THE INFLUENCE OF BIOLOGICALLY ACTIVE COMPOUNDS FROM *SALSOLA TUBERCULATIFORMIS* BOTSCH. ON STEROID PROTEIN INTERACTIONS

by

Fatima Allie

Thesis presented in partial fulfilment of the requirements for the degree of Master of Science (Biochemistry) at the University of Stellenbosch
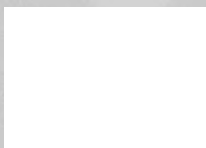
Study leaders: Ms. A. Louw

                        Prof. P. Swart

Department of Biochemistry, University of Stellenbosch

March 1998

Declaration:

I, the undersigned, hereby declare that the work contained in this thesis is my original work and has not previously in its entirety or in part been submitted at any University for a degree.

16/2/98

Date

OPSOMMING

Die studie vorm deel van 'n ondersoek van die sindroom van verlengde dragtigheid in skape en kontrasepsie in rotte, veroorsaak deur die Namibiese bos, *Salsola tuberculatiformis* Botsch. Bewyse was verkry vanaf studies met *S. tuberculatiformis* ekstrakte, toon dat verbindings in die bos adrenale steroiedgenese in die rot versteur en die binding van die natuurlike substraat, deoksikortikosteroon, aan skaap adrenale sitochroom P450c11 verhoed. Swaar beskryf die isolasie van een van die aktiewe verbindings in die bos, wat S2 genoem word. Daar is gevind dat die S2 fraksie uit 'n mengsel van 'n hoogs labiele verbinding plus een of meer stabiele komponente, waarvan sinefrien een is, bestaan. In 'n waterige medium is sinefrien 'n afbraak produk van die S2 fraksie. Derivatiserings studies het voorgestel dat die aktiewe komponent in S2 'n labiele hidroksie-feniel aziridien of die ooreenstemmende oop ketting voorloper mag wees. 'n Meer stabiele asetoksie-aziridien voorloper analoog, 2-(4-asetoksiefeniel)-2-chloro-N-metiel-etielammoniumchloried (Verbinding A) is gesintetiseer, en het kontraseptief getoets vir rotte en het adrenale P450c11 geïnhibeer, soos S2. 'n Etanoliese ekstrak van die plant, die aktiewe fraksie van die plant, S2, en Verbinding A, 'n analoog, is voorberei en getoets in die huidige studie.

Hierdie studie beskryf:

(a) die isolasie van die S2 fraksie uit *S. tuberculatiformis* en die karakterisering van die chemiese struktuur van die hoof komponent(e) in die S2 fraksie. Drie molekulêre ione, wat al voorheen beskryf is, is waargeneem by m/z 150, 166, 168 en geïdentifiseer as 'n hidroksie-aziridien, norsalsolinol en sinefrien, onderskeidelik. Daarby, is 'n nuwe molekulêre ioon by m/z 258 waargeneem en tentatief geïdentifiseer as die aziridien voorloper van die hidroksie-aziridien (m/z 150).

(b) die effek van Verbinding A, etanolekstrak en S2, op adrenale sitochroom P450c11. Die toets monsters het die Tipe I deoksikortikosteroon-geïnduseerde verskilspektrum geïnhibeer en 'n Tipe II substraat verskilspektrum ontlok. Verbinding A induseer gemengde inhibisie van die DOC-geïnduseerde verskilspektrum van sitochroom P450c11, met 'n sterk kompeterende element.

(c) die effek van Verbinding A, etanolekstrak en S2, op die verspreiding van endogene steroïde, kortisol en progesteroon, in skaapserum. Die toets monsters het kortisol verplaas van kortikosteroïed bindings globulin met die gevolglike verhoging in die persentasie vrye steroïed in skaapserum. Progesteroon verspreiding is egter nie noemenswaardig beïvloed deur die toets monsters nie.

(d) die toepassing van elektrosproei massapektrometrie om die verskillende faktore wat die siklisering van Verbinding A na die aziridien beïvloed te bestudeer. Hierdie het ingesluit die effek van versnellings energie, temperatuur en pH, en die teenwoordigheid van die P450c11 ensiem. Studies het getoon dat die siklisering van Verbinding A na die ooreenstemmende aziridien in buffer en skaap serum is temperatuur en pH afhanklik en dat daar 'n balans tussen kortikosteroïed bindings globulin binding, wat plaasvind as gevolg van die teenwoordigheid van skaapserum, en siklisering van Verbinding A bestaan. Die aziridien voorloper, Verbinding A, sikliseer na die ooreenstemmende labiele maar meer aktiewe aziridien by fisiologiese pH. Skaapserum het die tempo van siklisering vertraag terwyl die P450c11 ensiem die siklisering van Verbinding A na die ooreenstemmende aziridien katalities bevorder het.

SUMMARY

This study forms part of an ongoing investigation into the syndrome of prolonged gestation in sheep and contraception in rats, caused by the Namibian shrub, *Salsola tuberculatiformis* Botsch. Evidence was obtained from studies with *S. tuberculatiformis* extracts, that compounds in the shrub perturbed adrenal steroidogenesis in the rat and interfered with the binding of the natural substrate, deoxicorticosterone, to sheep adrenal cytochrome P450c11. Swart described the isolation of one of the active compounds from the shrub, named S2. The S2 fraction was found to consist of a mixture of an extremely labile compound plus one or more stable components, one of which is synephrine. In an aqueous medium synephrine is a breakdown product of the S2 fraction. Derivitization studies suggested that the active component in S2 was a labile hydroxyphenyl-aziridine or the corresponding open chain precursor. A more stable acetoxy-aziridine precursor analogue, 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammoniumchloride (Compound A) was synthesised, and found to be contraceptive in rats and to inhibit adrenal P450c11, like S2. An ethanolic extract from the plant, the active fraction from the plant, S2, and Compound A, the chemical analogue, were prepared and tested in the present study.

This study describes:

(a) the isolation of the S2 fraction from *S. tuberculatiformis* and the elucidation of the chemical structure of the main component(s) in the S2 fraction. Three previously described molecular ions were observed at m/z 150, 166, 168 and identified as a hydroxy-aziridine, norsalsolinol and synephrine, respectively. In addition, a new molecular ion at m/z 258 was observed and tentatively identified as the aziridine precursor of the hydroxy-aziridine (m/z 150).

(b) the effect of Compound A, ethanol extract and S2, on adrenal cytochrome P450c11. The test samples inhibited the Type I deoxicorticosterone-induced difference spectrum and elicited a Type II substrate induced difference spectrum. Compound A induced mixed inhibition of the DOC-induced difference spectrum of cytochrome P450c11, with a strong competitive element.

(c) the effect of Compound A, ethanol extract and S2, on the distribution of endogenous steroids, cortisol and progesterone, in sheep serum. The test samples displaced, cortisol from corticosteroid binding globulin with a resulting increase in the percentage free steroid in sheep serum. Progesterone distribution, however, was not affected to a great extent by the test samples.

(d) the application of electrospray mass spectrometry to investigate different factors which influence the cyclization of Compound A to aziridine. These included the effect of cone voltage, temperature and pH, and the presence of the P450c11 enzyme. Studies showed that cyclization of Compound A to the corresponding aziridine in buffer and sheep serum is temperature and pH dependant and that there is a balance between corticosteroid binding globulin binding, which occurs due to the presence of the sheep serum, and cyclization of Compound A. The aziridine precursor, Compound A, cyclises to the corresponding labile but more active aziridine at physiological pH. Sheep serum was shown to retard the rate of cyclization while, the P450c11 enzyme catalytically promoted cyclization of Compound A to produce the corresponding aziridine.

My family and Emille for your

love, support and encouragement.

Special poems for special seasons
are meaningful indeed,
But daily inspiration
is still man's greatest need
                    Helen Steiner Rice

ACKNOWLEDGEMENTS

I would sincerely like to thank the following persons for their help, guidance and support. Without their insight and contributions this particular study would not have been possible.

Ms. A. Louw. I'll be ever thankful for your continual encouragement, guidance and patience at all times. Without you all this would not have been possible.

Prof. P. Swart. Prof, for your assistance and guidance in this project.

My parents and brothers. For your support and encouragement, but above all for your love.

Emille. For your support, encouragement and faith in me, I'll never be able to thank you enough.

Zaino. Thanks, my friend for always being there when I needed you.

My roommates, Heidi and Janice. For putting up with my moods and always trying to understand.

Lucy. Thanks for your support and encouragement.

Fadlah and Zahir. For always showing an interest in my work.

Joanita, Edwina and Fiona. Thanks for your support and encouragement.

Dr. M. van der Merwe. For your everlasting patience and advice with the ES-MS work.

Mr. B.F. Rehder. For always being patient and always being willing to help.

Ms. M. Rautenbach. For always being willing to help and showing an interest in my work.

Neels, George, Velma and Anita. For technical assistance.

The FRD. For financial support.

For all those people not mentioned here that helped in one way or another.

TABLE OF CONTENTS

CHAPTER 4

THE INFLUENCE OF TEST SAMPLES ON THE PERCENTAGE FREE STEROID IN
UNDILUTED SERUM

CHAPTER 5

FACTORS WHICH INFLUENCE THE CYCLIZATION OF COMPOUND A TO THE
CORRESPONDING AZIRIDINE

CHAPTER 6

DISCUSSION   83

CHAPTER 7

EXPERIMENTAL

ABBREVIATIONS

ACTH	Adrenocorticotrophic hormone
BSA	Bovine serum albumin
CBG	Corticosteroid binding globulin
Compound A	2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammoniumchloride
DOC	Deoxycorticosterone
EI-MS	Electron impact mass spectrometry
ES-MS	Electrospray mass spectrometry
Ethanol extract	Ethanol extract isolated from *S. tuberculatiformis* Botsch.
FAB-MS	Fast atom bombardment mass spectrometry
^{1}H-NMR	Proton nuclear magnetic resonance spectroscopy
HPLC	High pressure liquid chromatography
Kic	Competitive inhibition constant
Kiu	Uncompetitive inhibition constant
LH	Lutenizing hormone
m/z	Mass over charge ratio
m	Modified
MS	Mass spectrometry
NMR	Nuclear magnetic resonance spectroscopy
N-TNP-MEA-SH	N-(2,4,6-trinitrophenyl)-mercaptoethanolamine
PBS	Phosphate buffered saline
RP HPLC	Reverse phase high pressure liquid chromatography
SHBG	Sex hormone binding globulin
S2	Active HPLC fraction from the shrub *S. tuberculatiformis*
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

Big lamb disease is a rare syndrome of prolonged gestation which occurs sporadically among karakul sheep in the Keetmanshoop area of Namibia [1]. The syndrome is characterised by foetal post-maturity and prolonged gestation which results in the fur of affected new-born lambs being overgrown at birth and leads to economically worthless pelts [1, 2]. The Namibian shrub, *Salsola tuberculatiformis* Botsch., has been established as the causactive agent of this syndrome and prolonged gestation can be experimentally reproduced by feeding the shrub to pregnant merino sheep [3]. Morgenthal conducted an extensive study on the effects of feeding *S. tuberculatiformis* Botch. to pregnant ewes [3]. He concluded that the hypoadrenocorticalism and hypothyroidism observed in the foetuses of ewes ingesting *S.tuberculatiformis* supports the theory that prolonged gestation is caused by a supression of the foetal hypothalamus or higher brain ce. res. Only two direct effects of the shrub on the ewe were identified namely, a marked under-development of the udder and also the development of a non-hemolytic anaemic condition.

The properties of plants belonging to the genus *Salsola* (Family: Chenopodiaceae) was first described by Ploss in 1902 [4]. He discribed the use, in Algiers, of an infusion of the leaves of the shrub as a contraceptive. San folklore also describe the use of *S. tuberculatiformis* or "gannabos" for contraceptive purposes [5].

In an attempt to reproduce the syndrome of prolonged gestation in rats, the shrub was fed to pregnant female rats, but in most cases it led to foetal resorption rather than prolonged gestation. If the plant was however, fed to non-pregnant female rats, the estrus cycles of these animals were blocked [2]. The above results suggest that *S. tuberculatiformis* contains contraceptive compounds. The contraceptive action of the shrub was however not observed in sheep [3].

Van der Merwe *et al*, exploited the estrus blocking activity of the shrub in rats and with the aid of vaginal smears and standard staining techniques succeeded in isolating active extracts and partly-purified fractions from *S. tuberculatiformis* [6]. By using this bio-assay as a monitoring system the extremely labile character of the extracts and fractions from the shrub was discovered. Specifically, their sensitivity towards pH, oxidation and direct light which result in autocatalytic degradation and polymerization. They isolated two less active, but more stable compounds which were identified by mass spectrometry (MS) and nuclear magnetic resonance spectrometry (NMR), as 4-hydroxy-acetophenone and 4-hydroxy-3-methoxy-acetophenone, respectively (Fig. 1.1). This indicated that the active compounds in *S. tuberculatiformis* were probably, small aromatic compounds containing phenolic hydroxyl groups.

(A) 4-hydroxy-acetophenone

(B) 4-hydroxy-3-methoxy-acetophenone
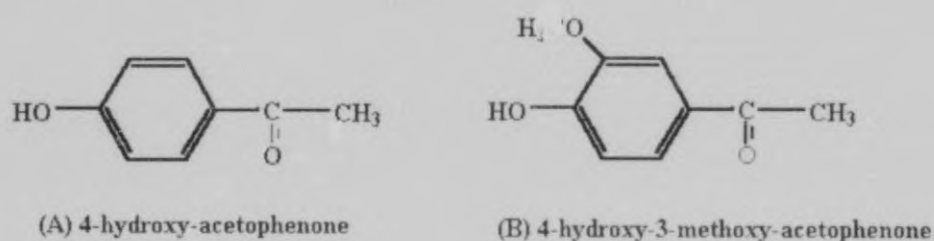
Figure 1.1 The chemical structures of (**A**) 4-hydroxy-acetophenone and (**B**) 4-hydroxy-3-methoxy-acetophenone.

Due to the considerable amount of active material needed per test and the long time required to complete the bio-assay it became necessary to develop biochemical micro-assays. The micro-assays would be more sensitive, and due to the shorter time required to complete the assays would reduce decomposition of the labile compounds. Evidence was obtained from studies with *S. tuberculatiformis* extracts, and with the two acetophenones mentioned above, that at least part of the prolonged gestation in ewes as well as the contraceptive action in rats may be related to changes in glucocorticoid hormone levels. Glucocorticoid hormones are actively produced in the adrenal glands and studies with rat adrenal tissues, showed that active extracts inhibited the synthesis of corticosterone from deoxycorticosterone (DOC) [6].

This assumption was verified by studies with metyrapone, a known inhibitor of cortisol and corticosterone biosynthesis [7]. Feeding of rats with metyrapone resulted in the blocking of the estrus cycle, in a manner similar to that observed with the shrub and led to the idea that active compounds in the plant affect adrenal steroidogenesis [6].

The biogenesis of steroid hormones are catalyzed by a group of cytochrome P450 dependent mono-oxygenases. Cytochrome P450 dependent enzymes are hemo-proteins with unique spectroscopic properties due to the presence of a heme moiety in the active centre. The interaction between the enzyme and its natural substrate, or inhibitor, can be monitored by UV difference spectra. This feature enabled Swart to develop two spectroscopic micro-assays with which the isolation of the active compounds from *S. tuberculatiformis* could be monitored [8]. The first one measured the direct interaction of an inhibitor with the cytochrome P450c11 enzyme, while the second monitors the interference of an inhibitor with the binding of the natural substrate, DOC, to the enzyme. A third micro-assay, added at a later stage, measured the effect of an inhibitor on the conversion of DOC to corticosterone by a reconstituted cytochrome P450c11 enzyme system [8].

The above assays were subsequently used to monitor the extraction and isolation of three extremely labile fractions from *S. tuberculatiformis*, namely S1(a), S1(b) and S2, which inhibits the hydroxylation of DOC to corticosterone [8, 9]. Of the three compounds only S2 has so far been investigated further. Swart observed that S2 stabilized the low spin state of the hemoprotein which results in a Type II difference spectrum and inhibited the binding of the natural substrate to the active site of cytochrome P450c11.

The elucidation of the chemical structure of S2 presented many problems. NMR studies of S2, dissolved in deuterium oxide (D_2O) or deuterated dimethyl sulphoxide (DMSOd6), revealed the presence of a 1,4-disubstituted benzene ring. NMR spectra also indicated that S2 dissolved in a mixture of DCl/D_2O is readily converted to 1-(4-hydroxyphenyl)-2-(methylamino)ethanol, commonly known as synephrine. Synephrine is a stable compound and showed only slight activity relative to the activity displayed by S2 with regard to the P450-based spectral assays. It also does not show the labile characteristics or contraceptive action normally observed with S2. These results indicated that synephrine could possibly be

regarded as a stable breakdown product of a highly active but more labile compound in the S2 fraction [6].

When S2 was subjected to electron impact mass spectrometry (EI-MS) and fast atom bombardment mass spectrometry (FAB-MS), the spectra showed no true molecular ion but instead an abundance of high molecular peaks [10]. This suggested that polymerization of S2 has taken place. Acetylation of S2 by van der Merwe yielded an unstable monoacetate while acetylated synephrine yielded a stable triacetate (Fig. 2.1). EI-MS of the acetylated S2 indicates a molecular mass of 191 in addition to fragment ions. From the MS data and other information it was deduced that the unacetylated compound might be a hydroxyphenyl aziridine.

Although S2 shares many of the labile characteristics exhibited by synthetic aziridines, a study was undertaken by de Kock to obtain chemical proof that a compound containing an aziridine moiety existed in the S2 fraction [11]. The aziridine ring, and its protonated form, the aziridinium ion, are reactive compounds which readily react with nucleophiles to form alkylated, ring opened products. De Kock developed a chemical probe, N-(2,4,6-trinitrophenyl)-mercaptoethanolamine (N-TNP-MEA-SH), which reacted under mild conditions with each of the model aziridines studied, but not with synephrine. When this probe was used to investigate the S2 fraction, the results clearly indicated that S2 contained a compound which reacted with the probe. This result was taken as proof that an aziridine, or its precursor is present in S2. During attempts to isolate this product by thin layer chromatography (TLC) on silica gel plates, it was discovered that the new product is unstable and rapidly decomposed to yield the original probe and a new compound, identified as 2-(4-hydroxyphenyl)-1-(aminomethyl)ethene.

From these results De Kock deduced that the active component in the biologically active S2 fraction may be a highly reactive hydroxyphenyl aziridine, or it's precursor. Aziridines are extremely labile and their precursors, which cyclise to the corresponding aziridine *in vivo*, are normally used in pharmacological studies. In a separate study, de Kock synthesized a series of structurally related phenyl aziridine precursors containing a 2-X-methyl- ethylammonium structure, in which X represents a good leaving group (Fig. 1.2) [12]. These precursor

molecules all cyclise to their corresponding aziridines at physiological pH. The 2-X-methyl-ethylammonium phenyl aziridine precursors could all be classified as mono-functional nitrogen mustards based on the resemblance of their chemical structures to these mustards. Interestingly enough nitrogen mustards are known to cause the same type of anaemia previously observed with the shrub in pregnant ewes [13].
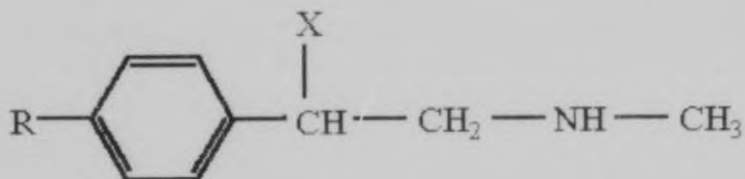

Figure 1.2 Chemical structure of open-chain aziridine precursors. X=good leaving group R=H, OH or AcO.

One of the aziridine precursors synthesized was 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammoniumchloride (Compound A), where R=acetoxy and X=chloride and which cyclises to the corresponding aziridine at physiological pH. In another study Louw *et al* found that this compound, when administered orally and intraperitoneally, had a contraceptive effect on female Wistar rats with a concomitant decrease in total body, uterus, and ovary mass and an increase in adrenal mass [14]. Spectrophotometric studies indicated that Compound A inhibited the Type I DOC-induced difference spectra of sheep adrenal cytochrome P450c11 and elicited a Type II difference spectrum in a manner similar to that of S2. Inhibition of the Type I difference spectrum was attenuated with time in buffer but not in native sheep plasma. Heated sheep plasma also showed attenuation of inhibition with time in a manner similar to that of Compound A in buffer.

ES-MS (electrospray mass spectrometry) studies indicated that in buffer the aziridine precursor (Compound A) cyclisises fully to the aziridine within 1 hour, while in sheep serum the cyclisization is retarded. It was found that it is the aziridine precursor, rather than the aziridine, which is the inhibiting agent in the cytochrome P450c11 system and that two high affinity steroid-binding proteins in serum, SHBG (sex hormone binding globulin) and CBG

(corticosteroid binding globulin) may bind and stabilize the aziridine precursor in sheep serum [14].

Binding of the biologically active aziridine precursor to the steroid binding globulins may simply be a transport mechanism, in which case the adrenal would be the primary site of action, or the binding itself, with concomitant displacement of endogenous steroids, may be part of the biological effect. Another study by Louw *et al* was under taken to establish which mechanism would predominate *in vivo* during contraceptive experiments in female rats with *S. tuberculatiformis* and the chemical analogue, Compound A [15]. From the results it was deduced that the contraceptive effect of the test compounds may be mediated by displacement of corticosterone from CBG as indicated by an increase in the percentage free corticosterone and a decrease in the percentage CBG bound steroid. Also, a decrease in adrenocorticotrophic hormone (ACTH), lutenizing hormone (LH) and CBG levels were observed, which may be due to the increase in bio-available corticosterone levels. It will also be of interest to examine whether the test compounds have an additional direct effect on hepatic CBG synthesis and ACTH and LH secretion by the pituitary or, whether the effect is primarily due to increased bio-available corticosterone.

The main objective of this study was to test the *in vitro* biological effects of Compound A, the ethanolic plant extract and the S2 fraction. Specifically the interaction of these samples with the steroid binding proteins, adrenal cytochrome P450c11 and plasma CBG, was investigated as well as the effect of these proteins on the cyclization of the test compound to aziridines. Chapter 2 deals with the methods applicable to the preparation of Compound A, the ethanol extract and the S2 fraction and the tentative identification of the chemical structure of the major component in S2, the active component in the shrub. The effect of the test samples on sheep adrenal mitochondrial P450c11 is described in Chapter 3. The influence of Compound A, the ethanol extract and the S2 fraction on the DOC-induced difference spectra and the ability of Compound A, the ethanol extract and the S2 fraction to elicit a Type II difference spectrum were tested in buffer, heated and native sheep plasma. The ability of Compound A, plant extract and S2, to displace the steroids, cortisol and progesterone, from CBG was also investigated (Chapter 4). In Chapter 5 ES-MS studies are described that investigate the equilibria between the open chain precursor (Compound A) and

the aziridine in PBS and sheep serum. The influence of different factors, namely cone voltage, temperature and pH, and the presence of the P450c11 enzyme on the cyclization of Compound A to the aziridine are also discussed. These studies are aimed at elucidating the interaction of the specified test samples with the plasma steroid binding proteins, CBG, and the adrenal substrate binding protein, cytochrome P450c11.

CHAPTER 2

PREPARATION OF TEST SAMPLES

2.1 Introduction

The study of labile natural products depends on quick and reliable methods by which these compounds can be extracted and purified. Swart developed such a method for the isolation of labile active compounds from *S.tuberculatiformis* [8]. The method consisted of ethanol extraction, ultrafiltration, ion-exchange separation and finally high performance liquid chromatography (HPLC). Using this method several compounds were isolated from *S. tuberculatiformis* of which the most active fraction was called S2.

Further investigations showed that the S2 fraction consisted of a mixture of an extremely labile compound plus one or more stable components, one of which is synephrine. The S2 fraction will decompose to synephrine (I in Fig. 2.1) in an aqueous medium. Derivitisation studies, however, showed that the S2 fraction is not synephrine, as acetylation of the S2 fraction yields the acetylated aziridine structure III in Fig 2.1, while acetylation of synephrine yields synephrine triacetate (II in Fig. 2.1) [10]. The above studies suggested that the active component in the S2 fraction might be 2-(4-hydroxy-phenyl)-1-methyl-aziridine (V in Fig. 2.2) or the corresponding open chain precursor (IV in Fig 2.1) [11,16,17]. De Kock obtained chemical proof of the presence of an aziridine as a component of the S2 fraction. The aziridine ring, and its protonated form the aziridinium ion, are reactive compounds which readily react with nucleophiles to form alkylated, ring-opened products. A sulfhydryl group containing the aziridine probe, N-TNP-MEA-SA, was synthesized. This probe would react with synthetic model aziridines, but not with synephrine. When N-TNP-MEA-SH was applied to the S2 fraction, it could be shown by TLC that a new product had formed in a way similar to what was observed with synthetic aziridines [11].

Phenolic aziridines are extremely labile and therefore not ideal test compounds, thus their open chain precursors, which cyclize to the aziridine *in vivo*, are normally used in pharmacological studies [17]. De Kock synthesised a range of phenolic aziridine precursors

and used FAB-MS to study these phenolic aziridine precursors and their cyclization to the corresponding aziridines [12]. The substituent on the phenyl ring appeared to be important as a determinant of the rate of cyclization as the 4-acetophenyl precursors could be detected by FAB-MS but not the hydroxyphenyl precursors. The FAB-MS spectra showed the peaks of the corresponding 4-hydroxyphenyl aziridines instead. These results suggested that the 4-hydroxyphenyl precursors were very labile and cyclisised quickly to the corresponding aziridine so that only the aziridine was observed by FAB-MS.
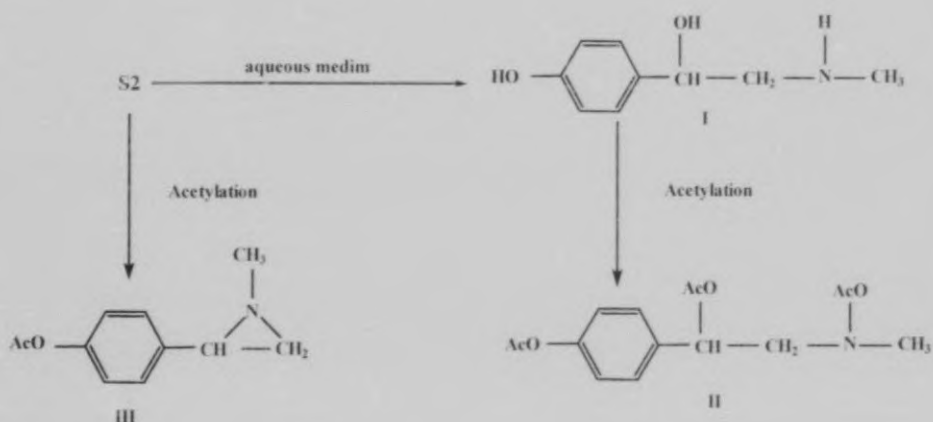
Figure 2.1 Reaction scheme for the formation of synephrine (I), synephrine triacetate (II) and acetylated S2 (III).

These results prompted the large scale synthesis of the more stable acetoxy phenyl aziridine precursor, Compound A (2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammoniumchloride; VI in Fig. 2.2), which cyclizes to the corresponding acetoxyphenyl methylaziridine (VII in Fig. 2.2) at physiological pH, to be used as a chemical analogue. Compound A, the chemical analogue, was subsequently used to test the hypothesis that the biologically active component of *S. tuberculatiformis* is a phenyl aziridine or its corresponding open chain precursor. Studies by Louw *et al* established that Compound A has the same effect as *S. tuberculatiformis in vivo* in that it is contraceptive in rats. *In vitro*, Compound A inhibited adrenal cytochrome P450c11 as does S2 which was isolated from the shrub [14]. Taken together the above results was seen as indicating that phenolic aziridines, or their precursors,

are the active agents in the shrub and that Compound A could be used as a more stable analogue to study the effects of the shrub.

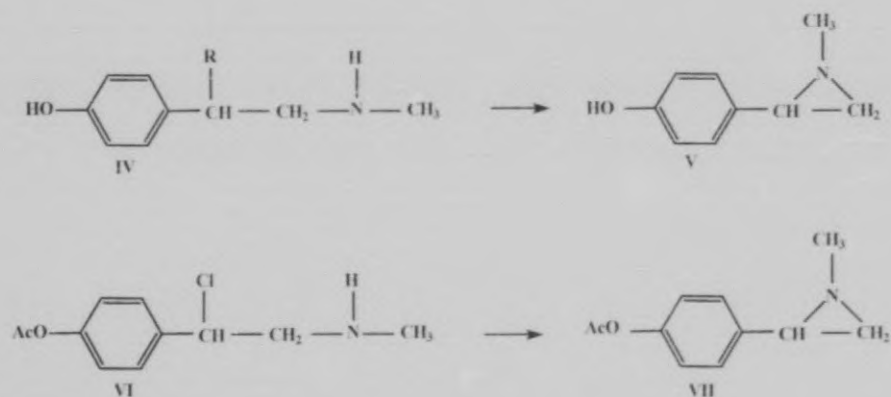**Figure 2.2** Chemical structures of aziridine precursors and their corresponding aziridines: 2-(4-hydroxyphenyl)-1-methylaziridine (V) and its open chain precursor (IV); 2-(4-acetoxyphenyl)-1-methylaziridine (VII) and its open chain precursor: Compound A (VI). R = good leaving group

2.2 Synthesis of Compound A

De Kock synthesised the aziridine precursor, Compound A, (2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammoniumchloride, using the following method. [12]. DL-Synephrine hydrochloride (10 g) was dissolved in glacial acetic acid (27 ml) and cooled to 10°C in an ice waterbath. Acetyl chloride (27 ml) was added slowly while stirring thoroughly, and the reaction temperature was kept below 30°C. A white precipitate formed from the resulting clear solution within 12 hour. The fine white precipitate, Compound A, was collected by filtration under nitrogen, washed with cold anhydrous ether, followed by cold anhydrous acetone, and kept in a vacuum desicator over silica gel for 12 hours before storage at -80°C. The yield of the reaction was 13.21 g or 83.6% of Compound A. The compound was highly hygroscopic and decomposed after the absorption of moisture or when left open at room temperature. The structure and purity of Compound A were verified by ^{1}H-NMR and FAB-MS, which gave the expected molecular ions at 228/230.

