with scattered Kerosene Grass (*Aristida contorta*). Cattle were observed twice-weekly for signs of *Pimelea* poisoning (diarrhoea, oedema of the head and brisket, body condition).

3.5.2 Phase II: Treatment of cattle with *Pimelea* poisoning

After approximately 4 weeks on pastures containing *Pimelea spp* plants, the introduced cattle were showing signs of *Pimelea* poisoning. The status (liveweight, diarrhoea, oedemas) of the cattle was recorded at 6 weeks, and they were then assigned at random to one of 3 groups and received one of the following treatments:

(i) Control (n = 21), no further treatment;

(ii) Gastric Stimulant Powder (n = 21), daily drench for 5 days; and

(iii) ARGT-Antidote (n = 9), two intraperitoneal injections of antidote 24 hours apart (150 ml on Day 0; 150 ml on Day 1).

Cattle were retained in yards on a diet of cereal hay (barley, oats and wheat) and were monitored for recovery from *Pimelea* poisoning. Measurements included changes in liveweight, regression of oedemas, and recovery from diarrhoea.

3.6 Statistical analyses

The data were analysed by analysis of variance procedures using the General Linear Models (GLM) procedure of SAS/STAT [19]. Results are presented as means $\pm$ SEM, unless otherwise specified.

4.0 RESULTS

4.1 Response to vaccination with the experimental vaccine

Cattle vaccinated with the experimental *Pimelea* vaccine developed significant circulating anti-*Pimelea* toxins antibodies (Figure 3.1).

4.2 Status of *Pimelea* plants and pasture species

At the time of transfer of cattle to the Marree District (April 1996), the only *Pimelea spp* plants present were from the previous growth period (May - October 1995). The *Pimelea*

plants were therefore present as dried plants, some with retained dried seed-heads. Remaining pastures were perennials (see 3.5.1 above). Within 6 weeks of the introduction of cattle, there was a decline in pasture availability, together with a decline in the occurrence of erect *Pimelea spp* plants (Plate 3.1).

4.3 Phase I: Occurrence of *Pimelea* poisoning

4.3.1 Changes in liveweight

The liveweight of the introduced cattle at the time of transfer to the Marree District was 285 ± 6 kg (Figure 3.2). Six weeks after transfer to the Marree District, the liveweight of these cattle had declined to 238 ± 5 kg, which represented an average liveweight loss during this period of approximately 0.8 kg/day (Figure 3.2). Liveweight loss was similar for control cattle and cattle vaccinated with the experimental *Pimelea* vaccine, and after 6 weeks on pastures containing *Pimelea spp* plants, there was no difference in liveweight between control and vaccinated cattle (Table 3.1).

4.3.2 *Pimelea* poisoning

The incidence of *Pimelea* poisoning was similar for introduced, control and vaccinated cattle 6 weeks after grazing pastures containing *Pimelea spp* plants (Table 3.2). The only significant ($P < 0.05$) difference in *Pimelea* poisoning status was a reduced incidence of ongoing clinical diarrhoea in introduced vaccinated cattle (Table 3.2). However, vaccinated cattle had the same incidence of oedemas of the head and brisket as

