SYNTHESIS AND PHARMACOLOGICAL ACTIVITY OF PREGNANE GLYCOSIDES

BY

TALAL HASAN ZEGLAM, B.Pharm., M.Sc.

A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfilment of the Requirements
for the Degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Naturally occurring cardiac glycosides have been used for many years as therapeutic agents for the treatment of the failing heart and are among the most widely prescribed drugs. They exert a powerful positive inotropic effect on the heart muscle. Nevertheless, cardiac glycosides show a narrow therapeutic index as well as wide differences in patient response when used for the management of cardiovascular disease.

This thesis is part of a structure activity relationship (SAR) study with respect to cardiac muscle receptor binding, inotropic activity and kidney effects of cardiac glycosides. Chlormadinone acetate (CMA) was previously shown by others to displace ouabain from the heart muscle receptor by means of a radiolabeled binding assay (RBA). The work describes the chemical synthesis of **(i)** C-6 chloropregnane derivatives related to CMA and **(ii)** 3ß,14-dihydroxy-5ß,14ß-pregnane and 21-nor-5ß,14ß-pregnane 3-glycosides derived from digitoxin and ouabain.

Chloropregnane derivatives were synthesized from steroid hormone derivatives employing established methods. Conversion of the α,ß-unsaturated γ-lactone of the cardiac glycosides into the 14-hydroxy-5ß,14ß-pregnane structure, was our primary objective for the synthesis of these pregnane derivatives. This was achieved by the discovery of an efficient method for conversion of the α,ß-unsaturated γ-lactone into an acetyl group in a "one pot" procedure by treatment with ozone, followed by excess zinc and acetic acid. This conversion takes place through an intermediate 21-hydroxy ester and the 21-hydroxy derivative also formed which was shown not to be reduced. A mechanism for these transformations is proposed. This procedure considerably shortens

the previous published method for this conversion and unlike the earlier method it can be carried out on an unprotected glycoside *e.g.*, the procedure makes the trisdigitoxoside derivatives readily available.

Mono- and bis-digitoxoside derivatives were also prepared to compare the effect of the number of sugar units on receptor binding potency and for comparison of the cardiotonic properties with other monoglycoside derivatives. Potency was not changed by the addition of the second and third digitoxose unit. Synthesis of the 20-carbonyl and the 20ß-alcohol showed that the alcohol was more potent in the RBA. Investigation of the SAR for the C-20 position was carried out in part by the synthesis of nitrogen derivatives of the C-20 ketone (*e.g.*, the 20-oxime, 20ξ-amine, 20ξ-nitro, 20-guanylhydrazone and 20-hydrazone). The latter derivatives were synthesized because of the reported association of the C-20 guanylhydrazone with cardiotonic properties.

Among the C-20 derivatives tested, the 20ξ-amine and 20ξ-nitro compounds showed the highest receptor binding in the RBA. Therefore, it was desirable to separate these pairs of epimers. However, because of their highly polar nature such a separation was extremely difficult. Consequently because of the difficult separation of the C-20 epimers containing the trisdigitoxoside structure, and also to investigate the importance of the 20-methyl substituent, alternatively both the 20-dimethyl and the 21-nor-20-alcohol were synthesized. Both derivatives remove the chiral nature of the C-20 carbon. These two derivatives differ in steric requirements which restrict the rotation around the C-17/C-20 bond limiting the angle at which the alcohol projects from the molecule. The 20α- and 20ß-pregnane alcohols would be intermediate in their steric requirements. The RBA

showed the order of potency as $-CH_2OH$ > -CH(ßOH)Me > $-C(OH)Me_2$. Because the primary alcohol was the most potent we synthesized further 21-norpregnane derivatives. The 20-nitro-14-hydroxy-3ß-trisdigitoxosyloxy-21-nor-5ß,14ß-pregnane (IC_{50} 0.07 µM) proved to be highly potent in the RBA (digitoxin 0.008 µM). This nitro derivative is the most potent pregnane derivative which has been synthesized and clearly demonstrates that in contradiction to classical SAR considerations the α,ß-unsaturated γ-lactone is not required for strong receptor binding.

It is documented that among the monoglycosides the α-L-rhamnoside showed the highest potency, therefore, analogous ouabain derivatives would be expected to show even higher potency. Furthermore, because ouabagenin is more highly hydroxylated than digitoxigenin it is more water soluble, a property which would be of value in pharmacological experiments. Therefore, in the present work some 21-norpregnane analogues of ouabain were synthesized. Although the above SAR appears straight forward, the potency was an order of magnitude less than anticipated, showing the necessity for ongoing SAR studies.

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INTRODUCTION

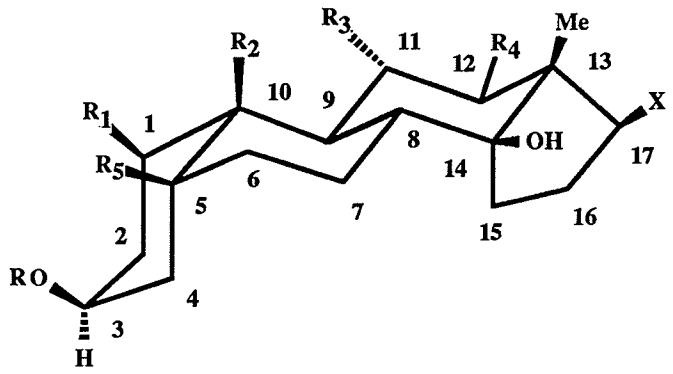
I. Nature of cardiac glycoside structure

Digitalis glycosides are naturally occurring steroid glycosides which have both beneficial and toxic effects on mammalian heart muscle (Thomas, 1981, 1987). They have been obtained from a variety of plant sources, especially the species known as *digitalis purpurea* and *digitalis lanata*, a source which has been known since 1841. Initially they were used as dried leaves or crude extracts. In 1930 one of the pure cardiotonic steroid glycosides, digoxin, was isolated and has been clinically used since in the treatment of congestive heart failure (CHF). The chemical structures of the plant glycosides mainly contain an α,ß-unsaturated γ-lactone (cardenolides), and the related toad poisons an α-pyrone ring (bufadienolides) at the C-17ß position of the steroid nucleus together with a glycoside moiety consisting of one or more units at the 3ß position (Figure 1). Frequently the steroid rings are hydroxylated. The C-17ß function can be classified into two groups:

(1) A five-membered α,ß-unsaturated lactone (butenolide or cardenolide) derived from 4-hydroxybutenoic acid.

(2) A six-membered di-unsaturated lactone (pentadienolide or bufadienolide) derived from 5-hydroxypentadienoic acid. This group, besides occurring in the toad poison, also occurs in a species known as *urginea maritima* and *urginea indica* from the Family *liliaceae*.

Mild acid hydrolysis of digitalis glycosides gives the genin or aglycone part which usually



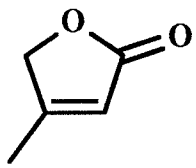
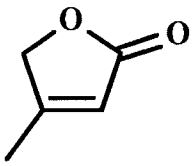
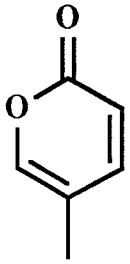
X	R	R_1	R_2	R_3	R_4	R_5	
	$(Digitoxose)_3$	H	CH_3	H	H	H	Digitoxin
	H	H	CH_3	H	H	H	Digitoxigenin
	Rhamnose	OH	CH_2OH	OH	H	OH	Ouabain
	H	OH	CH_2OH	OH	H	OH	Ouabagenin
	Glucose-rhamnose	H	CHO	H	H	OH	Hellebrin
	H	H	CHO	H	H	OH	Hellebrigenin

Figure 1. Structures of digitoxin, ouabain and hellebrin and their genin.

has *cis-trans-cis* ring junctions and ß-hydroxyl groups at the C-3 and C-14 positions. The sugar moiety is attached to the genin through the C-3 oxygen. The number of sugar units in the cardiac glycosides varies from one to four *e.g.*, digitoxin contains three ß-D-digitoxose units whereas ouabain contains one α-L-rhamnose unit (see Figure 1).

II. Nature of Na^+, K^+-ATPase.

Many research groups have described the excitability of the myocardial muscle on treatment with cardiac glycosides. However, this excitability is regulated by the enzyme sodium-potassium adenosine triphophatase (Na^+, K^+-ATPase) which is a plasma membrane protein which pumps sodium (Na^+) out of, and potassium (K^+) into the cell, the stoichiometry being (3 Na^+ : 2 K^+). The energy required for this process is derived from the hydrolysis of adenosine triphosphate (ATP). Thus, it maintains the Na^+ concentration outside the cell and provides energy for positive inotropy in excitable cells and couples the transport of ions and metabolites (Smith, 1989). The structure of Na^+, K^+-ATPase is thought to be composed of either two or three polypeptide subunits namely, α, ß and γ (Jorgensen, 1982a,b; Hansen, 1984; Anner, 1985; Shull *et al.*, 1986 and Ovchinnikov *et al.*, 1988). The ratio of α:ß has been determined to be 1:1 (Craig and Kyte, 1980; Peterson and Hokin, 1981) although one report favours a ratio of 2:3 (Freytag and Reynolds, 1981). The α subunit is a glycoprotein and its main function is for Na^+ pump activity but this mechanism is not completely understood. The γ subunit (Reeves *et al.*, 1980) is a proteolipid which has a smaller molecular weight compared to the α or ß subunit and it may not be essential for activity (Hardwicke and Freytag, 1981). Each active enzyme unit contains a high affinity ATP binding site and phospholipid both of

which may be required for full enzyme activity (Schwartz *et al.*, 1975 and de Pont *et al.*, 1978).

There have been extensive studies on the kinetics analysis of Na^+, K^+-ATPase. The steps involved in the reaction mechanism of Na^+, K^+-ATPase (Robinson and Flashner, 1979; Thomas, 1981; Fallon, 1987) can be summarized by the following equations:

$$E_1\text{-ATP} \overset{(Na^+)_i}{\rightleftarrows} (E_1P)\text{-}Na^+ + ADP$$

$$(E_1P)\text{-}Na^+ \rightleftarrows E_2P + (Na^+)_o$$

$$H_2O + (E_2P) \overset{(K^+)_o}{\rightleftarrows} E_2K^+ + P_i$$

$$E_2\text{-}K^+ + ATP \rightleftarrows E_1\text{-ATP} + (K^+)_i$$

E_1 and E_2 represent two different dephosphorylated forms of the enzyme. Na_i and K_i represent intracellular and Na^+_o and K^+_o represent extracellular ions, respectively, and P_i represents inorganic phosphate. The process begins with ATP bound to E_1. Na^+ add to high-affinity sites from the cytoplasm which results in reversible phosphorylation of the enzyme and release of ADP, leaving Na^+ bound to E_1P. The transport step causes an isomerization of E_1-P to E_2-P and the transformation of the Na^+ binding sites to low-affinity ones and Na^+ is released into the extracelluar fluid. K^+ will then be bound at high-affinity sites and dephosphorylation by H_2O followed by isomerization of E_2 to E_1 triggers the release of K^+ and rephosphorylation of the enzyme.

Recent evidence from several research groups has shown that cardiac glycosides interact with Na^+, K^+-ATPase when the conformation of E_1 has been changed to E_2. E_2 has a high binding affinity site for digitalis which acts as the receptor (Schoner *et al.*,

1977). Other studies by Yoda and Yoda (1987) showed that there are at least three phosphorylated forms of the enzyme according to the reaction sequence:

$$E_1P \rightarrow E^*P \rightarrow E_2P$$

suggesting that each step has the capability to undergo dephosphorylation and that the digitalis binding site is located on the E^*P cycle and not on the E_2P cycle (Hansen, 1984). It has also been shown that the interaction of digitalis with its binding site on Na^+, K^+-ATPase is irreversible at 0 °C and reversible at 37 °C (Tobin and Sen, 1970). For instance, under the reversible binding conditions the activity of a cardiac glycoside is dependent on the time required for the formation of E-P binding. Na^+ promotes the formation of E^*P while K^+ lessens it. This is consistent with the clinical experience that K^+ loss, through the action of K^+-depleting diuretics for example, enhances cardiac glycoside toxicity, whereas infusing K^+ leads to its breakdown. Studies by Akera *et al.* (1985) have shown that the effect of K^+ concentration on the inhibition of Na^+, K^+-ATPase is dependent on the particular glycoside structure. They found that the influence of K^+ concentration varied with the glycoside, species and tissue. The development of photoaffinity labelling techniques has led to their use in locating the digitalis receptor postulated as present in one of the three peptide units (*i.e.*, α, ß or γ). So far none of these units showed exclusively the main locus of the receptor site. The attachment of a photoaffinity label to the steroid nucleus or sugar part of a monoglycoside labels only the α unit (Rossi *et al.*, 1982; Goeldner *et al.*, 1983), but if the label is attached to the terminal sugar in a triose glycoside it labels the ß unit (Hall and Ruoho, 1980).

ACTIONS OF CARDIAC GLYCOSIDES

I. Direct inhibition of Na^+, K^+-ATPase.

Studies of the effect of cardiac glycosides on intracellular potentials and ion fluxes have led to the development of many theories to explain the ability of the digitalis compounds to increase the force of myocardial contraction. These theories indicate that all cardiac glycosides have a direct effect on the inhibition of the enzyme (Na^+, K^+-ATPase). This enzyme is located in the plasma membrane and provides the energy for active transport of Na^+ and K^+ across the myocardial cell. It is suggested that cardiac glycosides bind to its external face (Skou, 1957, 1960; Repke, 1963a). The earlier study by Schatzmann (1953) reported that cardiac glycosides inhibited the active transport of Na^+ and K^+ in erythrocytes. Two years later, Wilbrandt (1955) suggested a link between these effects and an increase in the intracellular concentration of calcium as a possible basis for the inotropic effect of digitalis. In 1957 Skou described a membrane-bound Na^+, K^+-activated ATPase which was specifically inhibited by cardiac glycosides. He suggested that this enzyme was the molecular basis for the active transport of Na^+ and K^+ across cell membranes. Repke (1963a) developed the proposal that the inotropic and toxic effects of cardiac glycosides were mediated via inhibition of Na^+, K^+-ATPase and that this was linked with increased intracellular Ca^{2+} concentration. Other investigators *e.g.*, Charnock and Post (1963) reported that digitalis did not affect the phosphorylation of the enzyme by adenosine triphosphate (ATP) but blocked dephosphorylation. However on the basis of studies using radioactively labelled glycoside, Matsui and Schwartz (1968) suggested that digitalis binds to a phosphorylated form of Na^+, K^+-ATPase. They also

found that binding to the phosphorylated conformer required Mg^{2+} and ATP or another nucleotide, and was stimulated by Na^+ and depressed by K^+. They subsequently found that binding would take place in the presence of only inorganic phosphate (P_i) and Mg^{2+}, but under this condition, Na^+ as well as K^+ inhibited binding (Schwartz *et al.*, 1968). Later, these two sets of conditions (*i.e.*, Na^+, Mg^{2+}, ATP and Mg^{2+}, P_i) were called type I and type II binding conditions, respectively. Originally it was thought that the digitalis-enzyme complexes formed under type I and type II conditions were identical, but this was shown later to be in error (Akera and Brody, 1971). More recently, as pointed out in a number of reviews (Schwartz *et al.*, 1975; Akera and Brody, 1978; Smith *et al.*, 1984a,b,c; Thomas *et al.*, 1990) the rate of increase in the calcium concentration is directly proportional to inhibition of the sodium pump by digitalis. It is generally agreed that the Na^+, K^+-ATPase acts as a receptor for cardiotonic steroids (Smith, 1989; Weiland *et al.*, 1991).

(i) Mode of action.

The direct positive inotropic action can be illustrated as shown in Figure 2. It appears that during the cardiac impulse there is a movement of Ca^{2+} and Na^+ into the myocardial cell and a resulting loss of K^+ (Figure 2). The hydrolysis of adenosine triphosphate (ATP) by Na^+, K^+-ATPase provides the energy for the so-called sodium pump (the system is located in the sarcolemma of cardiac fibres) that actively expels sodium and transports sodium into the fibres. Digitalis glycosides bind specifically to the Na^+, K^+-ATPase, inhibiting its enzymatic activity and impairing the active transport of these two monovalent cations. As a result, there is a gradual increase in intracellular

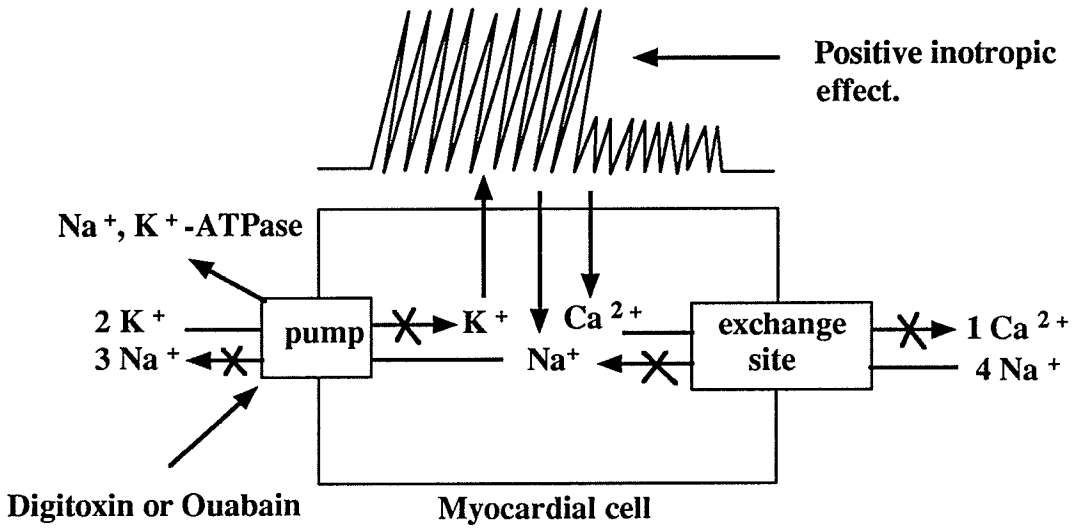


Figure 2. Schematic representation of myocardial function by cardiac glycoside.

sodium and a gradual but small decrease in potassium. These changes are small at therapeutic concentrations of the cardiotonic drug (digoxin). The increase of sodium inside the myocardial cell is judged to be crucially related to the positive inotropic effect of digitalis. This is so because in cardiac fibres intracellular Ca^{2+} is exchanged for extracellular Na^{+} by a transport system that is driven by the concentration gradients for these ions and the transmembrane potential (Blaustein, 1985). When the sodium ion is increased because of inhibition of the pump by digitalis the exchange of extracelluar Na^{+} for intracellular Ca^{2+} is diminished and Ca^{2+} increases. This exchange process is electrogenic with a stoichiometry of $3Na^{+}$:$1Ca^{2+}$ and is driven by the Na^{+} concentration due to inhibition of the Na^{+},K^{+}-ATPase which can give rise to a proportionally but much larger increase in Ca^{2+} concentration. This leads in turn to an increase in the force of contraction (see Figure 2). More recently, a report from Heller (1988) has raised the possibility that inhibition of Ca^{2+}, Mg^{2+}-ATPase may be responsible for the inotropic effects when therapeutic doses of digitalis are given. An extensive inhibition of the enzyme (> 60%) by cardiotonic steroids can lead to a sustained increase in Na^{+} concentration and an increase in Ca^{2+} concentration to produce a toxic effect (arrhythmia). Heller's study has been reported in a number of reviews (Thomas *et al.*, 1974a; Schwartz *et al.*, 1975; Thomas *et al.*, 1980; Thomas, 1981; Orrego, 1984; Repke and Schönfeld, 1984; Smith *et al.*, 1984a; Smith, 1989). The margin between therapeutic and toxic doses is small and for most patients there is no reliable index of optimal dosage. Toxicity is therefore frequently encountered (20-30% of patients) and many details of digitalis action thus become very important for its clinical use. The dissociation between inotropy and

inhibition of Na^+ and K^+ transport is therefore an area of major interest. Lüllmann *et al.* (1984) studied the different maximal effect of positive inotropy for a number of cardiotonic steroids. They suggest that when the receptor is occupied by the glycoside it does not produce inotropy but that inotropy is initiated by the Ca^{2+} already shed from the enzyme. The availability of Ca^{2+} depends on how long the glycoside remains on the receptor *i.e.*, there will be more Ca^{2+} if the glycoside dissociates and then recombines. When this process takes place more rapidly there will be more Ca^{2+}, but no more is shed if the glycoside remains on the receptor. Thus, such agents will have greater efficacy and elicit a higher level of inotropy before the development of toxicity. This is consistent with the earlier hypothesis, first proposed by Besch and Schwartz (1970), which suggests that the binding of digitalis to Na^+, K^+-ATPase may lead to shedding of bound Ca^{2+} and hence to inotropy without necessarily causing overall enzyme inhibition. More evidence in support of this proposal was reported by Gervais *et al.* (1977) who also concluded that the stored Ca^{2+} was most likely located on the phospholipids associated with the Na^+, K^+-ATPase. Other investigators have reported that in the range of therapeutic concentrations of glycosides there will be a decrease in Na^+ and increase in K^+. This has led to the suggestion that the inotropic effects of cardiotonic steroids are produced by stimulation of the Na^+, K^+-pump and the toxic effect by inhibition. This hypothesis has been reviewed by Noble (1980).

(ii) Relationship between the therapeutic and toxic effect.

Many compounds have been tested in this area *e.g.*, 4-amino sugar derivatives of digitoxigenin were considered to have an improved therapeutic ratio because the

amino groups (due to their ionization at physiological pH) would decrease uptake into the central nervous system (CNS). This was thought to be significant in minimizing CNS effects and decreasing cardiotoxic effects. However, the amino groups are largely non-ionized at biological pH so that lack of penetration into CNS can't be assumed. It has been believed (Thomas *et al.*, 1990) that the therapeutic effects of cardiotonic steroids are associated with increased Ca^{2+} and that an excessive amount of this ion will produce toxicity.

In summary, the separation between the therapeutic and toxic effect could be classified as follows:

(1) The inotropic effect could be mediated through Ca^{2+}, Mg^{2+}-ATPase and the toxic effect through Na^{+}, K^{+}-ATPase unless the effects on Ca^{2+}, Mg^{2+}-ATPase are secondary to an effect on Na^{+}, K^{+}-ATPase.

(2) The toxic effects may be mediated by the CNS (Levitt and Keefe, 1985), because digitalis has many effects on the CNS and peripheral nervous system (PNS) (Gillis and Quest, 1980; Smith *et al.*, 1984a,b,c).

(3) LaBella *et al.* (1987) stated that it is possible to separate therapeutic and toxic effects only if two different mechanisms or two different receptor sites are responsible. In their study, they found that chloromadinone acetate (CMA) (a modified progesterone structure, sterically unlike the cardiac glycoside structure and more similar to the natural hormones) can interact strongly with the cardiac glycoside receptor of heart muscle in a receptor

binding assay (RBA) and exert cardiostimulant (positive inotropy) and cardiodepressant effect (negative inotropy) and the balance between these two effects represents the difference between therapeutic and toxic effects.

II. Indirect effect on the neural system.

The existence of Na^+, K^+-ATPase is not limited to the myocardial cells but it is widely distributed in all tissues of the body. *In vivo* administration of cardiac glycosides will not only effect the cardiac muscle because of its high sensitivity but may also trigger toxic side-effects (indirect effect) in other tissues such as the renal tubule, which contains a high density of Na^+, K^+-ATPase. For instance, cardiac glycosides can inhibit the renal Na^+, K^+-ATPase up to 80%, which will increase Na^+ concentration to a higher level before the enzyme's reserve capacity to maintain the balance between Na^+ and K^+ ions comes into effect (Sejersted, 1986).

(i) Effects on afferent neural systems (baroreceptors and chemoreceptors).