2.3 Isolation of active compounds from S. tuberculatiformis

2.3.1 Preparation of ethanol extract

Active compounds from *S.tuberculatiformis* were extracted according to the method of Swart [8]. A schematic representation of the entire isolation process of active compounds from *S.tuberculatiformis* can be seen in Fig. 2.3.

Dried, ground plant material (500 g) was placed in a Soxhlet extraction apparatus fitted with a double wall condencer. All glassware were covered with aluminium foil to shield the plant material and the extract from light. A preliminary extraction was made with chloroform for 20 hrs to remove most of the non-polar components and a large fraction of the plant pigments. The chloroform extract, which was only slightly active, was removed and replaced by ethanol. After 20 hrs, extraction with ethanol was terminated, the extract removed and dried under reduced pressure on a rotary evaporator at room temperature. The resulting residue was taken up in de-ionized water and filtered through fine filter paper. The pH of the solution was normally between 3.5 and 4.0.

A fraction containing compounds with a molecular weight lower than 500 was prepared by ultrafiltration of the redissolved ethanol extract through a series of ultrafiltration membranes with molecular mass cut offs of 25 000, 10 000, 1000 and 500 respectively. Ultrafiltration was carried out at 4°C in a standard ultrafiltration cell pressurised with nitrogen. This procedure separated inactive higher molecular mass and pigments from the active lower molecular mass compounds in the extract. Ultrafiltration was preferred to gel filtration to avoid unwanted dilution of the active compounds. After ultrafiltration the ethanol extract was freeze dried (A in Fig. 2.3) and kept frozen at -20°C without lost of activity. The yield was 39.06 g ethanol extract from 500 g shrub. This sample is called the ethanol extract and is used in further studies.

Van der Merwe *et al* found that a considerable amount of activity was lost on exposure of the extract to light and care was therefore taken to shield the extract, as well as the filtrate, from light during the entire filtration process [6]. Activity was also lost after the extract was

exposed to an alkaline pH. The activity of the extract was tested during different stages of the extraction process using a cytochrome P450 based spectral assay (see Chapter 3).

500 g shrub

↓

chloroform extraction (5 L)

↓

ethanol extraction (5 L)

dried on rotary evaporator
↓

dissolved in 400 ml MQ H$_2$O

ultrafiltration (Mr<500)
↓

360 ml EtOH extract

A: freeze dried
(2.3.1)

B: kalignost treatment
freeze dried
redissolved in water
0.45 uM filtered
(2.3.2)

EtOH extract

S2

39,06 g / 500 g shrub

HPLC

10x 150 ul EtOH extract

↓

S2 fraction (16 ml)

kalignost treatment (2.3.4)
↓

S2 fraction (66 ml)
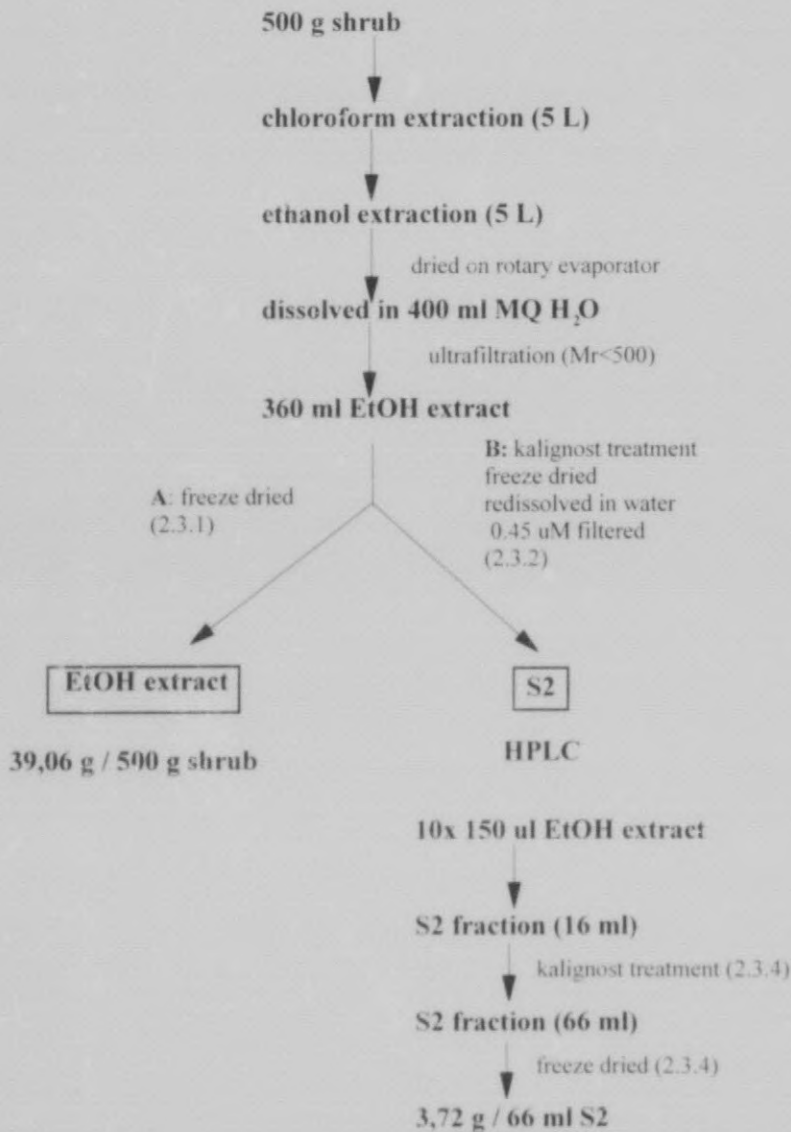
freeze dried (2.3.4)
↓

3,72 g / 66 ml S2

Figure 2.3 Schematic representation of the isolation of active compounds from *S.tuberculatiformis*.

2.3.2 Fractionation of active compounds with a liquid ion exchanger

After discovery that the active compounds in the extract were cations, a liquid ion exchanger sodium tetraphenylborate (kalignost) in benzyl alcohol, was employed to separate the cations from the mixture [8]. The plant extract, containing compounds with a molecular mass of less than 500, was shaken up in a separating funnel containing the kalignost solution (B in Fig. 2.3). The aqueous and organic phases were allowed to separate and the inactive aqueous phase discarded. The organic phase was washed three times with equal volumes of 1 M HCl solution which resulted in a 3x dilution. The active cations in the extract move from the organic phase to the aqueous phase due to the excess of cations provided by the HCl which compete with the cations of the extract. The HCl fractions were pooled, washed with an equal volume of dichloromethane to remove the benzyl alcohol, and freeze dried. The sample was redissolved in de-ionized water, filtered through a 0.45 μM membrane filter and then subjected to further separation on HPLC.

2.3.3 Isolation of S2 utilizing HPLC

In a previous study, Swart described the separation of the cationic fraction of the plant extract on an analytical C^{18} reverse phase (RP)-HPLC column [8]. Swart isolated three extremely labile fractions from *S. tuberculatiformis*, namely S1(a), S1(b) and S2, which inhibits the hydroxylation of DOC to corticosterone [8, 9]. Of the three compounds only S2 has so far been investigated further as S2 appeared to be a more homogeneous fraction and not as complex a mixture as S1. Swart observed that S2 stabilized the low spin state of the hemoprotein which results in a Type II difference spectrum and inhibited the binding of the natural substrate to the active site of cytochrome P450c11.

In this study, small scale separations of the active compounds in *S. tuberculatiformis* were achieved on an C^{18} reversed phase stainless steel column (8 mm × 250 mm), using the same method as Swart [8]. The elution buffers consisted of the following: Solvent A: 0.1 M trichloroacetic acid / trichloroacetate buffer, pH 3 and solvent B: methanol containing 1% glacial acetic acid. A linear gradient was run from 100% A to 50% A and 50% B in 30 min at a flow rate of 2.0 ml/min. The column effluent was monitored at 280 nm with a photodiode array (PDA) detector. The chromatogram obtained is shown in Fig. 2.4. This chromatogram confirmed that the S2 fraction obtained in the present study, which eluted after 18.5 minutes,

was the same as that previously isolated by Swart and Scnultz [8,18]. On line spectral analysis of this peak revealed a UV absorption band (maximum) at 284 nm (Fig 2.5). Fractions were collected and the pH of the collected fractions were adjusted to 4 with aqueous sodium hydroxide (0.1 M). The sodium trichloroacetate from the mobile phase was removed with the liquid ionexchange system as will be discussed in the next section.
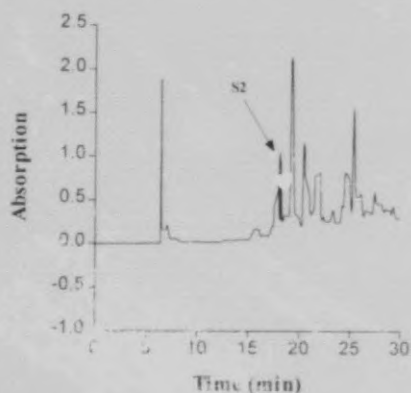
Figure 2.4 HPLC separation of the cation fraction on a C^{18} reverse phase column using a gradient elution method. A linear gradient was run from 100% A to 50% B in 30 min at a flow of 2.0 ml/min on a C^{18} column. The elution buffers consisted of the following: Solvent A: 0.1 M trichloroacetic acid/ trichloroacetate buffer. pH 3 and solvent B: methanol containing 1% glacial acetic acid. The column effluent was monitored with an ultra violet detector set at 280 nm. The sample volume was 150 µl. The S2 fraction was collected as indicated on the chromatogram.

Figure 2.5 The UV absorption spectrum of the S2 fraction exhibiting an absorption maximum at 284 nm.

2.3.4 Preparation of S2 free of TCA buffer salts

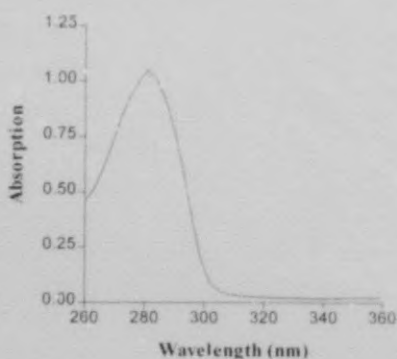S2 fractions obtained by HPLC still contain considerable amounts of crystalline sodium trichloroacetate (TCA) which stabilize the S2 fraction against autocatalytic degradation. The TCA buffer salts was removed and replaced with sodium hydroxide buffer salts because it would interfere with biological assays used to test the S2 fraction. Schultz applied two different methods to obtain TCA free S2 preparations [18]. The first involved an isocratic HPLC purification of the S2 fraction. The S2 fractions containing sodium trichloroacetate were freeze dried and then redissolved in de-ionized water, the pH adjusted to 4 with aqueous sodium hydroxide (0.1 M), filtered through an 0.45 micron filter and applied to the same C^{18} RP-HPLC column that was previously used. In the isocratic separation a water/methanol mixture (4:1) was used as the eluant at a flow rate of 7 ml/min. This system eluted S2 after 5 min. The active fraction was collected, lyophilized overnight and again subjected to the same isocratic HPLC separation to yield pure, but more unstable, S2. A serious drawback of this method is the low yield of pure S2. For this reason a second method using the liquid ion exchanger kalignost was later employed for the removal of sodium trichloroacetate from S2 samples following the same procedure as described previously in 2.3.2. The second method was used in this study because it produces a higher yield of S2. Fig. 2.6 shows the HPLC chromatogram of the S2 fraction dissolved in water after treatment with kalignost.
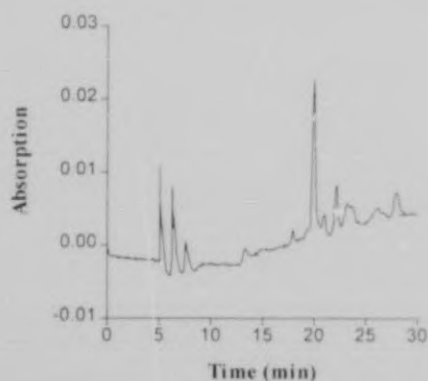
Figure 2.6 Chromatogram of S2 after treatment with kalignost. A linear gradient was run from 100% A to 50% B in 30 min at a flow of 2.0 ml/min on a C^{18} column. The elution buffers consisted of the following: Solvent A: 0.1 M trichloroacetic acid/ trichloroacetate buffer, pH 3 and solvent B: methanol containing 1% glacial acetic acid. The column effluent was monitored with an ultra violet detector set at 280 nm. The sample volume was 10 µl.

Spectral analysis of this peak revealed a UV absorption band (maximum) at 284 nm (Fig 2.7). These results suggest that S2 was not affected by the kalignost treatment. After use of the liquid ion exchanger, kalignost, to remove the sodium trichloroacetate from S2 samples, the HCl containing fractions were pooled and washed with an equal volume of dichloromethane to remove any residual benzyl alcohol, and freeze dried. The mass of lyophilised material S2 was 3.72 g from 1.5 ml ethanol extract. This yield is greater than the amount of ethanol extract added (0.16 g) and could be attributed to the NaCl salts.
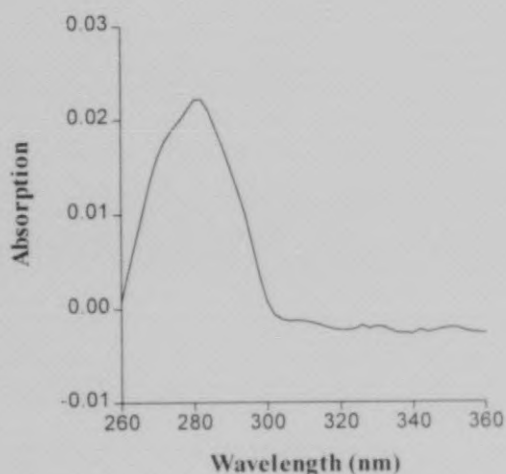
Figure 2.7 The UV absorption spectrum of S2 after treatment with kalignost exhibiting an absorption maximum at 284 nm.

2.3.5 ES-MS analysis of S2

The mild nature of electrospray ionization makes ES-MS ideally suited for the study of natural products and labile molecules. This technique was applied to re-investigate the components present in the cationic S2 fraction obtained from *S. tuberculatiformis*. In Chapter 5 the technique of ES-MS is discussed in detail. The S2 fraction was redissolved in de-ionized water and diluted 50:50 with acetonitrile and assayed immediately. Formic acid was not added as the sample was already very acidic. The cone voltage was set at 30 V, and the source temperature was kept at 75°C. The ES-MS spectrum of this sample is shown in Fig. 2.8. S2 contains molecular ions at m/z of 258, 168, 166 and 150.
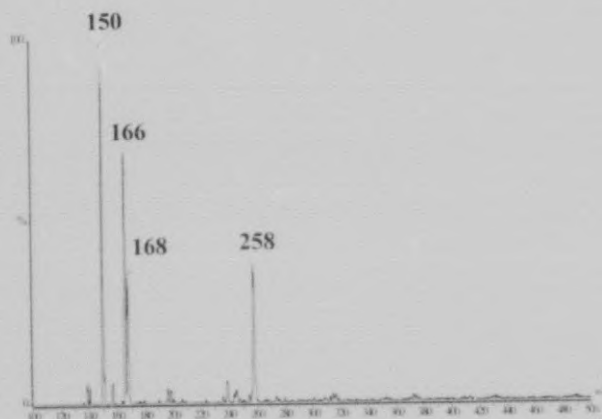
Figure 2.8 ES-MS spectra of the S2 fraction dissolved in water and diluted 50:50 with acetonitrile.

The ES-MS spectrum showed that S2 contained a molecular ion (M+H)⁺ at m/z 168 which could be attributed to synephrine. This was confirmed by comparing fragments obtained from 168 (Fig. 2.10 B) to those obtained from pure synephrine (Fig. 2.11 C). In Table 2.1, a summary of the fragments of the molecular ion at m/z 168 of S2 and pure synephrine (m/z 168) are presented. Both the molecular ion at m/z 168 of S2 and pure synephrine (m/z 168) contain identical fragments. From these results we can conclude that the molecular ion at m/z 168 is synephrine. This agrees with previous ^{1}H-NMR studies of S2 samples which, on occasion, showed considerable amounts of synephrine to be present in S2 samples. This observation eventually led to the discovery of the ready conversion of S2, under strong alkaline conditions, to synephrine.

Maritz, in a previous study, also used ES-MS to study the cationic fraction from *S. tuberculatiformis* [19]. The spectrum of the S2 fraction indicated the presence of three major peaks at m/z 168, 166 and 150. The peaks at m/z 168 and 150 were attributed to the (M+H)⁺ ion of synephrine and its dehydration product respectively. The interpretation of NMR

Table 2.1 Comparison of the fragments of the molecular ion at m/z 168 of S2 and pure synephrine obtained from spectra in Fig. 2.10 (B) and Fig. 2.11 (C) respectively.

m/z	S2 MOLECULAR ION (m/z 168)	PURE SYNEPHRINE
150	X	X
135	X	X
121	X	X
120	X	X
119	X	X
107	X	X
91	X	X
42	X	X
32	X	X

spectra resulting from the peak at m/z 166 suggested its chemical structure to be that of 6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline, commonly known as norsalsolinol (Fig. 2.9) The S2 fraction in this study also contained a molecular ion at m/z 166, like the S2 fraction of Maritz [19]. This was confirmed by comparing fragments of 166 (Fig. 2.10 C) with those of pure norsalsolinol (Fig. 2.11 D and Table 2.2). The molecular ion at m/z 166 of S2 ard pure norsalsolinol (m/z 166) contains similar fragments at m/z 149, 137, 132, 121, 103, 91, 77, 65, 44 and 30. From these results it can be concluded that the molecular ion at m/z 166 in S2 is indeed norsalsolinol.
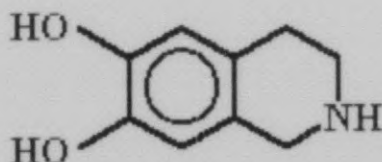

Figure 2.9 Chemical structure of norsalsolinol

The components chemically identified in the cationic fraction by Maritz all appeared to be stable compounds. The labile component(s), consistently observed in previous S2 samples, of which the EI-MS and FAB-MS spectra showed no true molecular ion but instead an abundance of high molecular peaks, appears to be absent. In this study the S2 fraction also contained the peaks at m/z 168, 166 and 150 observed by Maritz [19]. In addition, a peak at m/z 258 was also observed, which may be a labile component in S2. De Kock showed that S2 contained an aziridine moiety [12]. None of the peaks from Maritz's study, however, contained such a moiety [12]. Thus, the S2 isolated in our study, and specifically the molecular ion at 258, appears to be the most likely candidate to either contain the aziridine moiety or to be the precursor for an aziridine component. To investigate this, fragmentation of the molecular ions of S2 (m/z 258 and 150) was done, and compared with the fragments of pure synephrine (m/z 168), Compound A (m/z 228/230) and the aziridine formed from Compound A (m/z 192) (Table 2.3 and Fig. 2.10 A and D and Fig. 2.11 A, B and C respectively). Compound A has two chlorine isotopes at m/z 228 and 230.

Table 2.2 Comparison of the fragments of the molecular ion at m/z 166 of S2 and pure norsalsolinol obtained from spectra in Fig. 2.10 (C) and 2.11 (D) respectively.

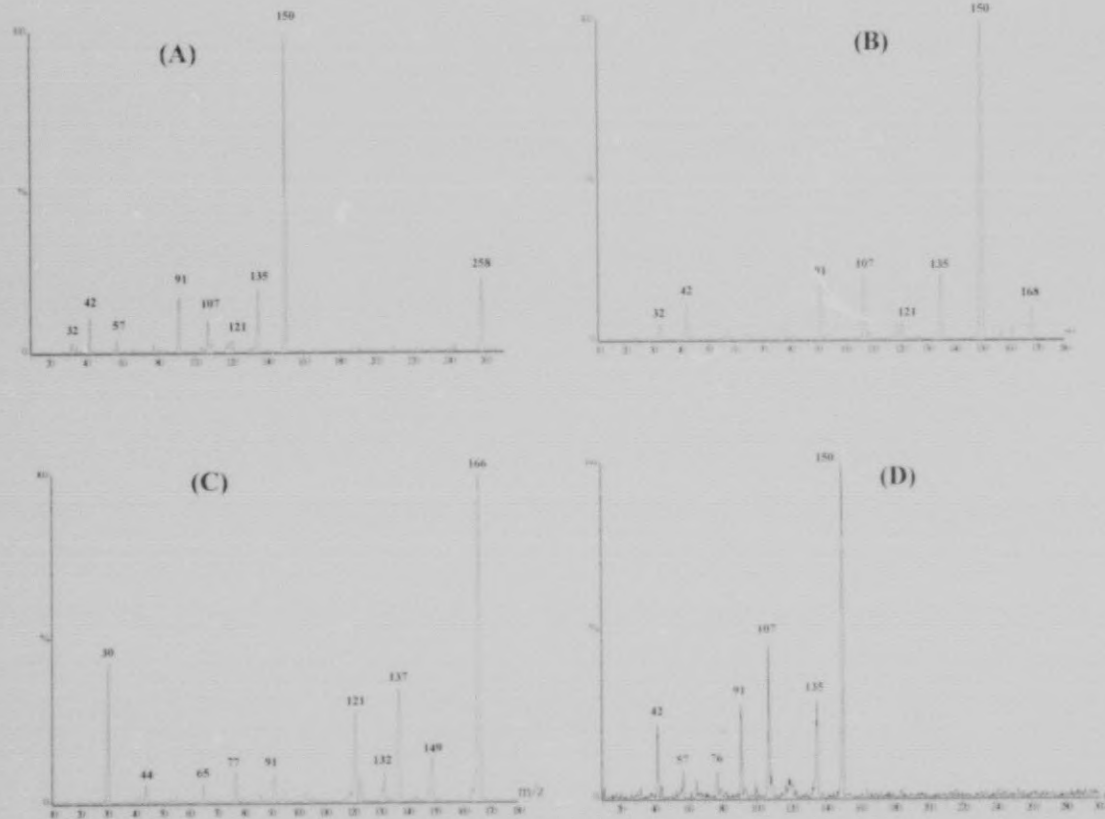| m/z	S2 MOLECULAR ION (m/z 166)	PURE NORSALSOLINOL
149	X	X
137	X	X
132	X	X
121	X	X
103	X	X
91	X	X
77	X	X
65	X	X
44	X	X
30	X	X

Figure 2.10 ES-MS spectra of the fragments of S2 molecular ions (**A**) 258 (**B**) 168 (**C**) 166 and (**D**) 150.
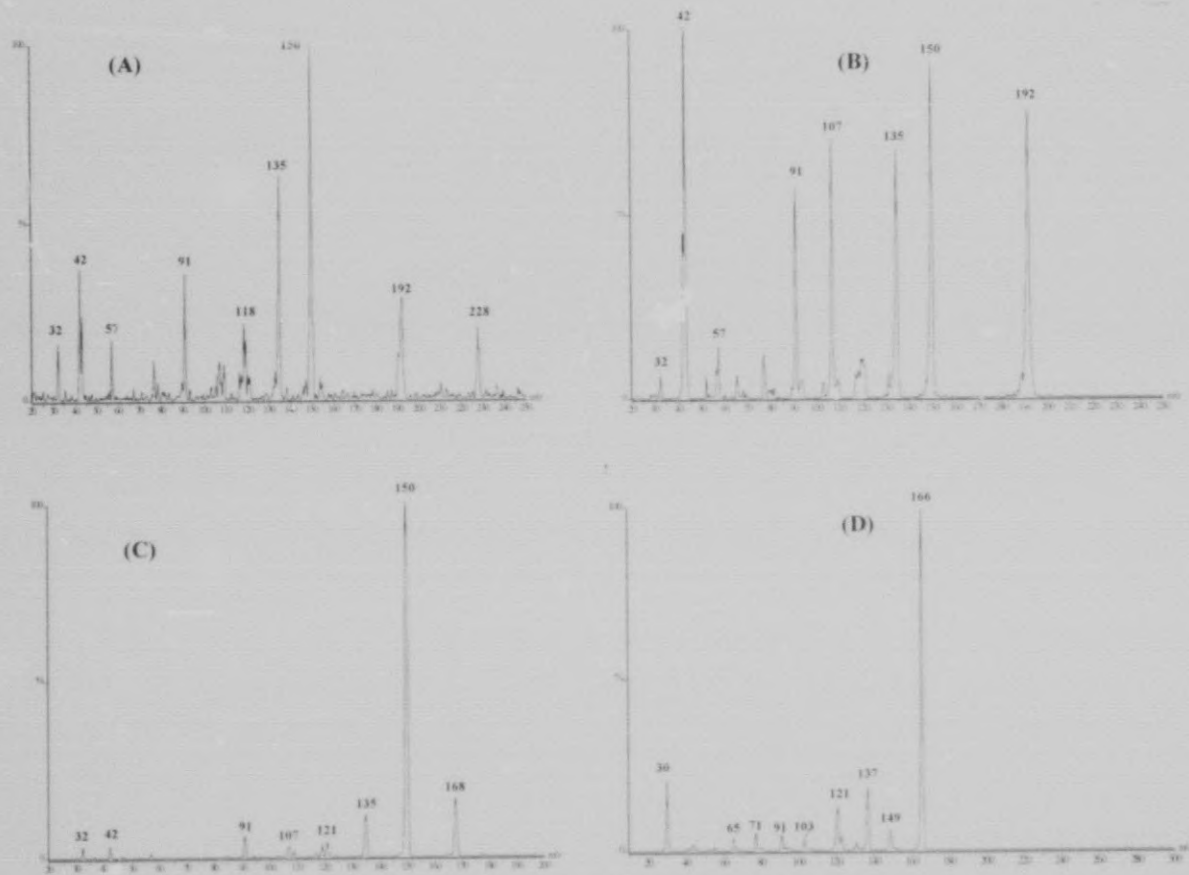
Figure 2.11 ES-MS spectra of the fragments of (**A**) Compound A (*m/z* 228/230), (**B**) the aziridine from Compound A (*m/z* 192), (**C**) pure synephrine (*m/z* 168) (**D**) pure norsalsolinol (*m/z* 166)

Previous work has established that Compound A (m/z 228/230) cyclisises to the corresponding aziridine (m/z 192) and then decomposes to synephrine (m/z 168), with time in an aqueous medium [14]. A comparison of the fragments of Compound A (m/z 228/230) and the corresponding aziridine (m/z 192) and synephrine (m/z 168) all exhibited a peak at m/z 150. The fragments ion at m/z 150 from Compound A, aziridine and synephrine is similar as can be seen from a comparison of the fragment ions in Table 2.3. We would like to suggest that this moiety is a hydroxy-aziridine (m/z 150) which is formed during electrospray ionization from aziridine (m/z 192) and synephrine (m/z 168) as indicated in Fig. 2.12. The fact that the fragment ion at m/z 150 is also observed in the Compound A (m/z 228/230) fragmentation pattern may be due to formation from the aziridine (192) as this daughter ion can clearly be seen in Fig. 2.11 (A). Although Maritz suggested that the m/z 150 molecular ion in S2 is a dehydration product of synephrine we would like to suggest, in the light of the above evidence, that this is not likely [19]. The fragmentation patterns of both Compound A (m/z 228/230) and aziridine (m/z 192) contain the m/z 150 fragment ion but not the synephrine (m/z 168) ion. Thus we conclude that a more likely senario is the one illustrated in Fig. 2.12 where both aziridine (m/z 192) and synephrine (m/z 168) forms the hydroxy aziridine at m/z 150.

Table 2.3 Comparison of the fragments of the molecular ion m/z 258 from S2, molecular ion m/z 150 from S2, pure synephrine (m/z=168), Compound A (m/z=228/230) and the aziridine formed from Compound A (m/z=192) obtained from spectra in Fig. 2.10 (A) and (D) and Fig. 2.11 (C), (A) and (B), respectively.

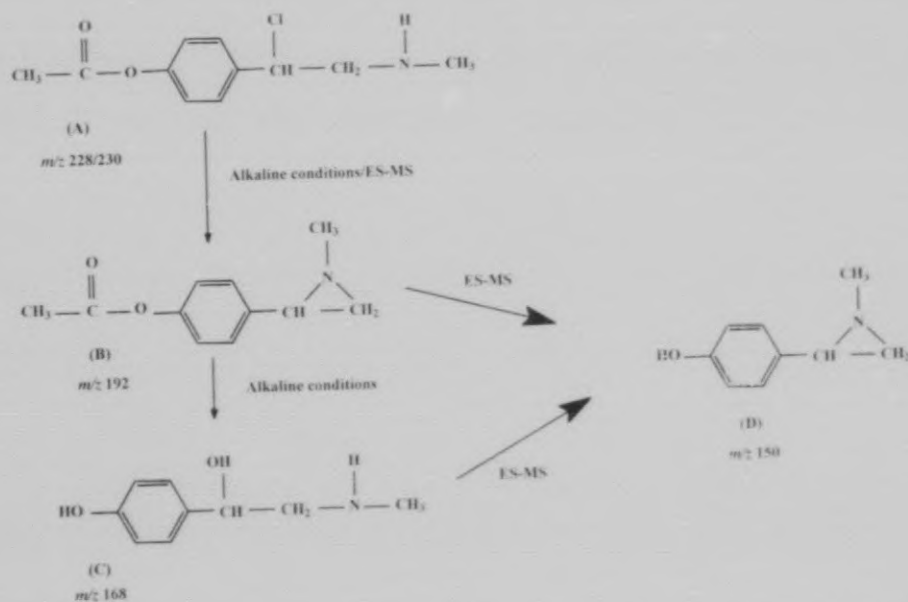| m/z	S2 (m/z =258)	S2 (m/z =150)	Synephrine (m/z =168)	Compound A (m/z =228/230)	Compound A (Az) (m/z =192)
192				X	
150	X	X	X	X	X
135	X	X	X	X	X
Cluster 118-120	X	X	X	X	X
107	X	X	X	X	X
91	X	X	X	X	X
76	X	X	X	X	X
57	X	X	X	X	X
42	X	X	X	X	X
32	X	X	X	X	X

Figure 2.12 Cyclization of Compound A (A) to the corresponding aziridine (B) and decomposition to synephrine (C), plus possible routes for formation of the hydroxy-aziridine (D) under ES-MS conditions.