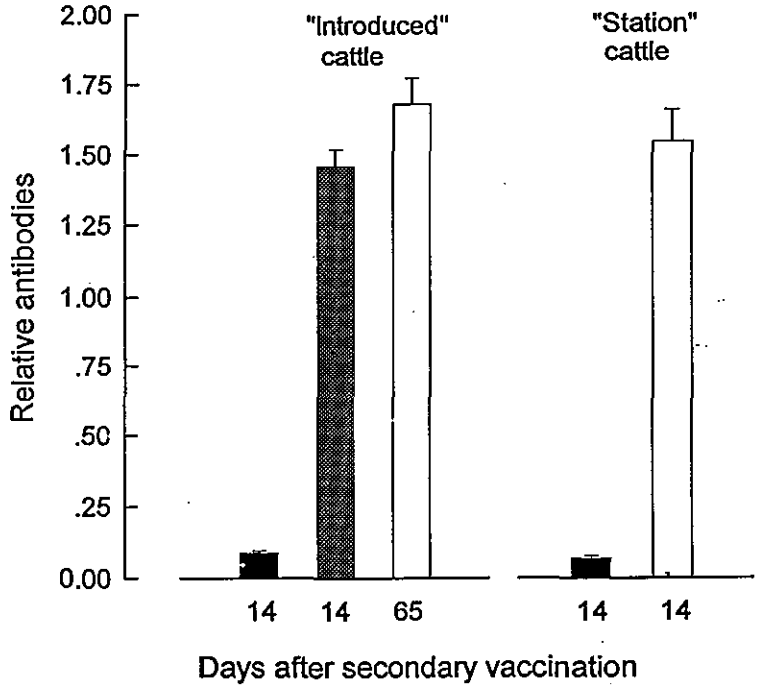

Figure 3.1 Relative circulating anti-*Pimelea* toxins antibody titres in unvaccinated control cattle (solid bars) and vaccinated cattle (open and hatched bars). Results for the "introduced" cattle are shown in the left panel and "station" cattle in the right panel. "Introduced" cattle had significant antibody titres at approximately the time of transfer to the Marree District (14 days after secondary vaccination) and 6 weeks after transfer to the Marree District (65 days after secondary vaccination).

Plate 3.1 Condition of pastures containing *Pimelea spp* plants. The top panel shows dried, erect *Pimelea trychostachia* in May 1996. The bottom panel is for the adjoining paddock in which the trial cattle had grazed for the previous 6 weeks. Note that the pasture is significantly depleted, and the density of *Pimelea* plants is also reduced in the grazed paddock. Broken *Pimelea* plants can be seen at centre-left in the bottom panel.