When cardiac glycosides are given to whole animals, they will affect the heart by a combination of direct and indirect effects, the latter involving the neuronal system, because the heart is very sensitive to neurological control and Na^+, K^+-ATPase plays a key role in the activity of all neuronal cells. Gillis and Quest presented an extensive review on the subject in 1980. In their review, they suggested that the effects of cardiac glycosides on certain reflexogenic sites (*e.g.*, baroreceptors, located in the carotid sinus and aortic arch and arterial chemoreceptors, located in the carotid and aortic bodies) can stimulate afferent

fibres in the IXth and Xth cranial nerve (Korner, 1978) and this may give rise to a considerable portion of the therapeutic benefit of digitalis whereas the toxic effects of cardiac glycosides on the heart are a result, at least in part, of indirect effects resulting from the increased sympathetic outflow from the central nervous system (CNS). It has been widely documented that digitalis causes excitation of carotid sinus and aortic arch baroreceptors leading to enhancement of parasympathetic efferent neural transmission to the atrioventricular node. This results in bradycardia and reflex withdrawal of sympathetic tone when a cardiac glycoside is administered in therapeutic doses (Heymans *et al.*, 1932; Chai *et al.*, 1967; Quest and Gillis, 1974; Gillis and Quest, 1980).

Studies employing the following techniques have provided much evidence that arrhythmogenic doses of cardiac glycosides can stimulate central sympathetic centres. As outlined by Gillis and Quest (1980), these include:

(1) monitoring of efferent neural activity during intravenous or local administration of cardiac glycoside;

(2) monitoring of end organ responses to cardiac glycoside either centrally or locally;

(3) monitoring of efferent neural activity during stimulation of CNS sites before and after treatment with digitalis.

Amongst these are a number of studies in which cardiac glycosides were shown to produce increased sympathetic outflow from the CNS. An early study by Abiko

et al. (1965) demonstrated that low doses of intravenous strospeside decreased sympathetic outflow in anaesthetized cats, but that when the dose was increased to arrhythmogenic levels, an increase in neural traffic was observed. This finding was later confirmed by Pace and Gillis (1976) with arrhythmogenic doses of digoxin.

(ii) Effects on efferent neural systems (sympathetic neuroeffector junctions and end-organ responses).

Another study by Gillis (1969) examined this phenomenon in decerebrated cats and found that arrhythmogenic doses of ouabain increased output from preganglionic cardiac sympathetic nerves with cardiovascular reflexes in intact animals and those in which the cardiac reflexes had been denervated. It was further observed that the decreased neural firing at lower, non-toxic doses reported by Abiko *et al.* (1965) could be abolished by denervation of the reflexogenic areas. Gillis concluded that reflex-mediated withdrawal of sympathetic tone occurred at lower doses of ouabain, while toxic doses caused excitation of central sympathetic sites and led to increased efferent activity. Furthermore, Gillis *et al.* (1972) found increased neural traffic in cardiac sympathetic efferent and phrenic nerves of anaesthetized cats following a constant infusion of ouabain. The increased activity was temporally associated with the onset of cardiac arrythmia and respiratory hyperactivity. However, Weaver *et al.* (1976, 1977) found that regular administration of digoxin did not have a central sympathetic excitatory effect. Digoxin was administered to anaesthetized cats every 15 min. until ventricle

fibrillation occurred. Preganglionic splanchnic and postganglionic inferior cardiac neural traffic were monitored in intact animals and in animals following denervation of both baroreceptors and chemoreceptors. Digoxin had an inhibitory effect on preganglionic nerve activity in the intact cats, but no change was observed in the denervated animals. Hence this effect was thought to be reflexed-mediated. The authors concluded that the primary site of sympathetic stimulation induced by cardiac glycosides was not in the CNS.

By measuring preganglionic sympathetic neural activity or end organ responses, many investigators observed stimulation of central sympathetic centres after administration of digitalis into the CNS. Pharmacological blockade or surgical intervention leading to selective denervation have often been employed to localize putative sites of digitalis action and hence yield indirect evidence of cardiac glycoside induced central sympathetic excitation.

An early study by Doggett and Spencer (1971) demonstrated general CNS excitation in mice following intracerebral administration of ouabain. A similar response was seen in anaesthetized rats by Iyoda *et al.* (1986) when increases in heart rate and sympathetic nerve activity were produced following injections of ouabain into the posterior hypothalamus. No response was seen when the injection site was located at the anterior preoptic hypothalamus or the ventromedial hypothalamus.

Administration of ouabain into the hypothalamus of anaesthetized dogs has

been shown to increase preganglionic sympathetic nerve activity coincident with the onset of cardiac arrhythmia, whereas similar treatment at medullary sites had no effect (Garvey, 1970).

(iii) Effect on neurotransmitters and histaminergic mechanism on cardiac glycoside toxicity.

Saxena and Bhargava (1974, 1975) comprehensively examined the central effects of cardiac glycosides in anaesthetized dogs. The response to intracerebroventricular and intracisternal administration of toxic doses of ouabain was observed following a variety of pharmacological and surgical interventions.

Systemic administration of ouabain to anaesthetized guinea pigs has also yielded indirect evidence of central sympathetic excitation by cardiac glycoside substances (Lechatt and Schmidt, 1982).

Studies have also been undertaken to investigate the role of histaminergic, dopaminergic and serotonergic mechanisms in central sympathetic stimulation by cardiac glycosides. Gillis and Quest (1980) have extensively reviewed the effects of cardiac glycosides on the release, storage, uptake, synthesis and degradation of various CNS neurotransmitters. Histaminergic mechanisms in cardiac glycoside toxicity have been examined by Tackett and Holl (1980). Intravenous infusions of ouabain were administered to vagotomized cats. Pretreatment with histamine administered into the fourth cerebral ventricle caused a 34% decrease in the cardiotoxic dose of ouabain. Furthermore, the onset of cardiac arrhythmia was

coincident with the observation of increased cardiac sympathetic outflow as measured from the left inferior cardiac nerve. The authors then studied the effects of a range of histamine agonists and antagonists. Dimaprit, a specific H_2-agonist, had a similar effect to pretreatment with histamine, while pretreatment with cimetidine, a specific H_2-antagonist, protected against ouabain cardiotoxicity in a dose-dependent manner. The H_1-antagonist diphenhydramine did not affect the response to ouabain. When histamine, dimaprit or either of the antagonists were administered intravenously, no potentiation or attenuation of ouabain responses was observed. The authors concluded that a central histaminergic mechanism may have a role in cardiac glycoside toxicity. Tackett and Holl (1980) suggested the following mechanism of action for ouabain-induced, centrally mediated sympathetic activation. Cardiac glycoside could stimulate hypothalamic neurons, *via* a mechanism mediated by central H_2-receptors, leading to centrogenic excitation of the sympathetic nervous system, increased efferent output and hence cardiotoxicity.

The roll of central dopaminergic receptors in the cardiac effects of cardiac glycoside is controversial. Helke (1978) reported that deslanoside, when administered systemically, produced an increase in the level of dopamine receptors in the hypothalamus. In a further study, Helke and Gillis (1978) examined the effects of the specific dopamine agonists apomorphine and piribedil (administered both intravenously and intracerebroventricularly) on the arrhythmogenic activity of deslanoside in anaesthetized cats. Systemic pre-treatment with both agonists

significantly increased the doses of deslanoside required to establish both ventricular arrhythmia and ventricular fibrillation. The authors were able to localize this protective effect to the fourth ventricle by studying the effect of selective intracerebroventricular administration with apomorphine. It was concluded that the protective effect of the dopamine agonist was probably due in part to an interaction with dopamine receptors since antagonism of these receptors with haloperidol significantly attenuated the antiarrhythmic effect of apomorphine on cardiac glycoside toxicity. The proposed mechanism involved deslanoside interacting antagonistically with the dopamine receptor thereby causing an increase in sympathetic outflow.

The findings of Plunkett and Tackett (1987) do not support such a mechanism. These investigators administered specific dopamine agonists and antagonists into the ventriculocisternal system of anaesthetized dogs and in these experiments, apomorphine did not protect against digoxin-induced cardiotoxicity. However, whilst the level of dopamine in the cerebrospinal fluid was elevated in control dogs after digitalization, this increase was much less in apomorphine-pretreated animals. Similar responses were observed in dogs pretreated with pimozide, a specific dopamine antagonist. Plunkett and Tackett proposed that dopamine receptors are not directly associated with the central effects of cardiac glycosides, but that they may influence central noradrenergic mechanisms mediating sympathetic efferent activity and hence cardiovascular function.

There is now strong evidence indicating that the interaction of cardiac glycosides with central sympathetic sites may induce coronary vasoconstriction and hence arrthythmogenesis at toxic doses (Tanz, 1986). Cross-perfusion techniques were employed by Garan *et al.* (1974a,b) to demonstrate a central effect of digoxin by monitoring α-adrenoreceptor-mediated coronary vasoconstriction. Digoxin was administered intravenously to a donor dog, the blood of which was used to perfuse the isolated head circulation of a recipient dog. The only connection between the head and body of the recipient dog was the nervous system. Coronary vasoconstriction was evident in the recipient following digoxin administration to the donor dog. This response was not produced when an equivalent dose of digoxin was administered systematically to the recipient. Intravenous administration of digoxin to intact control dogs did cause coronary vasoconstriction. Garan *et al.* also observed an increase in the level of digoxin in the cerebrospinal fluid concomitant with this response. The vasoconstriction could be abolished by pretreatment of the animal with the α-blocker phenoxybenzamine or following prior ganglion blockade with mecamylamine.

Thron *et al.* (1984) provide evidence against the localization of the excitation of central sympathetic neurons by cardiac glycoside at *area postrema.* Following discrete lesioning of the *area postrema* in anaesthetized cats, these investigators observed no attenuation of ouabain-induced cardiotoxicity when these animals were compared to control cases.

A further approach employed to evaluate the effect of cardiac glycosides on central sympathetic activity has involved the observation of the effects of cardiac glycosides on neural responses produced by electrical stimulation of hypothalamic regions in anaesthetized animals. Evans and Gillis (1975) electrically stimulated the posterior hypothalamus of the anaesthetized cat. This produced hyperactivity in cardiac efferent sympathetic fires and cardiac arrhythmia. Intravenous administration of small doses of ouabain attenuated these effects, but denervation of the baroreceptors abolished this attenuation. This response was therefore due to baroreceptor activation. Larger subarrhythmic doses, when combined with subthreshold electrical stimulation, caused increased sympathetic activity and arrhythmia. The authors concluded that ouabain acts at a posterior hypothalamic site to sensitize sympathetic neurons with concurrent enhancement of sympathetic activity.

The following summarizes the main conclusions regarding the indirect effect of cardiac glycosides on the CNS:

(1) *Therapeutic* doses of cardiac glycosides can reduce sympathetic outflow from the CNS, but this effect appears to be reflex-mediated (*i.e.*, not due to a direct CNS action).

(2) *Toxic* doses of cardiac glycoside can have a direct effect on the CNS, resulting in increased sympathetic outflow although this effect could also arise from a reflex-mediated-effect.

(3) Many studies, although inconclusive, have provided strong indirect evidence supporting the hypothesis that a direct action of cardiac glycoside on the CNS is responsible for much of the arrhythmia associated with *toxic* doses of cardiac glycosides (Thomas *et al.*, 1990). The site of action appears to be in the hypothalamus, possibly in the *area postrema*, a part of the brain not protected by the blood-brain barrier.

METABOLISM OF CARDIAC GLYCOSIDES

I. Introduction.

The therapeutic index of cardiac glycosides is so low that it is associated with toxic effects in the treatment of various forms of heart failure. Because of the low concentrations of these glycosides given to the patient and because of the difficulty in maintaining the plasma drug concentrations in the therapeutic range, there are difficulties in carrying out metabolic studies. These agents, due to their low plasma concentrations (in the ng/ml range), require a relatively simple, specific and sensitive assay procedure. The most commonly used technique for the clinical assay of plasma digitalis is radioimmunoassay. As reported by many investigators (Graves *et al.*, 1984a,b) this does not reflect the true drug concentration, as the antibody can interact with certain metabolites. Two glycosides, namely, digoxin and digitoxin, have been the most commonly used agents for studying the metabolism of cardiac glycosides. The metabolic studies carried out have involved

at least three or four steps, *i.e.*:

(1) Extraction of the biological sample with an organic solvent giving two fractions, namely, the organic solvent extractable material (usually made up of non-conjugated material) and the non-extractable material (conjugated and other highly polar metabolites).

(2) Enzymatic hydrolysis of the non-extractable fraction with ß-glucuronidase and/or aryl sulfatase and subsequent re-extraction of the deconjugated materials.

(3) Chromatographic separation of the extracted materials from **1** and **2** above.

(4) Assay of the separated fraction by either bioassay, immunoassay or counting of radioactivity.

One of the major practical difficulties encountered in the above steps is the efficient extraction and separation of the conjugated metabolites of the glycoside or partially degraded glycoside from the glycosides themselves because the acidic glucuronides cause hydrolysis of the glycoside on concentration (Eriksson *et al.*, 1981). One very significant point, not addressed in many of the metabolic studies, is the relationship between the concentrations of parent drug and metabolites in plasma, with those in the myocardium. For instance, in an exhaustive study by Storstein (1977b), the difference between the relative concentrations of parent drug and metabolites in plasma and myocardium was found to be significant. The data shown below are for patients treated chronically with digitoxin (Table 1).

Table 1. Relative concentrations of digitoxin and its metabolites in plasma and myocardium.

Type of metabolite	% of total drug or metabolite in serum	% of total drug or metabolite in myocardium
Unchanged DT_3	58	26
Non-conjugated DT_2, DT_1, DT_o	7	14
Conjugated DT_3	10.5	17
Conjugated DT_2, DT_1, DT_o	14.8	27

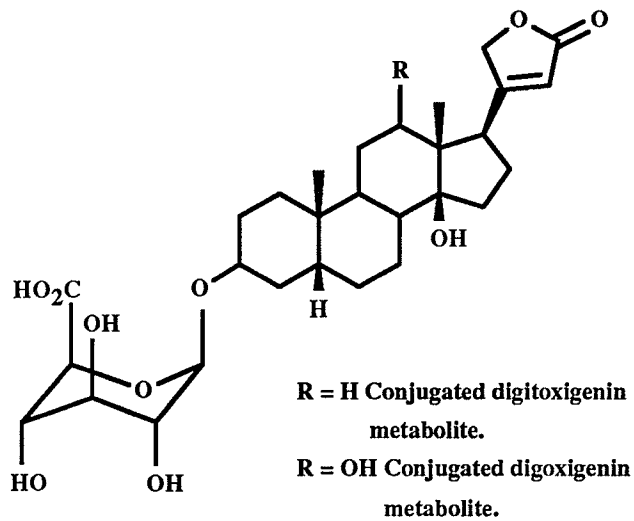
DT_3 = digitoxin tris-digitoxoside, DT_2 = digitoxin bis-digitoxoside, DT_1 = digitoxin mono-digitoxoside and DT_o = digitoxigenin.

These results demonstrate that uptake of active and inactive metabolites into the myocardium and also presumably into the central and peripheral nervous system is not linearly proportional to their serum concentration. This, coupled with the knowledge that the various metabolites do not have the same cardiotonic activity (see below), indicates that the plasma concentration of drug may, at best, only confirm an observed toxic or therapeutic effect. Furthermore, it may also be possible that some metabolites, while being cardioactive, may be able to bring about a centrally-mediated cardiotoxic action due to preferential combination with CNS receptors.

The inotropic actions of cardioactive metabolites of digoxin and digitoxin were first studied by Lüllmann and Peters (1971). Using isolated guinea pig atria, they found that the conjugated genins (Figure 3) of digitoxigenin and digoxigenin were inactive, whereas both the mono- and the bisdigitoxoside were more potent than digitoxin and for the digoxin series, the potency order was monodigitoxoside > bisdigitoxide > digoxin. Additionally, the aglycones of both series were very weak. Later, Bottcher *et al.*, (1973) found that digoxigenin monodigitoxoside was more potent than the bisdigitoxoside and digoxin when tested on the cat heart-lung preparation. They also reported differences in the therapeutic ratios. A different result was reported by Dutta *et al.*, (1976), who found, using the isolated perfused guinea-pig heart, that digitoxin was more potent than either the bis- or monodigitoxosides, or the aglycone (Figure 3), the potency decreasing with the loss of each digitoxose unit. Recently, Brown and Thomas (1983) confirmed the results of Lüllmann and Peters.

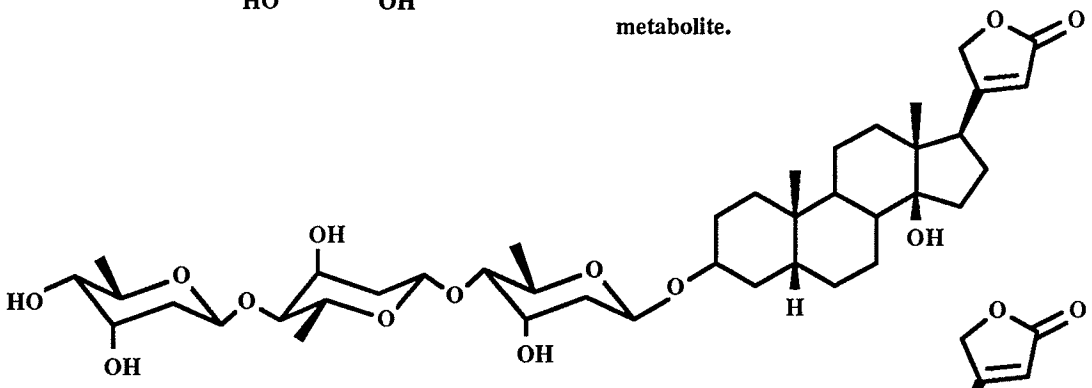
Flasch and Heinz (1978) tested some cardiac glycoside metabolites for cardiotonic activity on isolated guinea-pig papillary muscle and for inhibition of Na^+, K^+-ATPase from guinea pig heart, cat heart and ox-brain.

In general, the mono- and bisdigitoxosides of digoxin and digitoxin were found to be more potent than the trisdigitoxosides. The 20, 22-dihydrodigoxin was inactive, whereas the corresponding dihydrodigitoxin was about 30 times less potent than digitoxin. The aglycones of digoxin and digitoxin were about 10-20

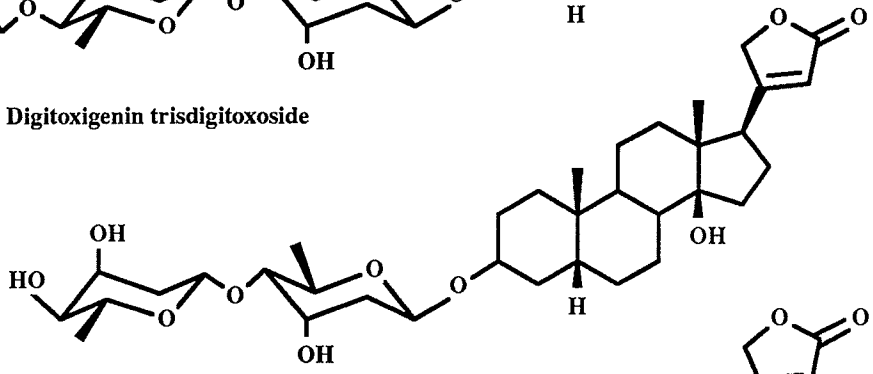


R = H Conjugated digitoxigenin metabolite.

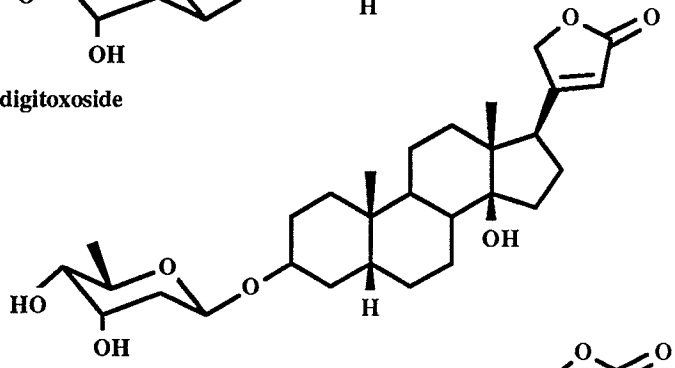
R = OH Conjugated digoxigenin metabolite.



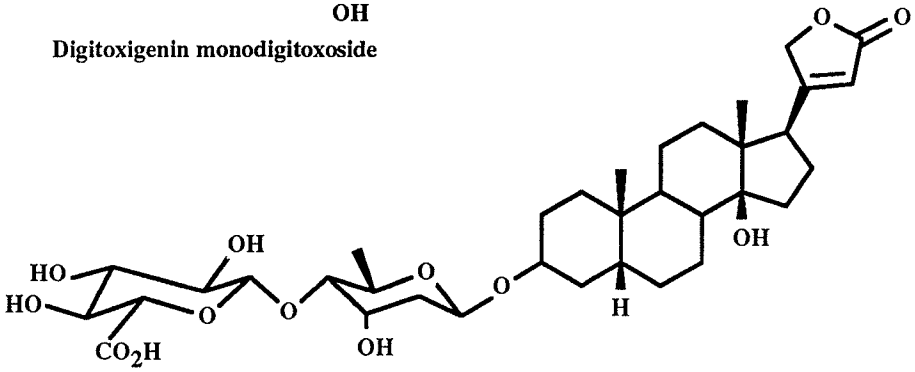
Digitoxigenin trisdigitoxoside



Digitoxigenin bisdigitoxoside



Digitoxigenin monodigitoxoside



Digitoxigenin monodigitoxoside-glucuronide

Figure 3. Structures of digitoxin metabolites.

times less potent than the trisdigitoxoside and epimerization of the hydroxy group at C-3 further reduced activity. These authors also found the conjugates to have some activity. The 4‘‘‘-glucuronides of digoxin and digitoxin were only 4 to 5 times less potent than their non-conjugated parents. In addition, the glucuronide of digitoxigenin monodigitoxoside (Figure 3) was about one sixth as active as digitoxin or, in other words, slightly more potent than digitoxigenin. The conjugates of the aglycones had very low activity. The 4‘‘‘-glucuronides of digitoxin and digoxin have been found, by Scholz and Schmitz (1984), to be active on isolated human ventricular strips. They found the conjugates were slightly less potent than the non-conjugated forms and cause a greater maximum inotropic effect.

An extensive review of the metabolism and disposition of cardiac glycosides has been published by Bodem and Dengler (1976), Greef (1981), and Erdmann *et al.*, (1986). Some relevant information in regard to absorption, metabolism and excretion of cardiac glycosides has been summarized by Thomas *et al.* (1980, 1990).

Cardiac glycoside metabolism is complicated by the diversity of experimental procedures used in the various studies. For example:

(1) **Use of different species.** There are marked species differences in metabolic activity (Schmoldt *et al.*, 1982).

(2) **Duration of study.** The metabolic pattern of cardiac glycosides can vary

depending on the duration of therapy (Storstein, 1977a). In some cases this may be due enzyme induction, but on other cases, prolonged dosing has led to a decrease in certain metabolites. Therefore, it is not correct to compare the results of single-intravenous-dose studies with those of multiple-oral-dose studies. Furthermore, there is also a potential problem in studies on patients with concomitant therapies that include enzyme-inducing or inhibiting agents.

(3) **Routes of excretion.** Not all studies have examined all routes of excretion (urine and faeces) as well as looking at changes in plasma-borne metabolites.

(4) **Assay method.** The use of non-specific assay methods without prior chromatographic separation give misleading data.

(5) **Duration and timing of sampling.** The duration and timing of sampling has sometimes been inadequate. This is especially true for compounds with long elimination half-lives such as digitoxin.