S2 isolated in this study also contained the m/z 150 peak (Fig. 2.8) which is also revealed in the fragmentation pattern of the S2 m/z 258 molecular ion (Fig. 2.10 A). Comparison of the fragmentation pattern of the fragment ions of S2, m/z 150 and m/z 258, with that of the m/z 150 peaks found in Compound A, aziridine (m/z 192) and synephrine show a great similarity (Table 2.3). We thus conclude that this peak in the S2 fraction is also a hydroxy-aziridine (150) and suggest that the m/z 258 is an aziridine precursor, which cyclises to the hydroxy-aziridine (m/z 150) which in turn, may decompose to synephrine (m/z 168) (Fig. 2.13).

The leaving group (X in Fig. 2.13) in S2's molecular ion at 258 is not shown in the fragmentation pattern of m/z 258 (Fig. 2.10 A), which suggests that this moiety is probably not positively charged and therefore not observed in the positive ES-MS mode. We did conduct a negative mode ES-MS analysis of m/z 258 (results not shown) and also did not find any group that could be attributed to this leaving group (X). Alternative chemical analysis would thus have to be employed to identify the leaving group (X) from the S2 molecular ion.

This group has been calculated to have a molecular mass of 108 (difference between 258 and 150).
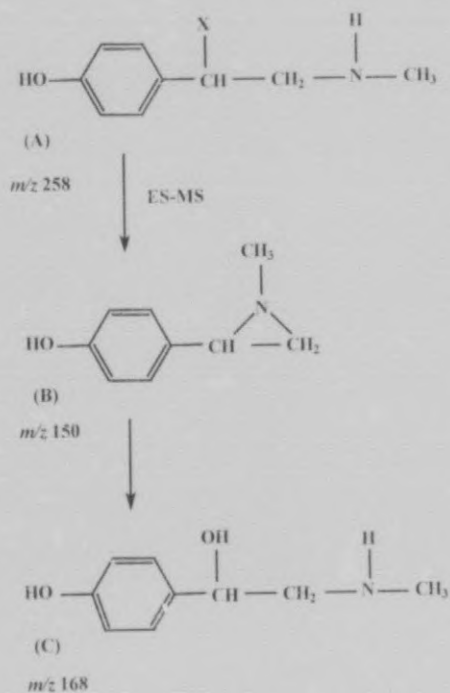
Figure 2.13 Suggested cyclization of S2 (A) to an hydroxy-aziridine (B), and decomposition to synephrine (C). X=good leaving group.

In summary, we conclude from the ES-MS analysis of S2, and comparison of this analysis with that of Compound A and synephrine, that the active component in S2 is the molecular ion at m/z 258. This molecular ion is probably the aziridine precursor for the hydroxy-aziridine formed at m/z 150, the latter which will decompose to synephrine. The m/z 166 peak observed in the S2 fraction is norsalsolinol. At present this is only a well informed hypothesis which would need to be investigated further in more detail.

CHAPTER 3

THE INFLUENCE OF TEST SAMPLES ON THE SPECTRAL ACTIVITY OF SHEEP ADRENAL CYTOCHROME P450C11

3.1 Introduction

Klingenberg and Garfinkel independently discovered the presence of a pigment in rat liver microsomes capable of binding carbon monoxide with an unique carbon monoxide-binding spectrum [20, 21]. They observed the appearance of a broad intense absorption band at 450 nm when carbon monoxide was bubbled through a dithionite-reduced suspension of liver microsomes. Investigation by Omura and Sato showed that the pigment had certain unique spectral properties and led to the conclusion that the pigment was a new b-type cytochrome [22]. The reduced versus the oxidized difference spectrum of the pigment showed a characteristic absorption maximum at 450 nm in the presence of carbon monoxide (Fig 3.1). Because of this unique spectral property the name P450 (pigment with an absorption at 450 nm) was given to the cytochrome. Omura and Sato also found that treatment of the P450-containing microsomes with detergent did not totally destroy the pigment, but converted it to another distinct solubilized form. The difference spectrum of the converted P450 in the presence of carbon monoxide, gave an absorbance maximum at 420 nm. This solubilized form was named P420 to distinguish it from the membrane bound form. It is assumed that the enzyme is catalytically active if the cytochrome P450 in a preparation
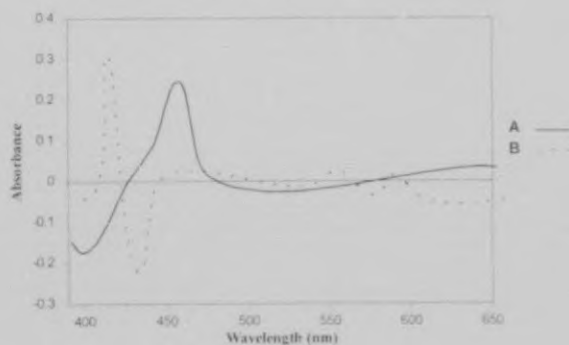

Figure 3.1. Carbon monoxide induced difference spectrum of cytochrome P450 (A) and P420 (B) (Redrawn from [22])

exhibits a difference spectrum with any given substrate. If the cytochrome P450 in the preparation is converted to cytochrome P420, the inactive form of the enzyme, no substrate induced difference spectrum can be obtained.

P450 enzymes are found in the adrenal glands, the liver and the gonads. The biosynthesis of adrenal steroid hormones take place in the mitochondria as well as in the endoplasmic reticulum of the adrenal cortex. Metabolic intermediates move back and forth between these two subcellular compartments where different types of cytochrome P450 enzymes are involved in the biosynthesis of glucocorticoids, mineralocorticoids and androgens from cholesterol. For example, deoxycortisol and deoxycorticosterone have to move back into the mitochondria from the endoplasmic reticulum for the final steps in glucocorticoid and mineralocorticoid biosynthesis to take place. Deoxycortisol and deoxycorticosterone are both hydroxylated at the C11-position by a cytochrome P450-dependant enzyme, P450c11, to give cortisol and corticosterone respectively. Corticosterone undergoes a further hydroxylation at the C18-position to yield 18-hydroxycorticosterone which is a direct precursor for aldosterone. Both C11- and 18-hydroxylation reactions are catalysed by the same enzyme, namely cytochrome P450c11. Cytochrome P450C11 is a steroidogenic P450 enzyme localised in the inner mitochondrial membrane [23]. A schematic representation of the conversion of cholesterol to different steroids hormones in the different organelles in the adrenal cortex is given in Fig. 3.2.

Various methods are used to study the interaction of P450's enzymes with differe. substrates. Substrate induced difference spectra is one of these methods which :: u d to study the interaction between the different types of adrenal cytochrome P450's and their substrates. This method is convenient, rapid and very sensitive to possible changes in specificity or affinity of any given ligand for the hemoprotein. A spectrum is obtained by measuring the optical difference between the enzyme-substrate complex in the sample cuvette and the enzyme in the reference cuvette.
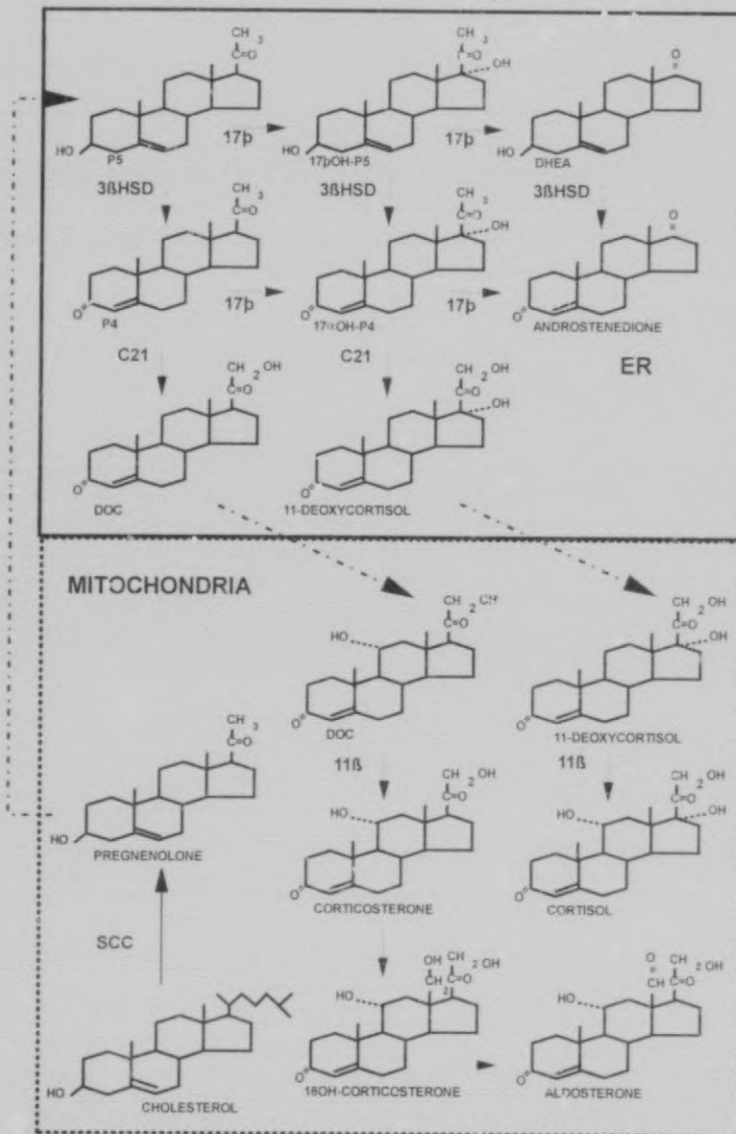
Figure 3.2 Schematic representation of adrenal steroidogenesis. The solid arrows indicate monooxygenase reactions catalyzed by cytochrome P450: (SCC) cytochrome P450scc; (C21) cytochrome P450c21, (17β) P450c17, (11β) cytochrome P450c11. The dotted arrows indicate reactions catalyzed by 3β-hydroxy δ⁵-steroid dehydrogenase-δ⁵-isomerase (3βHSD). The metabolic intermediates move back and forth between the two organelles (Redrawn from [23]).

The spectra are classified into three types namely, Type I, Type II and modified Type II (m) on the I , of maximum and minimum absorption values. Table 3.1. summarises the characteristics of the different types of substrate induced difference spectra of cytochrome P450 and the changes in the spin states involved [24].

Table 3.1 Characteristics of substrate induced difference spectra [24].

	Maximum	Minimum	Spin state change
Type I	385-390 nm	420 nm	low to high
Type II	430 nm	390 nm	high to low
Type II (m)	409-445 nm	365-410 nm	high to low

Yoshida and Kumaoka proposed the following classification for cytochrome P450-substrate interactions [25]. Type I spectra are induced by the interaction of large hydrophobic hydrocarbon residues on the substrate with the protein moiety of the   e (420 nm) cytochrome P450 which results in a shift to the high-spin type (3   nen the amino group on the substrate interacts with the heme iron of either the low-spin (420 nm) or the high-spin (390 nm) type of cytochrome P450 it results in a shift to the low-spin type (420 nm) and a Type II spectrum is obtained. Type II (m) spectra are observed when a hydroxyl group combines with the heme iron of the high-spin type (394 nm) which results in a shift to the modified low-spin type (416 nm). Typical examples of a Type I and Type II difference spectra are shown in Fig. 3.3.

Evidence was obtained from studies with *S. tuberculatiformis* extracts that at least part of the prolonged gestation in ewes as well as the contraceptive action in rats may be related to changes in steroid hormone levels [1, 2, 3, 15]. Steroid hormones are actively produced in the adrenal glands and studies with rat adrenal tissues, showed that active extracts inhibited the synthesis of corticosterone from deoxycorticosterone [6].
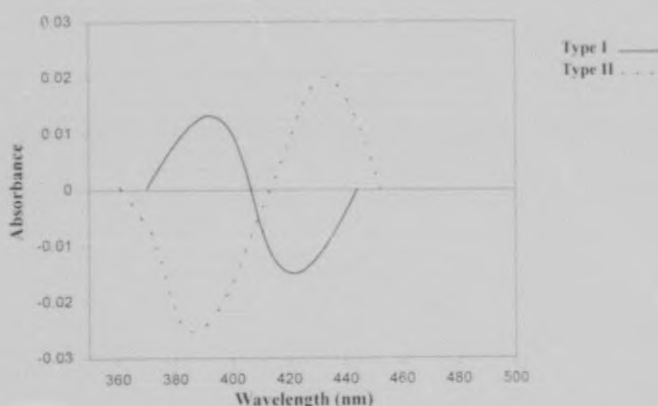
Figure 3.3 Schematic representation of Type I and Type II induced difference spectra of cytochrome P450. (Redrawn from [21])

Due to the considerable amount of active material needed per test and the long time required to complete bio-assays (prolonged gestation in sheep and contraception in rats) it became necessary to develop biochemical micro-assays. The micro-assays would be more sensitive, and due to the shorter time required to complete the assay it would minimise decomposition of the labile compounds. Swart developed two spectroscopic micro-assays, to study the interaction of labile compounds extracted fom *S. tuberculatiformis* with P450c11 [8]. The first measured the direct interaction of the test samples with the cytochrome P450c11 enzyme, while the second monitored the interference of the test samples with the binding of the natural substrate, DOC, to the enzyme. A third micro-assay, which was later added, determined the effect of the test samples on the conversion of DOC to corticosterone by a reconstituted cytochrome P450c11 enzyme system. Swart isolated an biologically active, but very labile, HPLC fraction called S2 from dried plant material of *S.tuberculatiformis* [8]. The above mentioned micro-assays were used to study the interaction of an Ethanol extract of the plant and S2 on cytochrome P450c11 enzymes. He found that the piant extract and S2 inhibited the Type I DOC-induced difference spectra of cytochrome P450c11, the enzyme respon. ble for the final step in the synthesis of corticosterone and cortisol, *in vitro*. In ad.itior. S2 elicited a Type II difference spectrum when reacting with cytochrome P450, indicating the probable presence of an amino group interacting with the heme in the active centre of the cytochrome P450 enzyme [25].

Previous studies showed that aziridines or their precursors, may be the active components in the biologically active S2 fraction [6]. Specifically a hydroxyphenyl methyl aziridine or its precursor appeared to be the most likely candidate for the active component in S2. De Kock synthesized a series of phenyl-methyl-aziridine precursors and found that the cyclization of the hydroxy-phenyl-methyl-aziridine precursor occurred at such a fast rate that only the corresponding aziridine could be detected by FAB-MS [12]. Replacing the hydroxy group on the phenyl ring with an acetoxy group resulted in a more stable chemical analogue, 2-(4-acetoxyphenyl)-2-chloro-N-methyl-ethylammoniumchloride. This compound was referred to as Compound A. Spectrophotometric studies indicated that Compound A inhibited the Type I DOC-induced difference spectrum of sheep adrenal cytochrome P450c11 and elicited a Type II difference spectrum. These results corresponded with those produced with S2, isolated from the shrub. Inhibition of the Type I spectrum by Compound A was attenuated with time in PBS but not in sheep serum. Native sheep serum appeared to stabilize the inhibitory effect of Compound A, while heated sheep serum showed attenuation of the inhibitory influence of Compound A with time, in a similar manner to that of Compound A in PBS. ES-MS studies indicated that in PBS the aziridine precursor cyclised fully to the aziridine within one hour, while in sheep serum the cyclization was retarded. It was found that it is the aziridine precursor, rather than the aziridine, that was the inhibiting agent in the cytochrome P450c11 system and that two high affinity steroid-binding proteins in serum, SHBG and CBG may bind and stabilise the aziridine precursor in sheep serum [14].

The aim of the present study was to test the *in vitro* biological effects of Compound A, the ethanol extract from the shrub and S2, on sheep adrenal mitochondrial cytochrome P450c11 in buffer, heated and native sheep serum. The work done by Louw *et al* with Compound A was repeated and the same assays used to test the ethanol extract and S2 [14]. These included the influence of the test samples on the DOC-induced difference spectra and the ability of the samples to elicit a substrate induced difference spectrum. Previous results showed that Compound A inhibited the DOC-induced difference spectra but that inhibition was abrogated with time in buffer. Native and heated serum was thus included in the assay because native sheep serum appeared to stabilize the inhibitory effect of Compound A while heated sheep serum (in which CBG and SHBG was destroyed) showed attenuation of the inhibitory influence with time in a manner similar to that of Compound A in buffer. Thus conducting

the assays in PBS, heated or native sheep serum would indicate whether the Ethanol extract and S2 are similarly stabilized by native sheep serum and whether the stabilization is due to CBG or SHBG.

3.2 Results: Spectral assays

3.2.1 Inhibition of the DOC-induced difference spectra

The spectral assays to study the influence of compounds from S.tuberculatiformis on the DOC-induced difference spectra of cytochrome P450c11 was developed by Swart [8]. Freeze dried mitochondrial powder, prepared by the method of Cheng and Harding [26], was used as the source of the P450c11 enzyme. In the assay the mitochondrial powder was sonicated at 4°C in pre-cooled phosphate buffer. After sonication the preparation was brought to room temperature by incubation at 25° C for 5 min. The concentration of the P450 in the pellet was determined from the CO induced difference spectrum (Fig. 3.4) using a millimolar extinction coefficient of 91, as reported by Omura and Sato [27].
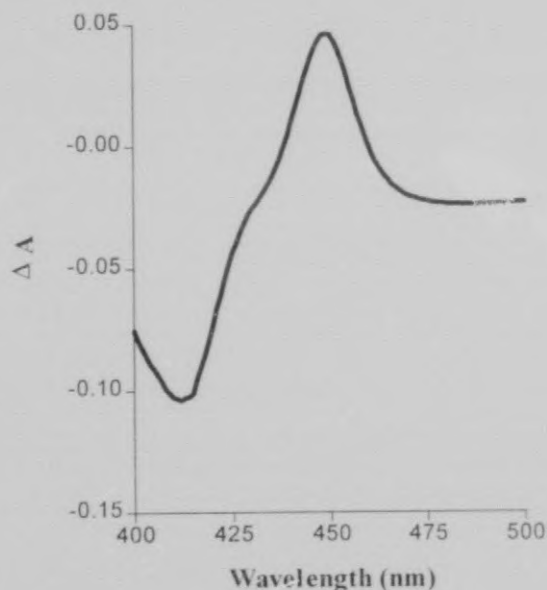

Figure 3.4 Carbon monoxide induced difference spectrum of cytochrome P450 in sheep adrenal mitochondrial powder reduced by sodium dithionate.

To record the DOC-induced difference spectra, equal volumes of the sonicate were pipetted into six matched cuvettes, and a baseline recorded between 500 and 360 nm. Deoxycorticosterone (DOC), dissolved in ethanol, was added to the sample cuvettes and an equal volume of ethanol to the reference cuvette. The final concentration of DOC was 5 uM and the final volume in each cuvette was 1 ml. The contents of the cuvettes were mixed for 1 min and a spectrum recorded between 500 and 360 nm.

DOC, the natural substrate for cytochrome P450c11, elicited a typical Type I difference (absorbance maximum at approximately 390 nm and an absorbance minimum at 420 nm) spectrum when incubated with mitochondrial powder prepared from sheep adrenals (Fig. 3.5). The binding of the substrate induces a shift in the soret peak (characteristic of the cytochrome P450 heme) from approximately 420 to 390 nm, and this shift can be measured by difference spectra. DOC induced difference spectra recorded in this manner remained unchanged for at least 60 min at room temperature, whether recorded in PBS, heated or native sheep serum.
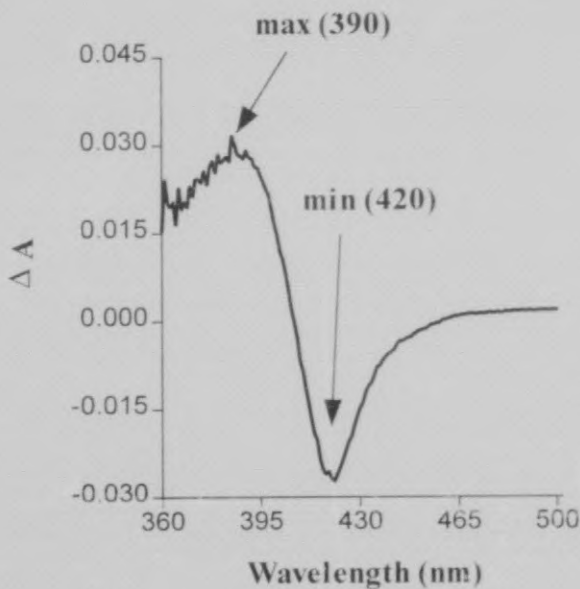
Figure 3.5 Schematic representation of Type I DOC-induced difference spectra of adrenal cytochrome P450c11. The absorbance maximum (390 nm) and minimum (420) are indicated.

The effect of the test samples on the Type I DOC-induced difference spectrum was assayed as described in Chapter 8. The test samples included Compound A, ethanol extract and S2 and were tested in buffer, heated and native sheep serum.

3.2.1.1 Compound A

Compound A (200 µM) was dissolved in the appropriate solution (PBS, native or heated sheep serum), and added to the P450c11 enzyme preparation and DOC, mixed well and spectra recorded every 5 minutes for 60 minutes from 360-500 nm.

The difference spectra seen in Fig. 3.6 shows that at 5 min, Compound A in PBS, or in native or heated sheep serum inhibited the Type I DOC-induced difference spectra. However, at 60 min the inhibition in PBS and in heated sheep serum was diminished, while in native sheep serum the inhibitory influence of Compound A seems to be stabilized.

Figure 3.6 Inhibition of DOC-induced (5 µM) difference spectra of an adrenal cytochrome P450 preparation by Compound A (200 µM) prepared in PBS, sheep serum (SS) or heated sheep serum (HSS). Spectra were taken at **(A)** 5 and **(B)** 60 min and are the average of six spectra taken.

The time studies that monitored the effect of Compound A prepared in PBS on the Type I DOC-induced difference spectrum over a period of 60 min showed that Compound A significantly inhibited ($P<0.01$) the Type I spectrum up to 25 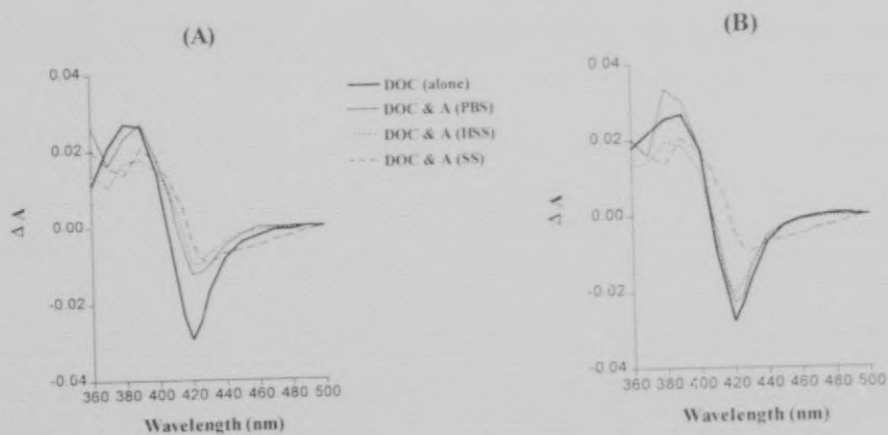min after addition of Compound A. Inhibition was, however, abrogated, from 30 min onwards (Fig. 3.7). Similar results were obtained with heated sheep serum. Native sheep serum in contrast, appeared to stabilize the inhibitory effect of Compound A over 60 min with significant inhibition ($P<0.01$) occurring throughout the time period.

	TIME (min)					
	5	20	25	30	40	60
PBS	**	**	**	NS	NS	*
SS	**	**	**	**	**	**
HSS	**	**	*	*	NS	NS

Figure 3.7 Time study of inhibition of the DOC-induced (5 µM) difference spectra of an adrenal cytochrome P450 preparation by Compound A (200 µM) in PBS, sheep serum (SS) and heated sheep serum (HSS). Spectra are the average of three runs, each consisting of the average of six determinations. The intensity of the substrate induced difference spectrum is expressed as the difference between the maximum and minimum values of the spectrum (Abs 390-420 nm)/ per µM P450). Statistical analysis was done using a one-way ANOVA and Dunnett's test as post test. The results are summarised in the insert. Statistical analysis compared the results with Compound A to the results without Compound A. (* = $P < 0.05$, ** = $P<0.01$, NS = not significant).

3.2.1.2 Ethanol extract

The ethanol extract (2 µl) was dissolved in the appropriate solution (PBS, native or heated sheep serum), added to the P450c11 enzyme preparation and DOC, mixed well and spectra recorded. In Fig. 3.8 a typical example of the inhibition of the DOC-induced difference spectrum by the ethanol extract can be seen.
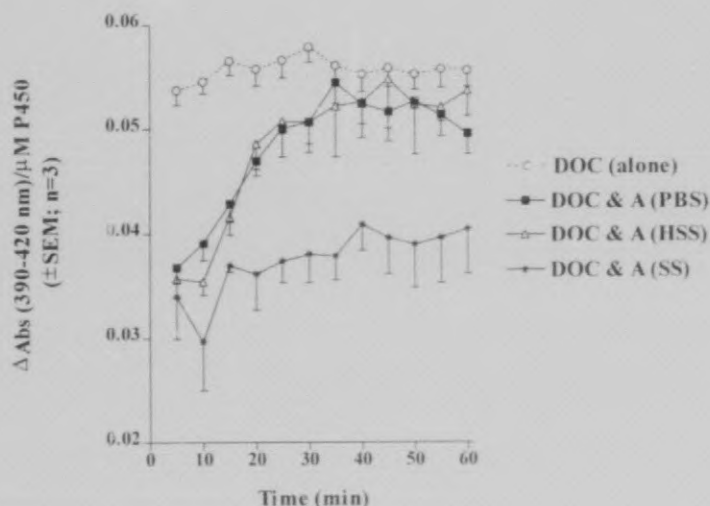

Figure 3.8 Inhibition of DOC-induced (5 µM) difference spectra of an adrenal cytochrome P450 preparation by ethanol extract (2 µl) prepared in PBS at time 60 min.

Time studies that monitored the effect of the ethanol extract prepared in PBS, native or in heated sheep serum on the Type I DOC-induced difference spectrum over a period of 60 min showed that it significantly inhibited ($P<0.05$) the Type I spectrum (Fig. 3.9). No attenuation of inhibition, as was seen with Compound A, was obtained with the ethanol extract in PBS, heated or native sheep serum. Statistical comparison of the results in PBS, native and heated sheep serum shows that there is no significant difference between these results.

	TIME (min)			
	5	**20**	**40**	**60**
PBS	**	**	**	*
SS	**	**	**	**
HSS	*	*	**	*

Figure 3.9 Time study of inhibition of the DOC-induced (5 μM) difference spectra of an adrenal cytochrome P450 preparation by ethanol extract (2 μl) in PBS, sheep serum (SS) or heated sheep serum (HSS). Spectra are the average of three runs, each consisting of the average of six determinations. The intensity of the substrate induced difference spectrum is expressed as the difference between the maximum and minimum values of the spectrum (Abs 390-420 nm)/ per μM P450). Statistical analysis was done using a one-way ANOVA and Dunnett's test as post test. The results are summarised in the insert. Statistical analysis compared the results with ethanol extract to the results without ethanol extract. (* = $P < 0.05$, ** = $P<0.01$, NS = not significant).

3.2.1.3 S2

S2 (10 μl) was dissolved in the appropriate solution (PBS, native or heated sheep serum), added to the P450c11 enzyme preparation and DOC, mixed well and spectra recorded. In Fig. 3.10 a typical example of the inhibition of the DOC-induced difference spectrum by the S2 fraction can be seen.
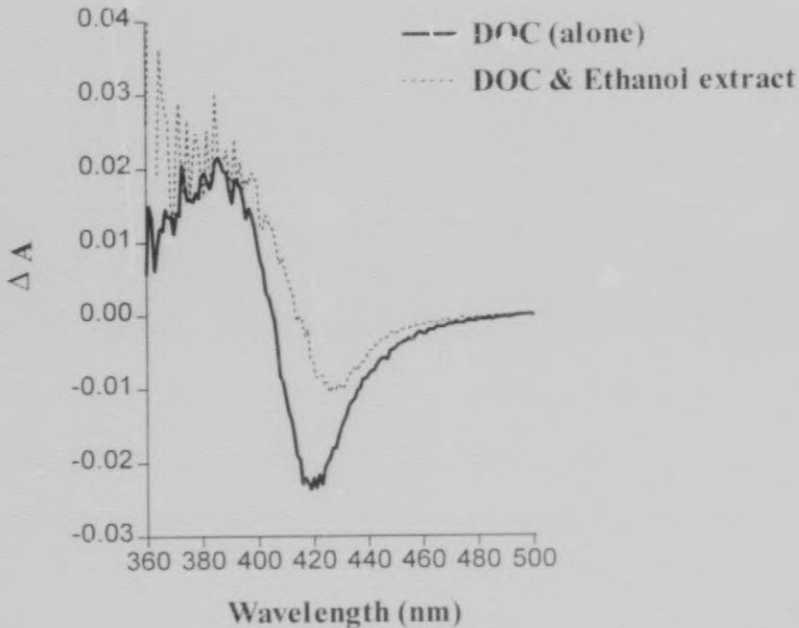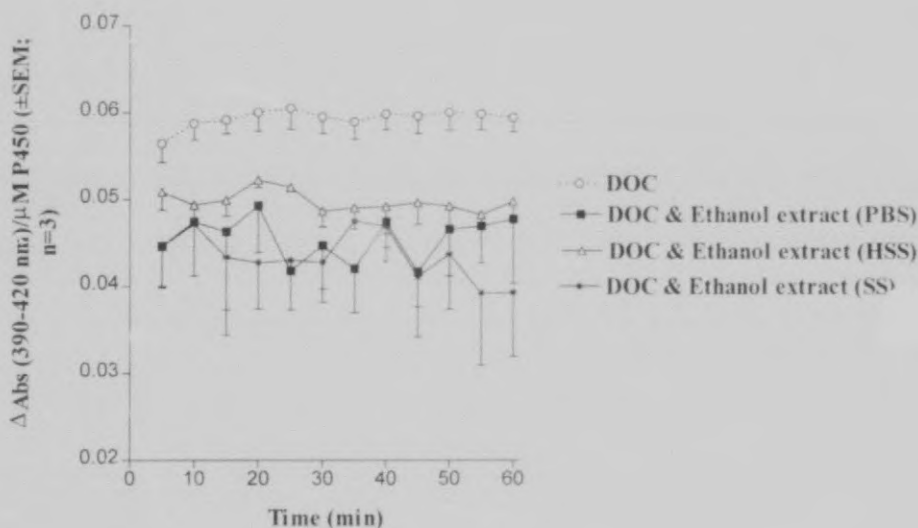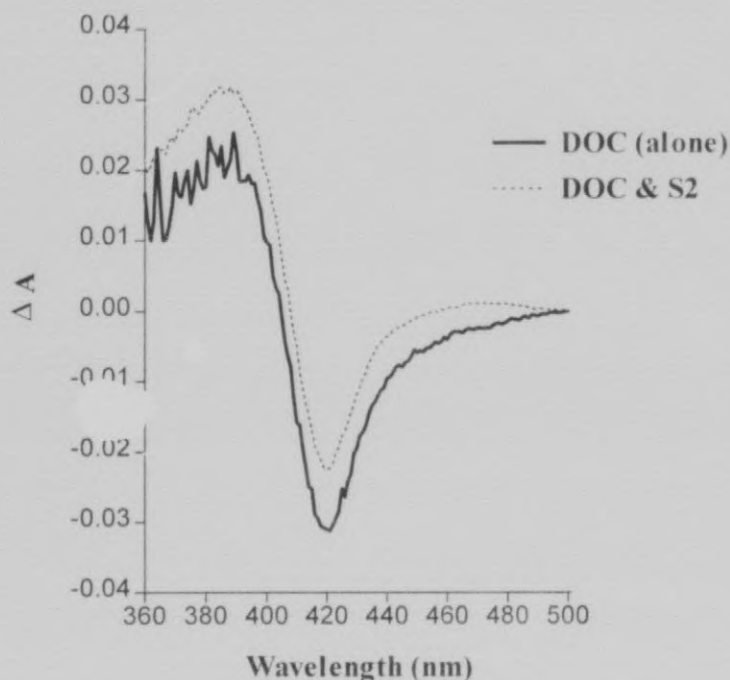

Figure 3.10 Inhibition of DOC-induced (5 μM) difference spectra of an adrenal cytochrome P450 preparation by S2 (2 μl) prepared in PBS at time 60 min.