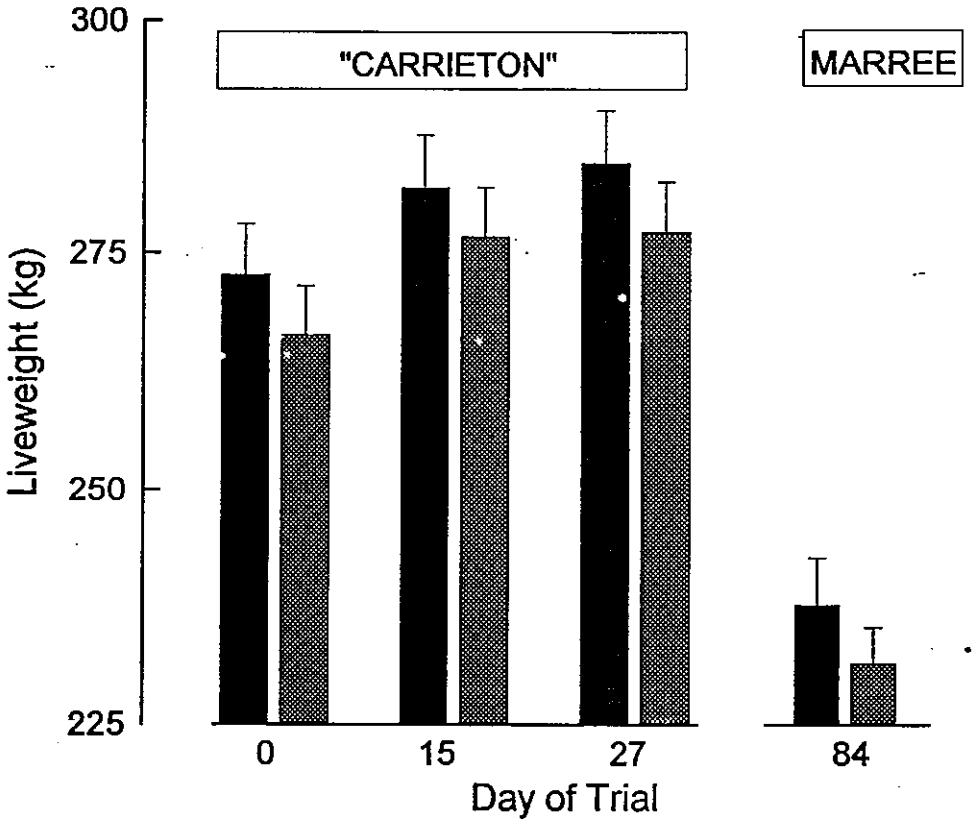

Figure 3.2 Longitudinal changes in liveweight for control (solid bars) and vaccinated "introduced" cattle during the period of vaccination at "Carrieton" and approximately 6 weeks after transfer to the Maree District, and introduction to patures containing *Pimelea spp* plants (see Plate 3.1).

unvaccinated control cattle (Tables 3.2 and 3.3). There was no apparent correlation between antibody titre and occurrence and size of oedemas ($r = 0.21$; $P = 0.23$) among vaccinated cattle, 6 weeks after grazing pastures with *Pimelea spp* plants (Figure 3.3).

Results for changes in liveweight and occurrence of *Pimelea* poisoning for the Santa Gertrudis station cattle are shown in Table 3.4. There was no difference ($P > 0.05$) in liveweight change between station control (- 2 kg) and vaccinated (+ 4 kg) cattle during the 6 week period after these cattle were joined with the introduced cattle. The incidence of *Pimelea* poisoning was relatively low for the station cattle, with 3/10 control and 0/10 vaccinated cattle showing clinical scouring (Table 3.4). There were no apparent signs of oedemas in either the control or vaccinated station cattle.

4.4 Phase II: Responses to Gastric Stimulant Powder and ARGT-Antidote

4.4.1 Selection of animals for Phase II

Because of the low incidence of *Pimelea* poisoning in the Santa Gertrudis station cattle, all except 3 of the cattle used for Phase II of the trial were selected at random from among the introduced cattle showing *Pimelea* poisoning.

4.4.2 Trial conditions during Phase II

In the initial design of Phase II of the trial, cattle showing *Pimelea* poisoning were to be treated with either Gastric Stimulant Powder or ARGT-Antidote, and then grazed in pasture that did not contain *Pimelea spp* plants. This proved not to be possible and the cattle were retained in yards and fed cereal hay. The quality of this feed was relatively poor and may have contributed, in part, to continuing diarrhoea in control and treated cattle. Temperatures were relatively low during Phase II and rain occurred at different times. Therefore, the cattle in Phase II of the trial were exposed to a range of environmental stresses.

Table 3.I Liveweight of control and vaccinated "introduced" cattle during the period of vaccination at "Carrieton" and after transfer to the Marree District.

	Liveweight (kg)			
	"Carrieton"			Marree District
	Day 0	Day 15	Day 27	Day 42[1]
Control (n = 36)	273 ± 5 [a]	282 ± 6 [a]	285 ± 6 [a, b]	238 ± 5[§, a, c]
Vaccinated (n = 38)	266 ± 5 [a]	276 ± 5 [a]	277 ± 5 [a, b]	231 ± 4 [a, c]

[1] Day 42 after transfer of "introduced" cattle to the Marree District

[§] One control heifer was calving and was excluded from further observation

[a] Means for control and vaccinated cattle, within days, do not differ

[b,c] Significant decrease in liveweight, within treatment group, between Day 27 and Day 42 ($P < 0.0001$)

Table 3.2 Status of control and vaccinated "introduced" cattle, 6 weeks after transfer to the Marree District[¶]

	Sub-mandibular and/or brisket oedema	Scouring[§]		No apparent sign of poisoning
		Previous	Present	
Control	13/36 [a]	10/36 [a]	12/36 [a]	9/36 [a]
Vaccinated	14/38 [a]	13/38 [a]	4/38 [b]	13/38 [a]

[¶] Some animals were scored for both oedema and scouring and contribute to more than one data set in the Table

[§] The scouring observed was not the dark coloured clinical scouring typically associated with *Pimelea* poisoning; however, the present scouring in the "introduced" cattle was considered to be related to exposure to *Pimelea* as it was not as prevalent in the "station" cattle

[a,b] Proportions within columns, with a different superscript, are significantly different ($P < 0.05$; Chi Square test)

Table 3.3 Distribution of oedema sizes in control and vaccinated "introduced" cattle, 6 weeks after transfer to the Marree District.