II. Metabolism of digoxin and digitoxin.

Okita *et al.* (1955), Wright (1960) and Okita (1964) established that digoxin and digitoxin underwent some form of stepwise degradation and that digitoxin and its metabolites could be converted to the corresponding digoxin (12ß-OH) analogues. Later, Repke (1959, 1963a, 1966 and 1970) reported a sequential cleavage of the digitoxose units for digoxin and digitoxin to give the genin which

was then converted to the 3-epi-genin. Additionally, there are conjugates of the C-3 epimerized and non-epimerized genins with glucuronic and sulphuric acids. Thomas and Wright (1965) confirmed the epimerization step using the reverse isotope dilution technique. In other hands, Aldous *et al.*, (1972) and Aldous and Thomas (1977) found that in human, lanatoside C and other higher analogues were converted to digoxin in the gut and that very little lanatoside C (digoxin 3‘‘‘-acetoxy-4‘‘‘-glucoside), as such, was absorbed. The excretion of digitalis glycosides and their metabolites in the bile was consistent with earlier reports (Katzung and Meyers, 1965, 1966) and enterohepatic recycling of some deconjugated materials can occur. It has been reported that the reduction of digitoxin to dihydrodigoxin is caused by gut flora, and 10% of patients in which it comprises 40% in the urine is an important metabolic pathway (Lindenbaum, 1986). So far, cardiac glycosides have been found to undergo a number of metabolic reactions *e.g.*, hydrolysis of the sugar moiety, hydroxylation and conjugation of the genin, epimerization of the C-3 hydroxyl group and finally, reduction of the unsaturated lactone ring which is mediated by bacteria that are present in the gut. The liver is however the major site of their metabolism.

Digoxin is usually excreted almost totally unchanged (Doherty, 1968, 1973 and Marcus *et al.*, 1966), but this may not necessarily be true in all patients as more recent evident indicates that gut flora may be responsible for the production of dihydrodigoxin (Doherty *et al.*, 1978). The differences observed between

excretion of digoxin and digitoxin are thought to be due to the increased polarity of digoxin resulting from the presence of the 12ß-hydroxyl group, which reduces its uptake in the liver. Furthermore, it has been shown that the two cardiotonic steroids have differences in metabolism. Digoxin and digitoxin were administered intravenously, to guinea-pigs. Six hours after dosing with digoxin, the percentages of chloroform-extractable material in plasma and myocardial tissue were 70.5% and 96.1%, respectively, of which 85-90% was unchanged digoxin. Six hours after administration of digitoxin, the percentages in the two tissues were 44.5 and 90.5% of which less than 10% was unchanged digitoxin (Dolphen and Lesne, 1981).

The results of other investigators for the metabolism of digoxin and digitoxin in human were somewhat different. It has been found that unchanged digoxin in plasma was around 64-90% and in urine 80-90%, whereas unchanged digitoxin in plasma was around 50-60% and in urine 60% (Doherty *et al.*, 1961; Marcus *et al.*, 1964; Rietbrock and Abshagen, 1973; Zilley *et al.*, 1975; Storstein, 1977a,b; Vohringer and Rietbrock, 1978; Gault *et al.*, 1979, 1984). The metabolites of digoxin in man are mainly genin, mono- and bisdigitoxosides (1-10% in urine; 20% in faeces) (Marcus *et al.*, 1966; Clark and Kalman, 1974) and "polar" metabolites, most likely glucuronides *e.g.*, 3-epi-digitoxigenin (0-20% in plasma and faeces, 0-5% in urine) (Gault *et al.*, 1984). These metabolites, with the exception of dihydrodigoxin, are produced in the liver. Dihydrodigoxin appears to be formed by *eubacterium lentum*, a bactrium which is a normal inhabitant of the colonic flora

(Reuning *et al.*, 1985; Lindenbaum, 1986). The site of inactivation is not known precisely, but it would appear to be lower down in the gut as reduction products are formed in greater quantities from poorly bioavailable preparations (Lindenbaum *et al.*, 1981a,b; Magnusson *et al.*, 1982). Reduction of digitoxin does not appear to be important in man (Strobach, 1986), probably because digitoxin is more readily absorbed.

III. Metabolism of ouabain.

The metabolism of ouabain, a more water-soluble glycoside, was studied by Farah (1946) and Cox and Wright (1959). In rats, 80-90% of an intravenous dose was found in the bile within 5 hours and only 5% was found in the urine after 24 hours. No metabolites were found. With a number of species, Russell and Klaassen (1972) examined ouabain excretion. Twelve hours after the administration of an intravenous dose, 54.6% of the dose was excreted in the bile of rats.

IV. General aspects of liver-mediated metabolism.

It is known that cardiac glycosides can be actively transported from plasma to bile (Farah, 1946; Kupferberg and Schanker, 1968). Cox and Wright (1959) reported that the rate of biliary excretion of cardiac glycosides was proportional to their polarity. This evidence was partially supported by Russell and Klaassen (1972, 1973) who found that two hours after administration of ouabain, digoxin and digitoxin to rats, the biliary excretion of the three agents was proportional to

polarity. After 12 hours however, this order had been reversed. Furthermore, they found that in dogs, biliary excretion was proportional to lipophilicity, only 1.31% of the administered dose of ouabain being excreted after 12 hours. Their conclusion was that the LD_{50} of glycosides could not be adequately explained by the biliary excretion rates, nor did they reflect the concentration of drug in the myocardium. The uptake of ouabain by rat liver slices was found to require oxygen and to be inhibited competitively by other cardiac glycosides, but not by organic acids and bases. However, there have been contested reports on the question of the mode of action by which ouabain is taken up by the liver and excreted into the bile. Kupferberg (1969) showed that in the rat, compounds with a common steroidal nucleus to ouabain, *e.g.*, dehydrocholate, inhibited ouabain accumulation in rat liver slices. Eventually, Erttmann and Damm (1975), were unable to find any cross inhibition between tritiated corticosterone, taurocholic acid and ouabain in uptake into rat liver slices. However, these investigators had shown that hepatic uptake of ouabain was a different process to the biliary excretory process, since probenicid non-competitively inhibited ouabain uptake, but had no effect on biliary excretion. A study of ouabain uptake into rat hepatocytes came later by Eaton and Klaassen (1978). They found the uptake to be mediated by a carrier at the sinusoidal site. Support for this came from their following observations:

(1) Accumulation was saturable.

(2) The uptake occurred against a concentration gradient.

(3) Transport was inhibited by four different metabolic inhibitors.

(4) Reduction in the incubation temperature resulted in a fall in uptake.

(5) The uptake was inhibited by structurally similar compounds.

In addition, the uptake mechanism was not specifically inhibited by compounds with a steroidal nucleus. Most organic bases had little effect, whereas a number of organic acids (*e.g.*, iopanoic acid) did inhibit uptake. However, the type of inhibition was not determined although it was shown not to be competitive inhibition. They concluded that, while uptake into rat liver was an active carrier-mediated process, it was not possible to say whether the same carrier was responsible for uptake of bile acids and other steroidal compounds. Schwenk *et al.* (1981) later found that ouabain uptake into hepatocytes was competitively blocked by taurocholate.

Figure 4 shows the absorption and distribution steps involved after oral administration of the cardiac glycoside, their absorption from the gastro-intestinal tract (GIT), liver uptake, metabolism and excretion. Highly polar molecules such as ouabain are so poorly absorbed from the GIT that they are administered only by the intravenous route. The metabolism of cardiac glycosides in the liver (Figure 4, cont'd) (Thomas *et al.*, 1990) can undergo three metabolic processes, namely, sugar cleavage, conjugation reactions and hydroxylation reactions (*e.g.*, hydroxylation of the 12ß position in digitoxin) and these have been studied in

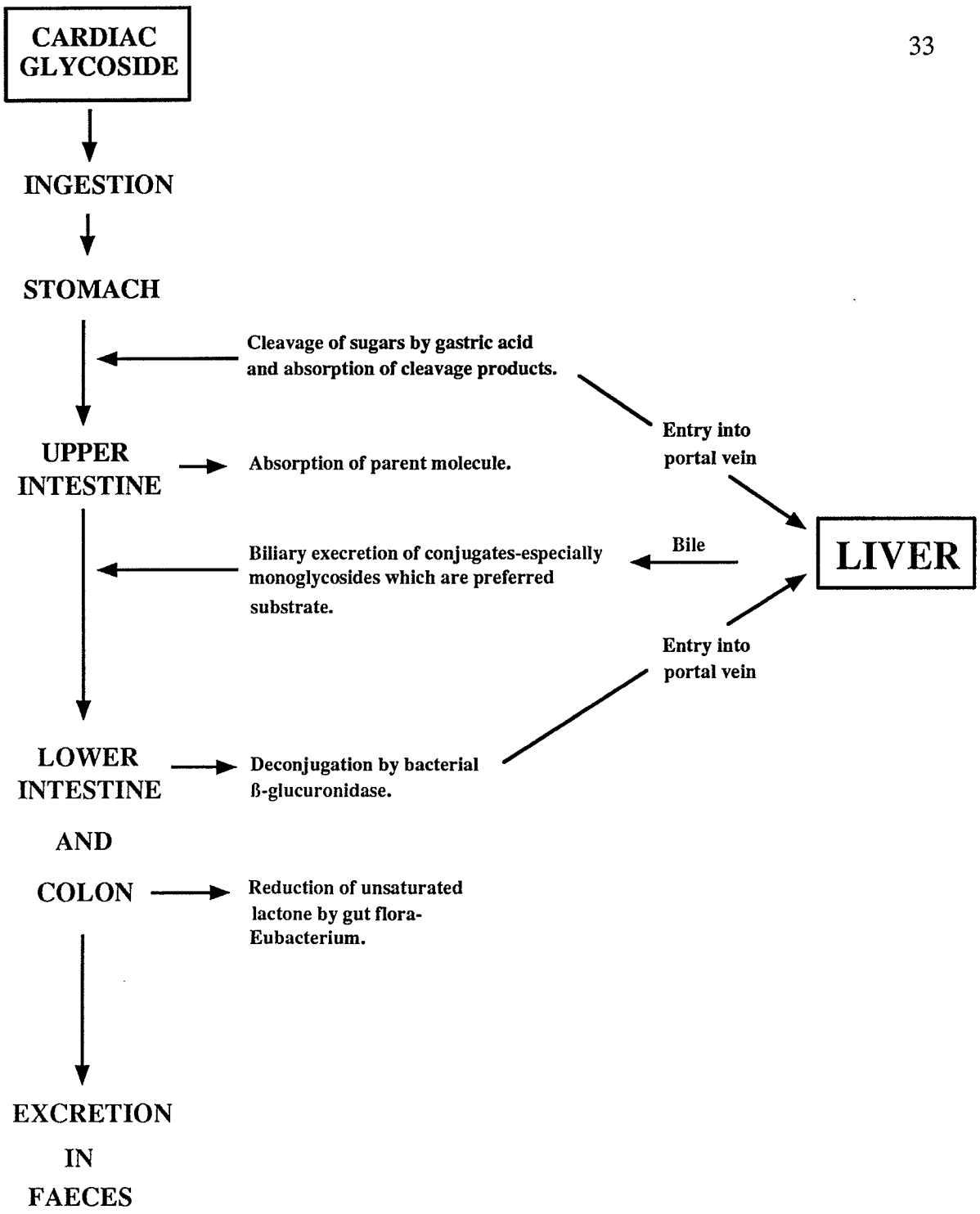


Figure 4. Metabolic pathways of cardiac glycosides in gastro-intestinal tract (GIT) in man (continued on next page).

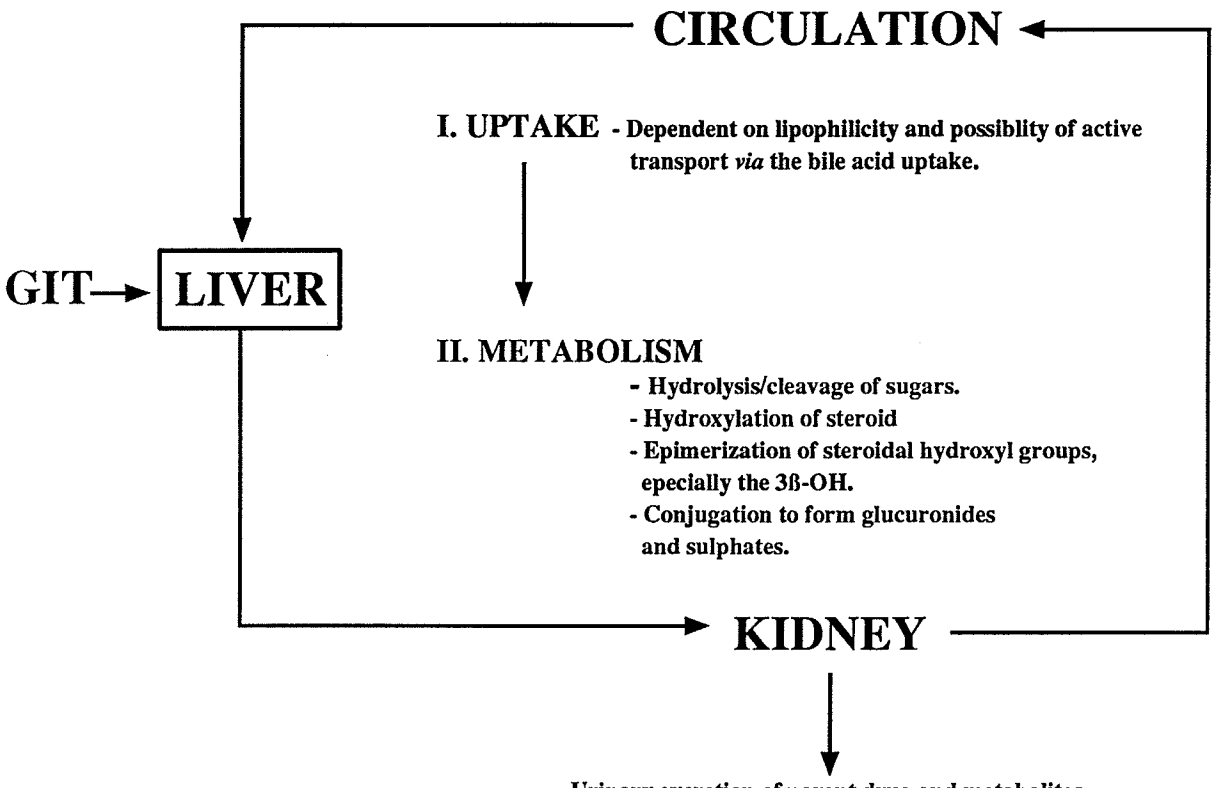


Figure 4 cont'd. Hepatic hydrolysis of the sugar linkages of bis- and trisdigitoxosides involves oxidation by cytochrome P450 (in the presence of NADPH and O_2) of the axial hydroxyl group of the terminal sugar to give 3‴ or 3‟ (15‘- or 9‘-) dehydro compound from which the terminal sugar unit is cleaved by microsomal enzymes (Thomas *et al.*, 1990).

detail. Cleavage of sugar units involves an initial oxidation of the axial hydroxyl group of the terminal sugar unit, giving a dehydrodigitoxoside derivative from which the terminal oxidized sugar unit is split. This sequence is brought about by cytochrome P450 in the presence of NADPH and oxygen (Schmoldt and Rohloff, 1978). Furthermore, Schmoldt (1978) found the rate of cleavage of the sugar units from both digitoxin and digitoxin bisdigitoxoside was increased by a factor of 4 by the liver microsomes of rats pretreated with spironolactone which induces the monoxygenase enzyme that is involved in the oxidation of the terminal sugar unit. Spironolactone was also found to increase the rate of the P450-catalyzed 12ß-hydroxylation of the steroid nucleus, whereas in pretreated rats with phenobarbitone, there was no increase in rates of cleavage or hydroxylation. Inhibition of digitoxin metabolism in rats pretreated with phenobarbitone has also been reported by Cardoso and Carvalhas (1980), possibly because of another type of P450 involved in this process. A more recent study by Kershaw *et al.* (1985) shows that the formation of digitoxin monodigitoxoside from the bisdigitoxoside was the rate limiting step, and that this transformation was the most affected by spironolactone pretreatment (>30-fold increase in rate). Cleavage of the last sugar to give the genin was a minor metabolic route as was 12ß-hydroxylation of the steroidal moiety. Schmoldt *et al.* (1986), found the metabolism of digitoxin in man followed the same pathways as described above for the rat, however, the initial oxidation, catalyzed by cytochrome P450, to give 3‘‘‘-dehydrodigitoxin was found

to be the rate-limiting step. Non-competitive inhibition by cimetidine, quinidine and metyrapone was observed, but only at concentrations much higher than would be encountered physiologically.

The third major metabolic pathway for cardiac glycoside metabolism is conjugation (either glucuronidation or sulphation). The primary conjugation reaction for cardiac glycosides is glucuronidation (Castle and Lage, 1973a,b, 1974; Richards *et al.*, 1977), the main conjugation partners both *in vivo* and *in vitro*, being the monodigitoxosides of digitoxin and digoxin. Richards *et al.* (1977) found that the conjugation of digitoxin monodigitoxoside in rat liver homogenates was greatly enhanced by pretreatment with spironolactone and greater levels of induction were obtained after pretreatment with pregnenolone-16α-carbonitrile (PCN) (Castle, 1980). Additionally, conjugation of the monodigitoxoside of digitoxin was 5 times greater than that of digoxigenin, the rates of both conjugations being increased by pretreatment with spironolactone or PCN (Watkins and Klaassen, 1982). The increased conjugation rate after spironolactone treatment was speculated as due to induction of a relatively specific P450-isocytochrome (Schmoldt, 1978). However, not all known liver inducers affect glucuronidation of digitoxin monodigitoxoside (Schmoldt and Promies, 1982). For instance, phenobarbitone, an inducer of glucuronyltransferase "form 2" did not affect glucuronidation of the monodigitoxoside in rat liver microsomes and also polycyclic aromatic hydrocarbons (*e.g.*, methylcholanthrene), known inducers of

glucuronyltransferase "form 1", did not affect glucuronidation of the monodigitoxoside. On the other hand, inhibition of digitoxin monodigitoxoside conjugation can only be accomplished by epi-digitoxin monodigitoxoside, digoxin monodigitoxoside and chenodeoxycholate. These authors concluded that the glucuronyltransferase involved in conjugation of digitoxin monodigitoxoside, like the isocytochrome P450 responsible for digitoxosyl oxidation of the terminal sugars in digitoxin and digoxin, had a very high level of substrate specificity. The rate of glucuronide formation from digitoxin monodigitoxoside was more than 10 times faster than that from digoxigenin and was selectively inducible by spironolactone. The preferred substrate for conjugation is the monodigitoxoside of either digitoxin or digoxin (Schmoldt *et al.*, 1982; Schmoldt 1984). In 1985, von Meyerinck *et al.* (1985) were able to isolate and characterize the glucuronyltransferase responsible for conjugation of digitoxin monodigitoxoside from spironolactone-treated rats. The highly purified enzyme, digitoxin monodigitoxoside uridine-diphosphate glucuronyltransferase, was found to have activity towards digitoxin mono-digitoxoside and slight activity towards the bisdigitoxoside, whereas it had no activity towards digitoxin.

STRUCTURE-ACTIVITY RELATIONSHIPS OF CARDIAC GLYCOSIDES

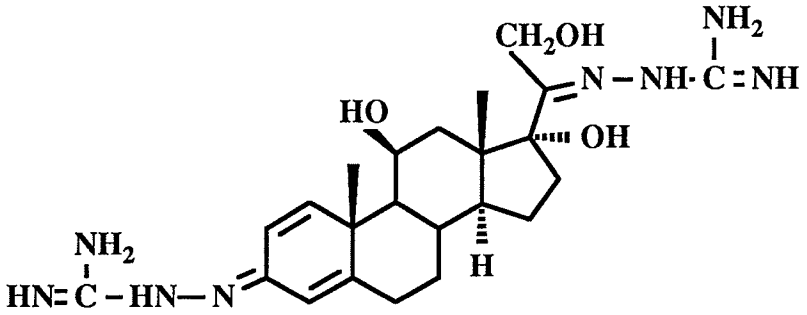
I. Introduction.

During the 1960's, the classical studies of structure-activity relationship (SAR) were reviewed by Tamm (1963) and Chen (1963) and were briefly discussed by Thomas *et al.* (1974a). In 1990, Thomas *et al.* reviewed the SAR studies of the previous 16 years to identify the most recent advances in research work in the field and indicated the principal conclusions of these studies. The traditional studies established the idea that significant digitalis-like activity is to be found only with 14ß-OH steroids substituted, in the 17ß position, with a lactone of the butenolide or pentadienolide type. These features were still being described as "essential for cardiac activity " as late as 1969 in spite of the known digitalis-like activity of the *erythrophleum* alkaloids such as cassaine or the synthetic analog such as the prednisolone 3,20-bisguanylhydrazone (Figure 5). These compounds do not necessarily need to possess a steroid ring system (cassaine) previously thought essential for biological activity (Schütz *et al.*, 1969). SAR studies were carried out to determine the principle regions of the molecule contributing to biological activity. They will therefore be outlined starting with the sugar moiety at the C-3 position of an aglycone, as follows:

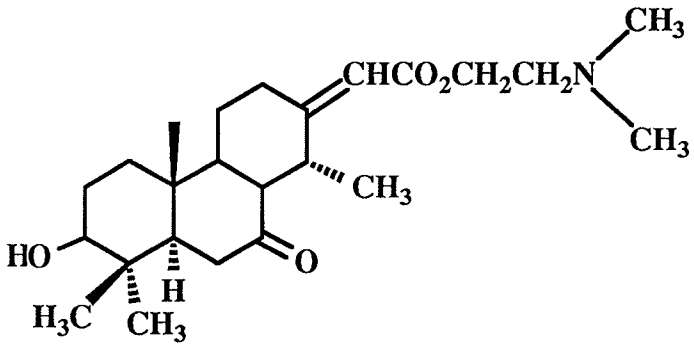
II. The role of the sugar in the action of cardiac glycosides.

(i) Pyranose-type glycosides.

Linkage of the sugar moiety to the C-3 position of the genin is known to



Prednisolone 3,20-bisguanylhydrazone



Cassaine (Erythrophleum alkaloid)

Figure 5. Nitrogen containing substances with digitalis-like inotropic activity.

increase the potency and alter both the pharmacokinetic and pharmacodynamic characteristics of the aglycone part of the molecule, although, the sugars *per se* are inactive. With respect to pharmacokinetics the free genins are more rapidly absorbed and more widely distributed in the body than the corresponding glycosides. Pharmacodynamically the genins are usually less potent than the glycosides and in contrast to the latter show rapid onset and reversal of receptor interaction. It has been recognized that the interaction between the sugar moiety of a cardiac glycoside and Na^+, K^+-ATPase increases the stability of the drug-enzyme complex (Wallick *et al.*, 1974; Schwartz *et al.*, 1975). These six-membered ring sugars or pyranose rings such as digitoxose, rhamnose or glucose may have either a L or D configuration and may have an α or ß attachment to the genin. The effect of the sugars on the stability of the drug-receptor complex has been studied by Yoda and co-workers (Yoda *et al.*, 1973; Yoda, 1974; Yoda and Yoda, 1975, 1977). They suggested (Yoda *et al.*, 1973; Yoda, 1974) that three steps were involved in the receptor binding depending on the nature of the steroid and on the sugar:

(1) The steroid binds to the receptor reversibly.

(2) The conformation of receptor active site changes to accommodate the bulk of the molecule.

(3) The sugar moiety, having been brought into close proximity, binds to the receptor.

They further showed that the cardiac monoglycosides containing a 3'-hydroxy group and a 5'-methyl group were the most potent of all glycosides and concluded that the 3'-hydroxyl was involved in hydrogen bonding whereas the 5'-α-methyl group was involved in a hydrophobic interaction. The 5'-methyl group was found to be more important when compared with the 5'-hydroxymethylene group in other glycosides and they concluded that the 5'-methyl group was the main factor in the interaction of the sugar with the receptor. Yoda and Yoda (1975) examined the effect of increasing the length of the sugar chain as found in many naturally occurring glycosides. They studied the interaction of both the second and third sugar units and the results indicated, as with the monoglucosides, that the 3'''-hydroxyl group on the third sugar was important but that the 4'''-hydroxyl group was not. The second sugar was shown to have a less important role in binding and the 3''-hydroxy group was perhaps involved in binding only to the type I enzyme form (*i.e.*, binding in the presence of Na^+, Mg^{2+} and ATP).