The time studies that monitored the effect of S2 in PBS, native or in heated sheep serum on the Type I DOC-induced difference spectrum over a period of 60 min showed that only in PBS is significant inhibition ($P<0.05$) of the Type I spectrum observed (Fig. 3.11). S2 in native and heated sheep serum appears not to inhibit the Type I DOC-induced difference spectrum. No attenuation of inhibition, as was seen with Compound A, was obtained with S2 in PBS.
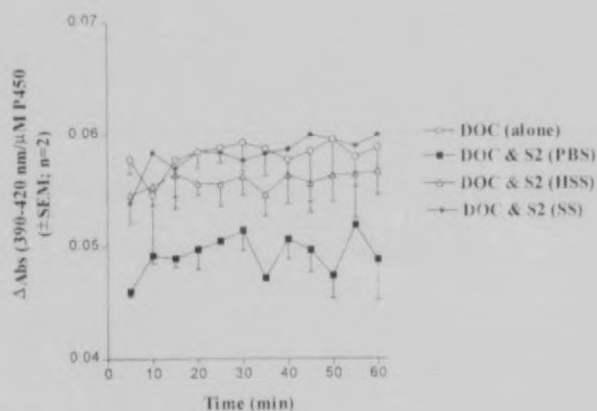
	TIME (min)			
	5	20	40	60
PBS	**	**	*	*
SS	NS	NS	NS	NS
HSS	NS	NS	NS	NS

Figure 3.11 Time study of inhibition of the DOC-induced (5 µM) difference spectra of an adrenal cytochrome P450 preparation by S2 (10 µl) in PBS, sheep serum (SS) or heated sheep serum (HSS). Spectra are the average of two runs, each consisting of the average of six determinations. The intensity of the substrate induced difference spectrum is expressed as the difference between the maximum and minimum values of the spectrum (Abs 390-420 nm)/ µM P450). Statistical analysis was done using a one-way ANOVA and Dunnett's test as post test. The results are summarised in the insert. Statistical analysis compared the results with S2 to the results without S2. (* = $P < 0.05$, ** = $P < 0.01$, NS = not significant).

3.2.2 Substrate induced difference spectra

All natural substrates for cytochrome P450 induce difference spectra. However, there are compounds that are not substrates of cytochrome P450, which can also induce difference spectra. These spectra are still referred to as substrate induced difference spectra. Compounds that are not substrates, for example Compound A, may also bind to the enzyme and induce a difference spectrum, a Type II difference spectrum in the case of Compound A, with an absorbance maximum at 430 nm and a absorbance minimum at 410 nm (Fig. 3.12).

Figure 3.12 Schematic representation of a Type II difference spectra elicited by Compound A from adrenal P450c11. The absorbance maximum (430 nm) and minimum (410 nm) are indicated.

Substrate induced difference spectra of P450c11 were obtained in the same manner and with the same mitochondrial preparation as for the deoxycorticosterone induced difference spectra, except for the DOC solution which was replaced by test sample. The test sample was added to the sample cuvettes and an equal amount of solvent to the reference cuvette. The final volume in the cuvettes were 1 ml. The contents of the cuvettes were stirred for 1 min and a spectrum recorded between 500 and 360 nm. A typical substrate induced difference spectrum is obtained by measuring the optical difference between the enzyme substrate complex in the sample cuvette and the enzyme alone in the reference cuvette.

3.2.2.1 Compound A

Compound A induced a Type II difference spectrum when it interacted with mitochondrial powder from sheep adrenals (Fig. 3.12), an indication of the presence of an amino group that reacts with the heme iron of the cytochrome [25]. A time study of the interaction of Compound A (200 µM) with an adrenal cytochrome P450 suspension in PBS, native or

heated sheep serum showed significant attenuation (P<0.01) of the binding of Compound A with time (Fig. 3.13). Attenuation appears to be significant at an earlier time for heated sheep serum than for PBS, and native sheep serum only showed significant attenuation after 20 min.
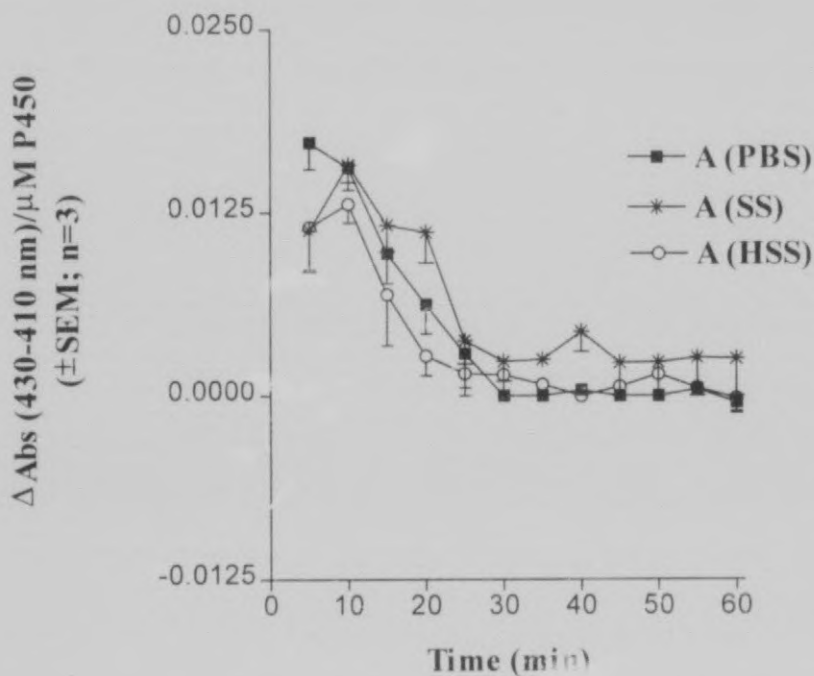
	A				B				
TIME	5	20	40	60	10	15	20	40	60
PBS	NS	NS	NS	NS	NS	NS	*	**	**
SS	NS	NS	NS	NS	NS	NS	NS	**	**
HSS	NS	NS	NS	NS	NS	NS	**	**	**

Figure 3.13 Time study of the interaction of Compound A (200 μM) with an adrenal cytochrome P450 suspension in PBS, sheep serum (SS) or heated sheep serum (HSS). Statistical analysis was done using a one-way ANOVA and Bonferroni (A) and Dunnett's (B) test as post test and the results are summarised in the insert. The Bonferroni (A) post test compares the results in PBS, native and heated sheep serum at indicated time points while the Dunnett's (B) post test compares initial values at 5 min with with results at 10, 15, 20, 40 and 60 minutes within each experimental group. (* = P < 0.05, ** = P<0.01, NS = not significant).

3.2.2.2 Ethanol extract

The ethanol extract induced a Type II difference spectrum when it interacted with mitochondrial powder from sheep adrenals (Fig. 3.14).
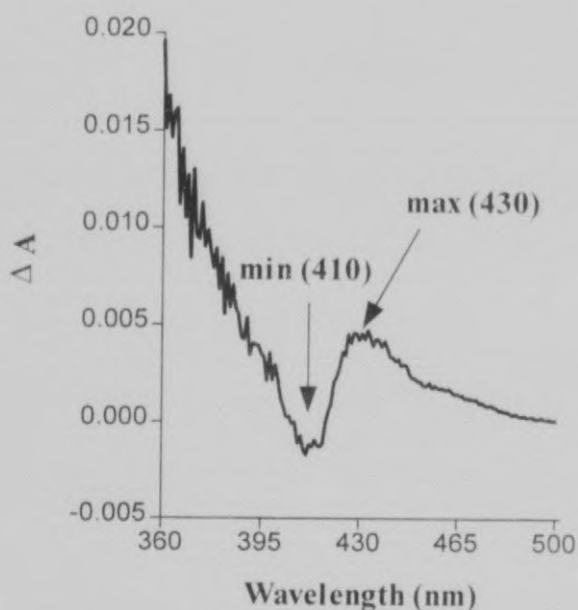

Figure 3.14 Schematic representation of a Type II difference spectra elicited by the ethanol extract from adrenal P450c11. The absorbance maximum (430 nm) and minimum (420) are indicated.

A time study of the interaction of the ethanol extract (2 µl) with an adrenal cytochrome P450 solution in PBS, native or heated sheep serum showed that the binding of the ethanol extract declines slightly, although not significantly, during the first 10 min and is then maintained with time (Fig. 3.15). The binding is maintained to the same degree in PBS, native and heated sheep serum with no significant differents between the groups.
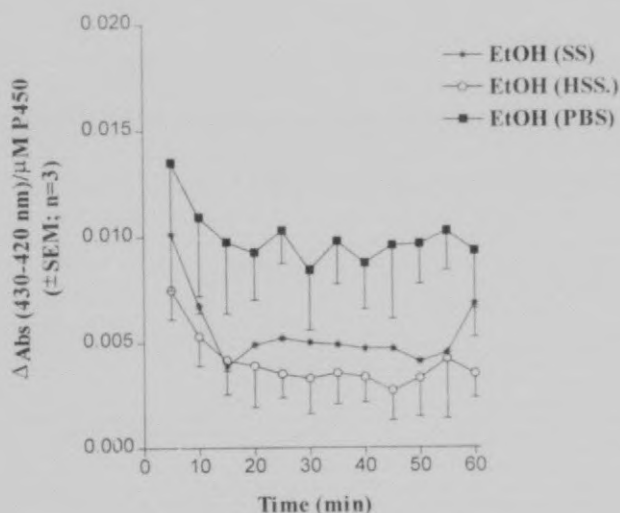
	A				B		
TIME	5	20	40	60	20	40	60
PBS	NS	NS	NS	NS	NS	NS	NS
SS	NS	NS	NS	NS	NS	NS	NS
HSS	NS	NS	NS	NS	NS	NS	*

Figure 3.15 Time study of the interaction of ethanol extract (2 µl) with an adrenal cytochrome P450 suspension in PBS, sheep serum (SS) or heated sheep serum (HSS). Statistical analysis was done using a one-way ANOVA and Bonferroni (A) and Dunnett's (B) test as post test and the results are summarised in the insert. The Bonferroni (A) post test compares the results in PBS, native and heated sheep serum at indicated time points while the Dunnett's (B) post test compares initial values at 5 min with with results at 2(i   (') and 60 minutes within each experimental group. (* = P < 0.05, ** = P<0.01, NS = no: significant).

3.2.2.3 S2

S2 induced a Type II difference spectrum when it interacted with mitochondrial powder from sheep adrenals (Fig. 3.16). A time study of the interaction of S2 (40 µl) with an adrenal cytochrome P450 solution in PBS, native or heated sheep serum showed that the binding of S2 is maintained with time (Fig. 3.17). The binding is maintained to the same extent in PBS,

native or heated sheep serum, with no significant difference between groups.
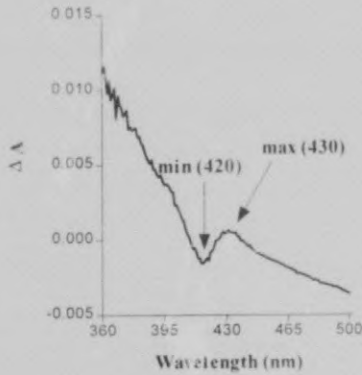
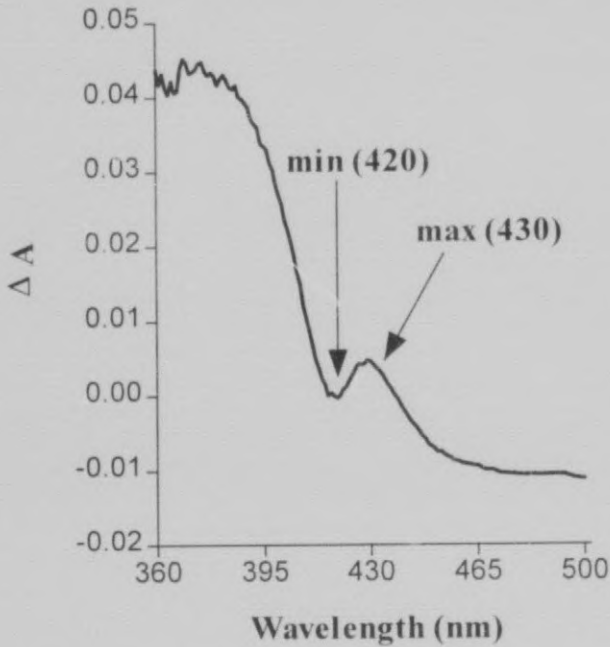**Figure 3.16** Schematic representation of a Type II difference spectra elicited by S2 from adrenal P450c11. The absorbance maximum (430 nm) and minimum (420) are indicated.
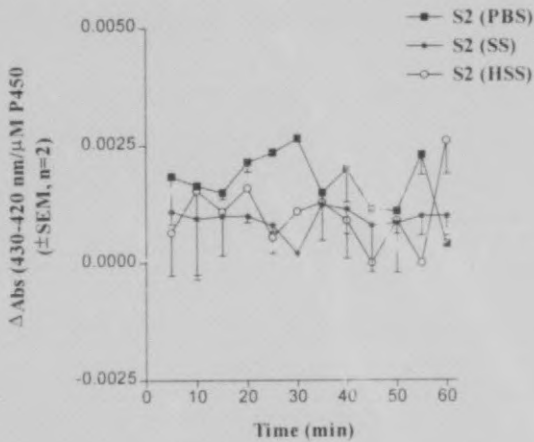
	A				B		
TIME	5	20	40	60	20	40	60
PBS	NS	NS	NS	NS	NS	NS	NS
SS	NS	NS	NS	NS	NS	NS	NS
HSS	NS	NS	NS	NS	NS	NS	NS

Figure 3.17 Time study of the interaction of S2 (40 μl) with an adrenal cytochrome P450 suspension in PBS, sheep serum (SS) or heated sheep serum (HSS). Statistical analysis was done using a one-way ANOVA and Bonferroni (A) and Dunnett's (B) test as post test and the results are summarised in the insert. The Bonferroni (A) post test compares the results in PBS, native and heated sheep serum at indicated time points while the Dunnett's (B) post test compares initial values at 5 min with with results at 20, 40 and 60 minutes within each experimental group. (* = $P < 0.05$, ** = $P < 0.01$, NS = not significant).

3.2.3 Determination of the type of inhibition induced by Compound A on the Type I DOC-induced difference spectra

The type of inhibition induced by Compound A on the DOC-induced difference spectrum of cytochrome P450c11 was investigated to determine if it is competitive or mixed inhibition. A competitive inhibitor would be a molecule that closely resembles the substrate of the enzyme, and that competes with the substrate at the binding site, of the enzyme. The inhibitor will thus bind only to the free enzyme and not to the enzyme substrate complex. The Km_{app} would be different (greater than without inhibitor) but the $Vmax_{app}$ similar, if the competition is competitive. A mixed inhibitor on the other hand, would bind to both the enzyme and enzyme substrate complex. For mixed inhibition both Km_{app} and $Vmax_{app}$ can change, with Km_{app} increasing and $Vmax_{app}$ decreasing, with increasing inhibitor concentration [29].

Spectral changes in the DOC-induced difference spectrum due to the addition of changing amounts of substrate (DOC) was measured in the presence of different concentrations of inhibitor (Compound A). A concentration range of 2-50 µM DOC was used with a range of concentrations (100 t) 250 µM) of the inhibitor, Compound A. The spectral data was then analyzed using three different methods. The first method illustrated in Fig. 3.18, is a non-linear regression plot to determine the Km_{app} and $Vmax_{app}$ values. In the second method, to determine the Km_{app} and $Vmax_{app}$, a direct linear plot was used (Fig. 3.19) while, in the third method a Lineweaver-Burk plot was used (Fig. 20) [28, 29, 30]. Of the three methods used, the Lineweaver-Burk plot is probably the least accurate as it gives a grossly misleading impression of the experimental error due to linearization of the data. The non-linear regression plot is an improvement over the Lineweaver-Burk plot but outliers can once again give inaccurate results. The direct linear plot however, is recommended to determine Km_{app} and $Vmax_{app}$, as it makes no assumption about the nature of experimental error and the graphical procedure requires no calculation, as inhibitor concentration and substrate concentration are plotted directly [29]. In addition outliers can very easily be identified and excluded from the analysis.

Figure 3.18 (A) Non-linear regression of the effect of changing concentrations of DOC on the amplitude of the LOC-induced difference spectrum in the presence of different concentrations of Compound A. (B) Comparison of Km_{app} values obtained for each of the curves in A. Statistical analysis was done using one-way ANOVA and Dunnett's test as post test. ($P < 0.05$ = *, $P < 0.01$ = **). (C) Comparison of $Vmax_{app}$ values obtained for each of the curves in A. Statistical analysis was done using one-way ANOVA and Dunnett's test as post test. ($P < 0.05$ = *, $P < 0.01$ = **).
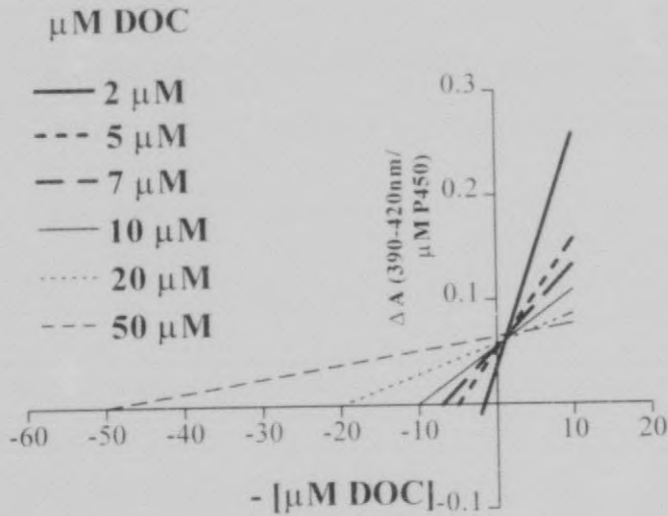
Figure 3.19 Example of direct linear plot used to determine the effect of changing concentrations of DOC on the amplitude of the DOC-induced difference spectrum in the presence of different concentrations of Compound A. In this example the concentration of Compound A was 0 μM.

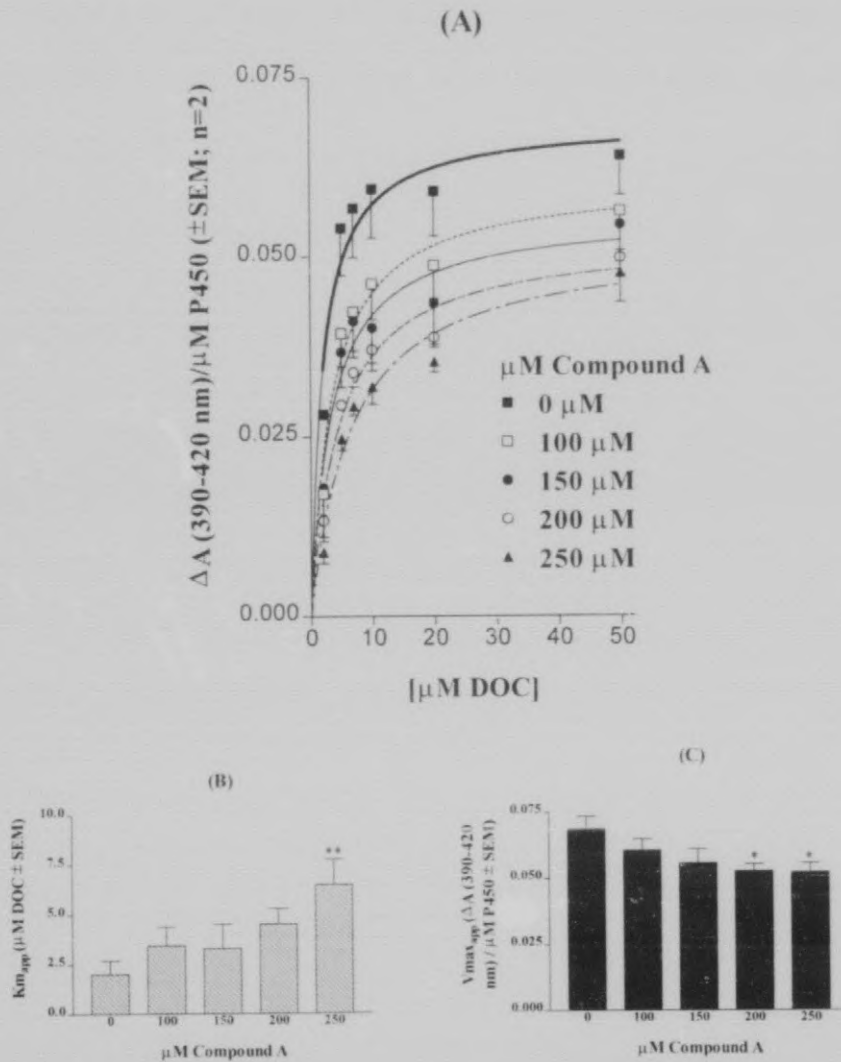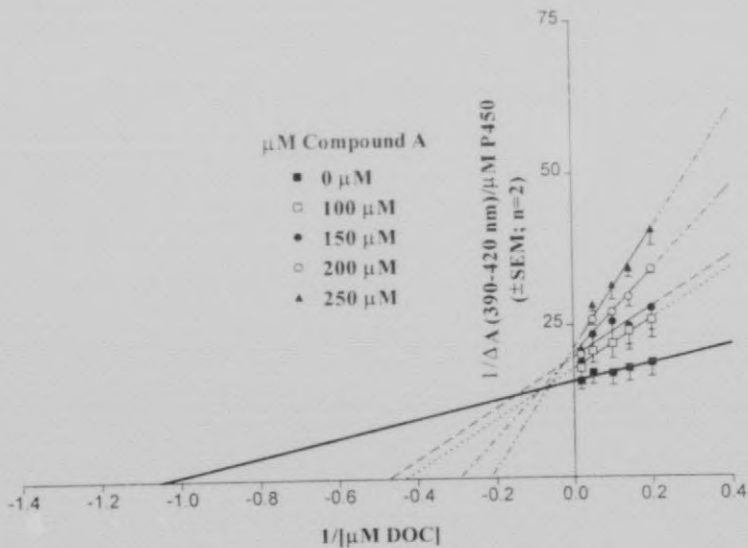**Figure 3.20** Example of Lineweaver-Burk plot of the effect of changing concentrations of DOC on the amplitude of the DOC-induced difference spectrum in the presence of different concentrations of Compound A.

In Table 3.2 the Km_{app} and $Vmax_{app}$ values, obtained by these three methods are presented. The results indicate that the values obtained using the three methods of analysis correlate well.

Table 3.2 Comparison of Km_{app} (μM) and $Vmax_{app}$ (ΔA (390-420 nm)/μM P450) values obtained by three methods. (A) Non-linear regression and (B) Direct linear plot and (C) Lineweaver-Burk plot.

µM Compound A	Vmax$_{app}$			Km$_{app}$		
	A:	B:	C:	A:	B:	C:
0	0.068	0.064	0.063	2.000	1.260	0.950
100	0.060	0.054	0.056	3.450	2.000	2.320
150	0.056	0.049	0.051	3.300	1.880	2.100
200	0.052	0.048	0.049	4.500	3.750	3.390
250	0.052	0.047	0.047	6.480	5.200	4.620

From the results we can clearly see that the $Vmax_{app}$'s decreases and the Km_{app}'s increases with increasing concentrations of Compound A, indicating mixed inhibition. Mixed inhibition is due to the fact that the inhibitor binds to both the enzyme and enzyme substrate complex as shown in Fig. 3.21 (A).

(A)

EI   ESI

$\updownarrow K_{ic}$ $\updownarrow K_{iu}$

$S + E \rightleftharpoons ES \longrightarrow E + P$
+   +
I   I

(B)

$$Vmax_{app} = \frac{Vmax\ S}{S\,(1 + I/Kiu) + Km(1 + I/Kic)}$$
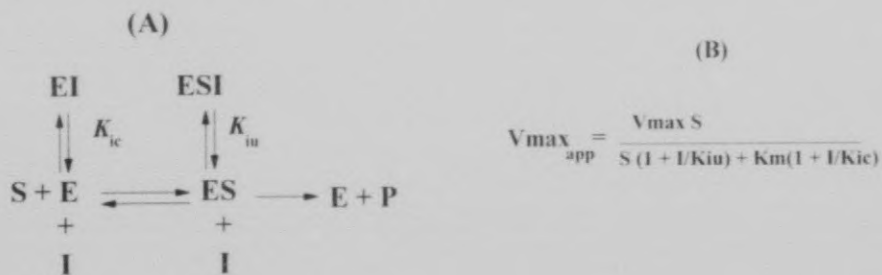
Figure 3.21 (A) Mechanism that produces mixed inhibition and (B) the formula used to describe this type of inhibition. S=substrate, E=enzyme, I=inhibitor, P=product, Kic=dissociation constant for EI complex or competitive inhibition constant and Kiu=dissociation constant for ESI complex or uncompetitive inhibition constant (Redrawn from [29]).

The formula used to describe this type of inhibition is shown in Fig. 3.21(B) and clearly shows that both a uncompetitive component (Kiu) and a competitive component (Kic) is involved in this type of inhibition [29]. The Kiu may be calculated by plotting $1/Vmax_{app}$ vs. [I] as shown in Fig. 3.22. The slope of the linear regression line is equal to $1/Vmax \times Kiu$. Once Kiu has been calculated Kic may be calculated using the following formula:

$$K_m^{app} = \frac{Km (1 + I/Kic)}{1 + I/Kiu}$$

The Kiu and Kic values for the inhibition of the DOC-induced difference spectra by Compound A are summarized in Table 3.3. From the results we can see that the Kic value obtained for the Lineweaver-Burk plot (62 µM) differs from the non-linear regression and direct linear plot values (110 and 106 µM, respectively). This may be due to the fact that the Lineweaver-Burk plot is not as accurate as the other two methods. The results also indicated that the competitive element in the present inhibition is greater than the uncompetitive element. Thus inhibitor binding to the enzyme alone is stronger than to the enzyme-substrate complex.
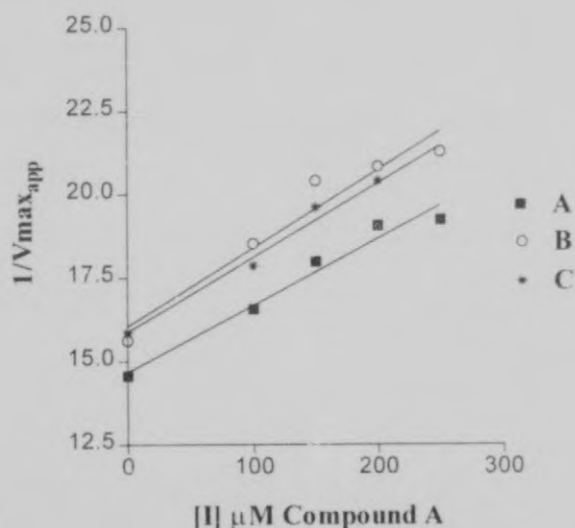
Figure 3.22 Determination of the Kiu for Compound A using values as determined by (A) Non-linear regression and (B) Direct linear plot and (C) Lineweaver-Burk plot.

Table 3.2 Comparison of Kiu and Kic values obtained by three methods. (A) Non-linear regression and (B) Direct linear plot and (C) Lineweaver-Burk plot.

	A	B	C
Kiu (μM)	737	667	706
Kic (μM)	110	106	62

3.3 Conclusion

The amplitude of the DOC-induced difference spectrum (difference between the maximum and minimum absorbance values) of P450c11 is an indication of the enzyme substrate interaction. Inhibitors of the enzyme will reduce the amplitude of the spectrum if they interfere with binding of substrate to the active site. Louw *et al* indicated that the resulting spectrum was the sum of a Type I DOC-induced spectrum and a Type II Compound A -induced spectrum, which suggests that the two substrates bind independently and that Compound A does not displace DOC upon binding [14]. This is consistent with the results obtained by Yoshida and Kumaoka for the simultaneous adding of Type I and Type II compounds [25].

From this study we can conclude that Compound A, the ethanol extract and S2 inhibited the Type I DOC-induced difference spectrum of mitochondrial powder containing P450c11 from sheep adrenals in a manner similar observed by Swart *et al.* with S2 [8].

The time studies that monitored the effect of Compound A prepared in PBS on the DOC-induced difference spectrum over a period of 60 min clearly showed that Compound A inhibited the Type I spectrum but that inhibition was abrogated with time. Previous ESMS time studies indicated that cyclization of Compound A to the corresponding aziridine was completed after 60 min and this correlated with the attenuation of inhibition found in the cytochrome P450c11 system if Compound A was prepared in PBS. Our results are thus in complete agreement with the previous results obtained by Louw *et al* [14].