	Number of cattle with different Oedema Score[¶]									
	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0	4.5	5.0
Control [§,‡] (n = 36)	5	1	2	3	1	1	0	0	0	0
Vaccinated [§,‡] (n = 38)	6	4	0	0	1	0	2	1	0	0

[¶] Oedema score on a scale of 0.5 to 5

[§] Total number of Control cattle showing oedema was 13/36, and Vaccinated cattle 14/38

[‡] 6/13 Control cattle had oedemas of score ≤ 1, compared with 10/14 Vaccinated cattle with oedemas of score ≤ 1; however, the 3 largest oedemas were observed in Vaccinated cattle

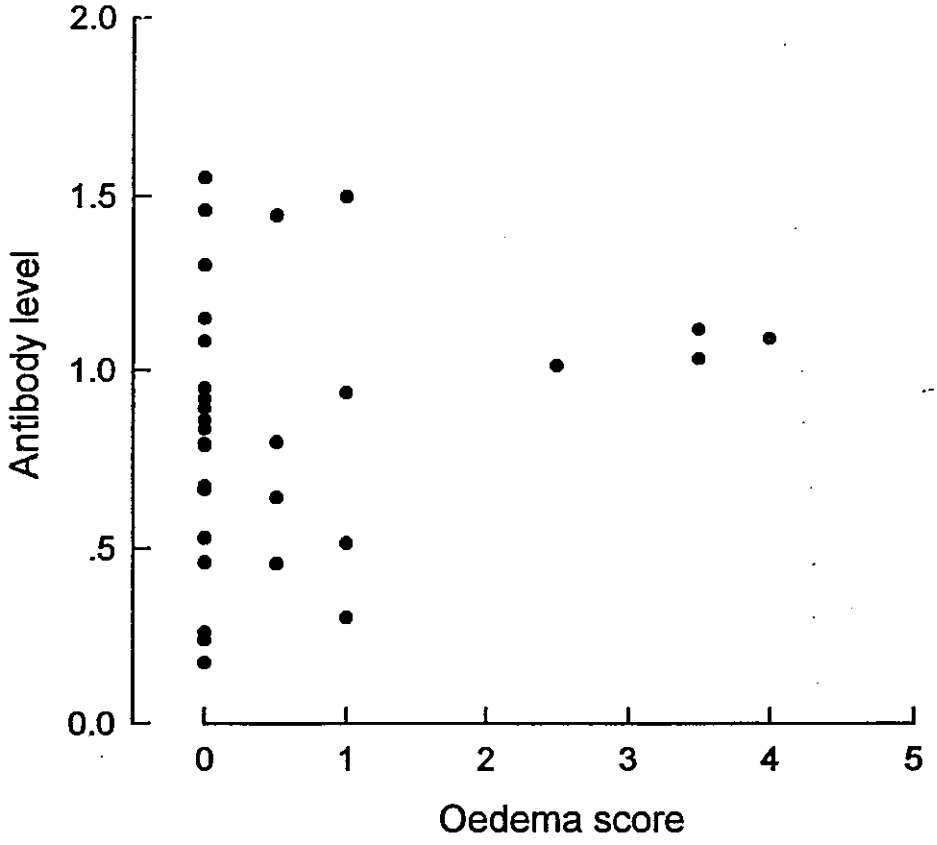

Figure 3.4 Relationships between anti-*Pimelea* toxins antibody levels in circulation and oedemas of the head and/or brisket (scale 0.0-5.0) in vaccinated "introduced" cattle, 6 weeks after transfer to the Marree District and grazing pastures containing *Pimelea spp* plants.