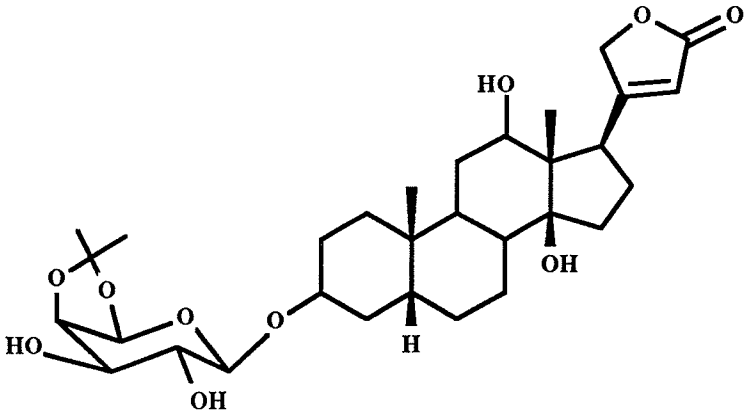
Erdmann and Schoner (1974) confirmed the importance of the 3'α-hydroxyl group and found that acetylation or methylation of the 4'''-hydroxyl group (*i.e.*, ß-acetyldigitoxin or ß-methyl digitoxin) caused a slight increase in binding. This finding was supported by Haustein (1974) who found that the acetate, propionate and nitrate derivatives of several steroids were less potent than the free sugar hydroxyl derivatives on isolated guinea-pig atria. The magnitude of the reduction was dependent on the size, number and structure of the substituents. On the other

hand, Brown and Thomas (1983, 1984) confirmed the importance of the 5'-methyl group, and also identified an equatorial 4'-hydroxyl group, an α-L-glycosidic linkage with the steroid, and an equatorial 5'-methyl group as being common features to all potent monoglycosides. Additionally, they found that the 2'-hydroxyl group was probably not involved in binding. Acetylation of the 2'-hydroxyl group gave drastically reduced potency, either because of the steric effect of the acetyl group, and/or disruption of hydrogen bonding. Furthermore, the presence of a 3'-methoxy group increased the potency, suggesting that the 3'-hydroxyl was not involved in an essential hydrogen bond. Also the 4'-hydroxy group formed the link with the second sugar in a bis-or polyglycoside, showing that it is not essential. Consequently, the sugar which is directly linked to the steroid may not be involved in hydrogen bonding. In a recent review Thomas *et al.* (1990) reported from unpublished studies that glycosides with the first sugar having a 5'-methyl function were more likely to give rise to persistent arrhythmia in intact animals than those with a 6-hydroxy group.

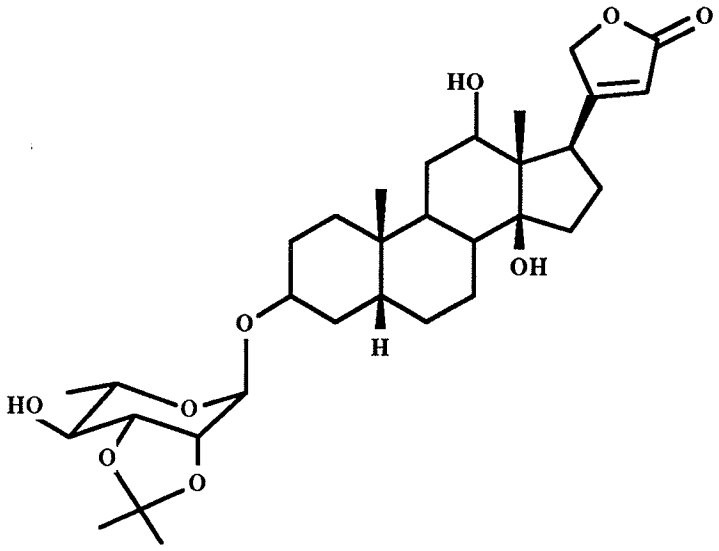
Fullerton *et al.* (1984) also made a systematic study of a series of monosides. They synthesized a number of digitoxigenin-type glycosides and tested these for inhibitory action on pig kidney Na^+, K^+-ATPase. Their results were in agreement with those of Thomas and Yoda. They confirmed that the 5'-methyl group is more potent and hence is more important in binding. Fullerton *et al.* (1984) also confirmed that an equatorial 4'-hydroxyl group and not the 3'-hydroxyl

group, had the greatest effect on activity. On the other hand an axial 4'-hydroxyl group, as found in galactose, was much less effective. In general, acetylation of sugar hydroxyls greatly reduced activity. For instance, acetylation of the 2'-hydroxyl group of the rhamnose moiety in digitoxin rhamnoside reduced activity by 98% making a compound that had 22 times the potency of the genin to one with less than half the activity. Acetylation of all three hydroxyl groups of the rhamnose moiety gave a compound with only one-fifth the potency of the parent genin which implied the dissociation of the drug-receptor complex was greater than with the free hydroxyl groups (Brown and Thomas, 1983).

Interestingly, the addition of an isopropylidene group to the sugar moiety did not appear to affect inotropy adversely. It has been found that digoxigenin (4', 5'-isopropylidene)galactoside and digoxigenin (2',3'-isopropylidene)rhamnoside (Figure 6) were equipotent with their corresponding unsubstituted glycosides (Thomas *et al.*, 1990). Flasch and Heinz (1978) examined the Na^+, K^+-ATPase inhibitory potential of a series of compounds that included glucuronide conjugates of the hydroxyl group at position 4 on the terminal sugar unit. They found that conjugates of digoxin, digitoxin trisdigitoxoside and digitoxin monodigitoxoside were 3-9 times less active than the parent compounds. Furthermore, El Masry *et al.* (1969) found that tetrahydropyranyl derivatives of digoxigenin and digitoxigenin that contained a methylene acetate group ($-CH_2OAc$) at C-5' were less active than the corresponding monoglycosides. Similarly, Zorbach *et al.* (1965) had previously



Digoxigenin (4', 5'-isopropylidene)galactoside



Digoxigenin (2', 3'-isopropylidene)rhamnoside

Figure 6. Isopropylidene analogues of different cardiac glycoside sugars.

found that the tetrahydropyranyl derivatives of digitoxigenin and strophanthidin were less potent than their corresponding aglycones. Other studies, reviewed by Watson *et al.* (1984) indicated that gomphoside (a 5α-compound with the sugar attached at both the C-2 and C-3 carbon) binds to the receptor through the 3'(axial)-hydroxyl group and the 5'(equatorial)-methyl group. The high potency and the fixed stereochemistry, *i.e.*, non-rotatable link between the steroid and sugar, made this compound an appropriate reference for systematic studies of the SAR of the cardiac glycoside sugar moieties, especially with respect to the important question of whether the receptor appears to change its conformation in order to accommodate glycosides of the 5α-H and 5ß-H series.

Chiu and Watson (1985) employed molecular graphics and conformational-energy calculations to examine the conformational factors that affect the SAR of the sugar component. For example, using computer graphics they showed that superimposition of the highly active 5ß-H digitoxigenin rhamnoside over the structure of the highly active 5α-H gomphoside, with the steroid rings B, C and D overlapping, brings about a close coincidence of the 3'-hydroxyl and 5'-methyl groups in both sugar moieties. This suggests that these two features of the sugar moiety are responsible for high biological activity. In a similar manner, they superimposed digitoxigenin monodigitoxoside over gomphoside, as is illustrated in Figure 7. These investigators concluded that the two main binding areas for the sugar were the 3'-axial hydroxyl group and the 5'-methyl. Chiu and Watson also

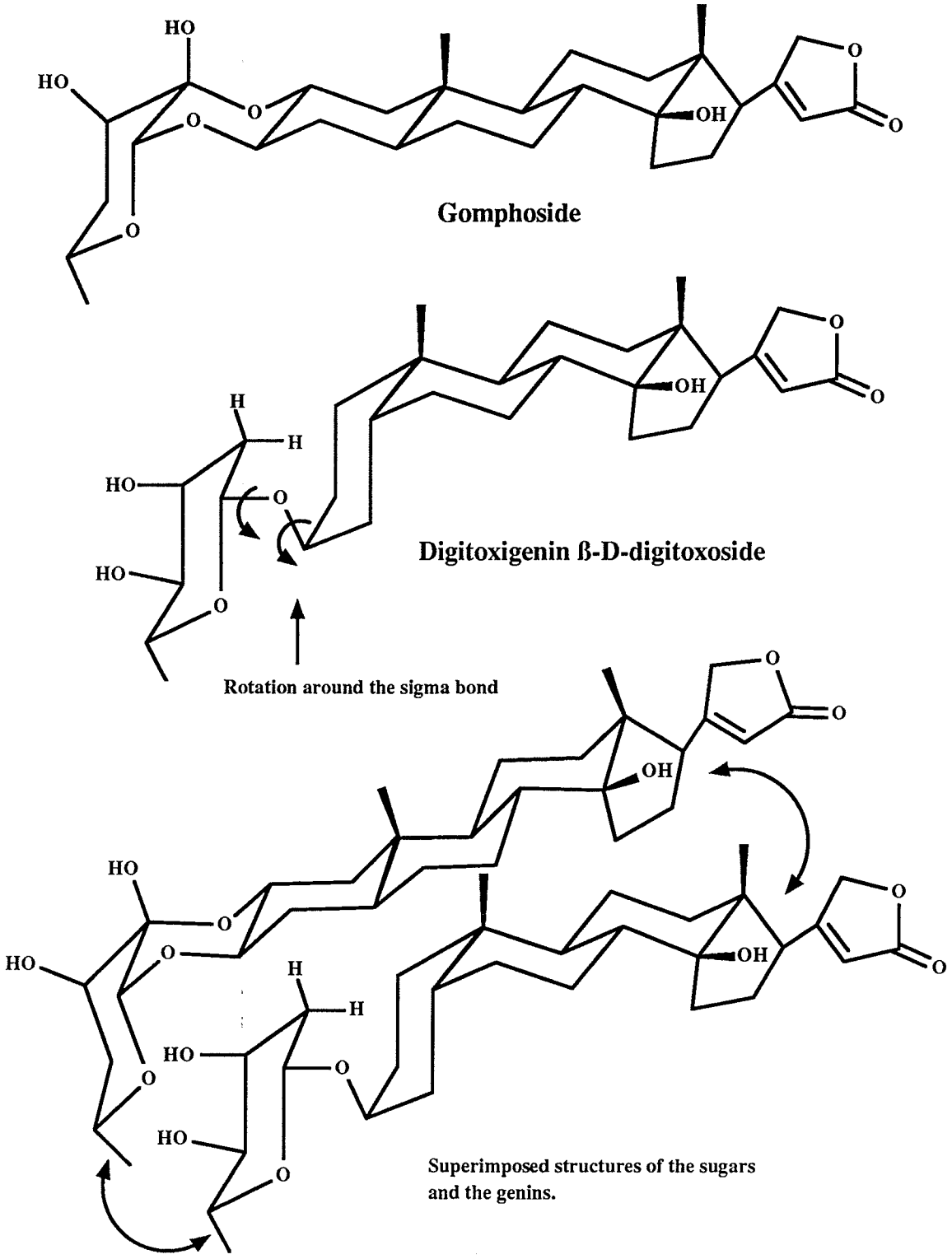


Figure 7. Superimposed structures of gomphoside and digitoxigenin ß-D-digitoxoside.

postulated that for highly active compounds the preferred conformation of the glycoside should be the same as its optimum binding conformation whereas in compounds of weak activity the optimum binding conformation is less energetically favourable. Furthermore, those compounds with wider rotational freedom would have lower potency as they would be able to take up "less optimum" positions with higher probability.

In another approach, Rathore *et al.* (1986) synthesized a series of digitoxigenin mannosides (Figure 8), glucosides and rhamnosides covering all four possible steroid glycoside linkages (α-D, α-L, ß-D, ß-L). The objective was to correlate biological activity with structural differences in the linkages. Their work was in agreement with the work of Brown and Thomas (1983, 1984), who found that a given sugar substituent may have a role in binding for some glycosides but not for others. For example, the stereochemistry of the 2', 3', 4' hydroxyl groups varies with the sugar type, such that the requirements of L-type sugars are different from those of the D-type sugars. The 5'-methyl and 4'-hydroxyl groups appeared to be the most important groups in α-L and perhaps ß-L-glycosides. Addition of a 6'-hydroxyl group to L-type sugars reduced potency. Interestingly, they found that the 5'-methyl group was not as important for the ß-D-glycosides. Consequently, these glycosides depend on contributions from the equatorial 4'-OH, axial 3'-OH and equatorial 2'-OH, for biological activity. However, there is much evidence to support a correlation between a 5'-methyl sugar moiety and cardiotonic

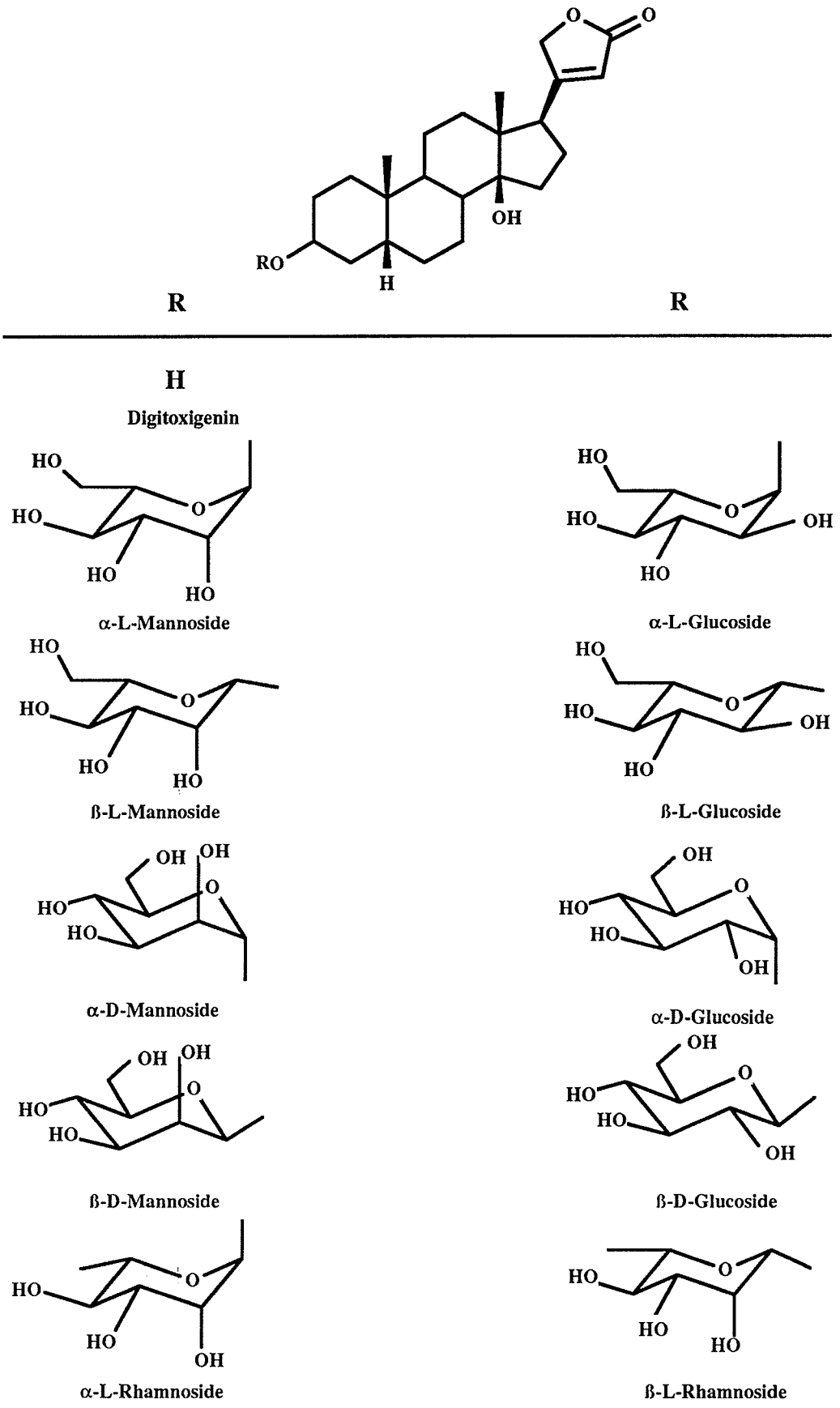
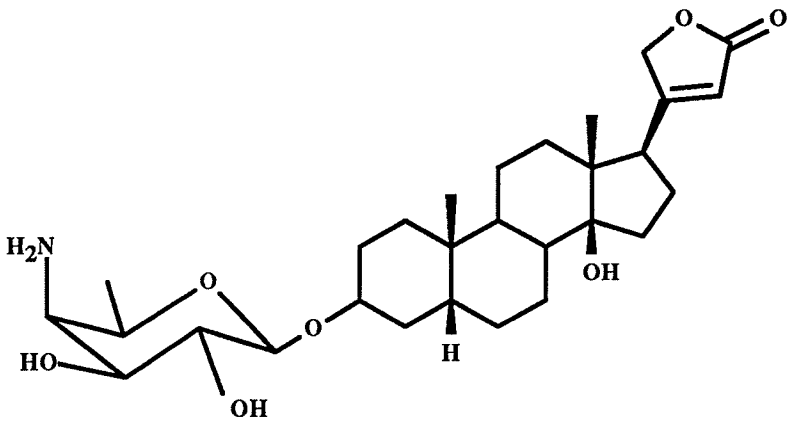


Figure 8. Representative glycoside sugars used to study sugar stereochemistry in cardiac glycosides (Rathore *et al.*, 1986).

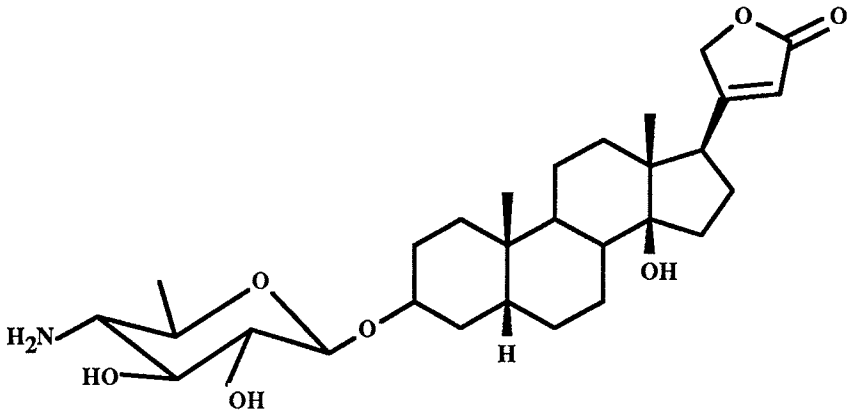
potency. Thomas *et al.* (1990) reviewed the unpublished results of Gray *et al.*, who found that the 5'-methyl group may be associated with a greater tendency to produce arrhythmogenic behaviour, particularly persistent arrhythmia (irregularity in the force or rhythm of the heartbeat). Their conclusion was based on studies of the cardiotonic and cardiotoxic effects of a series of digoxigenin and digitoxin mannosides in mice and anaesthetized dogs. They found that mannosides containing 5'-methyl sugars were significantly more potent than those with a 6'-hydroxyl group and, in comparison with digitoxigenin rhamnoside, digoxigenin galactoside and digitoxigenin glucoside, were around 14 and 10 times, respectively, less potent on isolated guinea-pig atria. On the other hand, there was a marked differences in the onset times for inotropic activity of the 5'-methyl and 6'-hydroxyl sugars. The 5'-methyl sugars took much longer, in comparison to the 6'-hydroxylated mannosides to reach a maximum inotropic effect after the administered dose.

Two aminoglycosides, digitoxigenin 4'-amino-6'-deoxygalactoside (ASI-222) and digitoxigenin 4'-amino 4',6'-dideoxylglucoside (ASI-254) (Figure 9) (Caldwell and Nash, 1976; Cook *et al.*, 1977), have shown interesting cardiotonic potential. In comparison with digitoxigenin, ASI-222 was found to produce more rapid and greater increases in cardiac contractility in intact dogs and rabbit atria, and both were almost equipotent in their Na^+, K^+-ATPase inhibitory potential and inotropic effect on isolated atria (Caldwell and Nash, 1978). This indicated that the



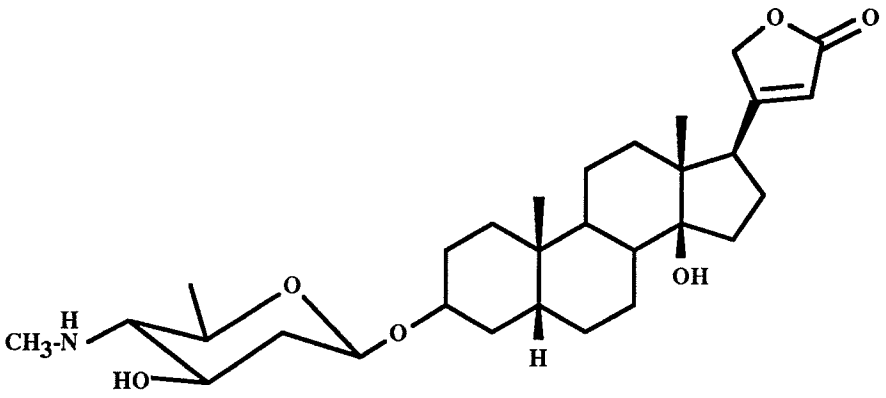
Digitoxigenin 4'-amino-6'-deoxygalactoside

(ASI-222)



Digitoxigenin 4'-amino-4',6'-dideoxylglucoside

(ASI-254)



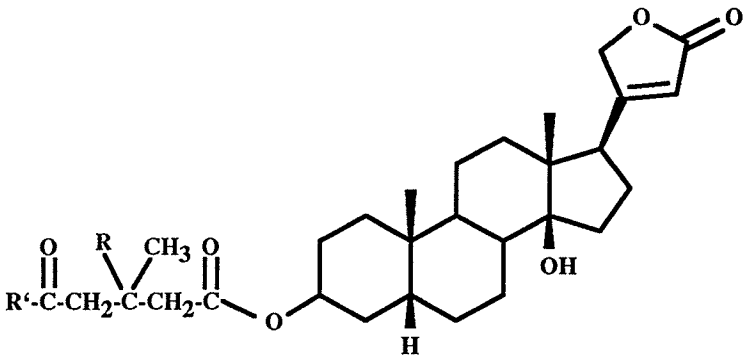
Digitoxigenin 4'-(N-methyl) aminodigitoxoside (mitiphylline)

Figure 9. Aminoglycosides of 3ß-substituted digitoxigenin.

conformation of an amino group at C-4' may not be important in increasing or decreasing the activity. Both ASI-222 and ASI-254 were more potent than digoxin, ouabain and digitoxigenin galactoside. The amino sugars were also reported to differ from the usual digitalis-type compounds as they appeared to have an increased therapeutic index (ratio of effect to toxic doses) (Thomas *et al.*, 1990).

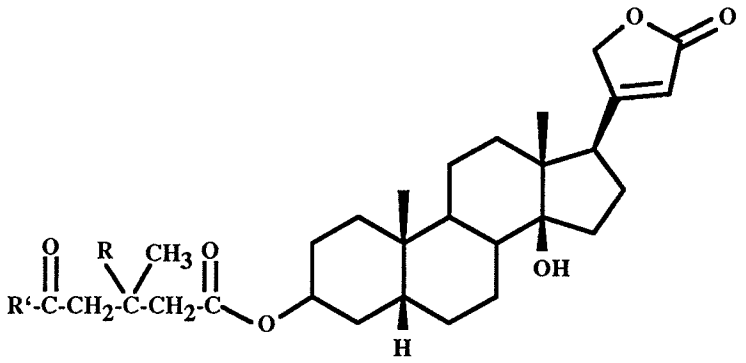
The introduction of non-sugar esters to the 3ß position of digitoxigenin was found to give active derivatives, the most active compounds belonging to the methylglutarate series. The methylglutarate methyl ester (Figure 10) increased the potency of the parent digitoxigenin by 350%, confirming the importance of the hydrophobic methyl side-chain in a position equivalent to the 5'-methyl group of potent glycosides such as the rhamnosides and digitoxosides. The above molecule has no proton-donating groups capable of forming hydrogen bonds (as in the case of the sugar molecules), which suggests that such hydrogen bonds may not be very important in binding the C-3 side-chain. Although the methylglutarate methyl ester side-chain does not increase activity to the same extent as the 5'-methyl sugars this may be due to the non-rigid structure of the side-chain and not to the lack of optimal binding groups. This would reduce activity because of the decreased statistical probability that the groups will be in the correct conformation for binding.