The time studies, with sheep serum showed that serum stabilized the inhibitory effect of Compound A in the P450c11 system. Heating of sheep serum, which destroys CBG and SHBG but not albumin, resulted in an inhibition pattern similar to that observed for PBS,

with no stabilizing of Compound A. Previous ES-MS studies showed that serum retarded the cyclization of Compound A to the corresponding aziridine and that the amount of Compound A present, correlated with the percentage inhibition of the DOC-induced difference spectrum [14]. ES-MS studies confirmed that CBG and SHBG may be involved in the stabilization of Compound A in sheep serum [14]. Thus heating of serum with concomitant destruction of CBG and SHBG, would not result in retardation of cyclization. From these results it was deduced that it is the aziridine precursor, Compound A, rather than the aziridine, which is the inhibiting agent in the cytochrome P450c11 system. Once again our studies showed similar results to those previously reported.

The time studies that monitored the effect of the ethanol extract on the DOC-induced difference spectrum in PBS and in native or heated sheep serum showed that these compounds inhibited the Type I spectrum over a period of 60 min and that no attenuation of inhibition occurred. This is in contrast with the time studies with Compound A where inhibition was abrogated with time in PBS and in heated sheep serum. The S2 fraction, like the ethanol extract, in PBS also showed no attenuation of inhibition with time. In native and heated sheep serum, however, no inhibition of the Type I DOC-induced difference spectrum was observed.

Compound A, ethanol extract and S2, all induced a Type II difference spectrum when interacting with mitochondrial powder from sheep adrenals, which indicated the presence of an amino group that reacted with the heme iron of the cytochrome [25]. A study of the interaction of Compound A with cytochrome P450c11 in PBS, native and heated sheep serum showed attenuation of the binding of Compound A with time. In native sheep serum the time course of attenuation of the binding of Compound A was slower than for PBS and sheep serum, which suggests that native sheep serum stabilized Compound A to some extent. The results however are not as clear as in the case of the inhibition of the Type I DOC-induced spectra. In contrast with the results obtained with Compound A, the ethanol extract and S2 showed no significant attenuation of binding with time. This agrees with the results obtained during the inhibition of the DOC-induced difference spectra for the ethanol extract and suggests that the lack of inhibition of the Type I spectrum by S2 in native and heated sheep serum may be an artefact as inhibition was observed in PBS.

The type of inhibition induced by Compound A on the DOC-induced difference spectrum of cytochrome P450c11, was also investigated to determine if it is competitive or mixed inhibition. The results indicated that mixed inhibition occurred. The Kic values for the inhibition of the DOC-induced difference spectra by Compound A were determined as 110, 106 and 62 µM for the direct linear plot, non-linear regression and the Lineweaver-Burk plot, respectively while the Kiu values were 737, 567 and 706, respectively. The fact that the Kic values were lower than the Kiu values indicated that the competitive element in the inhibition is stronger than the uncompetitive element.

These results are interesting in that mixed inhibition would suggest that binding of the inhibitor (Compound A) to the enzyme does not occur at the substrate site, but does however affect the binding of the natural substrate (DOC) to the enzyme in addition to inhibiting the catalytic activity of the enzyme. This agrees with the results obtained with the substrate induced difference spectrum of Compound A and DOC. DOC induced a Type I-difference spectrum, which according to Yoshida and Kumaoka, suggests binding to the protein moiety of the enzyme [25]. In contrast, Compound A elicited a Type II difference spectrum, which suggests binding to, or interaction with the heme moiety of the enzyme. Thus, the results are in agreement with the fact that Compound A asserts its affect on the P450c11 enzyme by binding to a different site than the substrate (DOC) binding site.

The inhibition of P450c11 by Compound A contains many of the elements identified by Ortiz de Montellano in reversible P450-enzyme inhibitors [31]. These include binding of the inhibitor to the heme iron and eliciting a Type II difference spectrum with a soret maximum at 430 and the fact that the most potent inhibitors are nitrogen-containing aliphatic and aromatic compounds. The most effective reversible inhibitors react strongly with both the protein and the prostetic heme iron and their activity is thus governed by both the hydrophobic character (interaction with protein) and by the strength of the bond with the heme iron.

CHAPTER 4

THE INFLUENCE OF TEST SAMPLES ON THE PERCENTAGE FREE STEROID IN UNDILUTED SHEEP SERUM

4.1 Introduction

Steroids in plasma bind to several transport proteins. The binding may be either low or high affinity. Sheep serum contains two glycoproteins that bind steroids with high affinity. They are corticosteroid binding globulin (CBG or transcortin), which binds primarily to cortisol and progesterone, and sex hormone binding globulin (SHBG; also known as testosterone-estradiol-binding globulin (TeBG) and steroid-binding protein (SBP)), which binds to testosterone, dihydrotestosterone and estradiol. Both occur in low concentrations in sheep serum, about 0.078 μM for CBG and about ten fold less for SHBG (0.0078 μM) [32]. The low affinity transport protein in sheep serum is albumin. Although the affinity of albumin for steroids is low the plasma levels are high enough to bind significant amounts of steroid ligand. Current theory holds that plasma steroid-binding proteins transport steroid hormones in the blood and regulate their access to target tissues [33]. Only the unbound fraction of steroid hormones can enter target cells. Thus, steroid hormones exists in two states in plasma, namely free and reversibly bound to proteins, either to the low affinity, high capacity binder, albumin, or to the high affinity, low capacity binders, CBG and SHBG .

It may be possible for test compounds with a high affinity for steroid binding proteins to displace endogenous steroids from the binding protein and in doing so, to increase the bio-available concentration of the steroids. Several such examples have been described in the literature. It has, for example, been shown that some of the extra-adrenal effects of metyrapone may be attributed to the displacement of cortisol from albumin [34]. In addition, Pugeat *et al.*, in an extensive study investigating the interaction of drugs with CBG and SHBG, showed that prednisolone, a potent synthetic glucocorticoid, binds to human CBG and causes a 32% decrease in the percentage cortisol bound [35]. Dunn *et al.* found that about 50% of the available CBG-binding sites is occupied by cortisol and that accounts for 94-96% of the binding to CBG in men and women [36]. Thus, CBG in the blood is largely occupied

with steroid ligand and provides a large reservoir of glucocorticoids that may be displaced by ligands with a higher affinity [37].

The liver is the major organ involved in the synthesis of CBG. The concentration of CBG in plasma, and thus synthesis by the liver, is regulated by the bio-available steroid concentration. It has been shown in the rat, that adrenalectomy results in increased CBG levels while exogenous corticosteroids results in decreased CBG levels [38]. An investigation into the effects of high levels of endogenous (Cushing's syndrome) and exogenous (patients receiving high doses of glucocorticoids) glucocorticoids indicated that this resulted in a down regulation of the circulating levels of CBG which may enhance glucocorticoid activity [39]. CBG has been shown to play an active role in determining the disposition of glucocorticoids levels and therefore reduced CBG levels will enhance the biological significance of existing glucocorticoid levels [40].

Spectrophotometric studies indicated that Compound A inhibited the Type I DOC-induced difference spectra of sheep adrenal cytochrome P450c11 and elicited a Type II difference spectrum in the same enzyme. The spectrophotometric effects with Compound A diminished with time in buffer but is stabilized in serum. In contrast to native sheep serum, heated sheep serum showed attenuation of the inhibitory influence of Compound A with time in a manner similar to that of Compound A in PBS, suggesting that CBG and SHBG (which would be destroyed by heating) play an important role in this stabilization. The role of CBG and SHBG in stabilizing Compound A and retarding cyclization to the corresponding aziridine was confirmed by ES-MS studies [14]. The above results posed the question of whether the binding of Compound A to CBG and SHBG was simply a transport mechanism or whether the binding, with possible displacement of endogenous steroids, could be part of the biological effect of Compound A.

Oral and intraperitoneal administration of Compound A and *S. tuberculatiformis* have a contraceptive effect on female Wistar rats and cause a decrease in total body, uterus, and ovary mass and an increase in adrenal mass. These results suggest a perturbance in the glucocorticoid profile of the rats. In addition Louw *et al.* demonstrated that the contraceptive action in female Wistar rats, of the shrub and Compound A, is associated with an increase in

the percentage free corticosterone in plasma. The increased free steroid results in an increase in the percentage of albumin bound steroid as binding to albumin is proportional to free steroid concentration [15]. The rise in the percentage free and albumin bound steroid is concurrent with a decrease in the CBG bound corticosterone and this may be due to the displacement of corticosterone from CBG by Compound A itself or by an active compound in the shrub. In the same study CBG concentrations were also found to decrease on administration of Compound A and *S. tuberculatiformis*. The decrease in CBG concentration, found with the study by Louw *et al.*, may be due to the increased levels of bio-available steroid which may inhibits CBG synthesis in the liver as increased endogenous and exogenous glucocorticoids have been shown to down regulate CBG synthesis in the liver [15]. This phenomena will result in a further amplification of the effects of existing glucogorticoid levels. Louw *et al.* concluded from these results that both Compound A and *S. tuberculatiformis* binds to CBG in rat serum and displaces bound corticosterone, which results in increased levels of free steroid in the plasma, which may contribute to the biological effects obseved in rats.

In the present study the influence of the test samples, Compound A, the ethanol extract from the shrub and S2, on the distribution of cortisol and progesterone in sheep serum was investigated. Previous work done by Louw *et al.* showed that Compound A binds to CBG and SHBG in sheep serum *in vitro*, and displaces endogenous steroids from CBG in rats *in vivo* [14, 15]. The present study was thus undertaken to determine if all the test samples binds to CBG in sheep serum and if binding of the test samples to the steroid binding globulins in sheep serum are simply a transport mechanism, or if the binding results in a concomitant displacement of endogenous steroids, which may be part of the biological effect as seen in rats with Compound A. The two steroids, cortisol and progesterone, which bind primarily to CBG, were monitored in sheep serum to determine the effect of the test samples on their binding to CBG.

The distribution of steroids in undiluted serum was determined by the ultrafiltration-dialysis method of Hammond *et al.* [41]. This method has several advantages over other existing methods. Opinion holds that only free steroid hormones can enter target cells, and quantitave estimations of free steroid concentration in serum appear to provide a more accurate

evaluation of their biological activity than measurement of their total concentration. None of the conventional methods of measuring steroids are sensitive enough to conveniently quantitate free steroid in blood samples. Methods developed for separation of bound and free steroids, include equilibrium dialysis, ultrafiltration through a dialysis membrane, zonal chromatography and steady state gel filtration. Although several of these methods give similar results, each one has one or more drawbacks, including large sample requirement, correction for variable increases in sample volume which occur during the course of dialysis, or the collection and measurement of radioactivity in numerous fractions for each sample. The method used in this study is rapid and convenient for the estimation of the percentage of various free steroids in small volumes of undiluted serum under conditions which mimic the *in vivo* situation.

Undiluted serum samples were incubated with ^{3}H-steroid and ^{14}C-glucose. The ^{14}C-glucose does not bind to any components in serum, can freely equilibrate across the membrane, and is used as internal standard to monitor the movement of unbound components across the dialysis membrane. Cortisol and progesterone bind to plasma steroid binding proteins leaving only the free cortisol and progesterone to equilibrate across the membrane as the protein molecules cannot pass through the membrane. Cortisol and progesterone bind primarily to CBG but also to SHBG with high affinity and to serum albumin with low affinity in human serum [36]. The percentage of free cortisol and progesterone can therefore be estimated by comparing the ratio of ^{3}H-steroid to ^{14}C- glucose in the ultrafiltrate with the corresponding ratio in the serum retained by the dialysis membrane as indicated in the following formula.

$$\% \text{ Free steroid } = \frac{(^3\text{H-steroid}/^{14}\text{C-glucose}) \text{ ultrafiltrate}}{(^3\text{H-steroid}/^{14}\text{C-glucose}) \text{ serum}} \times 100$$

The distribution of ^{3}H-cortisol and ^{3}H-progesterone between free, CBG bound and albumin bound may be calculated as described by Siiteri *et al.* [42]. Steroids occur either free or bound to albumin, CBG or SHBG, in serum. Heating sheep serum, at 60°C for 1 hour, destroys both CBG and SHBG but not albumin and thus heated sheep serum contains only free and albumin bound steroid [43].

Heated serum: 100% = % Free steroid + % Albumin bound steroid

Native serum: 100 %= % Free steroid + % Albumin bound steroid + % CBG bound
                      steroid

The percentage albumin bound cortisol and progesterone in heated serum can thus be obtained by subtracting the percentage free steroid in heated serum from 100%. The ratio of albumin bound to free cortisol/progesterone in heated and native serum is assumed to be the same, thus from percentage free steroid in native serum, percentage free steroid in heated serum and the percentage albumin bound steroid in heated serum the percentage albumin bound steroid in native serum can be determined. The percentage CBG bound cortisol/progesterone in native serum can then be calculated from the percentage free and percentage albumin bound cortisol/progesterone.

The concentration range of test samples were choosen to include the concentrations used in the *in vivo* contraceptive studies with female Wistar rats conducted by Louw *et al.* [15]. Louw administered 0.9 mg of Compound A/day (4.5 mg/kg bodyweight/day) and 40% of total feed of *S. tuberculatiformis*/day for 16 days. Assuming a volume of 8.6 ml of blood/200 g body weight of rat and using a molecular mass of 264 for Compound A this converst to 3.96×10^{-4} M Compound A/ day and 6.34×10^{-3} M Compound A cumulatively over 16 days [44]. In this study a concentration of 8.33×10^{-3} M Compound A was used as indicating physiological relevance. From the study by Louw we know that the rats consumed an average 14 g of feed/day (70 g/kg bodyweight/day) thus, a 40% replacement of Salsola would result in 5.6 g Salsola being ingested per day. The isolation results presented in Chapter 2 converts this amount of Salsola to 211 µl ethanol extract (2.17 g/20 ml) per 450 µl serum and 0.0174 g S2/450 µl serum.

4.2 Results

4.2.1 Cortisol

The distribution of cortisol in sheep serum was determined by the ultrafiltration-dialysis method of Hammond *et al.* as discussed above [41]. The results showed that in native sheep

serum, 62% of cortisol is bound to CBG, 22% is bound to albumin and 18% is free (Fig. 4.1 A). Thus, we can conclude that the largest proportion of cortisol is bound to CBG.

The effect of Compound A (83.35 - 16670 µM), ethanol extract (50-211 µl/450 µl serum) and S2 (0.0012 - 0.059 µg/450 µl serum) on the distribution of cortisol in sheep serum was determined (Fig. 4.1 B,C,D). With an increase in the concentration of Compound A (Fig 4.1 B) and S2 (Fig 4.1 C) and also an increase in volume of ethanol extract (Fig 4.1 D) a significant (P<0.01) increase in the percentage free cortisol was observed. The increased free cortisol resulted in a significant (P<0.01) increase in the percentage of albumin bound steroid as binding to albumin is proportional to free steroid concentration [42]. The rise in the percentage free and albumin bound steroid was concurrent with a significant (P<0.01) decrease in the CBG bound cortisol, which could be attributed to the displacement of cortisol from CBG by Compound A, ethanol extract and S2.

4.2.2 Progesterone

The distribution of progesterone in sheep serum was also determined according to the ultrafiltration-dialysis method of Hammond *et al.* as discussed in 4.1 [41]. The results showed that in native sheep serum 24% of progesterone is bound to CBG, 60% is bound to albumin and 21% is free. Thus, we can conclude that largest proportion of progesterone is bound to albumin (Fig. 4.2 A).

The effect of Compound A (83.35 - 16670 µM), ethanol extract (50-211 µl/450 µl serum) and S2 (0.0012 - 0.059 µg/450 µl serum) on the distribution of progesterone in sheep serum was determined (Fig. 4.2 B,C,D). With an increase in the concentration of Compound A and S2 a very slight, but not significant (P>0.05), increase in the percentage free progesterone was observed. A decrease in the percentage albumin bound progesterone was observed with both compounds. With Compond A this decrease was not significant (P>0.05) but with S2 at the highest concentration the decrease was significant (P>0.01). The percentage CBG bound stayed the same in the presence of Compound A, but with increasing concentrations of S2 appeared to increase significantly (P<0.01). With an increase in volume of the ethanol extract a significant (P<0.01) increase in the percentage free progesterone was observed. The rise in the percentage free progesterone was concurrent with a significant (P<0.01) decrease in the

CBG bound progesterone. This rise may be due to the displacement of progesterone from CBG by the ethanol extract. The percentage albumin bound progesterone stayed the same.
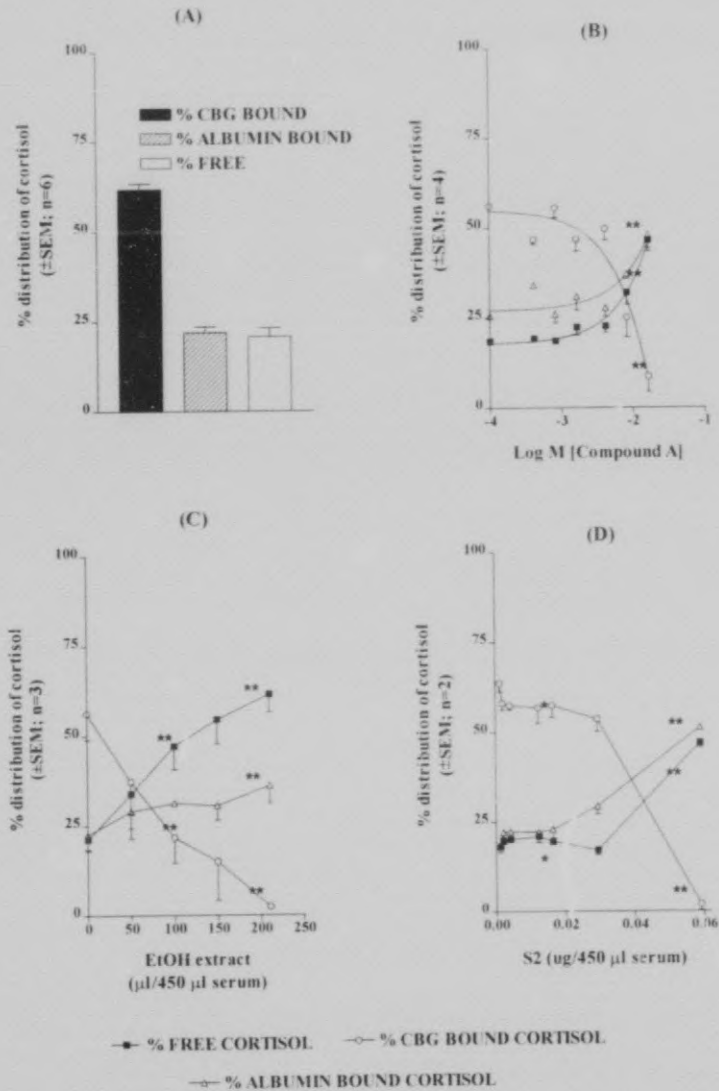
Figure 4.1 The distribution of the percentage free cortisol, CBG bound and albumin bound cortisol in sheep serum when (A) no competitor, (B) Compound A, (C) ethanol extract, and (D) S2 were added. Statistical analysis was done comparing the results when no competitor is present with when 834 and 16670 µM Compound A, 100 and 211 µl/450 µl ethanol extract and 0.0165 and 0.0591 µg/450 µl S2 is present. One-way ANOVA was used with Dunnett's test as a post test. (* = P < 0.05, ** = P<0.01, NS = not significant).

59
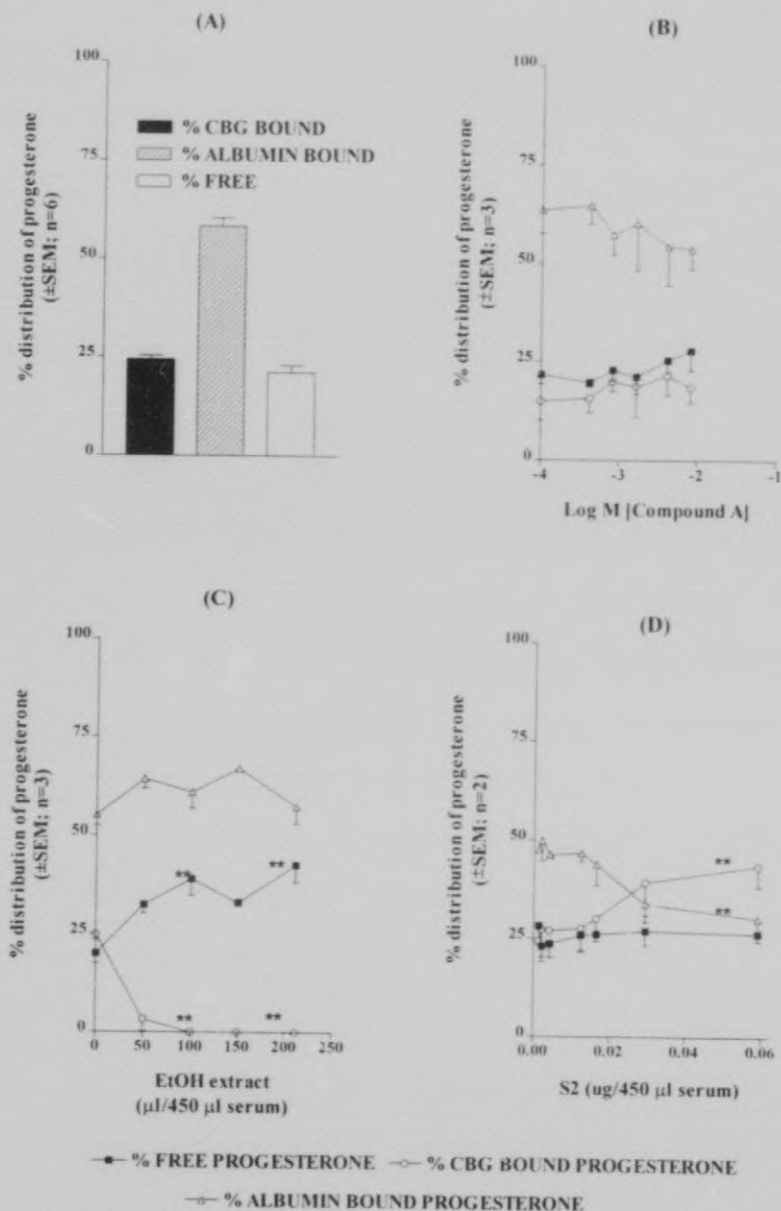
Figure 4.2 The distribution of the percentage free progesterone, CBG bound and albumin bound progesterone in sheep serum when (A) no competitor, (B) Compound A, (C) ethanol extract, and (D) S2 were added. Statistical analysis was done comparing the results when no competitor is present with when 834 and 16670 μM Compound A, 100 and 211 μl/450 μl ethanol extract and 0.0165 and 0.0591 μg/450 μl S2 is present. One-way ANOVA was used with Dunnett's test as a post test. (* = P < 0.05, ** = P<0.01, NS = not significant).

4.2.3 Physiological relevance

Table 4.1 Comparison of the distribution of cortisol (A) vs. progesterone (B) in sheep serum in the presence of Compound A (8335 µM), ethanol extract (211 µl) and S2 (0.0591 µg). Statistical analysis was done using one-way ANOVA and Dunnett's test as post test. (* = $P < 0.05$, ** = $P<0.01$, NS = not significant).

(A)

TEST COMPOUNDS	% FREE			% CBG			% ALB.		
	AVG	SEM	n	AVG	SEM	n	AVG	SEM	n
NONE	18	2.1	7	62	1.7	7	22	1.6	7
COMPOUND A	29**	2.3	3	25**	7.7	3	46**	7.5	3
ETHANOL EXTRACT	62**	5.1	2	2**	0.2	2	36**	4.9	2
S2	47**	1.2	2	2**	1.6	2	51**	0.4	2

(B)

| TEST COMPOUNDS	% FREE			% CBG			% ALB.		
	AVG	SEM	n	AVG	SEM	n	AVG	SEM	n
NONE	21	1.7	7	24	0.9	7	60	1.8	7
COMPOUND A	24	5.8	2	22	3.1	2	54	8.9	2
ETHANOL EXTRACT	43**	4.3	3	0**	0	3	57	4.3	3
S2	26	1.9	2	44**	5.4	2	30**	3.5	2

The previous results will now be discussed relative to the *in vivo* concentrations of the test compounds as used by Louw *et al.* to illustrate that physiologically significant results were obtained [15]. As discussed in the introduction to this Chapter this would entail the following concentrations: Compound A (8335 µM), ethanol extraxt (211 µl/450 µl serum) and S2 (0.0591 µg/450 µl serum).

The average percentage free cortisol before administration of the test compounds was 18% (Table 4.1 A). After administration of the test compounds, the percentage free cortisol rose significantly (P<0.01), to 29% for Compound A, 62% for the ethanol extract and 47% for S2. These changes in the percentage free cortisol was associated with a significant (P<0.01) decrease in CBG bound cortisol from 62% to 25%, 2% and 2% for Compound A, ethanol

extract and S2, respectively. In contrast, the percentage albumin bound cortisol after administration of the test compounds mimicked the results of the percentage free cortisol in that the percentage increased significantly (P<0.01) from 22% to 46%, 36% and 51% for Compound A, ethanol extract and S2, respectively, suggesting that the increased free cortisol was bound to albumin.

If these results are compared with those of progesterone in Table 4.1 (B), the average percentage free progesterone before administration of the test compounds was 21%, which is similar to the percentage free cortisol. After administration of the test compounds, a slight , but not significant (P>0.05), increase in the percentage free progesterone was observed in two cases, 24% for Compound A and 26% for S2, while a significant (P<0.01) increase of 43% for the ethanol extract was observed. A significant (P<0.01) decrease in the percentage albumin bound progesterone to 30% for S2 was observed, while with the ethanol extract and Compound A the percentage albumin bound progesterone did not change significantly (P>0.05). Only 24% of the progesterone was bound to CBG and a not significantly (P>0.05) decrease to 22% for Compound A was observed, while with the ethanol extract all the progesterone bound to CBG was displaced. With S2 a significant (P<0.01) increase to 44% in the percentage CBG bound was observed. This is a puzzling result in the light of the fact that S2 appears to displace cortisol from CBG and would thus appear to be unlikely to accumulate excess free progesterone bound to CBG.

From the above results, it can be concluded that the test samples, Compound A, ethanol extract and S2, displace cortisol more readily from CBG than progesterone, with the exception of the ethanol extract, which displaces both cortisol and progesterone from CBG. However, the rise in free cortisol is almost tripple the intial percentage cortisol, while the progesterone rise is only double the intial value. The albumin bound steroids appeared not to be easily displaced by the test compounds, with the exception of albumin bound progesterone which appeared to be displaced by S2. The albumin bound steroid rather appeared to be influenced by the percentage free steroid, with increases in albumin bound steroid occuring concomittantly with increases in free steroid.

4.3 Conclusion

In this study, the results showed that in native sheep serum, 18% of cortisol is free, 62% bound to CBG and 22% bound to albumin, whereas 21% of progesterone is free, only 24% bound to CBG and 60% bound to albumin. As can be seen from Table 4.2 our results with cortisol compare very well with the results obtained by Gayrard *et al.* who showed that 14% of cortisol is free, 66% bound to CBG and 20% to albumin [32]. Unfortunately, no results referring to the relative distribution of progesterone in sheep serum could be obtained from the literature. However, a comparison with results from human serum shows that most of the progesterone is bound to albumin (Table 4.2). A comparison of the percentage free cortisol values in humans in the Dunn and Gayrard studies suggests that there is an underestimation of the percentage free steroid in the Dunn study and therefore, the very low (2.4%) free progesterone in the Dunn study may well be higher [32, 36]. Progesterone results in sheep compare well with the results in humans. When CBG binding is, however, considered (24% vs 18%), results obtained in this study compare well with corresponding results obtained in humans.

Table 4.2 Comparison of the distribution of cortisol and progesterone in different species and in different studies. (A) cortisol and (B) progesterone.

SPECIES	A			B			Ref.
	% FREE	% CBG	% ALB	% FREE	% CBG	% ALB	
sheep	18	62	22	21	24	60	Present study
sheep	14	66	20				#32
human	4	90	6	2.4	18	79	#36
human	10	78	12				#32

The lower percentage binding of cortisol to CBG in sheep, relative to human serum, may be explained by (1) the lower concentration of CBG in sheep (Table 4.3) (2) the lower concentration of cortisol in sheep and (3) a similar order of affinity for cortisol in sheep and human CBG, as all these factors influence binding of cortisol to CBG [32]

CBG in the blood is largely occupied with steroid ligand and provides a large reservoir of glucocorticoids that may be displaced by ligands with a higher affinity [37]. Dunn *et al.*

found that about 50% of the available CBG-binding sites is occupied by cortisol and that accounts for 94-96% of the binding to CBG in men and women, while progesterone occupies less than 1% of the available CBG-binding sites [36]. This is due to the difference in concentration of these steroids in human plasma, (400 nM for cortisol and 0.65 nM for progesterone) and the difference in affinity between the two steroids (Kd of 1.3×10^{-8} M for cortisol and 4.2×10^{-8} M for progesterone). Binding of steroids to CBG is thus affected by the steroid concentration, the concentration of the CBG, the affinity of the steroid for CBG, and the affinity of an inhibitor for CBG [36].

Table 4.3 Comparison of difference in concentration (nM) of cortisol and progesterone and their affinities (Kd) for CBG as well as the CBG concentration (µM) in different species and in different studies. (A) cortisol and (B) progesterone.

SPECIES	A			B			Ref.
	µM CBG	Kd (nM)	nM	Kd (nM)	nM		
rat (female)	2.4	2.86					#45, #46
human	0.38	39	140				#32
human	0.7	13	400	42	0.65		#36
sheep	0.08	9.2	50		0.47		#32, #47

The effect of the test compounds, Compound A, the ethanol extract and S2 on the distribution of cortisol and progesterone in sheep serum was also determined in this study. Compound A, the ethanol extract and S2 caused a significant ($P<0.01$) increase in the percentage free cortisol. The increased free cortisol resulted in an significant ($P<0.01$) increase in the percentage of albumin bound cortisol, as binding to albumin was proportional to free steroid concentration [42]. The rise in the percentage free and albumin bound steroid was concurrent with a significant ($P<0.01$) decrease in the CBG bound cortisol. This rise may be due to the displacement of cortisol from CBG by Compound A, the ethanol extract and S2. Table 4.1 shows that this effect will also occur with physiological concentrations of the test samples. Pugeat et al., in an study investigating the interaction of drugs with CBG and SHBG, showed that prednisolone, a potent synthetic glucocorticoid, binds to human CBG and causes a 32% decrease in the percentage cortisol bound [35]. Thus previous studies have shown that cortisol may be displayed from CBG as we have found in the present study.