Table 3.4 Status of control and vaccinated "station" cattle, 6 weeks after joining with "introduced" cattle.

	Liveweight (kg)	Liveweight change (kg)[1]	Scouring	Not showing signs of *Pimelea* poisoning
Control (n = 10)	302 ± 21	+ 11.4 ± 7.9	3/10	7/10
Vaccinated (n = 10)	265 ± 17	+ 8.3 ± 6.5	0/10	10/10

[1] Liveweight change from March 16 - May 31, 1996

4.4.3 General health status of cattle during Phase II

A feature of the health status of cattle during Phase II was persistent diarrhoea. The persistence of diarrhoea appeared not to be related to treatment, and may have been caused by the relatively poor quality of cereal hay and other environmental factors. Approximately half of cattle that received Gastric Stimulant Powder over 5 days by drenching using relatively inflexible tubing developed a nasal mucous discharge on Day 3 to 4 of treatment. This discharge may have been caused by irritation of the throat during drenching.

4.4.4 Changes in liveweight

Changes in liveweight after treatment with Gastric Stimulant Powder or ARGT-Antidote are shown in Figure 3.4, and the results summarised in Table 3.5. Cattle that were treated with Gastric Stimulant Powder showed a greater ($P = 0.025$) increase in liveweight, 2 weeks after treatment, compared with control cattle. The liveweight change for cattle treated with ARGT-Antidote was similar ($P = 0.868$) to that of control cattle and less than that of cattle treated with Gastric Stimulant Powder, although the difference between ARGT-Antidote and Gastric Stimulant Powder cattle was not significant ($P = 0.088$). Cattle treated with Gastric Stimulant Powder maintained a liveweight change advantage 8 weeks after treatment, although at this time there were no significant ($P > 0.05$) differences in liveweight changes between the three treatment groups (Table 3.5).

4.4.5 Regression of oedemas

There was an initial small increase from Day 0 to Day 3 of Phase II in the incidence of oedemas, which may have been due to the persistence of *Pimelea* toxins after cattle were removed from pastures with *Pimelea spp* plants. The rate of regression of oedemas of the head and brisket appeared to be similar for control cattle and cattle treated with Gastric Stimulant Powder or ARGT-Antidote (Table 3.6). In all groups, oedemas tended to regress relatively quickly after cattle were removed from pastures with *Pimelea spp* plants. The rate of oedema regression is illustrated in Table 3.7 for cattle treated with Gastric Stimulant Powder.