The amides of the series were also found to be quite active. For instance, the amide of the dimethylglutarate (Figure 10) had three times the potency of the



Digitoxigenin methylglutarate methyl ester

(R = H; R‘ = OCH_3)



Digitoxigenin dimethylglutarate amide

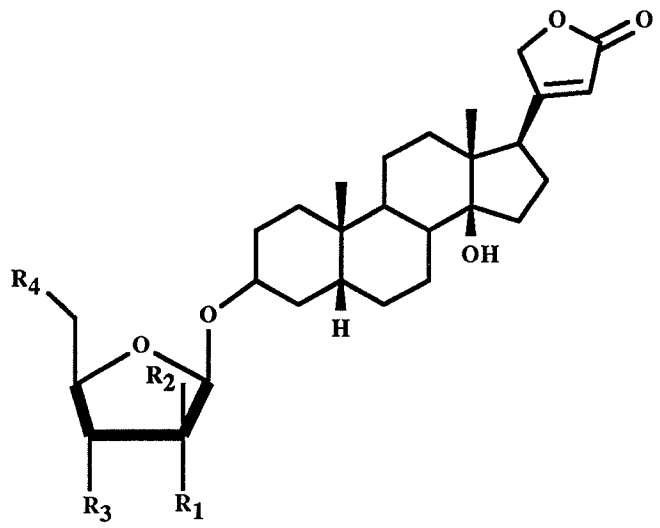
(R = CH_3; R‘ = NH_2)

Figure 10. 3ß-Substituted esters of digitoxigenin.

genin. The amide could be involved in hydrogen bonding but this is clearly not "essential" as the methyl ester also shows comparable potency. Another important finding of Fallon and Thomas (unpublished results) reviewed by Thomas *et al.* (1990) was that the free carboxylic acid anion (where R' = O^-) (Figure 10) was completely devoid of activity. This either implies repulsion by an anionic site in the receptor or that one of the receptor's many free carboxylic acid side-chains is located near the region that binds the first sugar. On this basis Fallon and Thomas suggested that the region of the receptor that binds the first sugar may contain a carboxylic acid moiety and if the sugar contains a 3'- or 4'-OH group, these may hydrogen-bond to the carboxylic acid moiety, thereby increasing potency. However, such binding may not be an essential requirement and the rest of the sugar-binding site appears to bind to the drug through distance-specific bonds, possibly of the van der Waals type.

Dzimiri and Fricke (1988) examined the relationship between the stepwise addition of digitoxose units to digoxigenin and their potency. They found the following:

Compound	Relative potency
Digitoxigenin	1.0
Digoxigenin monodigitoxoside	13.8
Digoxigenin bisdigitoxoside	14.9



R_1	R_2	R_3	R_4
H	OH	OH	OH
(α-L-arabinofuranoside)			
OH	H	OH	NH_2
(5'-aminofuranoside)			
OH	H	OH	N_3
(5'-azidofuranoside)			

Figure 11. 3ß-Substituted furanosides of digitoxigenin.

digoxigenin trisdigitoxoside	10.5
ß-methyl digoxin	7.0

The difference between the potencies of the mono- and bisdigitoxosides was not statistically significant implying that the digitalis receptor does not extend beyond the region of the first sugar. Yoda and Yoda (1975) and Templeton *et al.* (1991a) made a similar observation. This observation has been confirmed in this work for the 14ß-hydroxy-14ß,5ß-pregnanes using the [^{3}H]-ouabain radioligand binding assay (RBA, see below).

(ii) Furanose-type glycosides.

Investigations of the furanose-type glucosides for SAR have been limited when compared to the pyranose-type glycosides because of their lack of availability. Studies by Schwabe and Tschiersch (1982) and Schönfeld *et al.* (1985) showed that the inhibitory action of digitoxin α-L-arabinofuranoside (Figure 11) on human cardiac Na^+, K^+-ATPase was 3 times that of digitoxin. A number of ribofuranosides, 3,6-anhydroglucofuranoside and 3,6-iminoglucofuranosides have been synthesized by Prisbe *et al.* (1986) and found to have moderate to weak cardiotonic activity on isolated guinea-pig atria. The most potent compounds were the 5'-aminofuranoside and 5'-azidofuranoside (Figure 11), being about equipotent with digitoxigenin. Rotation around the C-4', 5' carbon bond of the furanoside would allow a 5'-substituent to approximate closely the position of either an axial

or equatorial substituent at the C-4' of a pyranoside and thus the substituent at the 5'-position of a furanoside may be as important in contributing to binding as is the C-4' position of a pyranoside.

(iii) Summary of the SAR of the glycosides.

The following generalisations regarding SAR of the glycosides have been recently summarized by Thomas *et al.* (1990).

(1) Attachment of the sugar moieties to the genins at the C-3 position generally enhances the overall potency.

(2) A 5'-methyl group present in most potent glycosides such as the digitoxosides, rhamnosides, thevetosides, and gomphosides is not an absolute requirement for high potency because some glycosides *e.g.*, glucosides with a 5'-hydroxymethylene group have also been shown to be highly active.

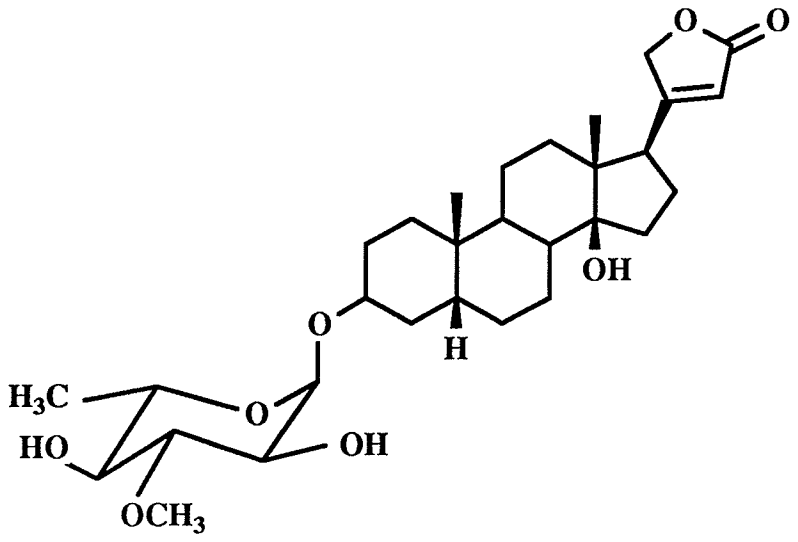
(3) Introduction of a methyl group at the 3α position greatly reduces the activity of both the genin and the corresponding glycoside. Schönfeld *et al.* (1985) concluded that the presence of the methyl group interfered with the binding of the drug to the receptor. Alternatively, a 3α-methyl group may reduce rotation around the glucoside link so that the sugar moieties are unable to take up their most favoured binding conformation.

(4) The exact role of C-3' and C-4' substituents (*e.g.*, OH) in the sugar moiety on binding activity is not clear. For example, the 3'-hydroxyl group in gomphoside should be in an axial position, because epimerization of this

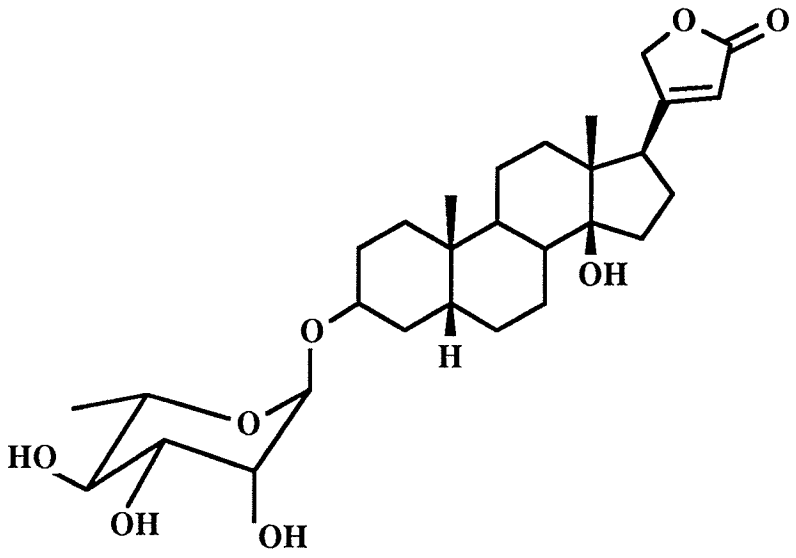
group leads to great loss of activity. Additionally, hydroxylation at C-4' also reduced activity. Alternatively, both the rhamnoside and the digitoxose sugars which have C-4' hydroxyl functions still possess high activity compared with their genins. Furthermore, replacement of the C-4' hydroxyl by an amino function (*e.g.*, ASI-222 and ASI-254) (Figure 9) greatly enhances the potency. In summary, it appears that the C-3' hydroxyl group plays a significant role in binding, especially for the glycosides with *fixed* sugar components, whereas the role of C-4'-OH or C-4'-amino group is at this stage unclear. Thomas *et al.* (1990) suggested that neither the 3'-nor the 4'-hydroxyl groups are essential for binding but both can contribute to binding if appropriately positioned by the overall conformation of the sugar moiety.

(5) It appears that the C-4' amino group as in ASI-254, ASI-222, the 4'-aminorhamnoside and holarosine B or an N-methyl amino group as in mitiphylline (Figure 9), has some advantage, in that these compounds are all stronger than, or equipotent to the corresponding 4'-hydroxy compounds.

(6) The role of the C-2' hydroxyl group in α-L linked sugars appears to be important for binding activity. For example, acetylation of the C-2' hydroxyl in digitoxigenin α-L-rhamnoside (Figure 12) caused a large decrease in activity (*ca.* 40-fold). Alternatively, methylation (C-3' hydroxyl group) and also formation of the 2',3'-isopropylidene linkage (Figure 6),



Digitoxigenin thevetoside



Digitoxigenin 3ß-α-L-rhamnoside

Figure 12. 3ß-Substituted glycosides of digitoxigenin.

only resulted in a loss of between 1/3 to 1/4 of the activity. The greater loss in activity found with the acetyl group may be due to its larger bulk. The thevetoside, however, is even more unusual in that the C-3' hydroxyl group is methylated and the C-2' hydroxy group is directed away from the receptor. At the present time, there appears to be no explanation as to why thevetoside has a significantly higher activity than digitoxin 3'-O-methylrhamnoside (Figure 12).

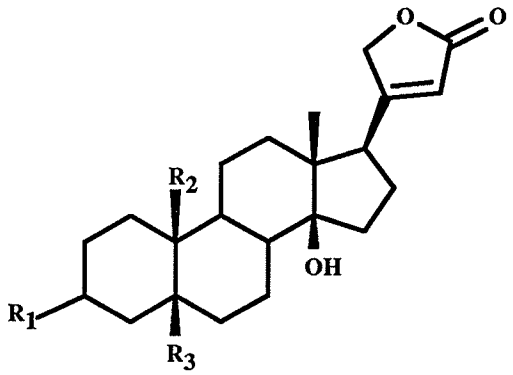
(7) Derivatization of any of the sugar hydroxyl groups (*e.g.*, acetylation, methylation) generally leads to loss of activity.

(8) Changes in the sugar type (*e.g.*, α-L-rhamnoside to α-D-mannoside) do not necessarily cause loss of potency.

(9) The furanose sugars do not appear to be as active as the pyranose sugars (Prisbe *et al.*, 1986). However, further investigation is required to elucidate SAR in this area.

III. Effect of non-sugar substituents at the C-3 position.

The aglycone formed by removal of the sugar units allows the synthesis of C-3 substituted derivatives. Usually this resulted in compounds with greater potency than the free genin. Epimerization of the 3α-hydroxyl group in digitoxigenin via 3-ketodigitoxigenin (Figure 13), resulted in a decrease in activity (Brown *et al.*, 1962; Tamm, 1963; Flasch and Heinz, 1978). 3- Deoxydigitoxigenin was found to be as potent a cardiotonic agent as digitoxigenin on the frog heart



R_1	R_2	R_3
=O	CH_3	H
3-Ketodigitoxigenin		
H_2	CH_3	H
3-Deoxydigitoxigenin		
$O-C(=O)-CH_2NH(CH_2)_4NH_2$	CHO	H
N-(4'-amino-n-butyl)-3ß-aminoacetylstrophanthidin		
OH	CHO	OH
Strophanthidin		
$O-C(=O)-CH_2Br$	CHO	OH
Strophanthidin 3-bromoacetate		
$O-C(=O)-CH_2Br$	CH_2OH	OH
Strophanthidol 3-bromoacetate		
$H_3C-C_6H_4-SO_2-O-CH_2-C(=O)-O$	CHO	OH
Strophanthidin-3-tosyloxyacetate		
$H_3C-C_6H_4-SO_2-O-CH_2-C(=O)-O$	H	H
Digitoxigenin-3-tosyloxyacetate		

Figure 13. 3ß-Substituted esters of digitoxigenin and strophanthidin.

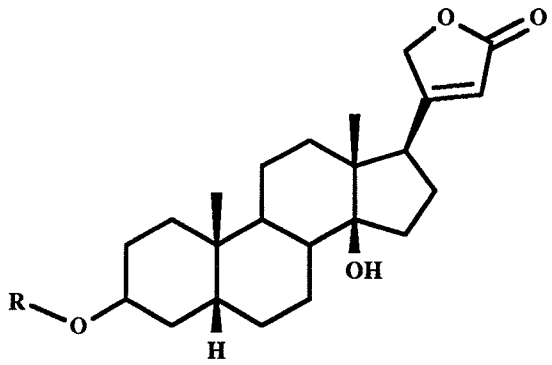
(Takeda *et al.*, 1970) and around 0.6 times as potent with respect to inhibition of Na^+, K^+-ATPase (Witty *et al.*, 1975). When acetylated at C-3, the potency is reduced (Brown *et al.*, 1962; Abramson and Cho, 1971). Valcavi *et al.* (1981) synthesized a series of 3ß-(α-aminoacid) esters of digitoxigenin that were found to have marked inotropic activity and a rapid onset of action in isolated guinea-pig heart. However, these compounds were found to be inactive following oral administration. A similar 3ß-amino compound, namely N-(4'-amino-n-butyl)-3ß-aminoacetyl strophanthidin (Figure 13) was found to bind strongly to rabbit kidney Na^+, K^+-ATPase (Kyte, 1972). It was proposed by Kyte that under the appropriate conditions the secondary amino group on the side-chain and the free carbonyl group in the open lactone interact with surface charges on the enzyme.

The work of Hokin *et al.* (1966) and Kupchan *et al.* (1967) showed that a number of 3ß-haloacetate and 3ß-azidoacetate derivatives of strophanthidin were active on both isolated guinea-pig heart and guinea-pig brain Na^+, K^+-ATPase and were found to inhibit Na^+, K^+-ATPase irreversibly. It was discovered that these compounds inhibit Na^+, K^+-ATPase by alkylating sulphydryl groups on the enzyme at or near the digitalis binding site. A similar study by Abramson and Cho (1971) found that haloacetate derivatives of digitoxigenin and digoxigenin inhibit Na^+, K^+-ATPase irreversibly.

The 3-bromoacetates of strophanthidin and strophanthidol (Figure 13) were extensively studied (Okita, 1977) and evidence to suggest a dissociation between

the inhibition of Na^+, K^+-ATPase and the inotropic effect (therapeutic effect) among these compounds was obtained. Thomas *et al.* (1990) stated that the inhibition of the enzyme (Na^+, K^+-ATPase) may be responsible for the toxic effect of these compounds but not for their inotropic action. Thomas *et al.* (1970) found that the positive inotropic effect of both compounds was readily reversible, but that there were relatively high concentrations of [^{3}H]-strophanthidol 3-bromoacetate in the myocardium after the inotropic effect had been washed out which also indicated that the inotropic response was not dependent on Na^+, K^+-ATPase inhibition. More recently, Fricke *et al.* (1979, 1981) concluded that the inotropic and toxic effects of a series of bromoacetates, and tosyloxyacetates of strophanthidin (Figure 13) and digitoxigenin were found to be fully reversible. This indicated a correlation between lipophilicity and alkylating potency such that the degree of irreversiblility of Na^+, K^+-ATPase increased with increasing lipophilicity. It was consequently suggested that the more lipophilic compounds may have easier access to the receptor site.

Another group of C-3 modified derivatives that contain ester moieties are the 3ß-hemiesters. The 3-hemisuccinate (Figure 14) of digitoxigenin and the 3-hemisuccinate methyl ester as well as other esters and salts of various cardenolides were reported by Zingg and Meyer (1957) to have no activity on cat heart. Thomas *et al.* (1990) reviewed the work of Kössler that involved digitoxigenin 3ß-hemisuccinate and its sodium salt, methyl ester and ethylene glycol ester (Figure

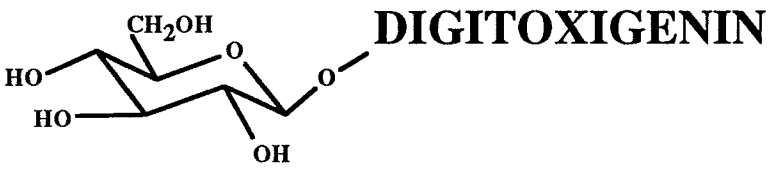


R = $O-\overset{\overset{O}{\|}}{C}-CH_2CH_2CO_2H$	Digitoxigenin 3-hemisuccinate
R = $O-\overset{\overset{O}{\|}}{C}-CH_2CH_2CO_2CH_3$	Digitoxigenin 3-hemisuccinate methyl ester
R = $O-\overset{\overset{O}{\|}}{C}-CH_2CH_2CO_2(CH_2)_2OH$	Digitoxigenin 3-ethylene glycol succinate
R = $O-\overset{\overset{O}{\|}}{C}-CH_2\overset{\overset{CH_3\ CH_3}{\diagdown\ \diagup}}{C}-CH_2CO_2H$	Digitoxigenin 3-dimethylglutarate acid
R = $O-\overset{\overset{O}{\|}}{C}-CH_2CH_2CH_2CONH_2$	Digitoxigenin 3-glutarate amide
R = $O-\overset{\overset{O}{\|}}{C}-CH_2OCH_2CONH_2$	Digitoxigenin 3-diglycolate amide

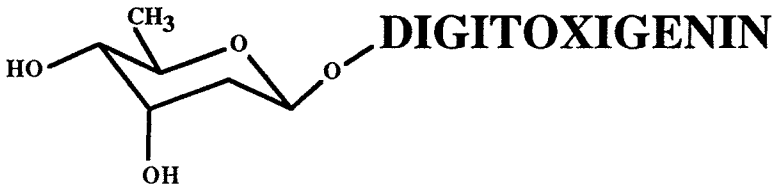
Figure 14. Further 3ß-substituted esters of digitoxigenin.

14). Although, the compounds were said to be active, no data were presented and when the toxicity of those compounds was examined their lethal doses were similar to that for digitoxigenin. The authors indicated that the therapeutic index of digitoxigenin ethylene glycol succinate was greater than that of digitoxigenin. Segel (1962, 1965) reported the synthesis of digitoxigenin 3ß-hemisuccinate and its sodium salt, methyl ester and ethylene glycol ester. Both Segel and Kössler suggested that the rationale behind the synthesis of the succinate derivative was to produce a compound with greater hydrophilicity in comparison with the standard digitalis-type compounds.

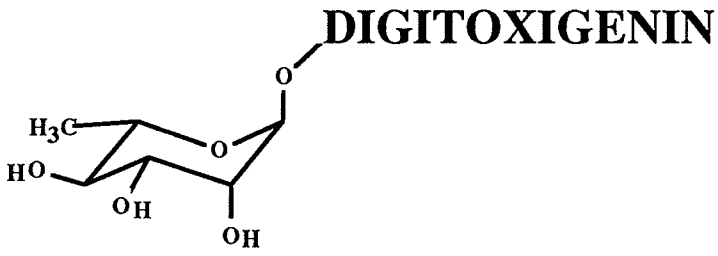
More recently, Thomas *et al.* (1990) reviewed recent unpublished work by Fallon and Thomas who synthesized a series of 3-hemiester free acids, their amides and methyl esters. These derivatives were then tested for cardiotonic activity on isolated guinea-pig atria and for their ability to inhibit guinea-pig cardiac Na^+, K^+-ATPase. Compared with digitoxigenin all the acid derivatives showed a significant reduction in inotropic activity on isolated guinea-pig atria and Na^+, K^+-ATPase. Among the esters the most potent compound was the dimethyl glutarate acid which had around 30% of the activity of digitoxigenin whereas the amides were at least equipotent to or more potent than digitoxigenin. The weakest compound was the glutarate amide (Figure 14) which had 80% of the activity of digitoxigenin. On the other hand, the strongest compound was the diglycolate amide which had almost twice the activity of digitoxigenin. The behaviour of the methyl ester derivatives



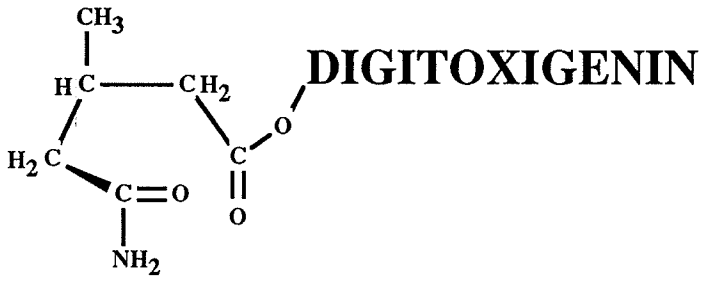
ß-D-Glucoside



ß-D-Digitoxoside



α-L-Rhamnoside



Methylglutarate amide

Figure 15. 3ß-Substituted glycoside and aminoacid derivatives of cardiac glycosides.

was interesting in that they produced an initial cardiotonic action, which was then followed by an apparent negative inotropy after each dose. This effect occurred over the range of concentrations tested and even at higher doses there did not appear to be a toxic effect. Similar behaviour but to a lesser extent did occur after dosing with the diglycolate. In general, addition of an acidic side-chain at the C-3 carbon resulted in marked reduction in potency in comparison to digitoxigenin. However, the methyl ester and amide derivatives were generally equipotent to digitoxigenin. The low activity of the acids was seen as supporting the suggestion of Kyte (1972) and Thomas *et al.* (1974b) that the C-3 binding region contains a nucleophilic group or a negative charge, since the acidic side-chain would be expected to be ionized at biological pH, resulting in a repulsive force between the molecule and the receptor. This repulsive force would not occur with the ester or amide derivatives and hence their ability to bind to the receptor was not decreased. Furthermore, it was suggested that since the side-chains have some degree of flexiblity it might be possible for the molecule to adopt a conformation that resembles that of the C-3 sugars in cardiac glycosides (Figure 15). It was further suggested that once in that conformation the methyl group(s) might take up a position approximating that of the 5'-methyl group of 6'-deoxy sugars. Hence the activities of the methyl- and dimethylglutarates support the conclusions of Yoda and others (discussed above) in relation to the important structural requirements for binding of the sugar moieties at C-3. The difference in potency between the 6'-

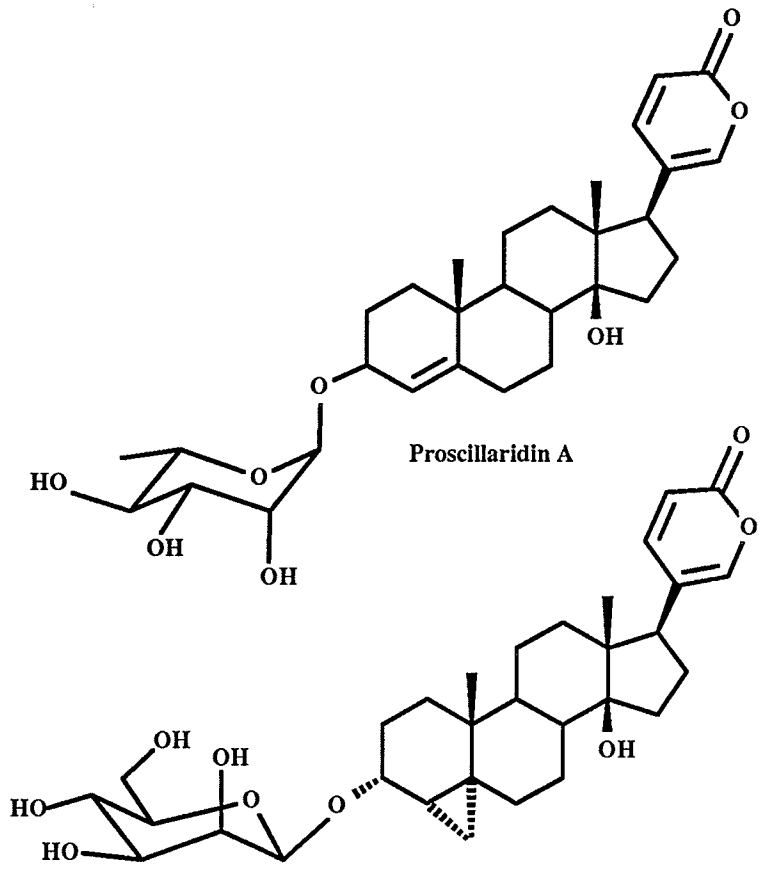
deoxyglycosides *e.g.*, digitoxigenin rhamnoside and digitoxigenin monodigitoxoside, and the methylglutarate derivatives was said to reflect the flexibility of the chain in the latter group of compounds, leading to the greater possibility of the side-chain taking up a less favourable conformation for binding. Another point noted by Fallon and Thomas was that the 3-methylglutarate side-chain had an asymmetric carbon. However, no attempt was made to resolve the two enantiomers and thus the results reported represent the activity of a racemic mixture.