Compound A and S2 did not cause a significant ($P<0.05$) increase in the percentage free progesterone (Fig. 4.2 B,C,D). The rise in the percentage free steroid in the case of the ethanol extract was, however significant ($P<0.01$), and concurrent with a significant ($P<0.01$) decrease in the CBG bound progesterone. This rise may be due to the displacement of progesterone from CBG by the ethanol extract. A significant ($P<0.01$) decrease in the albumin bound progesterone was seen with S2, which indicated that it displaced cortisol from albumin. The change in albumin bound progesterone observed with Compound A and the ethanol extract is not significant ($P>0.05$). Studies with metyrapone have shown that displacement of steroids from albumin can occur [34]. However, if we compare the effect of the physiological levels of the test compounds on the distribution of progesterone in sheep serum (Table 4.1) we see that both, Compound A and S2, had very little effect on this distribution. The ethanol extract appears to differ, in that free progesterone increased significantly ($P<0.01$). The fact that Compound A and S2 had little effect on the progesterone distribution while greatly affecting the cortisol distribution in sheep serum, may be explained by examining Table 4.3. We can see that the concentration of progesterone in sheep serum was much lower than cortisol and thus, we would expect very few of the steroid binding sites on CBG to be occupied by progesterone. Dunn *et al.* clearly illustrated this for human serum [36]. Thus, displacement of progesterone from CBG binding sites would be expected to be much less than that of cortisol.

We can conclude from this study that Compound A, the ethanol extract and S2 displace endogenous steroids, primarily cortisol, from CBG with a resulting increase in the percentage free steroid in sheep serum. The increase in free steroid would lead to a decrease in CBG synthesis by the liver which may amplify and enhance the biological effects of endogenous steroid levels [38]. The results obtained reconcile the facts that natural plant products, which are highly reactive and unstable *in vitro*, can be biologically active *in vivo* due to stabilization by binding to serum proteins. In addition, binding may result in displacement of endogenous steroids from serum binding proteins which may contribute to the biological effects of these compounds.

CHAPTER 5

FACTORS WHICH INFLUENCE THE CYCLIZATION OF COMPOUND A TO THE CORRESPONDING AZIRIDINE

5.1 Introduction

Previous studies have indicated that serum retards the cyclization of Compound A to the corresponding aziridine [14]. ES-MS is currently the only method that can be used to study the rate of cyclization of Compound A (Fig. 5.1). ES-MS is the mildest form of mass spectrometry and is ideally suited to the study of natural products and labile molecules.

Compound A:
2-(4-acetoxyphenyl)-2-chloro-N-
methyl-ethylammonium chloride
$m/z = 228/230$

Compound A's aziridine:
2-(4-acetoxyphenyl)-1-methylaziridine
$m/z = 192$
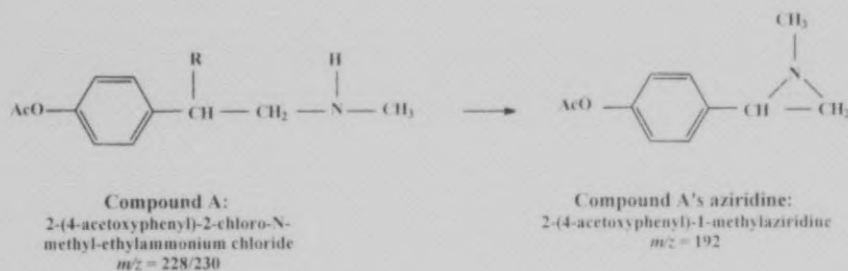
Figure 5.1 Cyclization of Compound A to the corresponding aziridine.

The current study was aimed at investigating different factors which may influence the cyclization of the aziridine precursor, Compound A, to the corresponding aziridine, both in buffer and in the presence of serum, which contains CBG to which Compound A binds. The effects of cone voltage, temperature and pH, and P450c11 enzyme concentration on the rate of cyclization were studied by using ES-MS.

The cyclization of Compound A to the corresponding aziridine was analyzed at a range of different cone voltages from 10-40 V, to select the best cone voltage for sensitivity and minimum fragmentation.

Secondly, the effects of temperature and pH were investigated on the rate of cyclization of Compound A, both in buffer and sheep serum. Temperature has been shown to have a

dramatic effect on the binding of cortisol and progesterone to human CBG. Scatchard analysis showed that the association constant decreased about 40-fold from 4 to 37°C, while the number of binding sites remained the same [48]. Slaunwhite and Sandberg in addition, found greater dissociation of the human CBG-cortisol complex at 37°C than at 4°C [49]. In a number of other species, the binding activity, as measured by gel filtration was also found to be considerably lower at 37°C than at 4°C [50]. Thus, binding and hence retardation of cyclization of Compound A in serum, may be affected by temperature. In addition we would expect increased temperature to increase the rate of cyclization in buffer.

It has been shown that the binding affinity between the various steroid hormones and CBG is dependent on the hydrogen ion concentration. Maximal binding was generally observed at approximately pH 8 in purified human CBG preparations as well as directly in serum [51]. Chan and Slaunwhite determined a pH optimum of 8 for the binding of cortisol and pH 8.5 for progesterone by using a purified human CBG preparation [52]. At pH 5 and below, irreversible denaturation of CBG appeared to take place with loss of the binding affinity. The effect of pH on the association constants for CBG bound to cortisol and progesterone were reinvestigated by Mickelson *at al.* [48]. Relatively broad pH profiles were obtained for both steroids with maximal binding between pH 8 and 11.5. In addition, the cyclization of Compound A to the corresponding aziridine is pH dependant with increased cyclization being observed at higher pH values [12].

Thus the influence of temperature and pH on the cyclisization of the aziridine precursor to the aziridine, in buffer and sheep serum was investigated. The cyclization of Compound A (m/z 228/230) to the corresponding aziridine (m/z 192) was monitored by ES-MS over a period of 30 min at 4, 22 and 37°C and also at pH 2.6, 4, 5, 6, 7, 7.4, and 7.9.

Finally, the effect of P450c11 enzyme concentration on the cyclization of Compound A to the aziridine was carried out. This was promted by an observation from the spectrophotometric studies which suggested that Compound A's cyclization may be accelerated by the enzyme. This study was also conducted in PBS and sheep serum to ascertain whether CBG influenced cyclization in the presence of the enzyme.

5.2 Electrospray mass spectrometry (ES-MS)

A mass spectrometer is an analytical device that determines the molecular weight of chemical compounds by separating ions according to their mass-to-charge ratio (m/z). Electrospray ionization (ESI) is a method used to produce gaseous ionized molecules from a liquid solution [53]. This method is mild enough to ionize macromolecules without fragmentation. A solution of analyte is passed through a needle that is kept at high electrical potential. At the end of the needle, the solution disperses into a mist of small, highly charged droplets containing the analyte. The small droplets evaporate rapidly and, by a process of either field desorption or residual solvent evaporation, protonated analyte molecules are released into the gas phase [54]. A typical mass spectrometer can be separated into three distinct components: the ion source, the mass analyzer and the detector. The instrument used for ES-MS will subsequently be discussed using the schematic representation in Fig. 5.2.
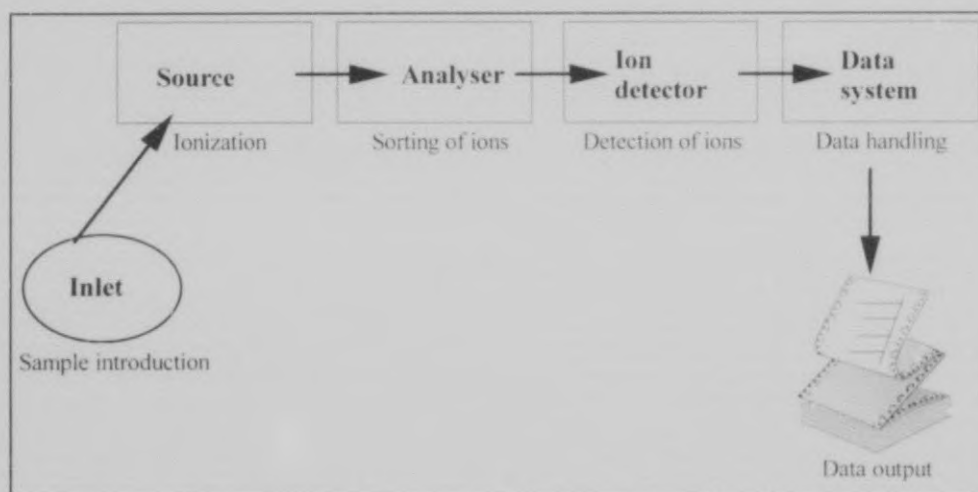
Figure 5.2 The components of a mass spectrometer.

5.2.1 Inlet and source

The sample inlet is the interface between the sample and the mass spectrometer. During electrospray ionization a dilute solution on an analyte in a stream of a suitable solvent or solvent mixture is pumped through a hollow needle at a flow of between 5-300 µl/min. The composition of the solvent mixture is critical for the type of ionisation that is to be achieved.

If a volatile acid, like formic acid, is added the molecules to be analysed will carry a positive charge. ES-MS is then said to be conducted in the positive mode. If a volatile base, like ammonium hydroxide is added to the solvent, the molecules to be analysed will carry a negative charge and ES-MS analyses is then done in the negative mode. Nitrogen gas is passed at a high flow rate in a sheath on the outside of the solvent stream to form a spray. On this needle a high potential of up to 5000 Volt (V) is applied relative to the analysing section of the instrument. This high potential causes the spray to break up into highly charged droplets of about 1 micron in size. This process is called the electrospray. The three principal steps of ionisation during ES-MS are illustrated in Fig. 5.3. The charged droplets are accelerated in the electric field towards the mass analyser which is held in a vacuum. Through the combined effects of a counter flowing drying gas and vacuum, the solvent present in the droplets starts to evaporate giving rise to smaller unstable droplets from which surface ions are deliberated into vacuum.

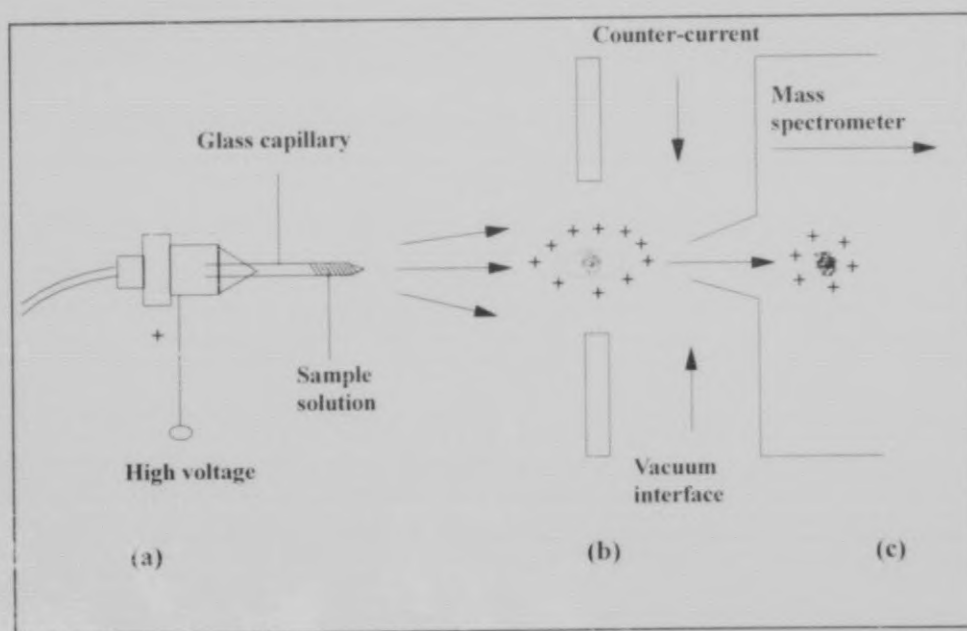**Figure 5.3** The three principals of electrospray mass spectrometry (ES-MS). **(a)** Formation of small, highly charged droplets by electrostatic dispersion of a solution under the influence of a high electric field **(b)** desorption of ions from the droplets into the gas phase (assisted by a countercurrent of hot N_2 gas). **(c)** Mass analysis of the ions in a mass spectrometer (Redrawn from [54]).

5.2.2 Mass analyzer

The mass analyzer contributes to the accuracy, range and sensitivity of the mass spectrometer. All mass analyzers distinguish ions according to their mass-to-charge ratios (m/z) or a related property. The instrument used in this investigation was equipped with a quadrupole analyzer which consists of four precisely parallel poles or rods. In this device, mass sorting depends on ion motion resulting from direct current (DC) and radio frequency (RF) electric fields. The field on the quadrupoles determines which ions are allowed to reach the detector. The quadrupoles thus function as a mass filter. Quadrupole mass spectrometers provide lower resolution, but are more readily interfaced to various inlet systems.

5.2.3 Ion detector

Passing through the mass analyzer, the final element of the mass spectrometer, the ion detector, is reached. In the instrument used in this study, ions are detected by means of a photomultiplier detection system. The maximum molecular mass range that can normally be measured accurately by a quadropole mass spectrometry is from 0 to 3000 daltons. It is a fact, however, that most large molecules, including biological polymers such as proteins, have more than one charge under electrospray mass spectroscopic conditions. As a result of multiply charging through the electrospray process, molecules with masses in excess of 3000 daltons, can be detected in the 3000 m/z detection range of the mass spectrometer.

5.3 The influence of cone voltage

Cone voltage in the ES-MS determines the acceleration of the sample particles in the ionisation source. It has a profound influence on detection and fragmentation of the sample molecules. In order to select the best cone voltage for sensitivity and minimum fragmentation, the cyclization of Compound A to the corresponding aziridine was analyzed at different cone voltages. A 200 µM solution of Compound A was prepared in PBS and diluted 100 times with acetonitrile containing 1% formic acid. The injection volume was 10 µl and the mobile phase, at a flow rate of 10 µl/min, was a 50:50 water:acetonitrile mixture containing 1% formic acid. The rate of cyclization of Compound A (m/z 228/230) to the corresponding aziridine (m/z 192) was monitored at 5 and 10 min by positive mode ES-MS analysis. The intensities of the mass peaks of the two ions were compared and expressed as

percentages of the highest intensity. Cone voltages of 10 V (Fig. 5.4), 15 V (Fig. 5.5), 20 V (Fig. 5.6), 25 V (Fig. 5.7), 30 V (Fig. 5.8) and 40 V (Fig. 5.9) were tested.

The ES-MS spectrum at a cone voltage of 10 V shows that this cone voltage was to low because there was no trace of the test compounds, Compound A or it's corresponding aziridine, at 1 and 10 min (Fig. 5.4). The peaks obtained may be due to adducts of the buffers used. At a cone voltage of 15 V the Compound A (m/z 228/230) peak and the aziridine peak (m/z 192) were present but the amount was very low (Fig. 5.5). The others peaks may be adducts of the test compound with the buffers used in this study.

The ES-MS spectra at a cone voltages of 20 and 25 V showed a greater amount of Compound A present at 1 min (Fig. 5.6 & 5.7). At 10 min, for both cone voltages, cyclization to the corresponding aziridine had occured although a considerable amount of Compound A was left. The spectrum of the cone voltage of 20 V contained a number of adducts of the test compounds and buffers used. In contrast, the spectrum at a cone voltage of 25 V was very clear, with both Compound A and the aziridine present and with the amount of adducts present reduced significally relative to what was observed at 20 V.

The ES-MS spectra at cone voltages of 30 and 40 V (Fig.5.8 & 5.9) showed that at 1 min a large amount of Compound A had already cyclisized to the aziridine. At 10 min, with a cone voltage of 30 V, a considerable amount of Compound A was still present but at 40 V, very little Compound A was left. At a cone voltage of 40 V fragmentation of the adducts was also occurring.

From these results it was deduced that cone voltages of 20 and 25 V would be ideally suited to study the cyclization of the aziridine precursor, Compound A, to the corresponding aziridine. At lower cone voltages the molecular ions in question (m/z 228/230 and m/z 192) were not observed in sufficient quantities, while at higher cone voltages (30 & 40 V) increased cyclization and fragmentation was observed (Fig. 5.10). A cone voltage of 25 V therefore gave the best results, as adducts were not observed. At a cone voltage of 20 V the adducts of Compound A and the aziridine had to be taken into accouat.

Figure 5.4 ES-MS spectra of the effect of a cone voltage of 10 V on the cyclization of Compound A to aziridine. (A) 1 min (B) 10 min.

Figure 5.5 ES-MS spectra of the effect of a cone voltage of 15 V on the cyclization of Compound A to aziridine. (A) 1 min (B) 10 min.

Figure 5.6 ES-MS spectra of the effect of a cone voltage of 20 V on the cyclization of Compound A to aziridine. (A) 1 min (B) 10 min.

Figure 5.7 ES-MS spectra of the effect of a cone voltage of 25 V on the cyclization of Compound A to aziridine. (A) 1 min (B) 10 min

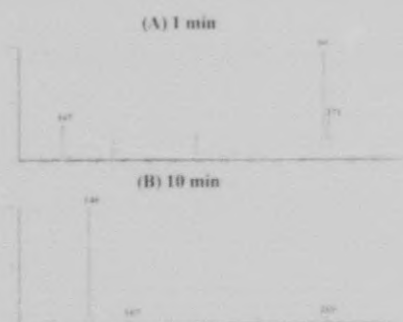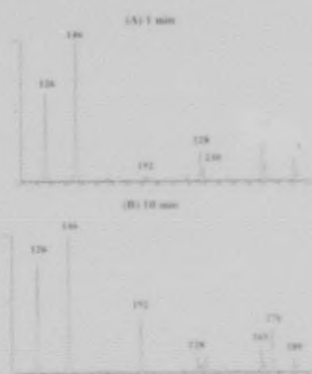**Figure 5.8** ES-MS spectra of the effect of a cone voltage of 30 V on the cyclization of Compound A to aziridine. (A) 1 min (B) 10 min.
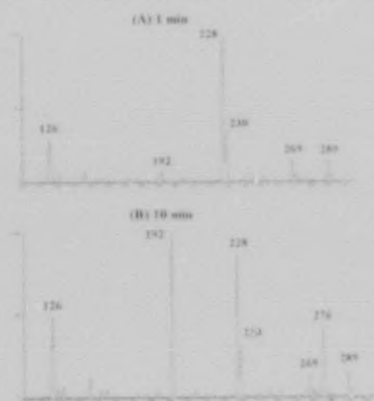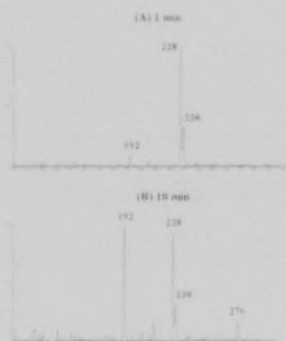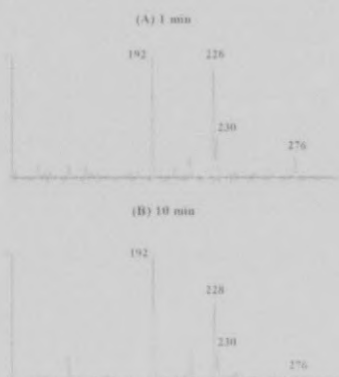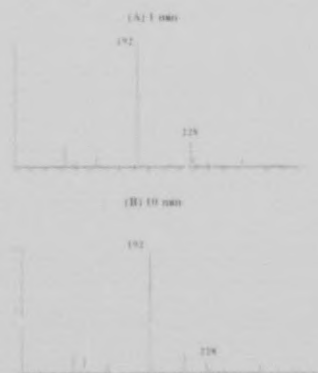

Figure 5.9 ES-MS spectra of the effect of a cone voltage of 40 V on the cyclization of Compound A to aziridine. (A) 1 min (B) 10 min.
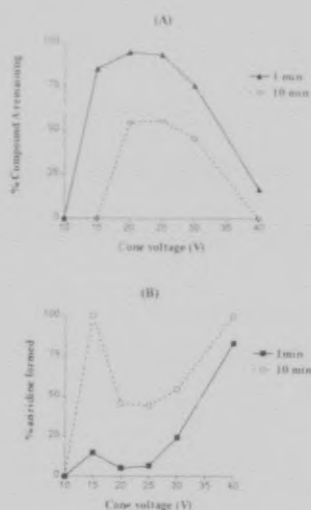
Figure 5.10 The influence of cone voltage on the cyclization of Compound A to aziridine in PBS.

5.4 The effect of temperature and pH on cyclization

ES-MS was used to study the influence of temperature and pH on the cyclization of Compound A to the corresponding aziridine. Compound A, at a concentration of 16.7 mM, was prepared in buffer or sheep serum at diffe   it pH values. It was diluted 100 times with a 50:50 water:acetonitrile mixture containing 1% formic acid, and analysed immediately. The injection volume was 10 ul and the mobile phase, at a flow rate of 10 ul/min, was a 50:50 water:acetonitrile mixture containing 1% formic acid. The percentage Compound A remaining (m/z 228/230) was monitored at 5 min intervals for 30 min by positive mode ES-MS analysis. The cone voltage was set at 20 V, and the source temperature was kept at 75°C. Compound A was made up in a range of citrate-phosphate buffers (0.05 M) with the pH ranging from 2.6 to 7.9 The sheep serum was dialysed overnight in these buffers to adjust the pH accordingly. The reason why temperature and pH values other than the physiological pH of 7.4 and temperature of 37°C were tested was to determine at which conditions Compound A is the most stable and must be stored, and to dtermine which conditions are ideal for use in biological assays. The cyclization of Compound A (m/z 228/230) to the corresponding aziridine (m/z 192) was monitored by ES-MS over a period of 30 min at 4, 22 and 37°C and also at pH 2.6, 4, 5, 6, 7, 7.4, and 7.9.

The results are presented as the percentage of Compound A remaining and was calculated using the following equation:

$$\frac{\text{Intensity of peak } m/z \text{ 228/230}}{\text{Intensities of peaks } m/z \text{ 192 and } m/z \text{ 228/230}} \times 100$$

The effect of temperature and pH on the rate of cyclization of Compound A to aziridine in buffer is shown in Fig 5.11. As can be seen from the figures the cyclization of Compound A to the corresponding aziridine in buffer was temperature and pH dependant. In buffer, the rate of cyclization increased with increasing temperature, with little cyclization occurring at 4°C for most pH-values, while at 37°C, cyclisation was complete after 30 min at pH 7 and above.

Figure 5.11 Effect of temperature and pH on the cyclization of Compound A to aziridine in buffer. Cyclization is presented as percentage Compound A remaining (A) 4°C, (B) 22°C and (C) 37°C.

The graphs illustrating cyclization of Compound A at pH 7 shows this very clearly. At 4°C more than 90% of Compound A was present after 30 minutes, while at 22°C this value decreased to about 20%, while at 37°C no Compound A was observed. The rate of cyclization is also increased with increased pH. At pH 4 and below little cyclization occured even at 37°C, while at pH 7.9 cyclization was completed after 30 min at 22 and 37°C, respectively.

Cyclization of Compound A to the corresponding aziridine in sheep serum was also temperature and pH dependant as can be seen in Fig. 5.12. In sheep serum, the rate of cyclization also increased with increased temperature and pH. Very little cyclization was observed at 4°C for most pH-values with the exception of pH 7.9. While at 37°C significant
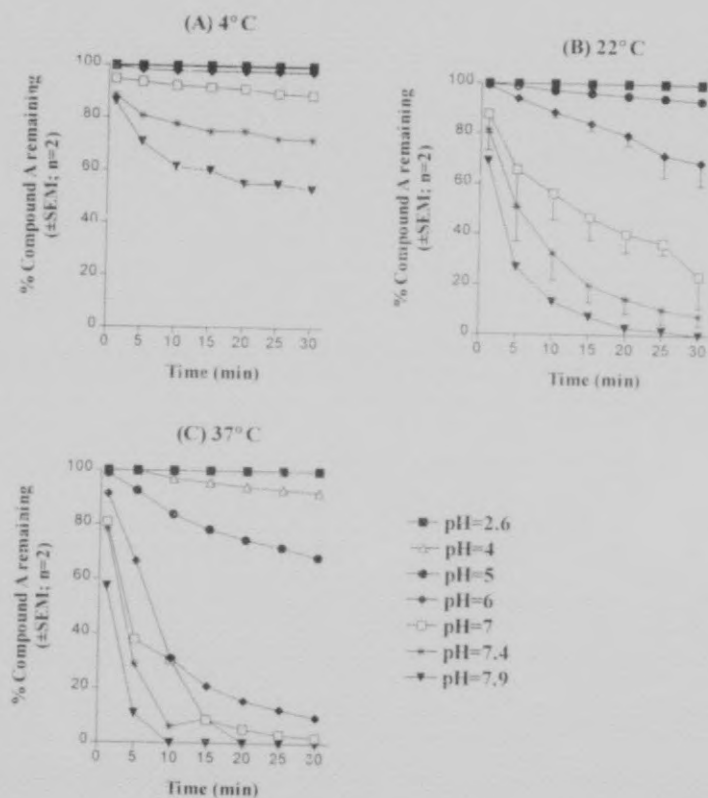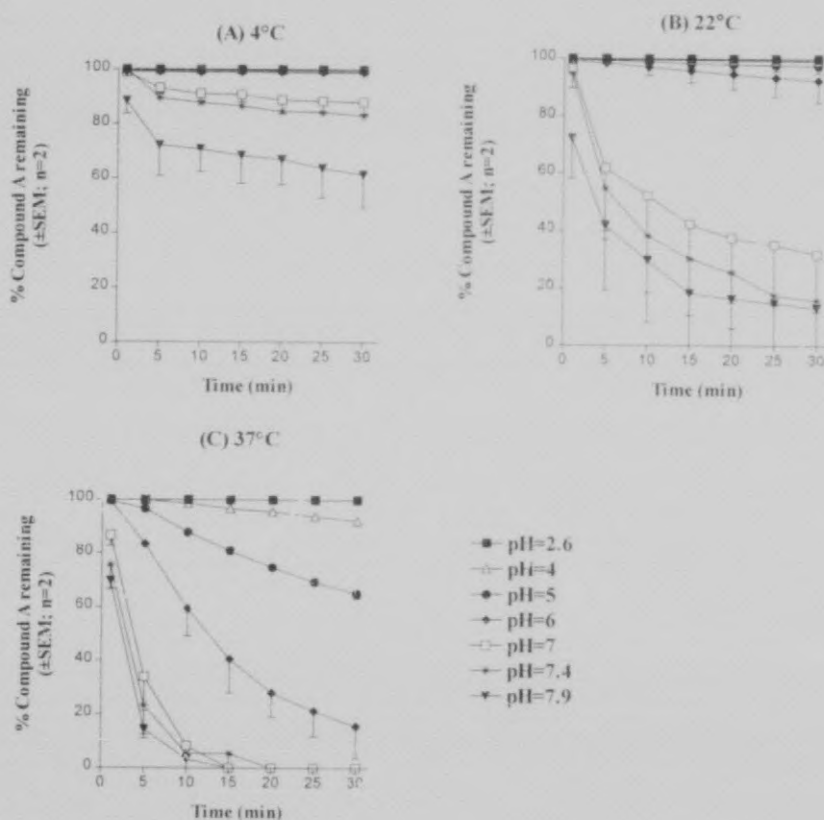

Figure 5.12 Effect of temperature and pH on the cyclization of Compound A to aziridine in sheep serum. Cyclization is presented as percentage Compound A remaining. (A) 4°C, (B) 22°C and (C) 37°C.

cyclization occured between pH 6 and 7.9 and even at pH 4 slight cyclization was observed after 30 minutes. The effect of pH can clearly be seen at 37°C (Fig. 5.12 C) where cyclization increased as the pH increased. This trend is observed to a lesser extent at 22° and 4°C.

As can be seen in Fig. 5.13 the cyclization of Compound A to the corresponding aziridine in buffer and sheep serum was temperature and pH dependant. In buffer cyclization increased significantly ($P < 0.01$) with increased temperature with little cyclization occurring at 4°C while at 37°C cyclization was completed after 30 min at pH 7 and above. This phenomena may be more clearly illustrated by comparing the pH values at which 50% cyclization of Compound A occured at the different temperature. At 4°C 50% cyclization occured at a pH > 7.9, at 22°C at pH 6.5 and at 37°C at pH 5.2. Cyclization was also increased with increased pH. at pH 4 and below little cyclization occurred at all temperatures, while at pH 7.9 cyclization was completed after 30 min at 22 and 37°C.
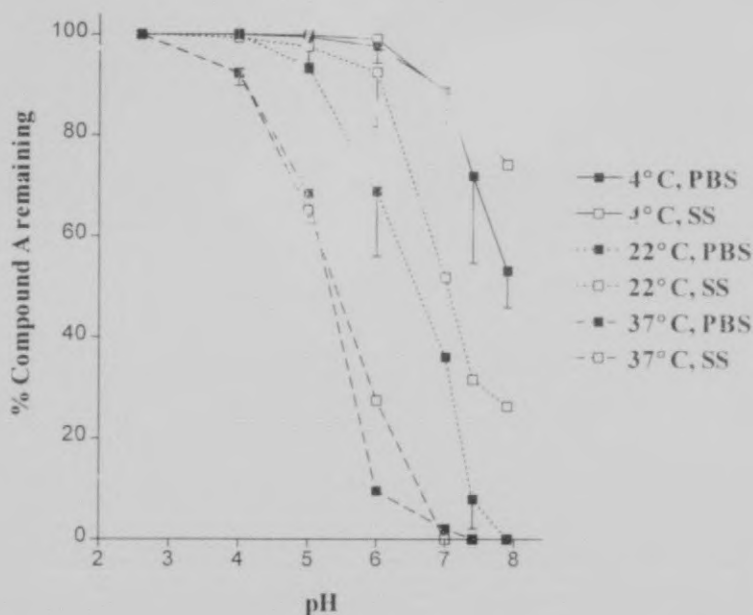
Figure 5.13 Effect of pH and temperature on the cyclization of Compound A to aziridine after 30 min in PBS and sheep serum. Statistical analysis was done using paired ANOVA and Bonferroni test as post test. A significant ($P < 0.01$) difference between 4 and 37°C in PBS and sheep serum was obtained. Also, when sheep serum was compared to PBS a significant ($P < 0.05$) difference was observed at 22°C.