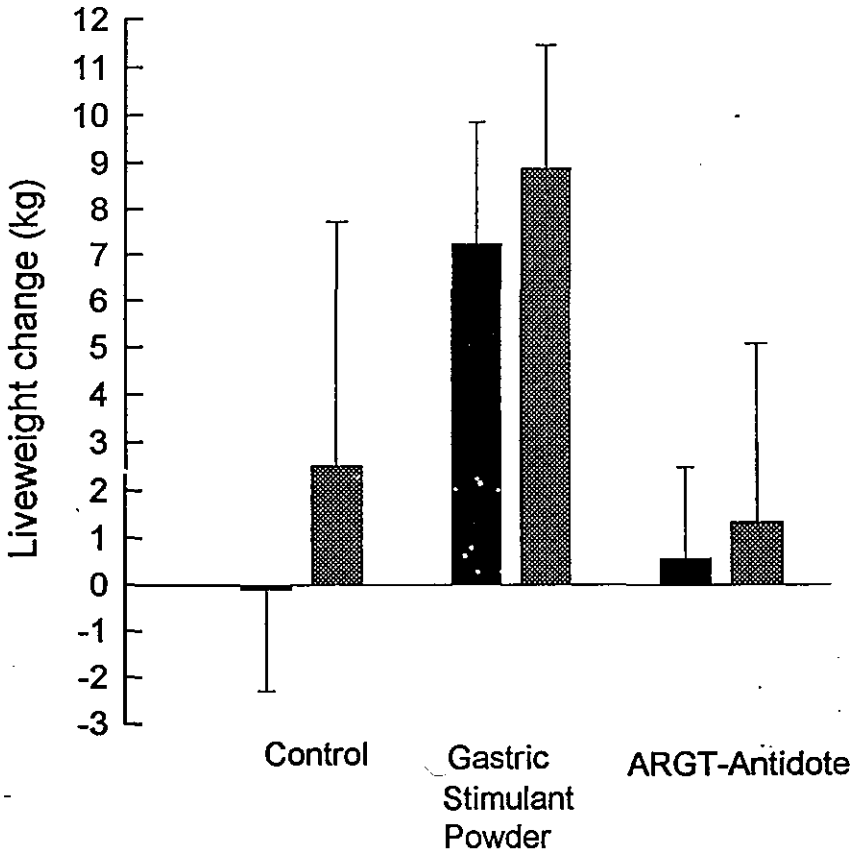

Figure 3.4 Changes in liveweight 2 weeks (solid bars) and 8 weeks after the start of Phase II of the trial, for Control cattle showing *Pimelea* poisoning that received no further treatment (left panels), cattle treated with Gastric Stimulant Powder (centre panels) and cattle treated with the experimental ARGT-Antidote (right panels).

Table 3.5 Changes in liveweight after the start of **Phase II** of the trial (Day 0).

	Day after the start of **Phase II**		
	Day 0	Day 13	Day 51
Control			
Liveweight (kg)	223 ± 4 (n=24)	222 ± 5 (n=21)	231 ± 9 (n=14)
▲liveweight	------	- 0.1 ± 2.2 [a,c]	+ 2.5 ± 5.2 [¶]
Gastric Stimulant Powder			
Liveweight (kg)	243 ± 6 (n=24)	247 ± 6 (n=21)	246 ± 7 (n=16)
▲liveweight	------	+ 7.2 ± 2.6 [b,e]	+ 8.9 ± 2.6
ARGT-Antidote			
Liveweight (kg)	226 ± 9 (n=14)	223 ± 10 (n=11)	229 ± 15 (n= 9)
▲ liveweight	------	+ 0.5 ± 1.9 [d,f]	+ 1.3 ± 3.7

[a,b] $P = 0.026$; [c,d] $P = 0.868$; [e,f] $P = 0.088$

[¶] There were no significant ($P > 0.05$) differences among groups in ▲ liveweight on Day 51

Table 3.6 Proportions of cattle with oedema of the head and/or brisket after the removal of cattle from pastures containing *Pimelea spp* plants (Day 0), and start of **Phase II** of the trial.

	Day after the start of **Phase II**			
	Day 0	Day 3	Day 13	Day 27
Control	12/24	15/24	5/24	0/24
Gastric Stimulant Powder	9/24	5/23	4/23	0/23
ARGT-Antidote	5/14	9/14	0/14	0/14
TOTAL	26/62 (42%)	29/61 (48%)	9/61 (15%)	0/61 ----

Table 3.7 Regression of oedemas of the head and/or brisket in cattle removed from pastures containing *Pimelea spp* plants (Day 0) and treated with Gastric Stimulant Powder (**Phase II**).

	Oedema score[1]		
	Day after the start of **Phase II**		
	Day 0	Day 3	Day 13
Mean±SEM[1]	1.4 ± 0.4	1.3 ± 0.7	0.6 ± 0.2
Range	0.5 - 4.0	0.5 - 4.0	0.5 - 1.0

[1] Oedema score using arbitrary scale from 0.5 (mild oedema) to 5.0 (severe oedema)

5.0 DISCUSSION

An important feature of the present trial was that cattle not previously exposed to *Pimelea spp* plants were vaccinated using the experimental *Pimelea* vaccine and, after the cattle had developed circulating anti-*Pimelea* toxins antibodies, they were transferred to a station in the Marree District that had a history of *Pimelea* poisoning.