Other analogues with polar substituents at C-3 studied were the conjugated metabolites of digitoxigenin and digoxigenin. The sulphate and glucuronide conjugates of these genins were found by Flasch and Heinz (1978) to be ysignificantly less potent than the aglycones when tested on isolated Na^+, K^+-ATPase. A related series of compounds *i.e.*, the alkyl- and cycloalkylsulphonates of digitoxigenin, were prepared by Siemann *et al.* (1978) and, although the compounds were said to produce a weaker cardiotonic effect than digitoxigenin no biological data were given.

IV. Effect of 3α-methyl substitution on the action of cardiac glycosides.

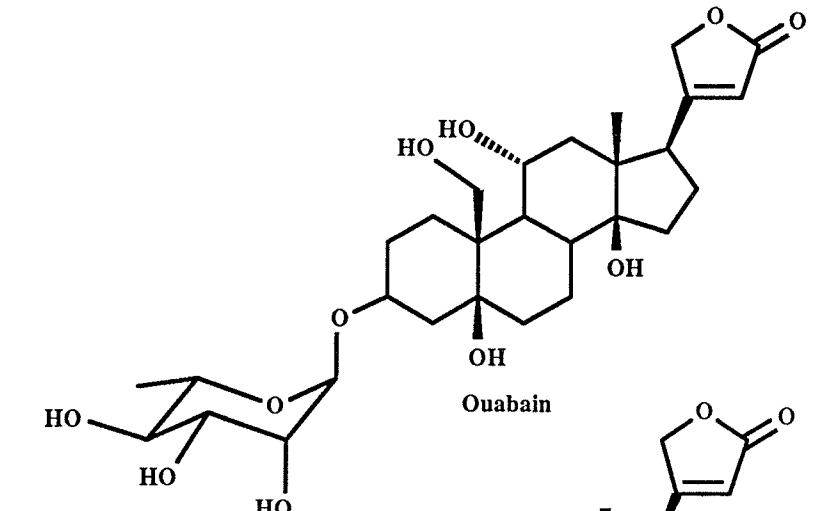
The effect of branched groups attached at C-3 has also been investigated. Siebeneickh and Hoffmann (1978) examined a series of 3α-substituted derivatives of digitoxigenin 3ß-substituted derivatives of epi-digitoxigenin for inhibition of Na^+, K^+-ATPase from beef brain and dog heart. For both series the presence of a branched chain generally reduced the inhibitory potency relative to that of the

unbranched compound. It was suggested that increased steric shielding of the 3ß-hydroxyl resulted in reduced activity, although it should be noted that epi-digitoxigenin derivatives with branched groups containing a hydroxyl moiety appear to have somewhat higher activity. This indicated that a hydroxyl group in the 3ß-side-chain mimicked the action of either the 3ß-hydroxyl or glycoside hydroxyl groups. Zahorsky (1980) found that 3α-methyldigitoxigenin glucoside was 1/10 as active as digoxin but its maximum inotropic effect was greater, indicating an improvement in therapeutic ratio. Lüllmann and Mohr (1982) and Lüllmann and Peters (1982) found that 3α-methyldigitoxigenin glucoside bound to guinea-pig heart muscle at the same receptor site as ouabain but with a 10-fold lower affinity. The lower affinity was said to be a result of a very fast dissociation rate (Mohr, 1983). Further work by Lüllmann *et al.* (1984) examined the degree of correlation between the inotropic effect on isolated guinea-pig atria and the extent of inhibition of Na^+, K^+-ATPase for a series of four glycosides *e.g.*, proscillaridin A, 3α-hydroxy-4α,5α-cyclopropanoscillarenin ß-D-mannoside, ouabain and 3α-methyldigitoxigenin glucoside (Figure 16). They found that the inotropic response did not correlate with the degree of Na^+, K^+-ATPase inhibition. Furthermore, compounds such as 3α-methyldigitoxigenin glucoside which exhibit a fast onset of action produced greater maximal increases in their force of contraction before toxicity was observed. Hence it was postulated that the inotropic effect of cardiac glycosides was a result of the number of associations and dissociations (between

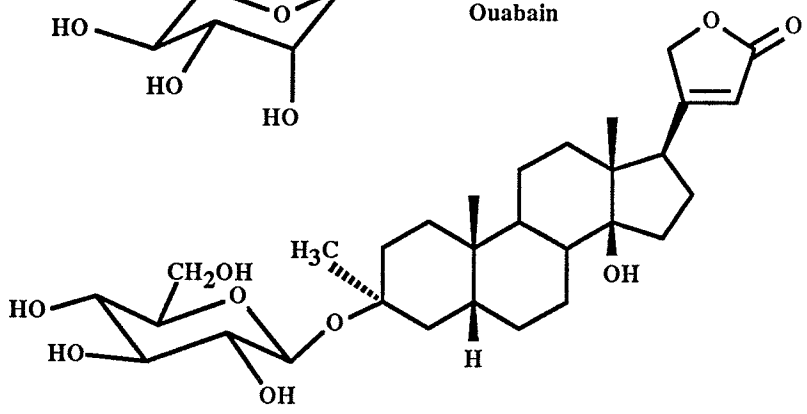


Proscillaridin A

3α-Hydroxy-4α,5α-cyclopropanoscillarenin ß-D-mannoside



Ouabain



3α-Methyldigitoxigenin 3ß-ß-D-glucoside

Figure 16. Strucures of bufadienolides and butenolides.

the receptor and the inhibitor) whereas the toxic effects were due to irreversible binding of the drug to the receptor. However, this theory was not supported by Brown and Erdmann (1984), who found that both ouabain and 3α-methyldigitoxigenin glucoside caused the same maximal increase in force on isolated guinea-pig atria. There is evidence to suggest that the oxygen at C-3 is not essential but may be replaced by other electron-rich groups. Abramson *et al.* (1976, 1977) found that the 3ß-thiocyanate and 3ß-thioacetate derivatives of digitoxigenin were cardioactive. In fact, the 3ß-thioacetate had the same cardiotonic activity as digitoxigenin but appeared to be less toxic (on intact rat heart) and hence possessed a greater therapeutic index. A considerably different steroid structure, prednisolone-3,20-bisguanylhydrazone (Figure 5) showed a strong cardiotonic activity and yet did not have the structure normally associated with cardiac glycosides (Schütz *et al.*, 1969). The activity of these type of compounds seems to be independent of the substituents on the steroid and the location of the guanylhydrazone groups does not appear to be critical. Thomas *et al.* (1974b) proposed that the region of the receptor that binds the C-3 position of digitalis might contain a strong nucleophilic group. They suggested, that because these compounds are positively ionized at physiological pH, they might be able to bind to the receptor via an ion-pair type interaction. In addition, because the charge in the guanylhydrazone side-chain can be distributed over three atoms, there is an allowance for flexibility in the position of the group.

V. Effect of structural changes to the genin.

The classical SAR studies for cardiac glycosides (Tamm, 1963; Chen 1963) concluded that the following three features present in the digitoxigenin ring system (Figure 1) were all essential for activity:

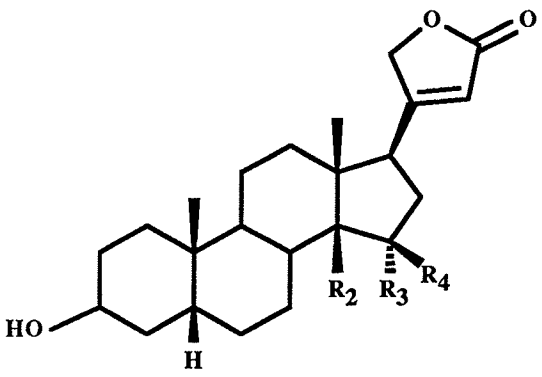
(1) The 17ß-lactone.

(2) The cis configurations of the A/B and C/D ring junctions.

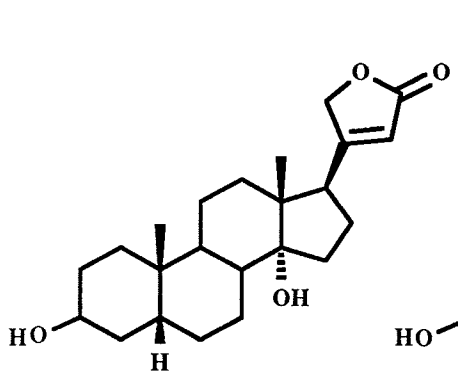
(3) The 3ß- and 14ß-hydroxyl groups.

(i) Changes at C-14/C-15.

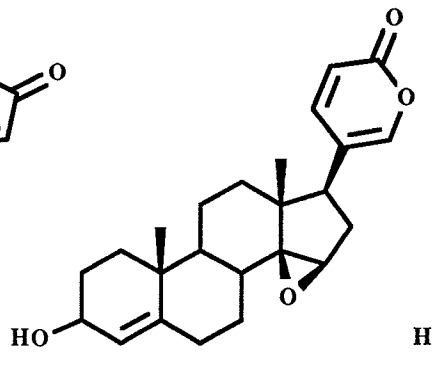
A series of digitoxigenin analogues substituted with a 15-hydroxyl group were tested on isolated frog hearts by Shigei *et al.* (1973). 15ß-Hydroxy-digitoxigenin was around 1/10 as active as digitoxigenin, whereas the 15α-hydroxyl or 15-keto derivatives were inactive (Figure 17). Although removal of the 14ß-hydroxyl group does not abolish activity, its presence significantly increases activity and it probably has a direct binding role. Naidoo *et al.* (1974) suggested that the 14ß-hydroxyl group may be involved in hydrogen bonding to the receptor surface. Replacement of the 14ß-hydroxyl group by a chlorine atom, for example, in 3ß-acetoxy-15α-hydroxy-14ß-chlorodigitoxigenin resulted in a slightly increased inhibition of myocardial Na^+, K^+-ATPase. Alternatively, the 14ß-dehydro compound (14α-H or 14ß-H) has 1/10 the potency of digitoxigenin. Zurcher *et al.* (1969) found that 14-epi-digitoxigenin (Figure 17) was inactive on guinea-pig myocardial Na^+, K^+-ATPase. Similarly, Schmidt *et al.* (1979) found that a series



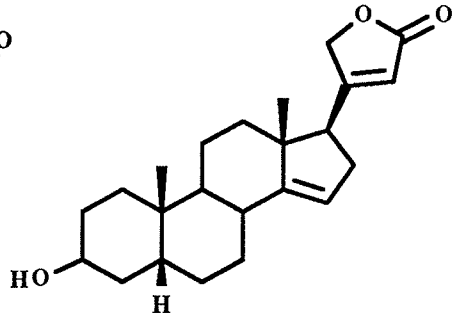
R_2	R_3	R_4	
OH	OH	H	15ß-Hydroxydigitoxigenin
OH	H	OH	15α-Hydroxydigitoxigenin
OH	-------- O --------		15-Ketodigitoxigenin
H	H	H	14ß-Hydrodigitoxigenin



14-Epidigitoxigenin



14ß,15ß-Epoxyproscillaridigenin



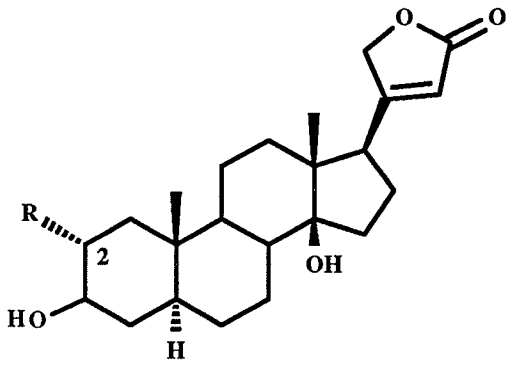
14-Enedigitoxigenin

Figure 17. Structures of digitoxigenin analogues that involve structural alterations of 14- and 15-positions.

of 14- and 15-amino analogues of digitoxigenin were around 100 times less potent than digitoxigenin in inhibiting Na^+, K^+-ATPase. Other changes to the C-14 region of the molecule were described by Lindner *et al.* (1979), who found the 14,15-epoxide analogue of proscillaridin (Figure 17) had approximately 1/4 of the activity of the parent. The same results were reported by Schönfeld *et al.* (1985), who tested a number of genins with a 4,5-double bond and the 17ß-lactone ring replaced by a pyridine structure. In these compounds, the change from the 14ß-hydroxyl group to a 14,15-epoxide reduced activity 14-fold. Additionally, a change from the 14,15-epoxide to the 14,15-ene further reduced activity 7-fold. Finally, Fullerton *et al.* (1977) found that the 14-ene of digitoxigenin (Figure 17) was devoid of cardiotonic activity when tested on isolated guinea-pig atria.

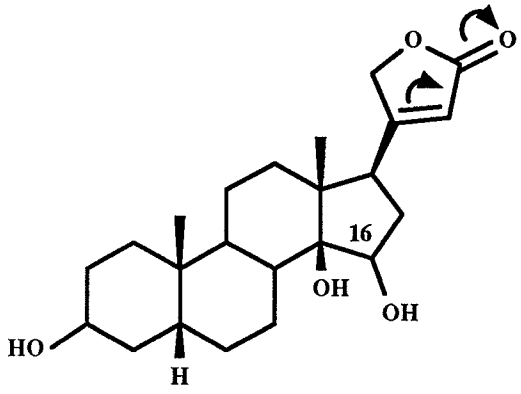
(ii) Hydroxylation and esterification at C-16.

In support of the above observations, it was found that further hydroxylation of the steroid generally decreased activity (De Pover and Godfraind, 1982; Brown *et al.* 1983). For example, the dissociation constant (K_D) values for inhibition of beef heart Na^+, K^+-ATPase for digitoxin, digoxin (12ß-hydroxydigitoxin) and gitoxin (16ß-hydroxydigitoxin) were 1.1, 3.6 and 15 $\times$ 10^{-9} M with parallel potencies for inotropic activity on guinea-pig atria (Brown *et al.*, 1983). In another example, the hydroxylated derivatives of uzarigenin had significant loss of activity. Brown and Thomas (1984) found that 2α-hydroxyuzarigenin (Figure 18) had 1/20 the potency of uzarigenin. In a further study, these authors and others (Schönfeld

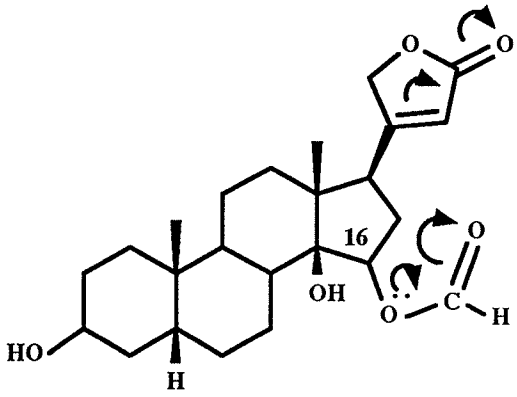


R = H Uzarigenin

R = OH 2α-Hydroxyuzarigenin

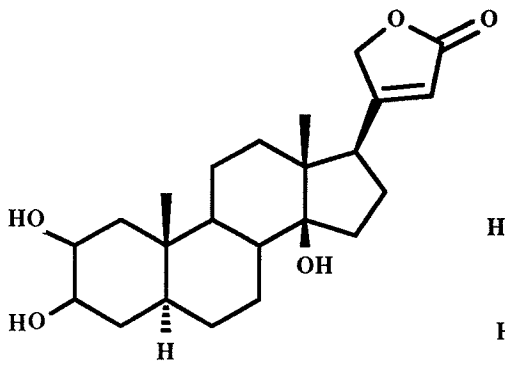


Gitoxigenin

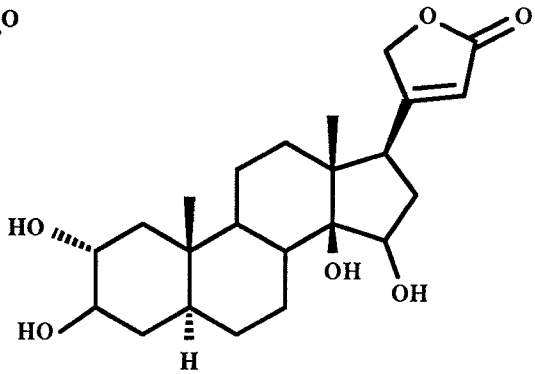


Gitaloxigenin

[Molecular model and structure of gitaloxigenin, (De Pover and Godfraind, 1982)]



Gomphogenin



Afrogenin

Figure 18. Cardiac glycoside genin structures.

et al., 1985), also reported that hydroxylation of digitoxigenin led to a loss of activity. For example, digoxigenin, ouabagenin, diginatigenin, and gitoxigenin are all at least 5-10 times less potent than digitoxigenin. In addition, it would appear that acetylation or nitration of these hydroxyl groups further reduces activity (Haustein, 1974). Thomas *et al.* (1990) suggested that the loss of activity upon further hydroxylation of the steroid may mean that either the portion of the receptor that binds the steroid has hydrophobic characteristics, or that additional hydroxyl groups interfere with the tight fit of the molecule in the receptor cleft.

An interesting observation was made by De Pover and Godfraind (1982) who formylated the 16ß-OH of gitoxin to produce gitaloxin, which increased activity by a factor of 41. The corresponding 16α-OH on formylation also showed increased activity but to a lesser degree. These authors suggest that the formylated side-chain is involved directly in binding to the receptor because of its strong dipole character (Figure 18) and speculated that it may be binding to the same site as the lactone. More recently, however, Griffin *et al.* (1986) and Hashimoto *et al.* (1986) proposed a second binding site on the enzyme that interacts specifically with the 16ß-formyl group. Fullerton *et al.* (1986) found that the 16ß-hydroxyl and corresponding formate and acetates alter the conformation of the D-ring, bringing the lactone carbonyl into a position that is more favourable for hydrogen bonding. However, this effect was not enough to account for the increased potency and Fullerton *et al.* (1986) have suggested that the formyl group may bind to an

additional site separate from the lactone-binding site and thus it can reinforce the overall binding. Alternatively, studies on the sensitivity of the proposed C-16 binding site to changes in configuration and also to the bulk of the substituted group at this position showed that increases in the bulk of the ester group led to loss of potency. For example, the 16ß-acetyl compounds have usually about 1/2 the potency of the corresponding formyl derivatives (Haustein and Glusa, 1980).

(iii) Effect of the C-3 glycoside on the genins.

The enhancement of potency was found to increase much more if the genins were linked with the sugars through the 3ß-hydroxyl group. The 3ß-hydroxyl group does not contribute to binding but provides an essential point of attachment for the sugar residues. For instance, the addition of a 6'-deoxy sugar (*e.g.*, ß-D-digitoxose or α-L-rhamnose) component to most genins leads to a 10-fold increase in activity. For extremely weak genins (*e.g.*, gomphogenin, afrogenin) (Figure 18) the magnitude of the increase was of the order of 100-400 fold.

(iv) Stereochemistry of the A/B ring junction.

It was generally reported by reviewers that the presence of the A/B ring *trans* configuration was associated with a significant loss of biological activity. However, earlier toxicity studies in the cat had identified some 5α-H steroids such as frugoside with potent cardiotonic properties. Using guinea-pig atria, Brown and Thomas (1984) found that uzarigenin (a 5α-H cardiotonic steroid) which differs only in the configuration of its A/B ring junctions which was approximately

equipotent with digitoxigenin. However, these authors found that the attachment of sugars to the 3ß position of both genins produced very dissimilar results. For example, they found that glucosidation of uzarigenin reduced activity in the following order: 63% for the monoglucoside, 90% for the bisglucoside and 82% for the trisglucoside, whereas glucosidation of digitoxigenin increased activity by almost 300%. On the other hand, the 6-deoxyalloside and rhamnoside of uzarigenin were respectively, 2.2 and 7.8 times as potent as the parent. Rhamnosidation also increased the potency of digitoxigenin by an even greater amount (22 times) but the results show that 5α-H steroids can be very potent glycosides. For example, uzarigenin rhamnoside is 4 times as potent as digoxin and twice as potent as ouabain, although the latter is also a rhamnoside. These results were confirmed by [^{3}H]-ouabain displacement studies using beef heart Na^+, K^+-ATPase. The authors concluded that the A-ring of the cardiotonic steroids probably does not play an important role in the binding of cardiotonic steroids to the receptor but is important in positioning the sugar moiety.

In another study, Gelbart and Thomas (1978) attached guanylhydrazone groups to a series of C/D *trans* steroid systems having a variety of A/B ring junction types (*cis*, *trans*, quasiplanar). All compounds were shown to be weak inhibitors of guinea-pig myocardial Na^+, K^+-ATPase and all had "negative" inotropic effects on isolated guinea-pig atria. It is significant that the attachment of lactone isostere side-chains were more effective than the guanylhydrazone group

when attached to the "classical" cardiotonic steroid but were inactive (up to concentration of 1×10^{-4} M) when attached to any of the C/D *trans* steroids. This suggests that the close approach to the receptor is more critical for the lactone isosteres than for the guanylhydrazone group and hence that a key role of the C/D *cis* configuration of the classical steroid is to position the lactone so that it can become very closely aligned with the receptor. These studies also imply that the C/D *cis* configuration is not an absolute requirement for digitalis activity at least with respect to inhibition of Na^+, K^+-ATPase, but that it does play an important role in positioning the 17ß side-chain.

Conclusions regarding changes in the genin, based on various synthetic and semi-synthetic glycosides tested for cardiotonic action, were reported by Haustein and co-workers (Haustein *et al.*, 1973; Haustein and Hauptmann, 1974; Haustein, 1974) and can be summarized as follows:

(1) Substitution of groups into those parts of the steroid that may face the receptor site reduce the potency, *e.g.*, digitoxigenin to digoxigenin.