In sheep serum cyclization also increased significantly (P<0.01) with increased temperature. At 22°C it can however clearly be seen that cyclization in sheep serum was significantly (P<0.05) retarded relative to cyclization in buffer. Cyclization of Compound A in sheep serum also increased with increased pH. Retardation of cyclization in serum, relative to buffer appeared to be pH and temperature dependant with maximal retardation occurring at pH 7.9 and 22°C.

5.5 The effect of the P450c11 enzyme on cyclization

ES-MS was used to study the influence of P450c11 enzymes on the cyclization of Compound A to the corresponding aziridine. Compound A, at a concentration of 5.102 mM, was prepared in PBS and in native sheep serum. To study the effect of P450c11 enzymes on cyclization, two separate experiments were done.

Firstly, the enzyme and Compound A (in PBS or sheep serum) were incubated together or separately for one hour and Compound A dissapearance and aziridine formation was monitored at 10 min intervals by positive mode ES-MS analysis. Freeze dried mitochondrial powder containing the cytochrome P450c11 enzymes was prepared by the method of Cheng and Harding as described in 9.2.1 and used as the source of enzyme [26]. The mixture of Compound A and enzyme mimicked the preparation used in the spectrophotometric assays with 98 µl Compound A (in PBS or sheep serum) and 2 µl ethanol added to 900 µl of enzyme preparation (1 mg/ml). The only exception was the concentration of Compound A which had to to be increased from 200 µM to 5.102 mM to allow detection by ES-MS in the presence of the enzyme. Before injection into the ES-MS the Compound A and enzyme preparation was diluted 10 times with a 50:50 water:acetonitrile mixture containing 1% formic acid, and assayed immediately. The injection volume was 100 µl and the mobile phase, at a flow rate of 10 µl/min, was a 50:50 water:acetonitrile mixture containing 1% formic acid. The cone voltage was set at 20 V, and the source temperature was kept at 75°C.

The percentage Compound A remaining (Fig. 5.14 A) over a period of 60 min was determined. If Compound A and the P450c11 enzyme were incubated together, cyclization of Compound A to aziridine, in PBS and sheep serum, was significantly (P<0.05) faster than when it was incubated separately. When incubated together in PBS and sheep serum,

cyclization of Compound A was completed after 40 min with no significant (P>0.05) difference in cyclization in PBS or sheep serum. However, when cyclization in PBS and sheep serum is compared it can be seen that sheep serum retards cyclization significantly (P<0.01) when the enzyme and Compound A are incubated separately. To conclude, sheep serum appeared to retard cyclization, while the presence of P450c11 accelarated cyclization of Compound A to the corresponding aziridine.

(A)
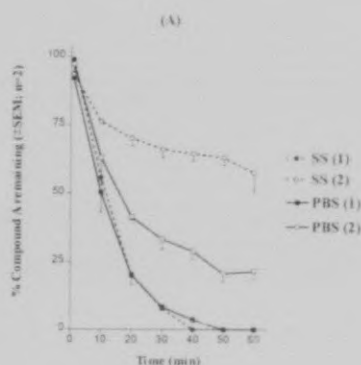
(B)

PBS (1) vs. PBS (2)	*
SHEEP SERUM (1) vs. SHEEP SERUM (2)	**
PBS (1) vs. SHEEP SERUM (1)	NS
PBS (2) vs. SHEEP SERUM (2)	**

Figure 5.14 Effect of P450c11 enzymes on the cyclization of Compound A. (A): percentage Compound A remaining (1) Enzyme and Compound A incubated together. (2) Enzyme and Compound A incubated separately. (B) Statistical analysis was done using paired ANOVA and Bonferroni test as post test (* = P<0.05, ** = P<0.01 and NS = not significant).

In the second series of experiments the influence of an increase in DOC concentration (natural substrate for P450c11 enzyme) and enzyme concentration, on the cyclization of Compound A to the corresponding aziridine was investigated. In this experiment the concentration of Compound A was lowered from 5.102 mM to 2.487 mM. Thus, the previous experiment was repeated, to once again establish that the presence of the enzyme accelerated cyclization (Fig. 5.15). The results again showed that when Compound A and P450c11 were

incubated together, cyclization of Compound A to aziridine in PBS was faster than when it was incubated separately. If we, however, compare the cyclization of Compound A incubated separately, with the values observed in previous experiments, cyclization was a lot slower. This was due to the lowering in the concentration of Compound A from 16.7 mM for the temperature and pH studies (Fig. 5.11), and from 5.102 mM for the enzyme studies (Fig. 5.14) to 2.487 mM, used in this study. The lowering in the concentration of Compound A was necessitated to correlate with the concentrations of DOC used in the next part of the experiment. Thus we can once again conclude that P450c11 accelerated the cyclization of Compound A to aziridine.
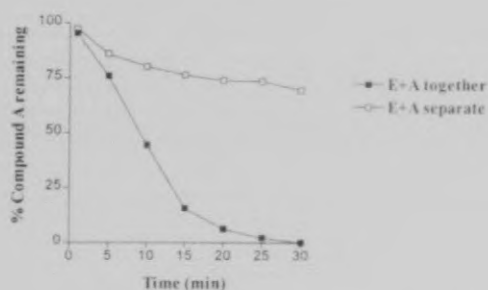
Figure 5.15 Effect of P450c11 enzymes on the cyclization of Compound A.

In the second part of this experiment, the influence of different concentrations of DOC, the natural substrate for the P450c11 enzyme, on the cyclization of Compound A was investigated. The conditions for this experiment was the same as above, except for the 2 µl ethanol which was replaced with DOC (0 to 500 µM made up in ethanol). The spectrophotometric results in Chapter 3 showed that when Compound A and P450c11 were incubated together, mixed inhibition of the Type I DOC-induced difference spectra was obtained, characterised by a high competitive element. The results in Fig 5.16, however, showed that no change in the cyclization of Compound A was obtained when DOC was incubated with Compound A and the enzyme preparation. It could be argued that this might be due to the low DOC concentration (500 µM) relative to the Compound A concentration (2.487 mM). However, a comparison of the concentration of DOC devided by its Km value (307), with the concentration of Compound A devided by its Kic value (23), indicates that sufficient DOC is present to inhibit the cyclization of Compound A, if in fact DOC had an effect. Km and Kic values were obtained from the spectrophotometric results in Chapter 3.

From these results we can thus conclude that DOC did not inhibit the cyclization of Compound A, although Compound A inhibited the Type I DOC-induced difference spectra of P450c11.
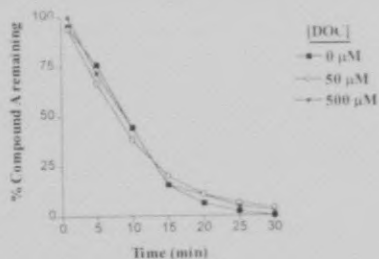
Figure 5.16 Effect of a increase in DOC (0-500 µM) concentration, in the presence of enzyme, on the cyclization of Compound A.

Finally, the effect of enzyme concentration on the rate of cyclization of Compound A was investigated. An increase in the concentration of P450c11 enzyme (0-4 mg/ml) caused an increase in the cyclization of Compound A to the aziridine as can be seen in Fig. 5.17 A and B. When the reaction rate (V_0) for Compound A remaining and aziridine formed was plotted against the enzyme concentration a straight line was obtained (Fig. 5.17 C and D, respectively). From these results it can be concluded that the cyclization of Compound A to the corresponding aziridine is enzymatically accelerated by the P450c11 enzyme.

Figure 5.17 Effect of a increase in P450c11 enzyme concentration on the rate of cyclization of Compound A. (A) percentage Compound A remaining (B) percentage aziridine formed in PBS (C) reaction rate for Compound A vs enzyme concentration and (D) reaction rate for aziridine vs enzyme concentration. The units of V_0 are % Compound A remaining/% aziridine formed per minute.
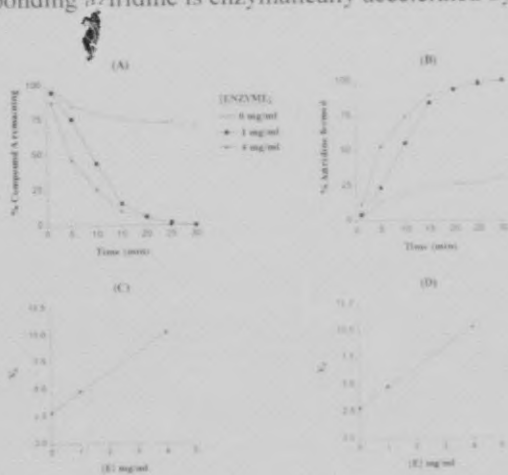
5.6 Conclusion

When the effect of cone voltage on the cyclization of Compound A to aziridine was determined it clearly showed that 20 and 25 V would be ideally suited for the present study. Lower cone voltages did not result in the presence of clear molecular ions, while higher cone voltages lead to increased cyclization and fragmentation.

Evidence was obtained that cyclization of Compound A to the corresponding aziridine in buffer and sheep serum was temperature and pH dependant. In buffer and sheep serum the cyclization increased significantly ($P<0.01$) with a rise in temperature, with little cyclization occurring at 4°C, while at 37°C cyclization was completed after 30 min at pH 7 and above. Cyclization was also increased with increased pH. At pH 4 and below little cyclization occured while at pH 7.9 cyclization was completed after 30 min at 37°C. In sheep serum the cyclization was slower than in buffer at all temperatures, but only significantly ($P<0.01$) so at 22°C. The results suggested that Compound A bound to CBG, which retarded cyclization, and that binding was maximal at pH 7.9 and 22°C.

From the above results it can be concluded that there is equilibrium between CBG binding, which occured due to the presence of the sheep serum, and cyclization of Compound A. With an increase in temperature, a decrease in CBG binding was obtained, and thus, at 37°C there was little difference between cyclization rate in buffer or sheep serum. These results concurred with those of previous studies which indicated that CBG binding decreased dramatically at 37°C relative to the binding at 4°C [48, 49]. In addition, with an increase in pH, an increase in cyclization of Compound A was also observed. At pH 4, where only slight cyclization was obtained, there was no significant ($P>0.05$) difference between buffer and sheep serum. This may be attributed to the fact that little cyclization of Compound A occured at this pH, whether in buffer or serum, and to the fact that previous studies have shown that CBG is denatured at pH 5 and below [48]. Maximal binding of cortisol to CBG has been shown to occur at pH 8. Thus, at 22°C and pH 7.9, where CBG binding is maximal, the difference between cyclization in buffer and sheep serum was most significant ($P<0.01$).

ES-MS investigation into the influence of the P450-11 enzyme on the cyclization of Compound A to aziridine yielded interesting results. The results obtained showed an increase in aziridine formation, when Compound A and the enzyme were incubated together. In sheep

serum when Compound A and enzyme were incubated separately, the rate of aziridine formation was significantly ($P<0.01$) reta.ded relatively to that in buffer. However, little significant ($P>0.05$) difference was observed between sheep serum and PRS ··hen Compound A and enzyme was incubated together. Thus, the enzyme promoted cyclization while sheep serum retarded cyclization. The fact that the presence of the enzyme overrides the stabilization effect of sheep serum would suggest that the enzyme has a higher affinity for Compound A than CBG.

To characterise the effect of the enzyme on Compound A cyclization, we examined the influence of DOC (the natural substrate for the P450c11 enzyme) and different enzyme concentrations. From the results it is clear that DOC did not interfere with the cyclization promoting activity of the enzyme. Increasing enzyme concentrations however, clearly indicated that the P450c11 enzyme catalytically promotes cyclization of Compound A to produce the corresponding aziridine. Metabolism of drugs, which include metabolic activation of various carcinogenic compounds, by P450 enzymes is well documented, but occurs primarily in the liver [24]. In adult mammals the adrenal is not involved in detoxification, although it has been indicated that the fetal adrenal liver has detoxification capabilities [55]. Future elucidation of the interaction of Compound A with the P450 enzyme clearly warrants attention. Firstly, to establish the kinetic parameters of the interaction and secondly, to establish whether bio-activation of Compound A to the corresr .ding aziridine occurs only in the adrenal or whether other P450's, especially those in the liver, are involved.

CHAPTER 6

DISCUSSION

This study forms part of an ongoing investigation into the syndrome of prolonged gestation in sheep and contraception in ra.., caused by the Namibian shrub, *Salsola tuberculatiformis* Botsch. Evidence was obtained from studies with *S. tuberculatiformis* extracts, that compounds in the shrub perturbed adrenal steroidogenesis in the rat and interfered with the binding of the natural substrate, DOC, to sheep adrenal cytochrome P450c11 [6, 8]. Swart described the isolation of one of the active fractions from the shrub, named S2, and developed two spectroscopic micro-assays with v .ich the isolation of t. active fractions could be monitored [8].

The elucidation of the chemical structure of the main component(s) in the S2 fraction presented many problems. This was mainly due to its highly labile nature and the presence of impurities in samples. The S2 fraction was found to consist of a mixture of an extremely labile compound plus one or more stable components, one of which is synephrine. In aqueous medium synephrine is a breakdown product of the S2 fraction [6]. Derivitazation studies showed that S2 is not synephrine as acetylation of S2 yields a acetylated aziridine structure, while acetylation of synephrine yielded a synephrine triacetate. These results promted the suggestion that the active component in S2 might be 2-(4-hydroxy-phenyl)-1-methyl -aziridine or the corresponding open chain precursor [6, 11, 16, 17]. De Kock proved that an aziridine, or its precursor was present in S2 by developing a chemical probe, N-TNP-MEA-SH, which reacted under mild conditions with each of the model aziridines, but not with synephrine [11]. In a separate study, de Kock synthesized a series of structurally related aziridine precursors [12]. Although it was suggested that a hydroxy-aziridine or its precursor was present in S2, de Kock found that this precursor cyclicised very rapidly and could not be detected by FAB-MS [12]. Thus a more stable acetoxy-aziridine precursor analogue, 2-(4-acetoxyphenyl) -2-chloro-N-methyl- ethylammoniumchloride (Compound A), was synthesised to be used in further studies.

In previous ES-MS studies with S2, Maritz found molecular ions at m/z 168, 166 and 150 [19]. The molecular ions at m/z 166 and 168 were identified by NMR as norsalsolinol and synephrine, respectively. It was also suggested that the molecular ion at m/z 150 was a dehydration product of synephrine. To conclude, previous studies suggest that an aziridine moiety may be a component of S2, specifically that S2 may be a hydroxyphenyl-aziridine or the corresponding precursor, which decomposes to synephrine in aqueous medium. In addition the S2 fraction may contain norsalsolinol and a dehydration product of synephrine.

During the present study, S2 was again isolated by using the methods of Swart, namely, ethanol extraction, ultrafiltration and RP-HPLC. The isolation process was monitored with the above mentioned micro-assays. Reinvestigation of the components   S2 was done with the aid of ES-MS. The ES-MS spectra of the S2 fraction showed molecular ions at m/z 258, 168, 166 and 150. Comparison of the fragmentation patterns of the S2 molecular ions at m/z 168 and 166 obtained in our study, with that of synephrine and norsalsolinol, indicate that the molecular ions at m/z 168 and 166 are indeed synephrine and norsalsolinol, respectively.

Maritz identified the S2 molecular ion at m/z 150 as a dehydration product of synephrine [19]. Our study, which compared the fragmentation patterns of Compound A (m/z 228/230), its corresponding aziridine (m/z 192), and synephrine (m/z 168) with that of the m/z 150 and 258 ions from S2, however, suggest that this is not so. Previous work has established that Compound A (m/z 229/230) cyclisises to the corresponding aziridine (m/z 192) and then decomposes to synephrine (m/z 168) with time in an aqueous medium [14]. A comparison of the daughters of Compound A and the corresponding aziridine and synephrine all show a peak at m/z 150. The daughter ions at m/z 150 from Compound A, aziridine, and synephrine, are similar as can be seen from a comparison of their fragment ions. We would like to suggest that this moiety is a hydroxy-aziridine (m/z 150) which is formed during ES-MS fragmentation from aziridine (m/z 192) and synephrine (m/z 168) and not a dehydration product of synephrine, like Maritz suggested, as the fragmentation pattern of the molecular ions at m/z 228/230 and 192 do not indicate the presence of synephrine.

S2 also contains the m/z 150 peak as does the fragmentation pattern of S2's m/z 258 molecular ion. Comparison of the daughter ions of S2's molecular ions at m/z 150 and 258 with that of

the m/z 150 peaks found in Compound A, aziridine and synephrine show a great similarity. We conclude that this peak in the S2 fraction is also a hydroxy-aziridine. The components chemically identified in S2 by Maritz were all stable compounds, the labile components consistently observed in previous S2 samples were, however, absent. In this study, the S2 fraction also contains the peaks at m/z 168, 166 and 150 like in the Maritz study, but a peak at m/z 258 is also observed which may be the labile component. The fact that the labile component was absent in the Maritz study may be due to exposure of the S2 fraction to light or an alkaline pH. Also, in the present study, unlike in the study by Maritz, the activity of the S2 fraction was tested during different stages of the isolation process, using a cytochrome P450 based spectral assay. We suggest that the m/z 258 is an aziridine precursor which cyclises to the hydroxy-aziridine, which in turn may decompose to synephrine. De Kock proved that an aziridine or its precursor is present in S2, thus the molecular ion at m/z 258 may be the active component in S2. The leaving group in S2's molecular ion at m/z 258 is not shown in the fragmentation pattern of 258 which suggests that this moiety is probably not positively charged and therefore not observed in the positive ES-MS mode. Other chemical analysis would thus have to be employed to identify the leaving group from the S2 molecular ion at m/z 258.

To conclude, the current study obtained a new peak in the S2 fraction, m/z 258, which may be the active component in S2 and probably is the aziridine precursor of the molecular ion at m/z 150. The molecular ion at m/z 150 is proposed to be that of an hydroxy-aziridine and not a dehydration product of synephrine as previously suggested. Finally, the ions at m/z 168 and 166, were confirmed to be synephrine and norsalsolinol, respectively.

Previous spectrophotometric studies indicated that Compound A and S2 inhibited the Type I DOC-induced difference spectrum of sheep adrenal cytochrome P450c11 and elicited a Type II difference spectrum [8, 14]. With Compound A, inhibition was attenuated with time in buffer, but not in native sheep plasma. Heated sheep plasma also showed attenuation of inhibition with time in a manner similar to that of Compound A in buffer. ES-MS studies indicated that in buffer the aziridine precursor cyclisises fully to the aziridine within 1hour while in sheep serum the cyclisization is retarded. It was found that it is the aziridine precursor, rather than the aziridine, which is the inhibiting agent in the cytochrome P450c11

system and that two high affinity steroid-binding proteins in serum, SHBG and CBG, may bind and stabilize the aziridine precursor in sheep serum [14].

In the present study the influence of Compound A, the ethanol extract from *S. tuberculatiformis* and the S2 fraction, on the Type I DOC-induced difference spectrum, and the ability of the test samples to elicit a substrate induced difference spectrum (Type II) with sheep adrenal cytochrome P450c11, was investigated. All three test samples inhibited the Type I difference spectrum of P450c11. The effect of the ethanol extract , in PBS and in native or heated sheep serum, and S2 in PBS, on the DOC-induced difference spectrum showed that these samples inhibited the Type I spectrum over a period of 60 minutes with little or no attenuation of inhibition with time. This is in contrast with the time studies with Compound A, where inhibition was abrogated with time in PBS and in heated sheep serum, but not in sheep serum, probably due to stabilization of Compound A in serum. Thus, the active components in the ethanol extract and S2 appear not to be significally affected during the time studied by the presence of sheep serum, as is the case with Compound A. All three test samples induced a Type II difference spectrum, when interacting with mitochondrial powder from sheep adrenals, which indicates the presence of an amino group that reacts with the heme iron of the cytochrome [25].

The type of inhibition induced by Compound A on the DOC-induced difference spectrum of cytochrome P450c11, was spectrophotometricly investigated. The results indicate that mixed inhibition, with a strong competitive element, occurs. Mixed inhibition suggests that binding of the inhibitor (Compound A) to the enzyme does not occur at the substrate site, but does, however, affect the binding of the natural substrate (DOC) to the enzyme in addition to inhibiting the catalytic activity of the enzyme. This agrees with the spectrophotometric results obtained with Compound A which elicits a Type II difference spectrum and suggests binding to, or interaction with, the heme moiety of the enzyme. In contrast, DOC induces a Type I-difference spectrum, which suggests binding to the protein moiety of the enzyme [25]. The results suggests that Compound A asserts its effect on the P450c11 enzyme by binding to a different site than the substrate (DOC) binding site.

The inhibition of P450c11 by Compound A contains many of the elements identified by Ortiz de Montellano in reversible P450-enzyme inhibitors [31]. These include binding of the inhibitor to the heme iron and eliciting a Type II difference spectrum with a soret maximum at 430, and the fact that the most potent inhibitors are nitrogen-containing aliphatic and aromatic compounds. The most effective reversible inhibitors react strongly with both the protein and the prostetic heme iron and their activity is thus governed by both the hydrophobic character (interaction with protein) and by the strength of the bond with the heme iron. The mechanism of Compound A's inhibition of the DOC induced-difference spectrum will, however, have to be examined in more detail by using additional test systems, such as the *in vitro* conversion of DOC to corticosterone.

The distribution of cortisol and progesterone in sheep serum and the effect of the test samples, Compound A, the ethanol extract from the shrub and S2, on this distribution was also investigated. The distribution of cortisol in sheep serum compares well with previous studies [32]. Cortisol binds mainly to CBG (62%), 18% of the cortisol was free, and 22% was bound to albumin. We could not find any results referring to the distribution of progesterone in sheep serum. However, a comparison with results from human serum shows that our results are in agreement [36]. Most of the progesterone in our study was bound to albumin (60%), 21% of the progesterone was free, and 24% was bound to CBG.

Compound A, the ethanol extract, and S2 caused a significant ($P<0.01$) decrease in the percentage CBG bound cortisol. A significant ($P<0.01$) increase in the free cortisol results, with a concommitant significant ($P<0.01$) increase in the percentage of albumin bound cortisol. The rise in the percentage free cortisol is probably due to displacement of cortisol from CBG by Compound A, the ethanol extract, and S2. Previous studies have shown that cortisol may be displaced from CBG by high affinity ligands [33]. Pugeat *et al.* for example, showed that prednisolone, a potent synthetic glucocorticoid, binds to human CBG and causes a 32% decrease in the percentage cortisol bound [33]. These results strongly support the concept that the administration of certain drugs can affect the transport and disposition of endogenous steroid hormones by competing for binding or transport proteins, like CBG.

Compound A and S2 caused only a slight but not significant ($P > 0.05$) increase in the percentage free progesterone. The ethanol extract, however, significantly ($P < 0.01$) increased the percentage free progesterone. The fact that Compound A and S2 had little effect on the progesterone distribution while greatly affecting the cortisol distribution in sheep serum may be because the concentration of progesterone in sheep serum is much lower than cortisol and thus, we would expect very few of the steroid binding sites on CBG to be occupied by progesterone. Dunn *et al* clearly illustrates this for human serum [36]. Thus, displacement of progesterone from CBG binding sites would be expected to be much less than that of cortisol.

We can conclude from this study that the test samples displace endogenous steroids, primarily cortisol, from CBG with a resulting increase in the percentage free steroid in sheep serum. The increase in free steroid could lead to a decrease in CBG synthesis by the liver, which may amplify and enhance the biological effects of endogenous steroid levels [39]. The results obtained reconcile the facts that natural plant products, which are highly reactive and unstable *in vitro*, can be biologically active *in vivo* due to stabilization by binding to serum proteins. In addition, binding may result in displacement of endogenous steroids from serum binding proteins which may contribute to the biological effects of these compounds.

Factors which may influence the cyclization of the aziridine precursor, Compound A, to the corresponding aziridine was investigated. These included the effect of cone voltage, temperature and pH and the presence of the P450c11 enzyme. Cone voltage was found to have a significant effect on the cyclization of Compound A to aziridine and the results clearly showed that 20 and 25 V would be ideally suited for the present study. Lower cone voltages do not result in the presence of clear molecular ions while higher cone voltages leads to increased cyclization and fragmentation. With an increase in temperature a increase in cyclization of Compound A to the corresponding aziridine is observed. At 37°C, however, little difference between cyclization in buffer and sheep serum is observed. This may be due to a decrease in CBG binding as previous studies have indicated that CBG binding decreased dramatically at 37°C relative to the binding at 4°C [48, 49]. With an increase in pH, an increase in cyclization of Compound A is also observed. At pH 4 only slight cyclization is obtained and little difference is observed between buffer and sheep serum. This may be due to the fact that little cyclization of Compound A occurs at this pH, whether in buffer or serum.

and to the fact that previous studies have shown that CBG is denatured at pH 5 and below [48]. At physiological conditions namely pH 7.4 and 37°C, cyclization in sheep serum and PBS occurs at a fast rate but their is little significant (P>0.05) difference between cyclization in sheep serum and PBS. At 22°C and pH 7.9, where CBG binding and cyclization of Compound A is maximal, the difference between cyclization in buffer or sheep serum is significant (P<0.01). These temperature and pH studies showed that cyclization of Compound A to the corresponding aziridine in buffer and sheep serum is temperature and pH dependant and that there is a balance between CBG binding, which occurs due to the presence of the sheep serum, and cyclization of Compound A.

Results obtained from the ES-MS studies into the influence of the P45c11 enzyme on the cyclization of Compound A to aziridine, showed a significant (P<0.01) increase in aziridine formation, when Compound A and the enzyme were incubated together. When incubated separately, the rate of aziridine formation was significantly (P<0.01) retarded in sheep serum relatively to that in buffer. This difference in cyclization in the presence of sheep serum was not observed when the enzyme and Compound A were incubated together. We conclude from these results that the enzyme promotes cyclization while, sheep serum retards cyclization. The fact that the presence of the enzyme overrides the stabilization effect of sheep serum would suggest that the enzyme has a higher affinity for Compound A than CBG. The effect of the enzyme was further investigated by examining the influence of DOC (the natural substrate for the P450c11 enzyme) and different enzyme concentrations on the cyclization of Compound A. The results showed that DOC does not interfere with the cyclization promoting activity of the enzyme. However, increasing enzyme concentrations clearly indicated that the P450c11 enzyme catalytically promoted cyclization of Compound A to produce the corresponding aziridine.

Metabolism of chemicals by cytochrome P450 usually leads to the formation of inactive metabolites, however, chemical carcinogens or prodrugs like, cyclophosphamide, can be activated by cytochrome P450 to form chemically unstable reactive intermediates [24]. The resulting reactive intermediates may bind covalently to DNA and initiate cancer. The aziridine may be considered to be the more active but labile form of Compound A. Thus, we could propose that the adrenal P450c11 enzyme bio-activates Compound A by promoting

cyclization to the corresponding azridine. Mammalian P450's may be classified into two groups, those metabolizing endogenous substrates (CYP 7-24) and those metabolizing exogenous compounds like, xenobiotics, drugs and carcinogens (CYP 1-4). Metabolism of drugs occurs primarily in the liver [24]. In adult mammals, the adrenal is not involved in detoxification although it has been suggested that the fetal adrenal has detoxification capabilities [55]. Future elucidation of the interaction of Compound A with the P450 enzyme is required. Firstly to establish the kinetic parameters of the enzymatic interaction, and secondly to establish whether bio-activation of Compound A to the corresponding aziridine, occurs only in the adrenal or whether other P450's, especially those in the liver, are involved.

CHAPTER 7

EXPERIMENTAL

7.1 Isolation of active compounds from S. tuberculatiformis

7.1.1 Preparation of ethanol extract

Active compounds in *S.tuberculatiformis* were extracted according to the method of Swart [8]. A schematic representation of the entire isolation process of active compounds from *S.tuberculatiformis* can be seen in Fig. 2.3. Dried, ground plant material (500 g) was placed in a standard glass heat extractor fitted with a double wall condenser. The extractor was connected to a round bottomed flask containing chloroform (5 L). All glassware were covered with aluminium foil to shield the plant material and the extract in the flask from light. A preliminary extraction was made with chloroform for 20 hrs. The chloroform extract was removed and replaced by ethanol (5 L). After 20 hrs, extraction with ethanol was terminated, the extract removed and dried under reduced pressure on a rotary evaporator at room temperature. The resulting residue was taken up in de-ionized water (400 ml), filtered and the pH measured. The activity of this fraction was tested with spectral assays.

A fraction containing compounds with a molecular weight lower than 500 was prepared by ultrafiltration of the redissolved ethanol extract through a series of ultrafiltration membranes (Millipore) with molecular mass cut offs of 25 000, 10 000, 1000 and 500 respectively. Ultrafiltration was carried out at 4°C in a standard ultrafiltration cell (50 mm x 40 mm) pressurised with nitrogen. The extract, as well as the filtrate, were shielded from light during the entire filtration process. The yield of extract after filtration was 360 ml. The ethanol extract with a molecular mass of less than 500 was freeze dried, kept at -20°C and used for subsequent assays (this will be referred to as the ethanol extract in future assays). The yield of the ethanol extract was 39,06 g/500 g shrub.

7.1.2 Isolation of S2 with a liquid ion exchange system and HPLC

The ethanol extract, containing compounds with a molecular mass of less than 500 (100 ml), was shaken up in a separating funnel containing a solution (100 ml) of sodium tetraphenylborate (kalignost) dissolved in benzyl alcohol (10 mg/5 ml) for one minute. The aqueous and organic phases were allowed to separate and the inactive aqueous phase discarded. The organic phase was washed three times with HCl (250 ml, 1M). The HCl fractions (750 ml) were pooled and then washed with an equal volume of dichloromethane (750 ml) and freeze dried. The sample was then redissolved in de-ionized water (2 ml), filtered through a 0.45 μM membrane filter (Millipore) and chromatographed on a C^{18} reversed phase stainless steel column (polygosil® 60-7C^{18}, Machery-Nagel, 7.5 μM particle size, 60 Å pore size, 8 mm × 250 mm). The injection volume was 150 μl and the elution buffers consisted of the following: Solvent A: (0.1 M trichloroacetic acid/trichloroacetate buffer, pH 3), solvent B (methanol containing 1% glacial acetic acid). A linear gradient was run from 100% A to 50% A and 50% B in 30 min at a flow rate of 2.0 ml/min. The column effluent was monitored with an ultra violet detector set at 280 nm. Fractions were collected and the pH of the collected fractions were adjusted to 4 with aqueous NaOH (0.1 M). S2 eluted at 18.5 minutes and the volume of this fraction was 1.6 ml. The S2 fractions of 10 runs (16 ml) were pooled for the next step. The liquid ion exchanger kalignost, was again employed for the removal of sodium trichloroacetate from S2 samples, following the same procedure as described previously and then subjected to the ES-MS for further analysis. The volume of the S2 fraction after kalignost treatment was 66 ml. This was freeze-dried and yielded 3.72 g S2/ 1.5 ml ethanol extract.