After 6-weeks exposure to *Pimelea spp* plants, fewer vaccinated cattle were judged to have persistent clinical diarrhoea compared with unvaccinated cattle. However, the presence of anti-*Pimelea* toxins antibodies in circulation did not prevent the development of oedemas of the head and brisket, or the dramatic decline in body condition that occurs in cattle with *Pimelea* poisoning. Furthermore, there were no apparent relationships among vaccinated cattle between titres of anti-*Pimelea* toxins antibodies in circulation, and the occurrence or size of oedemas of the head and brisket.

It was concluded, therefore, that the experimental *Pimelea* vaccine does not reduce the susceptibility of cattle to *Pimelea* poisoning, and vaccinated cattle show the typical spectrum of pathophysiological responses to exposure to *Pimelea spp* plants. It should be noted, however, that this conclusion was based on observations in lines of cattle from areas free of *Pimelea spp* plants. The possibility of an interaction between vaccination and some form of acquired "resistance" to *Pimelea* poisoning in lines of cattle from areas with a history of *Pimelea* poisoning cannot be excluded, although at the present time this type of interaction would appear unlikely.

With regard to the latter comments, 52/74 (70%) of the total cattle introduced into the Marree District showed at least one symptom of *Pimelea* poisoning. In contrast, only 3/20 (15%) of the station cattle showed evidence of *Pimelea* poisoning when grazed as contemporaries to the introduced cattle. This finding provided conclusive evidence that cattle which originate from an environment free of *Pimelea spp* plants have an increased susceptibility to *Pimelea* poisoning. This observation has important implications for re-stocking of pastoral country containing *Pimelea spp* plants following a period of reduced stock numbers. The basis for an apparent reduced susceptibility to *Pimelea* poisoning of "local" cattle is not understood, but may reflect selection for "resistance" to *Pimelea* poisoning. The mechanism(s) of any "resistance" to *Pimelea* poisoning could include an increased ability to detoxify *Pimelea* toxins (either in the rumen or systemically), or decreased sensitivity of target tissues (e.g. lungs, gastro-intestinal tract) to *Pimelea* toxins. The metabolism of *Pimelea* toxins by contemporary animals that show different susceptibility to *Pimelea* poisoning warrants further study.

A possible breed component in the susceptibility of cattle to *Pimelea* poisoning has not been excluded. However, *Pimelea* poisoning has been demonstrated in both *Bos taurus* (British and European) and *Bos indicus* (Zebu) breeds, and it is possible that differences among individuals within a breed are as important as differences between breeds. A systematic study on the susceptibility to *Pimelea* poisoning of different families of cattle within a breed has not been carried out, and should be considered.

In the present trial, introduced cattle were transferred to the Marree District in April 1996. At the time of transfer, the only *Pimelea spp* plants present were represented by dried plants from the previous growth period (May - October 1995). It was anticipated,

therefore, that *Pimelea* poisoning was unlikely to occur until rain stimulated the growth of new *Pimelea spp* plants. Contrary to expectations, introduced cattle started to show signs of *Pimelea* poisoning (clinical diarrhoea, early oedemas, loss of body condition) approximately 4 weeks after grazing pastures with *Pimelea spp* plants and, after 6 weeks, had developed the full symptoms of *Pimelea* poisoning. Dried *Pimelea spp* plant material was therefore capable of inducing typical *Pimelea* poisoning. This observation was consistent with the recent finding that toxins are present in *Pimelea spp* plants during the senescent and dying stages of the plant life-cycle [3, 15, 16].

It is not known if the introduced cattle accumulated toxins progressively over a period of several weeks, and showed signs of *Pimelea* poisoning after toxin levels had surpassed a threshold required for poisoning, or whether introduced cattle started to ingest/inhale toxins as the amount of pasture decreased. With regard to the latter, the height of pasture was reduced progressively during the trial and it is possible that, after several weeks, the cattle were grazing relatively close to the surface and inadvertently ingested/inhaled increased amounts of *Pimelea* plant material. If the latter scenario prevailed, then *Pimelea* poisoning may have been precipitated relatively quickly.