(2) Functional groups located on the α-side of the steroid may lead to increased potency by interacting with a complementary binding site as yet undetermined.

(3) Acetylation of the hydroxyl group at C-16 (either α- or ß-configurations) causes a marked increase in potency, whereas acetylation of hydroxyl groups in other positions reduces activity.

(4) Alterations of the 14ß-hydroxyl group in the classical cardiac glycosides results in a significant loss of activity.

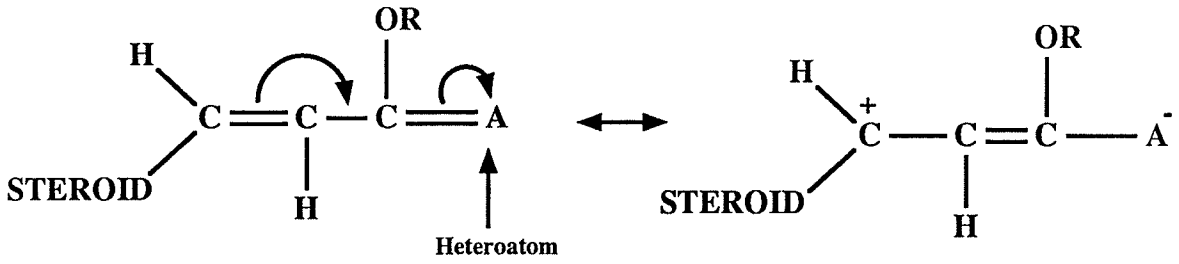
VI. Effect of the C-17 group in 14ß-OH derivatives.

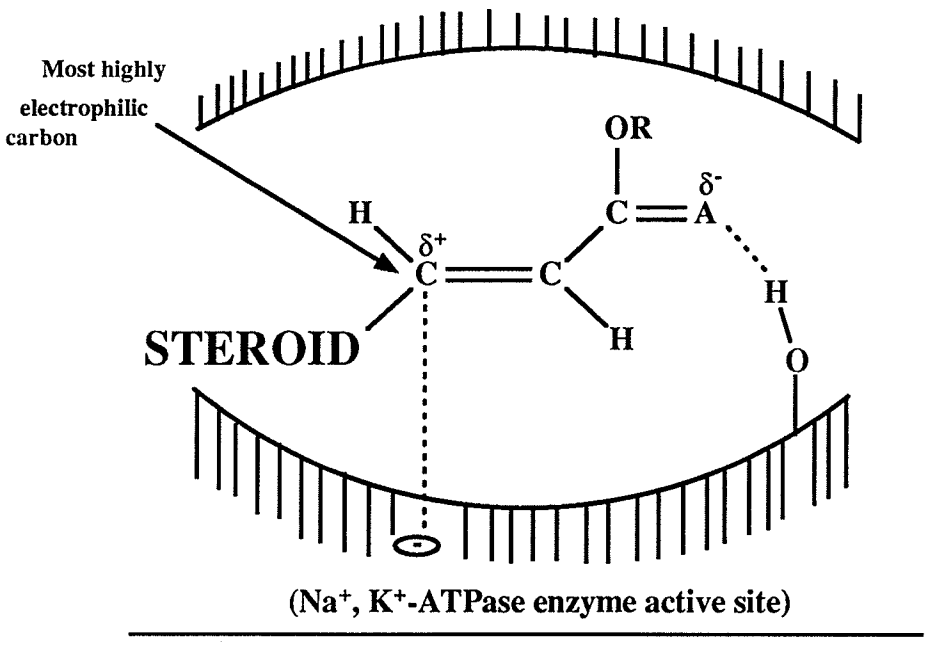
(i) Nature of the α,ß-unsaturated γ-lactone ring interaction.

It has been thought that the C-17 α,ß-unsaturated γ-lactone ring was the first point of attachment between the steroid and the receptor. It should therefore be the most important functional group of the cardiac glycosides for cardioactivity (Tamm, 1963). Repke and Portius (1963b) also believed that cardiac glycosides should have an unsaturated lactone ring and it is known that reduction of the 20,22-ene or epimerization at the C-17 position results in almost complete loss of activity. For example, Tamm (1963) found that the 17α-isomer of digitoxigenin was devoid of activity. Portius and Repke (1964) suggested that the lactone was bound to a receptor by a hydrogen bond, However, this idea could not explain all the structure-activity data and suggests that forces in addition to hydrogen bonding must be involved (Thomas *et al.*, 1974a). Fullerton *et al.* (1986) reviewed the work of their co-workers who used X-ray crystallography and computer graphics to establish that the common feature that correlated with activity was the position of the carbonyl group. From this they concluded that the hydrogen-bonding potential of the lactone carbonyl and its bioisosteres was the only molecular feature relevant to the binding of the 17ß side-chain to the receptor.

Thomas *et al.* (1974a,b) found that, apart from hydrogen bonding, the

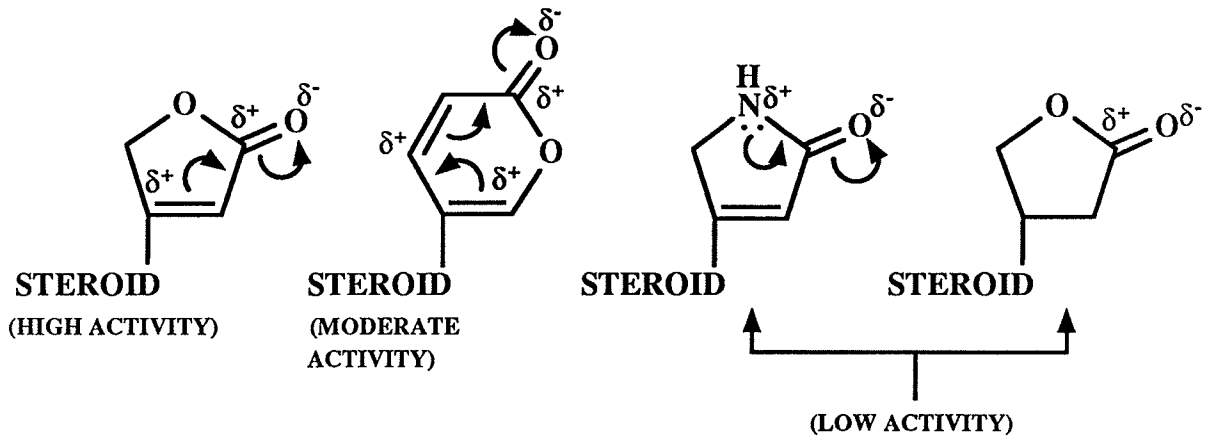
lactone can be involved in charge transfer complex formation and ion-dipole and dipole-dipole interactions, and that the lactone could be replaced by an open chain α,ß-unsaturated group. They proposed that the α,ß-unsaturated γ-lactone or its open-chain analogues were bound to the receptor by a "two-point attachment" involving a hydrogen bond and an ion-dipole interaction on the ß-carbon (Figure 19). In subsequent studies, Thomas *et al.* (1990) further suggested that a dipole with the positive pole on C-20 was a feature of all active compounds. They tested this by determining the chemical shift in the ^{13}C NMR spectrum at C-20 of a series of 17ß side-chains, and found that there did appear to be a correlation between the size of the shift and activity (Figure 19 cont'd). This correlation exists because the size of the chemical shift is determined by the degree to which electron density has been withdrawn from the atom, *i.e.*, it determined by the size of the fractional positive charge. Those compounds with a small chemical shift at C-20 proved to be inactive. However, steric factors also were present that could have prevented the close approach needed for significant ion-dipole bonding. The possible importance of these is illustrated by the low activity of the C-21 substituted ester (Figure 19 cont'd) which has *ca.* 10% the activity of the non-substituted ester (Thomas *et al.*, 1974b). The presence of the C-21 methyl group skews the planarity of the conjugated system, and thus impedes the flow of electrons from C-20 to the carbonyl group. The methyl group may also provide steric hindrance to binding.



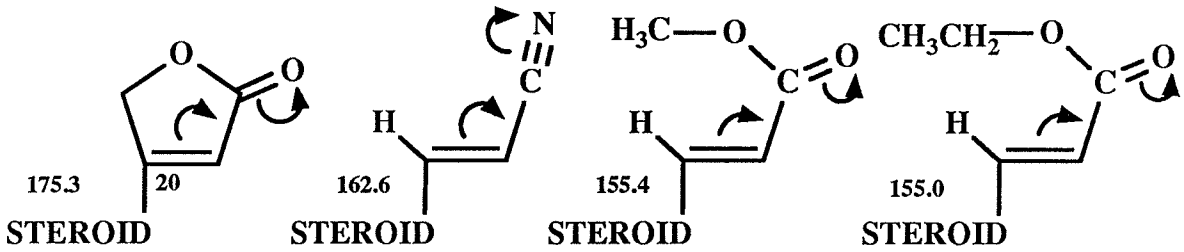


(Proposed model of interaction of α,ß-unsaturated γ-lactone with receptor active site of Na^+, K^+-ATPase enzyme by Thomas *et al.* (1974a,b))

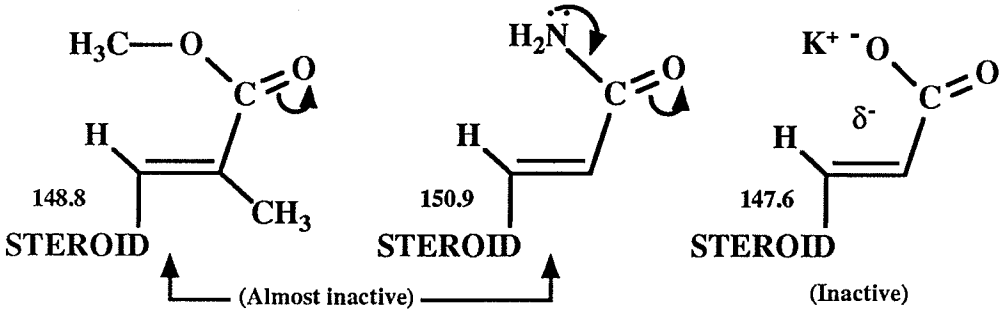
Figure 19. Model for α,ß-unsaturated γ-lactone interaction with the receptor active site of Na^+, K^+-ATPase.



(The resonance effects in 17ß-lactone and the relationship of these to biological activity. Thomas *et al.* (1990))



(Decreasing order of activity with decreasing the chemical shift of C-20 in ^{13}C NMR (ppm))



(The higher the value, the greater is the fractional positive charge. The fractional charge is negligible at values below 150 pmm. Thomas *et al.* (1990))

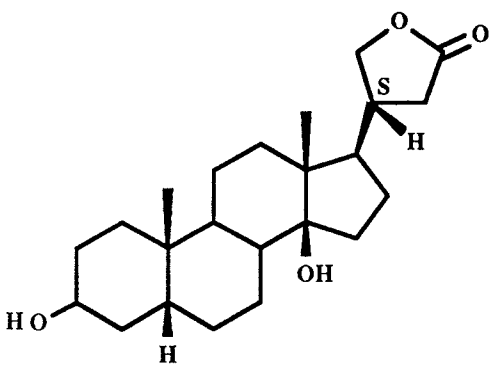
Figure 19 cont'd. Relative potency and C^{13} NMR data for digitoxigenin analogues.

More recently, Thomas *et al.* (1990) reviewed unpublished work by Gray (1988) who obtained unexpected results when he tested the bis-nitrile derivative of the mono-nitrile shown in Figure 19 cont'd and the 21-cyano-21-ethoxycarbonyl analogue, both of which were inactive. Both of these showed chemical shifts of 175.9 and 169.6 ppm respectively in their ^{13}C NMR spectra. The low activity of these compounds did not support the previous suggestion that a correlation existed between electron deficiency on C-20 (as measured by the shift in the ^{13}C NMR spectrum) and biological activity (Smith *et al.*, 1982). On the other hand, the saturated γ-lactone has very low activity and also lacks the dipole arrangement that exists in the active α,ß-unsaturated γ-lactone. The low activity could be due to repositioning of the carbonyl group so that its ability to hydrogen bond to the receptor is greatly reduced (Thomas *et al.*, 1974a,b). For example, Fullerton *et al.* (1977, 1979) synthesized the 20R and the 20S isomers of the 20, 22-dihydrodigitoxigenin (Figure 20) and found them to be about 1/30 the potency of digitoxigenin.

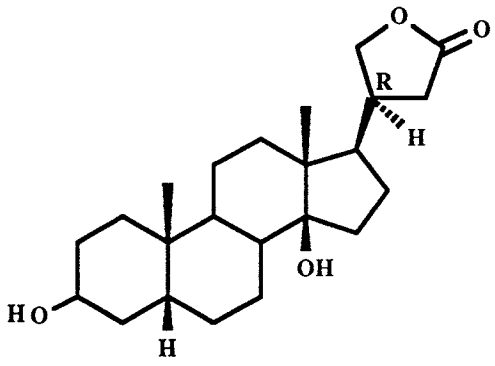
(ii) γ-Lactone analogues.

The first synthetic analogues with an altered ring structure at the C-17 position was actodigin (Figure 20) (Deghenghi, 1970) which was subsequently tested by Mendez *et al.* (1974), who found that actodigin was less potent, had a more rapid rate of onset and a greater margin of safety than the classical cardiac glycosides. This compound has been also tested *in vitro*, where it was found to

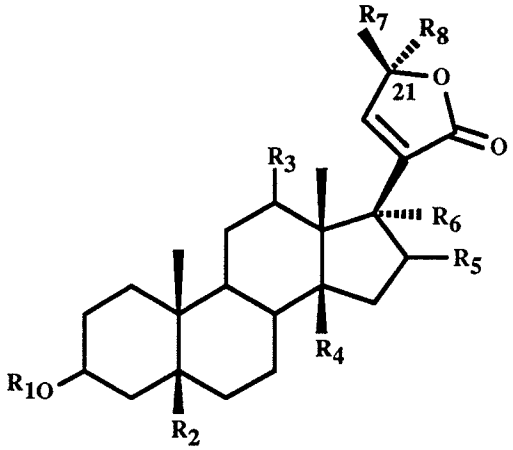
have a relatively low potency for the inhibition of human heart Na^+, K^+-ATPase. Fullerton *et al.* (1980) found that the genin of actodigin was an extremely weak inhibitor of Na^+, K^+-ATPase. More recently, Pastelin and Mendez (1983) tested a series of actodigin analogues (Figure 20). Of these, only two compounds, those with a C-21 methyl groups showed activity; the first ($R_7 = CH_3$) being 2.5 times as potent as actodigin and the second ($R_8 = CH_3$) being equipotent to actodigin, respectively (Figure 20). Fullerton *et al.* (1980) believed that the low potency of actodigin and its genin was due to displacement of the lactone carbonyl group from its "ideal" position, as found in the lactone rings of the classical glycosides such as digitoxin. Schönfeld *et al.* (1985) tested a series of digitoxigenin 17ß-γ-lactone analogues (Figure 21) for the inhibition of human myocardial Na^+, K^+-ATPase all of which were found to be significantly less active than digitoxigenin. The most potent of these was the 17ß-furanyl compound. The unsubstituted ring was found to give the highest activity and substitution at C-21 had a greater weakening influence than substitution at C-22 because of a differential effect upon the conformational flexibility and the spatial relationship between the butenolide ring and the steroid nucleus. Fullerton *et al.* (1976, 1977, 1979, 1980) have examined a series of aglycones in which the double bond found in the normal γ-lactone ring was moved to the C-22 position in an exocyclic position (Figure 22). These were tested and found to be relatively inactive (Ahmed *et al.*, 1983; Rohrer *et al.*, 1984). The only compound of significant activity was the open-chain α,ß-



(20S,22)-Dihydrodigitoxigenin

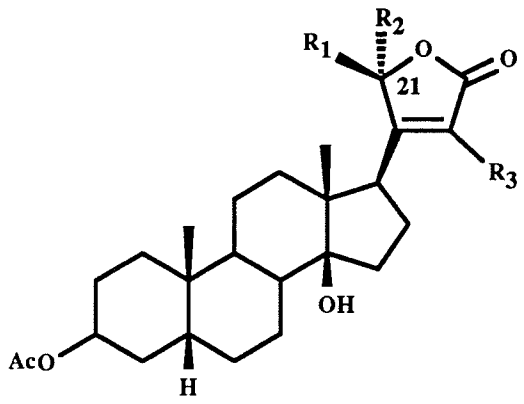


(20R,22)-Dihydrodigitoxigenin



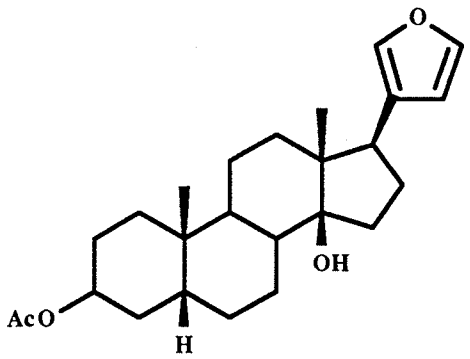
R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	
ß-D-glucose	H	H	OH	H	H	H	H	
(Actodigin)								
ß-D-glucose	OH	H	H	H	OH	H	H	→ Actodigin analogues
ß-D-glucose	H	H	H	H	OH	H	H	
ß-D-glucose	H	OH	OH	H	H	H	H	
ß-D-glucose	H	H	OH	OH	H	H	H	
ß-D-glucose	H	H	OH	H	H	CH_3	H	
ß-D-glucose	H	H	OH	H	H	H	H	
H	H	H	OH	H	H	H	CH_3	

Figure 20. Structures of the 20(R) and 20(S) isomers of 20,22-dihydrodigitoxigenin and synthetic analogues of actodigin and its genin.



R_1	R_2	R_3
CH_3	H	H
H	H	OH
H	H	$OC(O)CH_2CH_3$
H	H	OCH_3
H	H	$OC(O)CH_3$

(Digitoxigenin 17ß-γ-lactone analogues Schönfeld *et al.* (1985))



(17ß-Furanyl derivatives of digitoxigenin acetate)

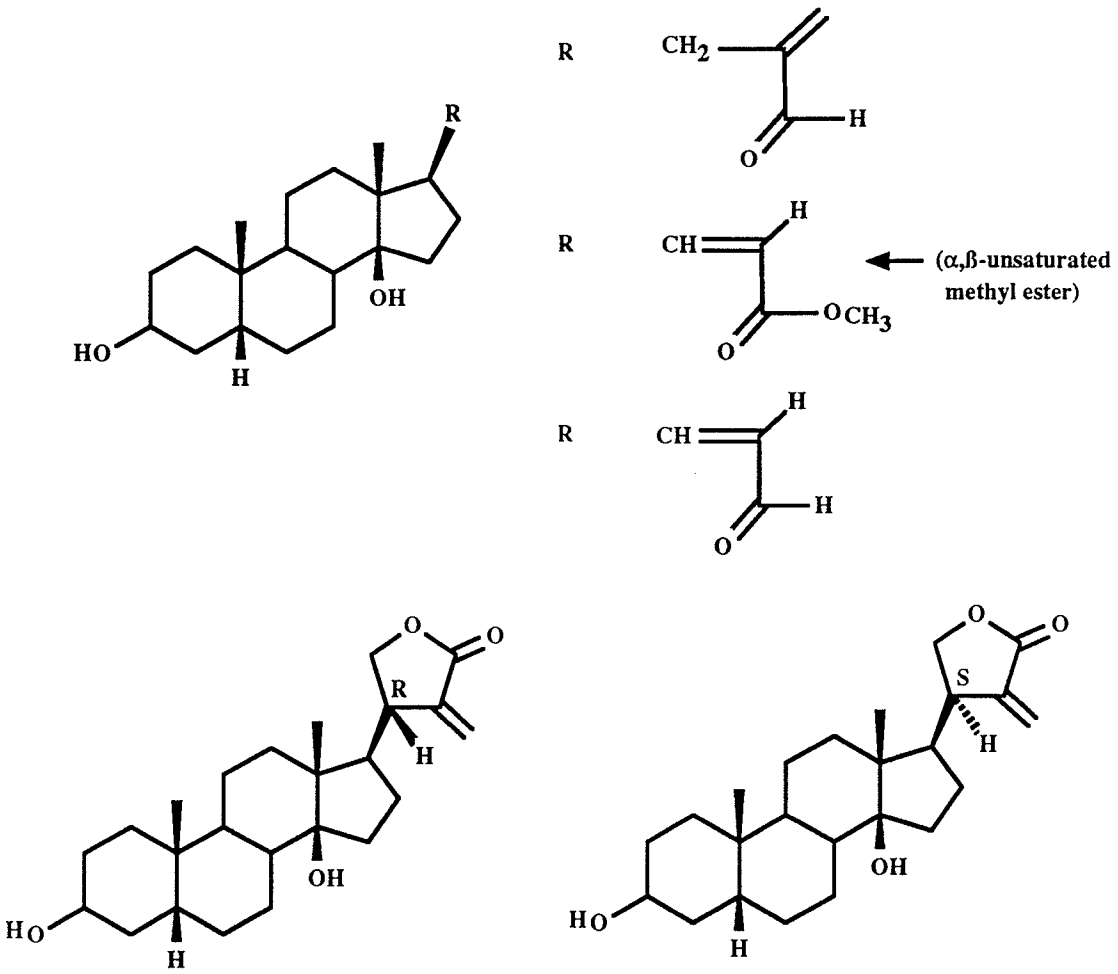
Figure 21. Lactone analogues of digitoxigenin acetate.

unsaturated methyl ester (Figure 22) whose relative potency of 0.9 was increased to 4.3 after addition of a 3ß-digitoxose sugar. The addition of the digitoxose sugar to the 22-methylene analogues (20R and 20S) (Figure 22) only increased their activity to 0.05 and 0.14 respectively, still well below that of digitoxigenin (relative potency = 1). Examination of the position of the carbonyl groups in this series of compounds confirmed for these investigators that this was the important structural requirement for this region, such that each displacement of 2.2 Å from the "ideal" position led to a loss in potency of one order of magnitude.

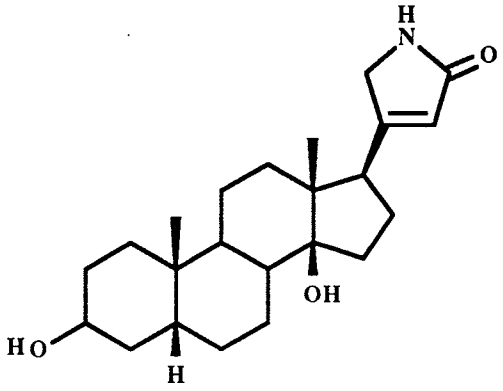
(iii) Nitrogen analogues of butenolides.

Other analogues of the unsaturated butenolides are those in which the ether oxygen has been replaced with a nitrogen atom. Katzung *et al.* (1970) found the unsaturated cyclic amide (Figure 22) to be inactive. In 1979, Megges *et al.* (1979) synthesized and tested a number of cardenamides (Figure 23) that were found to be less than 1/10 the activity of the corresponding cardenolides. It was suggested that there was probably no hydrogen bond between the amide nitrogen atom and the receptor corresponding to a lactone oxygen interaction in the cardenolides.