7.1.3 ES-MS analysis of S2

The S2 fraction was analysed using ES-MS. The sample was redissolved in de-ionized water and diluted 50:50 with acetonitrile and assayed immediately. Formic acid was not added as the sample is already very acidic. The cone voltage was set at 30 V (25 V was tested but did not produce adequate molecular ions), the carrier solvent was 50:50 acetonitrile:water containing 1% formic acid delivered at a flowrate of 10 μl/minute and the source temperature was kept at 75°C.

Fragments of selected ions were produced by collisionally induced fragmentation. During this process the parent ion was ionized in the electrospray source using identical conditions to that described above, except that the flow rate of the solvent was decreased to 5 μl/minute. The first analyzer of the instrument was set at the *m/z* value of the parent ion which was passed into the fragmentation cell with the exclusion of all other ions. In the fragmentation cell argon was introduced at a pressure of 3×10^{-3} mbar to effect collisions between the parent ion and the argon molecules. Fragmentations was augmented by increasing the collision energy stepwise from 10 to 20 to 40 eV during the period in which the parent ion was present in the fragmentation cell. The resultant product ions were observed by scanning the second analyzer through the desired *m/z* range. The data produced in this way was acquired in the MCA mode, during which process all sequential scans were added to produce one representative scan at the conclusion of the analyses.

7.2 Spectral assays

7.2.1 Preparation of cytochrome P450 enriched mitochondrial powder from sheep adrenals

Freeze dried mitochondrial powder was prepared by the method of Cheng and Harding [26]. The entire preparation was done in a cold room at 4°C. Decapsulated adrenals from freshly slaughtered sheep were homogenized with a Potter Elvejhem homogenizer in a sucrose solution (0.25 M, pH 7.4) containing EDTA (0.1 M), and the resulting homogenate centrifuged at 1000 g for 20 min. The supernatant was decanted and centrifuged at 12 000 g for 20 min to yield the mitocondrial pellet. The pellet was subsequently washed three times by resuspension and centrifugation at 12 000 g for 15 min, once in a sucrose-EDTA-solution containing 1% BSA, and twice in the sucrose-EDTA-solution. The resulting pellet was freeze dried and the powder used as a source of cytochrome P450 in subsequent assays. The mitochondrial powder was stable for months when kept at -20°C.

7.2.2 Determination of cytochrome P450 content

The cytochrome P450 content of the mitochondrial powder was determined by the carbon monoxide (CO) method of Omura *et al.* [27]. Sheep adrenal mitochondrial powder (1 mg/ml) was dissolved in sodium phosphate buffer (0.1 M, pH 7.4, containing 10% (m/v) ethylene

glycol) by sonicating the solution six times for one minute at 4°C, with one-minute cooling intervals. The sonicate was saturated with CO and a baseline reading was taken between 500 and 360 nm. A few granules of sodium dithionite were added to the sample and the difference spectrum was monitored between 490 and 450 nm. The P450 concentration was then calculated, using a millimolar extinction coefficient of 91 for the absorption difference between 450 and 490 nm. The final P450 content is expressed as µM P450/mg protein (protein content determined by Bradford).

Example of cytochrome P450 concentration determination:

$$\Delta Abs_{290-450} \, nm = 0.0712$$

$$E_{mm} = 91 \; (1 \; mM \; of \; P450 \; gives \; an \; \Delta Abs_{290-450} \; of \; 91)$$

$$0.0712/91 \; X \; 1 \; mM \quad = 7.824 \times 10^{-4} \; mM$$
$$= 0.782 \; \mu M$$
$$= 0.000782 \; \mu mol \; P450 \; in \; 1 \; ml$$

900 ml used in spectral assays:
$$0.000782 \; X \; 900/1000$$
$$= 7.038 \times 10^{-4} \; \mu mol$$

Finale volume in assay is 1 ml:
$$7.038 \times 10^{-4} \; \mu mol \; in \; 1 \; ml$$
$$= 0.7038 \; \mu M \; P450$$

7.2.3 The Bradford method for the determination of protein concentration

In accordance with the Bradford method, Coomassie Brilliant Blue G-250 (100 mg) was dissolved in 95% ethanol (50 ml) [56]. To this solution was added 85% (w/v) phosphoric acid (100 ml). The solution was diluted to a final volume of 1 L with distilled water. The colour reagent was filtered and stored in a dark, airtight container. For the protein assay a standard series (0 mg/ml to 1.0 mg/ml) was made up from a bovine serum albumin (BSA) stock solution (1 mg BSA/ml sodium phosphate buffer (0.1 M, pH 7.4, containing 10% (m/v)

ethylene glycol)). Colour reagent (2.5 ml) was added to 50 ul BSA or mitochondrial pellet solution (1 mg/ml) and vortexed. The coloured solutions (200 ul) were pipetted into microtitre plates and the absorption was read at 620 nm (after at least 2.5 min). A standard curve was generated from the absorption readings of the standards and the unknown protein concentrations calculated from the standard curve.

7.2.4 Spectral assays with sheep adrenal cytochrome P450c11

Inhibition of the DOC-induced difference spectra and induction of difference spectra by test compounds were performed by using the method of Swart *et al.* [8]. All spectra were recorded on a Beckman DU650 spectrophotometer equipped with a six-cell attachment.

7.2.4.1 Inhibition of the DOC-induced difference spectra

Freeze dried mitochondrial powder (1 mg/ml) was sonicated at 4°C in pre-cooled phosphate buffer (0.1 M, pH 7.4, ethylene glycol (10% m/v)). After sonication the preparation was brought to room temperature by incubation at 25°C for 5 min. Equal volumes (900 µl) of the sonicate were pipetted into seven cuvettes. DOC (5 µM), dissolved in ethanol (2 µl), was added to the six sample cuvettes and an equal volume of ethanol (2 µl) to the reference cuvette. The volume was made up to 1000 µl with PBS. The reference cuvette was used as blank. The contents of all samples cuvettes were mixed for 1 min and a spectrum recorded between 500 and 360 nm for 1 hour at 5 minute intervals.

To test the influence of the test compounds on the DOC-induced difference spectra, DOC (5 µM), dissolved in ethanol (2 µl), was added to the six sample cuvettes and an equal volume of ethanol (2 µl) to the reference cuvette. All cuvettes contained 900 µl enzyme preparation (1 mg/ml). The test samples, Compound A (200 µM), the ethanol extract (2 µl), or S2 (10 µl), were prepared in PBS, native or heated sheep serum, and immediately added. In Table 7.1 the amounts of all components which were added are summarized. Compound A (0.022 g) was dissolved in 1000 µl PBS or sheep serum (native or heated) and sonicated. Of this solution 200 µl was diluted 5x with PBS or sheep serum. This solution was further diluted 83x by taking 12 µl of Compound A prepared in PBS or sheep serum and adding it to 86 µl PBS, 2 µl ethanol and 900 µl of the enzyme in the assay. The ethanol extract (0.1085 g) was dissolved in 1000 µl PBS or sheep serum and sonicated. Of this solution 2 µl was diluted

500x by adding to 96 µl PBS, 2 µl ethanol and 900 µl of the enzyme. S2 (0.095 g) was dissolved in 150 µl PBS or sheep serum and sonicated. Of this solution 10 µl was diluted 100x by adding it to 86 µl PBS, 2 µl ethanol and 900 µl of the enzyme. All samples were mixed and spectra obtained between 500 and 560 nm at 5 minute intervals for 1 hour. For the time studies with the test samples, the absorption difference between 390 and 420 nm, at every 5 minute interval, was devided by the cytochrome P450 concentration, and plotted against time.

Table 7.1 Volumes of all components in inhibition of DOC-induced difference spectral assay. (A) DOC (B) DOC & Compound A (C) DOC & ethanol extract (D) DOC & S2. (* in PBS/SS/HSS)

	A: Ref	A: Sample	B: Ref	B: Sample	C: Ref	C: Sample	D: Ref	D: Sample
Enzyme	900 µl	900 µl	900 µl	900 µl	900 µl	900 µl	900 µl	900 µl
PBS	86 µl	86 µl	86 µl	86 µl	96 µl	96 µl	88 µl	88 µl
EtOH	2 µl	-	2 µl	-	2 µl	-	2 µl	-
DOC	-	2 µl	-	2 µl	-	2 µl	-	2 µl
PBS/SS/ HSS	2 µl	2 µl	2 µl	-	2 µl	-	10 µl	-
* Test compound	-	-	-	2 µl	-	2 µl	-	10 µl

7.2.4.2 Substrate induced difference spectra

Substrate induced difference spectra of cytochrome P450c11 were obtained in the same manner as the DOC-induced difference spectra, except for the DOC solution which was replaced by the test samples. Compound A (200 µM), the ethanol extract (2 µl) and S2 (40 µl) were prepared in PBS, native or heated sheep serum and added to the sample cuvettes. In Table 7.2 the amounts of all components which were added can be seen.

Table 7.2 Volumes of all components in substrate induced difference spectral assay. (A) Compound A (B) ethanol extract (C) S2. (* in PBS/SS/HSS)

	A: Ref	A: Sample	B: Ref	B: Sample	C: Ref	C: Sample
Enzyme	900 µl	900 µl	900 µl	900 µl	900 µl	900 µl
PBS	86 µl	86 µl	96 µl	96 µl	58 µl	58 µl
EtOH	2 µl	2 µl	2 µl	2 µl	2 µl	2 µl
PBS/SS/HSS	12 µl	-	2 µl	-	40 µl	-
* Test compound	-	12 µl	-	2 µl	-	40 µl

7.3 Determination of the percentage free steroid in undiluted sheep serum by ultrafiltration-dialysis or isodialysis

The percentage free steroid in undiluted sheep serum was determined according to the ultrafiltration-dialysis method of Hammond *et al.* [41]. Sheep serum contains free, albumin bound and CBG bound steroid. Heated sheep serum on the other hand, contains only free and albumin bound steroid because heating at 60°C for 1 hour destroys CBG and SHBG but not albumin [43]. Radioactive compounds, [1,2,6,7-^{3}H] cortisol (60 Ci/mmol), [1,2,6,7-^{3}H] progesterone (60 Ci/mmol) and [^{14}C] glucose (55 Ci/mmol), were obtained from Amersham Life Science. The solvent of the ^{3}H-steroids, were evaporated and the steroids reconstituted in 1 ml ethanol to give 250 µCi/ml ethanol and frozen. Working solutions were made up in ethanol immediately before use. The [^{14}C] glucose (200 µCi/ml), was stored frozen as supplied, and working solutions were made up in distilled water immediately before use.

Native and heated serum samples were prepared and stored at 4°C until further use. Heated sheep serum was prepared by heating for one hour at 60°C and then sentrifuging for 15 minutes at 3000 g. Dialysis membranes were also prepared by washing with distilled water and stored overnight at 4°C. Aliquots of 3×10^5 dpm of ^{3}H-steroid (cortisol/progesterone) in ethanol (10 ul) were added to disposable glass tubes as indicated in Table 7.3 and evaporated overnight in a fume cabinet.

On the next day 12×10^3 dpm [^{14}C] glucose/5 ul distilled water was added to the indicated glass tubes (see Table 7.3). The test samples were made up as shown in Table 7.4, in both native and heated serum. The stock solutions consisted of 0.020, 0.1085 and 0.59 g per 1000 µl for Compound A, ethanol extract and S2, respectively. Serum samples (450 µl) were pipetted into the specified tubes (Table 7.3), briefly mixed on a Vortex mixer, and incubated under an atmosphere of 95% O_2: 5% CO_2 at 37°C for 60 minutes. The pH of the serum samples after addition of Compound A or the ethanol extract was 6.5 and after addition of S2 was 6.

Table 7.3 Preparation of standards and samples for ultrafiltration-dialysis assay. NS=native serum, HS=heated serum and BKG=background.

GLASS TUBES	^{3}H-steroid	^{14}C-glucose	NATIVE SERUM	HEATED SERUM
1. BKG	-	-	450 µl	-
2. ^{3}H-STANDARD	10 µl	-	450 µl	-
3. ^{14}C-STANDARD	-	5 µl	450 µl	450 µl
4. NS	10 µl	5 µl	450 µl	-
5. HS	10 µl	5 µl	-	450 µl
6. TEST COMPOUNDS (NS)	10 µl	5 µl	450 µl	-
7. TEST COMPOUNDS (HS)	10 µl	5 µl	-	450 µl

Ultrafiltration vials comprised of a glass tube (internal diameter: 10 mm), one end over which a single piece of wet dialysis membrane was evenly stretched and held in place by a small elastic band, was used. The glass tubes with the dialysis membrane attached (inner tubes) were blotted dry before being inserted into glass scintillation vials (6 ml), which contained three filter paper discs (diameter: 13 mm; Whatman No. 1). The position of the inner tube inside the scintillation vial was such that the latter could be closed with a plastic cap. In Fig. 7.1 a schematic representation of the ultrafiltration vial can be seen.

Table 7.4 Preparation, in native or heated serum, of the test compounds, (A) Compound A, (B) ethanol extract and (C) S2, for ultrafiltration-dialysis assay.

	(A)		(B)		(C)		
μM	STOCK (μl)	SERUM (μl)	STOCK (μl)	SERUM (μl)	μg/450 μl	STOCK (μl)	SERUM (μl)
16,670	100	350	50	400	0.001	2.1	447.9
8,335	50	400	100	350	0.002	3.5	446.5
4,168	25	425	150	300	0.004	7	443
1,667	10	440	211	239	0.012	21	429
834	5	445			0.017	28	422
417	2.5	447.5			0.03	50	400
83	0.5	449.5			0.059	100	350

Duplicate aliquots (200 μl) of the serum incubations were then pipetted on to the dialysis membrane at the bottom of the inner tubes (Fig 7.1). The 6 ml scintillation vials, containing the inner tubes, were then capped and put back in the incubator overnight.

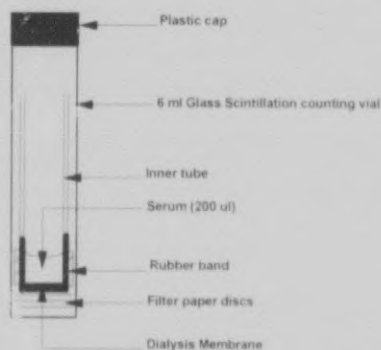Figure 7.1 Diagram of an ultrafiltration vial. Aliquots (200 ml) of serum incubations are pipetted into the inner tubes. The vials are capped and kept overnight at 37°C. The free ^{3}H-steroid and ^{14}C-glucose pass through the dialysis membrane together with the ultrafiltrate, which is absorbed by three filter paper discs beneath the dialysis membrane. The bound ^{3}H-steroid is retained by the dialysis membrane.

Early the next morning, the inner tubes were carefully removed from the scintillation vials. The vials contain the free ^{3}H-steroid and ^{14}C-glucose which passes through the dialysis membrane together with the ultrafiltrate and is absorbed by the filter paper disc and are referred to as the bottom vials. From the inner tubes, 30 ul of the serum remaining inside was pipetted on to a filter paper disc within another 6 ml scintillation vial, referred to as the top vials. These top vials contain the bound ^{3}H-steroid which is retained by the dialysis membrane. Distilled water (350 µl) was then dispensed into all scintillation vials, which were mixed on a Vortex mixer (5 s). Scintillation liquid (3 ml) was added to the vials, which were capped, mixed on a Vortex mixer, and counted on a Beckman LS 5000 Td liquid scintillation spectrophotometer adjusted for the simultaneous measurement of both ^{3}H and ^{14}C. The amount of ^{3}H and ^{14}C in each channel can be determined according to the method of Friefelder, a example of which is shown in Table 7.5 [57]. The distribution of counts from the ^{3}H and ^{14}C standard samples in channel 1 and channel 2 indicates the distribution of ^{3}H and

Table 7.5 Calculation of ^{3}H/^{14}C ratio in a double labelled sample, counted in two channels of a scintillation counter. Radioactivity recorded in cpm [Redrawn from 57].

SAMPLE	CHANNEL 1	CHANNEL 2	2/1	1/2
EXPERIMENTAL	158.55	128.5		
^{3}H-STANDARD	5,524.95	110.4	0.019	
^{14}C-STANDARD	236.05	920.3		0.26

Calculations for samples:

1. ^{3}H in channel 2 = 0.019 x 158.55 = 3.01

2. ^{14}C in channel 2 = 128.5 - 3.01 = 125.49

3. ^{14}C in channel 1 = 0.26 x 128.5 = 33.41

4. ^{3}H in channel 1 = 158.55 - 33.41 = 125.14

5. Total ^{3}H = 128.15

6. Total ^{14}C = 158.9

7. ^{3}H/^{14}C = 0.81

^{14}C radioactivity in the experimental sample assuming that there is no quenching of the sample with respect to the standard.

The percentage free cortisol and progesterone can be calculated by comparing the ratio of ^{3}H-steroid to ^{14}C- glucose in the ultrafiltrate with the corresponding ratio in the serum retained by the dialysis membrane. The following is an example of such a calculation:

	^{3}H/^{14}C ultrafiltrate	^{3}H/^{14}C serum	% Free
NATIVE SERUM	0.98	7.2	13.55
HEATED SERUM	3.13	7.76	40.35

$$\% \text{ Free} = \frac{^3\text{H}/^{14}\text{C ultrafiltrate}}{^3\text{H}/^{14}\text{C serum}} \times 100$$

Heated serum (H): 100% = % Free + % Alb bound

%Alb bound steroid in heated serum: 100% -% F(H)

$$= 100 - 40.35$$
$$= 59.65\%$$

$$\frac{\% \text{ Alb (H)}}{\% \text{ Free (H)}} = \frac{\% \text{ Alb (N)}}{\% \text{ Free (N)}}$$

$$\% \text{ Alb (N)} = \frac{\% \text{ Alb (H)}}{\% \text{ Free (H)}} \times \% \text{ Free (N)}$$

$$= 59.65/4.35 \times 13.55$$
$$= 20.03\%$$

Native serum (N): 100% = % Free + % Alb bound + CBG bound

$$100\% = 13.55\% + 20.03 \% + \% \text{ CBG bound}$$

$$\% \text{ CBG bound} = 66.42\%$$

7.4 ES-MS studies

All ES-MS studies were carried out on a VG-Biotech mass spectrometer.

7.4.1 Cone voltage

Compound A with a concentration of 200 µM, was prepared in PBS (0.1 M, pH 7.4, containing 10% (m/v) ethylene glycol). It was diluted 100 times with acetonitrile containing 1% formic acid. The injection volume was 10 µl and the mobile phase at a flow rate of 10 µl/min was 50:50 water:acetonitrile containing 1% formic acid. The rate of cyclization of Compound A to the corresponding aziridine was monitored at 5 and 10 min by positive mode ES-MS analysis at different cone voltages. Cone voltages of 10, 15, 20, 25, 30 and 40 were tested and the source temperature was kept at 75°C.

7.4.2 Temperature and pH studies

Compound A with a concentration of 16.700 mM, was prepared in buffer or native sheep serum at different pH's. It was diluted 100 times with 50:50 water:acetonitrile containing 1% formic acid, and assayed immediately. The injection volume was 10 µl and the mobile phase at a flow rate of 10 µl/min was 50:50 water:acetonitrile containing 1% formic acid. The % Compound A remaining (m/z 228/230) was monitored at 5 min intervals for 30 min by positive mode ES-MS analysis. The cone voltage was set at 20 V, and the source temperature was kept at 75°C. Compound A was made up in a range of citrate-phosphate buffers (0.05 M) with values ranging from pH 2.6 to 7.9 and the sheep serum was dialysed overnight in these buffers to adjust the pH accordingly. Cyclization was monitored at 4, 22 and 37°C and also at pH 2.6, 4, 5, 6, 7, 7.4 and 7.9

7.4.3 The influence of P450c11 enzyme on cyclization

Two experiments were performed to test the effect of the P450c11 enzyme on the cyclization of Compound A to the corresponding aziridine.

For the first experiment, Compound A, at a concentration of 5.102 mM, was prepared in PBS (0.1 M, pH 7.4, containing 10% (m/v) ethylene glycol) and in native sheep serum. Compound A (0.067 g) was dissolved in 1000 µl PBS or sheep serum and sonicated. Of this solution 200 µl was diluted 5x with PBS or sheep serum. The mixture of Compound A and enzyme

mimicked the mixture used in the spectrophotometric assays with 98 μl Compound A (in PBS or sheep serum) and 2 μl ethanol added to 900 μl of enzyme preparation (1 mg/ml). Freeze dried mitochondrial powder containing the cytochrome P450c11 enzymes was prepared by the method of Cheng and Harding as described in 8.2.1 [26]. Compound A was incubated with or without the enzyme for one hour and samples were taken every 10 minutes for ES-MS analysis. Before injecting in the ES-MS, the Compound A and cytochrome P450c11 enzyme mixture was diluted 10 times with a 50:50 water:acetonitrile mixture containing 1% formic acid, and assayed immediately. The injection volume was 100 μl and the mobile phase at a flow rate of 10 μl/min was a 50:50 water:acetonitrile mixture containing 1% formic acid. The rate of aziridine ($m/z=192$) formation and Compound A remaining (m/z 228/230) was monitored at 10 min intervals for 60 min by positive mode ES-MS analysis. The cone voltage was set at 20 V, and the source temperature was kept at 75°C.

For the second experiment conditions were kept the same as for the previous experiment, with the exception of the concentration of Compound A which was decreased to 2.457 mM. The first part of the second experiment investigated the effect of the presence of the enzyme on the cyclization of Compound A in PBS, with the lower concentration of Compound A. Compound A was either added to the enzyme and then assayed over a period of time, or Compound A was prepared and only added to the enzyme just before ES-MS analysis. During the second part of the experiment, increasing concentrations of DOC (0-500 μM prepared in 2 μl ethanol) was added to the Compound A and enzyme mixture and the effect on the cyclization of Compound A monitored. The final part of the experiment examined the effect of the enzyme concentration on the cyclization of Compound A. The same conditions as above was used except for the enzyme concentration which was varied from 0-4 mg/ml.

REFERENCES

1. De Lange, M. (1961) *Proc 4th Internat Congress Anim Reprod*, The Hague, **3**, 590-592.

2. Basson, P.A., Morgenthal, J.C., Bilbrough, R.B., Marais, J.L., Kruger, S.P. & van der Merwe, J.L.deB. (1969) *Onderstepoort J Vet Res*, **36**, 59-103.

3. Morgenthal, J.C. (1988) A study on the effect of *Salsola tuberculatiformis* Botsch. on certain physiological aspects of the ewe, her fetus and neonate. *Ph.D thesis*, Department of Human an Animal Physiology, University of Stellenbosch.

4. Ploss, H. (1902) *Das Weib ind. Natur-u. Völkerkunde* **1**, 670, 1902 as cited by Brondegaard V.J. (1973) *Planta Med*, **23**, 167-172.

5. Maritz, K. (1969) *Dagbreek en Landstem*, **33**, 1.

6. Van der Merwe, K.J., Hofmeyr, J-H.S., Swart, P., Parkin, D.P., Rossouw, J., Hartshorne, J., Van Rensburg, S.J., Morgenthal, J.C. & Basson, P.A. (1976) *SA J Sci*, **72** (6), 184.

7. Williamson, D.G. & O'Donnel, V.J. (1969) *Biochemistry*, **8**(4), 1306-1311.

8. Swart, P. (1986) The isolation of a novel inhibitor of adrenal cytochrome P-450 from *Salsola tuberculata Ph.D thesis*, Department of Biochemistry, University of Stellenbosch.

9. Swart, A.C. (1986) A biochemical study of two natural products from *Salsola tuberculata*. *M.Sc thesis*, Department of Biochemistry, University of Stellenbosch.

10. Van der Merwe, K.J., De Kock, S.S., Swart, P. & Fourie, L. (1991) *Biochem Soc Trans*, **19**, 432s.

11. De Kock. S.S. (1995) Die sintese en gebruik van chemiese merkers vir biogene
     amienderivate wat oor aziridiene verval *M.Sc thesis*, Department of
     Biochemistry, University of Stellenbosch.

12. De Kock. S.S. (1995) A study of phenolic aziridines and their precursors. *Ph.D thesis*,
     Department of Biochemistry, University of Stellenbosch.

13. Jackson. K.E. (1934) *Chem Rev*, **15**, 425-461.

14. Louw, A., Swart, P., de Kock, S.S. & van der Merwe, K.J. (1997) *Biochemical
     Pharmacology*, **53**, 1-8.

15. Louw, A. & Swart, P. (1998) Contraceptive effect of *Salsola tuberculatiformis*
     Botsch. and a chemical analogue mediated by displacement of corticoserone from
     Corticosteroid binding globulin (CBG) in female Wistar rats (submitted for
     publication).

16. Van der Merwe, K.J., De Kock, S.S., Swart, P. & Fourie, L. (1992) *Biol Mass
     Spectrom*, **21**, 672-674.

17. Fourie, L., van der Merwe, K.J., Swart, P. & De Kock, S.S. (1993) *Anal Chim Acta*,
     279, 163-166.

18. Schultz. H.W. (1993) A spectroscopic investigation of aziridines and natural products
     occurring in *Salsola tuberculatiformis* Botsch *M.Sc thesis*, Department of
     Biochemistry, University of Stellenbosch.

19. Maritz, M. (1993) A study of labile natural products occurring in *Salsola
     tuberculatiformis* Botsch. *M.Sc thesis*, Department of Biochemistry, University of
     Stellenbosch.

20. Klingenberg, M. (1958) *Arch Biochem Biophys*, **75**, 376-386.

21.   Garfinkel, D. (1958) *ibid*, **77**, 493-509.

22.   Omura, T. & Sato, R. (1962) *J Biol Chem*, **237**, 1375-1376.

23.   Takemori, S. & Kominami, S. (1984) *TIBS*, **9**, 393-396.

24.   Omura, T. & Sato, R. (ed) (1978) *Cytochrome P-450*, Kodansha Ltd. and
        Academic Press Inc., Tokyo.

25.   Yoshida, Y. & Kumaoka, H. (1975) *J Biochem*, **78**, 455-468.

26.   Cheng, S. & Harding, B.W. (1973) *J Biol Chem*, **248**, 7263-7271.

27.   Omura, T. & Sato, R. (1964) *J Biol Chem*, **239**, 2379-2385.

28.   Cornish-Bowden, A. (ed) (1995) *Analysis of Enzyme Kinetic Data*, Oxford University
        Press, Oxford, New York and Tokyo.

29.   Cornish-Bowden, A. (ed) (1995) *Fundamentals of Enzyme Kinetics*, Portland Press
        Ltd, Londen.

30.   Zeffren, E. & Hall, P.L. (ed) (1973) *The study of enzyme mechanisms*, John Wiley &
        Sons, Inc.

31.   Ortiz de Montellano, P.R. (ed) (1995) *Cytochrome P450*, second edition, Plenum
        Press, New York and Londen.

32.   Gayrard, V., Alvinerie, M. & Toutain, P.L. (1996) *Domestic animal endocrinol*,
        13 (1), 35-45.

33.   Rosner, W. (1990) *Endocrine Rev*, **11**, 80-91.

107

34.   Kehlet, H. & Binder, Chr. (1976) *Acta endocrinol.* **81**, 787-792.

35.   Pugeat, M.M., Dunn, J.F. & Nisula, B.C. (1981) *J ClinEndocrinol and Metab.* **53**, 69-75.

36.   Dunn, J.F., Nisula, B.C. & Rodbard, D. (1981) *J Clin Endocrinol Metab.* **53**, 58-67.

37.   Hammond, G.L. (1995) *TEM*, **6**, 298-304.

38.   Westphal U. (ed) (1986) *Steroid -Protein Interactions*, vol II., Springer-Verlag, Berlin.

39.   Schlechte, J.A. & Hamilton, D. (1987) *Clin Endocrinol*, **27**, 197-203.

40.   Bright, G.M. (1995) *J o´ Clin _ndocrinol Metab*, **80**, 770-775.

41.   Hammond, G.L., Nisker, J.A., Jones, L.A. & Siiteri, P.K. (1980) *J Biol Chem*, **255**, 5023-5026.

42.   Siiteri, P.K., Murai, J.T., Hammond, G.L., Nisker, J.A., Raymoure & Kuhn, R.W. (1982) *Recent progress in hormone research*, **38**, 457-478.

43.   Hammond, G.L., Lähteenmäki, P.L.A., Lähteenmäki, P. & Luukkainen, T. (1982) *J Steroid Biochem*, **17**, 375-380.

44.   Rowett, H.G.Q. (1960) *The Rat*, third edition, John Murray Publishers, Ltd., Londen

45.   Smith, C.L & Hammond, G.L. (1991) *J Biol Chem*, **226** (28), 18555-18559.

46.   Tinnikov, A.A. (1993) *Horm metab Res*, **25**, 88-89.

'47.   Knobil, E. & Neil, J.D. (ed), (1994) *The physiology of reproduction* vol 2, second edition, Raven Press., New York.

48.   Mickelson, K.E., Forsthoefel, J. & Westphal, U. (1981) *Biochemistry*, **20**, 6211-6218.

49.   Slaunwhite, W.R. & Sandberg, A.A. (1959) *J clin Invest*, **38**, 384.

50.   Seal, U.S. & Doe, R.P. (1963) *Endocrinol*, **73**, 371.

51.   Westphal U. (ed) (1971) *Steroid -Protein Interactions*, vol I., Springer-Verlag, Berlin.

52.   Chan, D.W. & Slaunwhite, W.R. (1977) *Arch Biochem Biophys*, **182**, 437-442.

53.   Siuzdak, G. (ed) (1996) *Mass Spectrometry*, Academic Press, San Diego, New York, Boston, Londen, Sydney, Tokyo and Toronto.

54.   Mann, M. & Wilm, M. (1995) *Trends Biol Sci* **20**, 219-224.

55.   Juchau, M.R., Chao, S.T. & Omiecinski, C.J. (1980) *Clin Pharmacokinet*, **5** (4) 320-339

56.   Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248-254.

57.   Freifelder, D.M. (1982) *Physical Biochemistry- Applications to Biochemistry & Molecular Biology*, WH Freeman & Co, San Francisco