Treatment of cattle showing *Pimelea* poisoning with Gastric Stimulant Powder was associated with an increased liveweight change 2 weeks after treatment, compared with untreated cattle showing *Pimelea* poisoning. It is unlikely that the increased liveweight change of the former cattle represented increased somatic tissue deposition over 2 weeks. Rather, treatment with Gastric Stimulant Powder may have increased rumen fill by increasing food and water intake. Nevertheless, this increased intake would be significant, as facilitation of food intake is an important first step in the recovery from *Pimelea* poisoning of emaciated animals with possible rumen stasis. The utility of Gastric Stimulant Powder in cases of *Pimelea* poisoning warrants further study.

The ARGT-Antidote had no apparent influence on the rate of recovery of cattle from *Pimelea* poisoning. The ARGT-Antidote used was recently shown to significantly reduce the incidence of deaths in cattle suffering from Annual Ryegrass Toxicity (ARGT) [J. Edgar, unpublished results]. The toxins that cause ARGT share chemical properties with toxins that cause *Pimelea* poisoning, and hence the ARGT-Antidote was tested in the present trial. Based on recent success of the antidote approach in the treatment of ARGT, other candidate antidotes should be tested for their potential to facilitate recovery from *Pimelea* poisoning. Potential *Pimelea*-antidotes should initially be evaluated in laboratory studies before further testing in cattle with *Pimelea* poisoning.

A feature of the recovery of cattle from *Pimelea* poisoning was the relatively rapid rate of regression of oedemas of the head and brisket. A similar rapid regression of oedemas was reported previously in cattle showing *Pimelea* poisoning [3]. This rapid regression of oedemas is likely to contribute, in part, to the recovery of cattle from *Pimelea* poisoning after removal from pastures with *Pimelea spp* plants and provision of an appropriate diet.

In summary, vaccination of cattle with the experimental *Pimelea* vaccine did not reduce the susceptibility of cattle to *Pimelea* poisoning, and vaccinated cattle showed all the typical signs of *Pimelea* poisoning. Treatment of cattle showing *Pimelea* poisoning with Gastric Stimulant Powder had a positive effect on the acute liveweight change after treatment, and the practical use of Gastric Stimulant Powder to encourage re-alimentation in emaciated cattle should be further evaluated.

6.0 INDUSTRY RECOMMENDATIONS

At present, there is no veterinary treatment that reduces the susceptibility of cattle to *Pimelea* poisoning. Prevention of *Pimelea* poisoning must, therefore, continue to rely on management. Wherever possible, sufficient pasture should be retained to minimise the likelihood that *Pimelea spp* plants will predominate in the event of a seasonal rainfall pattern that favours growth of *Pimelea spp* plants. It would appear that significant outbreaks of *Pimelea* poisoning are more likely to occur if pastures are limiting; however, it should be recognised that this does not necessarily apply in all circumstances of critical outbreaks of *Pimelea* poisoning. In areas susceptible to *Pimelea* poisoning, a management decision may be required between short-term relatively high stocking rates, and longer-term reduced stocking rates that may decrease the occurrences of *Pimelea* poisoning. The two management strategies could be tested in Producer Demonstration Site trials. In conjunction with these trials, surveys could be carried out to describe general relationships between pasture conditions and histories of critical outbreaks of *Pimelea* poisoning for different stations.

Cattle with *Pimelea* poisoning should be transferred to pastures without *Pimelea spp* plants. Additionally, the cattle should be provided with good-quality supplementary feed (can be good pastures). Molasses, or an alternate source of energy, can be included, although this may not be essential. Gastric Stimulant Powder could be given for 3 to 5 days to encourage feeding and water intake in emaciated animals. The Gastric Stimulant Powder could be administered with molasses, or mixed with other feed that will be consumed in a relatively short period.

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