Another approach was to carry out testing for those compounds with a C-17 6-membered ring, *i.e.*, analogues with the pentadienolide ring. Schönfeld *et al.* (1985) tested a bufadienolide (Figure 23) and its 3-rhamnoside derivative and found them to be 8.3 and 21.7 times more potent than digitoxigenin in inhibiting human heart Na^+, K^+-ATPase. Bohl and Sussmilch (1986) reported a relative potency of

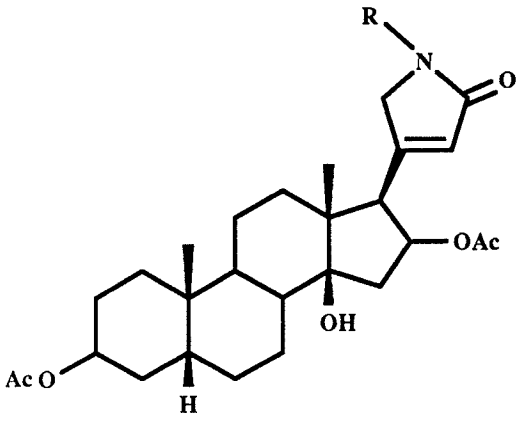


22-methylene analogues (20R and 20S). (Fullerton *et al.*, 1977)



17ß-unsaturated cyclic amide (Katzung *et al.*, 1970)

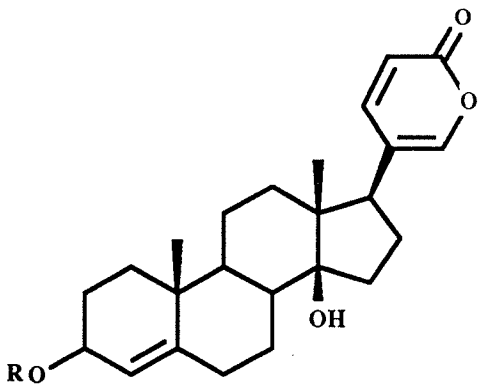
Figure 22. Lactone analogues of digitoxigenin.



R = H

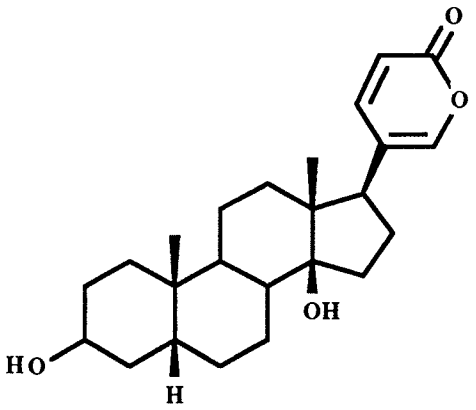
R = CH_3

Cardenamide derivatives
(Megges *et al.*, 1979)

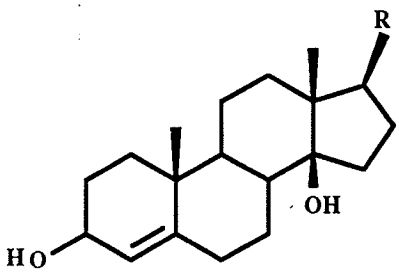


R = H Bufadienolide (genin) (Schönfeld *et al.*, 1985)

R = rhamnose

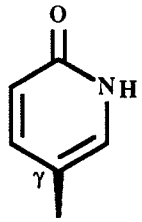


Bufalin

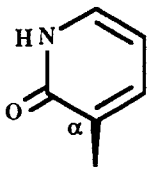


Pentadienamides derivatives
(Schönfeld *et al.*, 1985)

R =



R =



R =

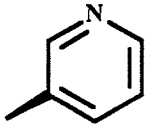


Figure 23. Cardenamide, bufadienolide and pentadienamide genin derivatives.

10.7 for bufalin (Figure 23). These results indicate that the pentadienolide may be a preferred structure since compounds with this group appear to be significantly more potent than the corresponding butenolides.

(iv) Heterocyclic analogues of butenolides.

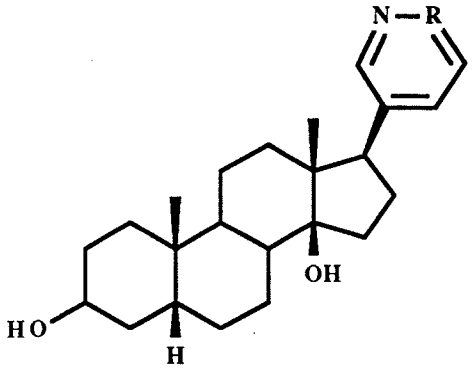
Derivatives of 6-membered heterocyclic rings have been used as substitutes for the butenolide ring. Schönfeld *et al.* (1985) tested compounds with pentadienamide (Figure 23) and pyridine rings for inhibition of human heart Na^+, K^+-ATPase. The pyridyl analogue had a relative potency of 0.38. The pentadienamides were interesting in that they had vastly different activities, a γ-steroidyl pentadienamide had about 1/3 the activity of digitoxigenin, whereas an α-steroidyl pentadienamide (Figure 23) was inactive. No reason for this difference was given. Bohl and Sussmilch (1986) reported the inhibitory actions of pyridazinyl (Figure 24) and pyridyl substituted compounds on guinea-pig heart Na^+, K^+-ATPase, to be equal to that of digitoxigenin. Godfraind and Ghysel-Burton (1979) found that the 3ß-trisdigitoxosyl pyridazine was equipotent with digoxin in inhibition of guinea-pig heart Na^+, K^+-ATPase. The high activity of this compound was rationalized on the basis of two binding sites for the lactone ring, which are able to be satisfied by the pyridazine ring structure.

(v) Open-chain lactone analogues.

Studies on derivatives in which the α,ß-unsaturated γ-lactone ring was replaced by a series of analogous open-chain structures, with steric and electronic

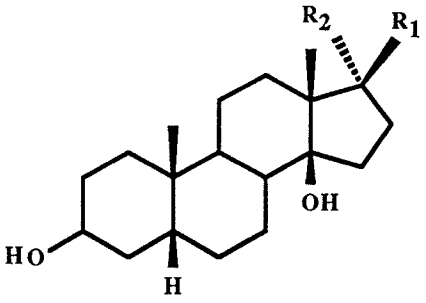
properties similar to those of the lactone, were first carried out by Boutagy and Thomas (1971, 1972, 1973) and Thomas *et al.* (1974b). Initially, they synthesized a series of α,ß-unsaturated side-chain analogues of digitoxigenin and tested those compounds for inhibition of guinea-pig heart Na^+, K^+-ATPase and cardiotonic action on isolated guinea-pig atria. These results are given in Figure 24. From these observations, a number of conclusions were drawn concerning the requirements of the 17ß side-chain needed for biological activity. Attention was drawn to the difference in activity between the 17ß-guanylhydrazone group and the semicarbazone group, as shown in Figure 24. To explain the activity of the former, it was suggested that the guanylhydrazone moiety, being a strong base, is protonated under physiological pH and thus was be able to form an ion-pair with the anionic group of the receptor (Figure 19). Thomas *et al.* (1974b) suggested that the charge on the guanylhydrazone is not at the optimal distance from the proposed receptor anion to give maximum activity. The lack of activity of the similar semicarbazone, was thought to be due to its inability to ionize.

Further studies on the C-17 open-chain analogues were carried out by Fullerton *et al.* (1976) who tested the α,ß-unsaturated aldehyde (Figure 22) on isolated guinea-pig atria and found a relative potency of 0.035. Later, Ahmed *et al.* (1983) tested the 21-ene (Figure 22) for the inhibition of Na^+, K^+-ATPase in the cat heart and found a relative potency of 0.08. Rohrer *et al.* (1984) have also tested the inhibitory potential of the unsaturated methyl ester (Figure 22) and found



R = H Pyridazine

R = CH Pyridyl derivative



R_1	R_2	Relative potency
$CH(OH)CH_2OH$	H	Inactive
CHO	H	Inactive (negative inotropy)
$CH=N-NHC(NH_2)=NH$	H	0.2
$CH=C(CH_3)C(O)OCH_3$	H	0.04
$CH=CHC(O)OCH_2CH_3$	H	0.03
$CH=CHC(O)OCH(CH_3)_2$	H	0.005
CH=CHC(O)OPh	H	0.004
$CH=CHCH=CHC(O)OCH_3$	H	0.004
$CH_2CH_2C(O)OH$	H	Inactive
$CH=CH(CH_3)C(O)OH$	H	Inactive
$CH=N-NH-C(O)NH_2$	H	Inactive
CH=CHC(O)Ph	H	Inactive (negative inotropy)
H	CH=CHCN (17α)	0.04
H	$CH=CHC(O)OCH_3$ (17α)	0.002

17-α,ß-Unsaturated open-chain groups with various steric and electronic resemblance ot the γ-lactone group and with potency compared to digitoxigenin for inhibition of Na^+, K^+-ATPase. The relative potency of digitoxigenin = 1 (Thomas *et al.*, 1974b).

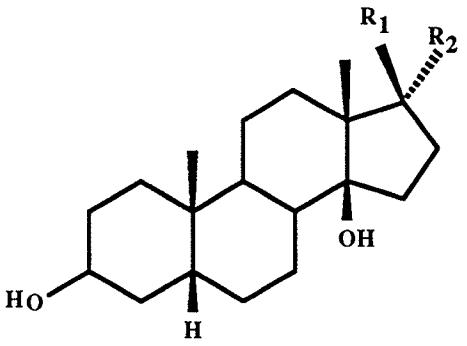
Figure 24. Pyridazine and pyridyl derivatives of digitoxigenin. Pharmacological activity of a series of C-17 analogues of digitoxigenin.

a relative potency of 0.9. All of the above results suggested the greater potency for compounds whose carbonyl groups were closest in position to that found in the usual butenolide ring. The further the displacement from the "ideal", the lower the potency.

Schönfeld *et al.* (1985) have also examined some replacements for the butenolide ring. These derivative are shown in Figure 25 and were found to be relatively inactive when tested on human heart Na^+, K^+-ATPase, with the exception of the 17ß-guanylhydrazone, which had a relative potency of 0.003. The reason for this compound having a higher activity than the parent ketone was attributed to an ion-pair association between the cationic group and the anionic binding site of the receptor.

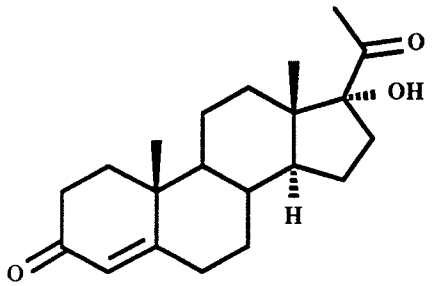
VII. 14α-Pregnane analogues.

It has long been recognized that the conformation of the steroid nucleus in cardiac glycosides differs considerably from the more or less planar nucleus of the mammalian steroid hormones. However, the large number of steroid metabolites identified in biological systems suggests that metabolic transformation might generate derivatives (non-digitalis like steroids) conformationally similar to the cardiac glycoside structure. For instance, the first steroid compound to show significant activity in a [^{3}H]-ouabain radioligand binding assay was cyproterone acetate, a derivative of 17α-hydroxyprogesterone, a major metabolite of progesterone. On the basis of the structure of cyproterone acetate, significant

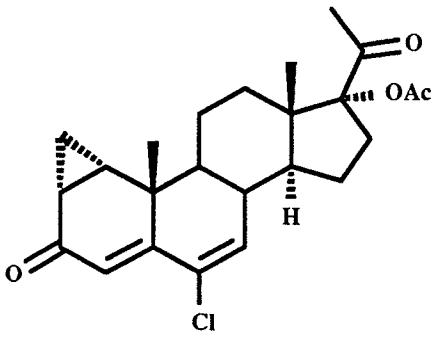


R_1	R_2
H	H
OH	H
---------- O ----------	
$OC(O)CH_3$	H
H	$OC(O)CH_3$
$=NNHC(NH_2)=NH_2^+$	

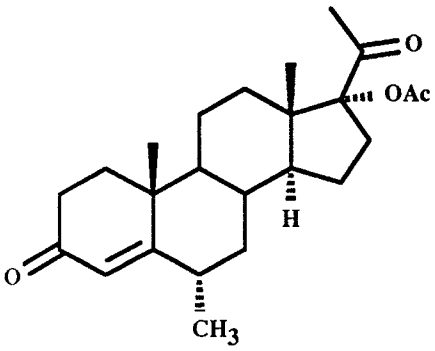
(Modification of the butenolide ring, Schönfeld *et al.* (1985))



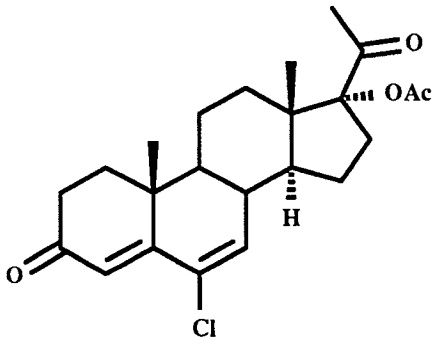
17α-Hydroxyprogesterone



Cyproterone acetate



Medroxyprogesterone acetate



Chlormadinone acetate

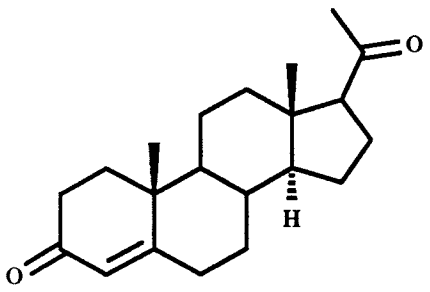
Figure 25. Androstane and pregnane derivatives for SAR studies.

determinants of activity in the [^{3}H]-ouabain radioligand binding assay were defined as a 17α-acetoxy group and unsaturation and/or C-6 substitution in the B-ring (Kim *et al.*, 1980). LaBella *et al.* (1985) found that in the absence of unsaturation in the B-ring, the C-6 substituent was effective only in the α configuration *e.g.*, medroxyprogesterone acetate. The most potent steroid identified was chlormadinone acetate (CMA) having from 1/20-1/50 the activity of ouabain in the [^{3}H]-ouabain radioligand binding assay. In competition with [^{3}H]-ouabain binding, CMA is more potent than ouabagenin and almost as potent as digoxigenin. Structures of these compounds are shown in Figure 25. Wehling *et al.* (1981) concluded that CMA binds to the same site on Na^+, K^+-ATPase as does ouabain because CMA displaced radiolabel from the enzyme-[^{3}H]-ouabain complex at the same rate as did the cardiac glycoside. The basis for the activity of CMA comes from comparison of the crystallographic structure of CMA with the structures of glycosides, genins and related compounds that inhibit Na^+, K^+-ATPase. They found that when the structures of digitoxigenin and CMA are superimposed and the distances between corresponding atoms were calculated to provide a direct measure of the geometric differences, the results indicated that the position of the 20-oxygen closely approximated the 23-oxygen in the γ-lactone ring of the genin. The relative positions of the C-17 side-chain carbonyl oxygen showed a nearly perfect correlation with potencies of the compounds to inhibit Na^+, K^+-ATPase. Consequently, the position of the 17ß side-chain was in the right place for

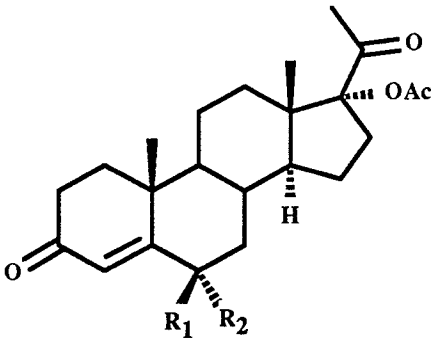
interaction with the receptor active site. Further study on the structure activity relationships (SAR) of CMA was carried out by Templeton *et al.* (1987a) who synthesized a number of progesterone derivatives having a 17α-acetoxy group and various functions at C-3 and C-6 carbons and tested them using a [^{3}H]-ouabain radioligand binding assay (as a screening test) with membranes from dog myocardium. They concluded the following with respect to SAR based on progesterone which itself shows weak binding to Na^+, K^+-ATPase and weak cardiodepressant activity as does CMA:

(1) The presence of a C-6 vinyl substituent (R = Me, Cl, Br) or a C-4 unsaturated 6 substituent (R = Me, Cl, Br) (Figure 26) was necessary for strong binding.

(2) Replacement of the 17α-OAc in CMA by a 17α-Me gave moderate binding. The relative potency of these derivatives are shown in Figure 26. CMA and related steroids may elicit transient or, occasionally, sustained enhancement of cardiac contractility. The major cardiac effect of these compounds is generally cardiodepressant in contrast to that of cardiac glycosides . Progesterone itself has long been known to depress contractility in a variety of isolated cardiac tissues (Tanz, 1963). Thus, these observation have given rise to the speculation that the binding site which mediates inhibition of the sodium pump may not be identical to that which mediates positive inotropy. This led LaBella *et al.* (1989) to suggest a hypothetical model

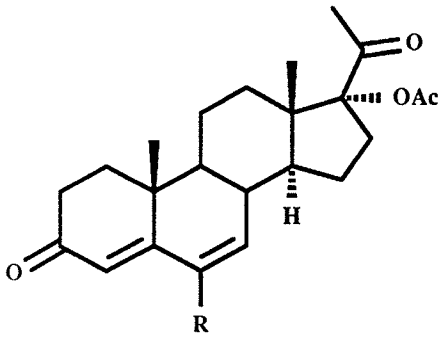


Progesterone
(IC_{50} *ca.* 100 μM)[a]



17α-Acetoxyprogesterone

R_1	R_2	Relative potency (IC_{50} in μM)[a]
H	H	93
Cl	H	Inactive
Br	H	87
H	Me	12
H	F	49
H	Cl	3
H	Br	2



R	Relative potency (IC_{50} in μM)[a]
H	59
Cl	0.3

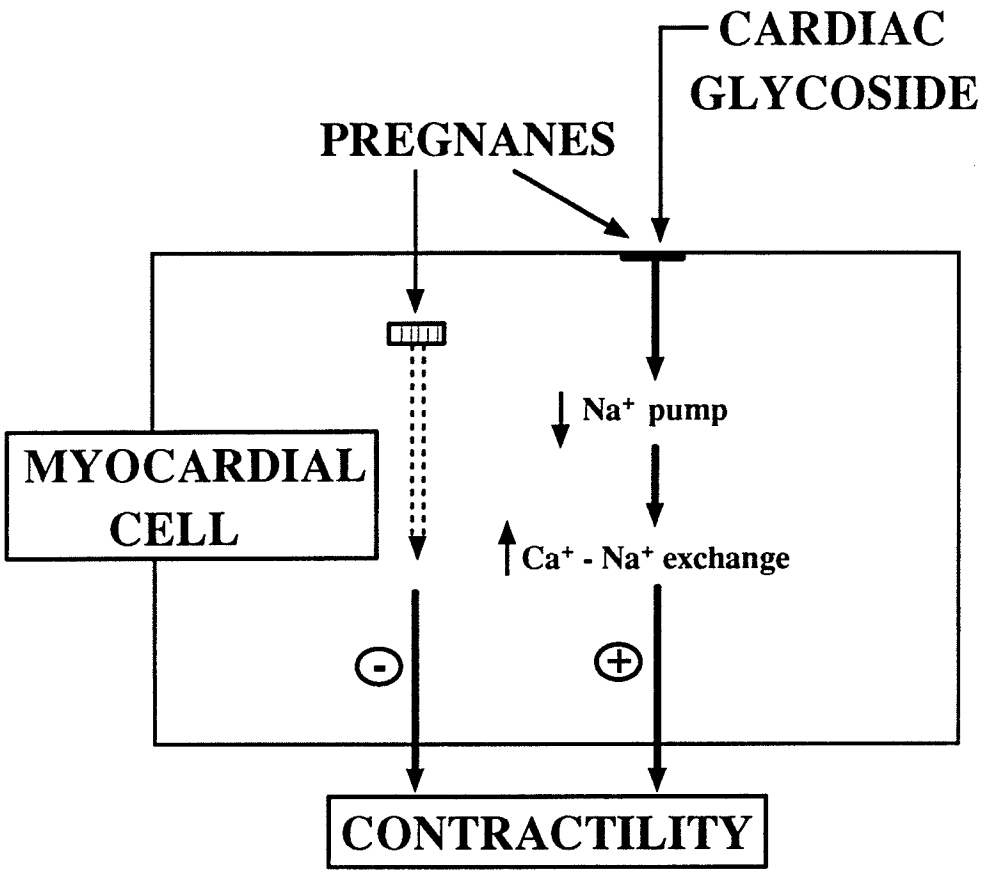
[a]The IC_{50} is the concentration of the steroid that inhibits displaceable binding of [^{3}H] ouabain by 50% (Templeton *et al.*, 1987a).

Figure 26 Relative potencies of progesterone derivatives in a [^{3}H]-ouabain RBA.

(Figure 27) to account for the paradoxical cardiodepression by pregnanes which bind to the receptor active site of the Na^+, K^+-ATPase. These authors indicated that the pregnanes and cardiac glycosides compete for a binding site on Na^+, K^+-ATPase whose occupation results in enzyme inhibition, as a consequence, enhancement of cardiac contractility. In addition, pregnanes interact with an intracellular site to promote processes that counteract the concomitant positive inotropic action.

VIII. 14ß-Pregnane analogues.

Because the 14ß-hydroxy function is an important determinant of biological activity of the cardiac glycoside (Guntert and Linde, 1981), Templeton *et al.* (1987b) synthesized 14ß-hydroxyprogesterone (Figure 27) which has a C/D cis ring junction and possessed the possibility of enhancing cardiac contractility instead of a negative inotropic response (LaBella *et al.*, 1984, 1985; Bose *et al.*, 1988). Incorporation of a C-3 glycoside in CMA strongly decreased binding to the receptor while introducing cardiostimulant activity. An increase in cardiac contractility was also found in 14ß-hydroxyprogesterone 3ß-glucoside (Templeton *et al.*, 1987a). This strongly suggests that the inotropic mechanism of action of steroids is not identical with their interaction with Na^+, K^+-ATPase (Templeton *et al.*, 1988). More potent compounds have been made in this series, for example, the 20ß(R)-alcohol of 14ß-hydroxprogesterone C-3 glucoside (Figure 28). This glycoside is 1/20 as potent as ouabain and elicits prominent, sustained, positive



(Hypothetical model proposed by LaBella *et al.* (1989), that shows the paradoxical cardiodepression by pregnanes that bind the "digitalis receptor" and inhibit the Na^+, K^+-ATPase enzyme (Solid bar). The pregnanes interact, in addition, with an intracellular site (hatched bar) to promote processes that counteract the concomitant positive inotropic action).

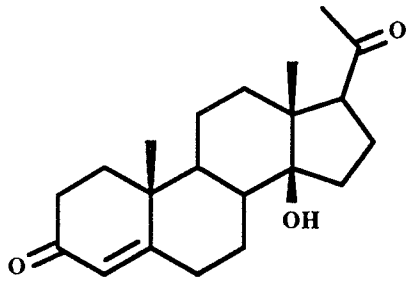
Figure 27. Model to explain the cardiostimulant/cardiodepressant activity of cardiac glycosides on myocardial cells.

inotropy in isolated cardiac muscle (Templeton *et al.*, 1988). The α-L-rhamnose derivatives show improved activity in the 14ß-hydroxypregnanes. On the other hand, Jarreau *et al.* (1984) found that the 20ß(R)-alcohol, 14-amino-5ß,14ß-pregnane-3ß,20ß-diol α-L-rhamnoside (Figure 28) was highly active for inotropic activity (*i.e.*, more potent than ouabain). The reason suggested for the high activity was that it was able to undergo an ion-pair association between the 14-amino group and a postulated anionic binding site.

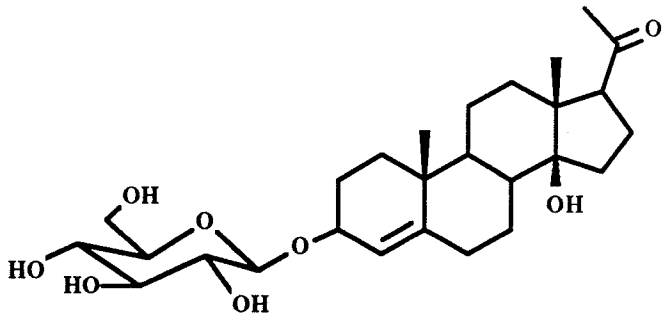
Attention is drawn here to the hypothesis made by Thomas *et al.* (1974a,b) that the 17ß side-chains of active compounds were taken up into a cleft on the enzyme surface where they were bound by means of a two-point attachment involving hydrogen bonding to the heteroatom and electrostatic interaction or a charge transfer complex formation between the conjugated system in which the partial positive charge on the C-20 carbon was considered to be a key feature (see Figure 19). This hypothesis does not explain the activity of many of our derivatives which are discussed below.

OBJECTIVES

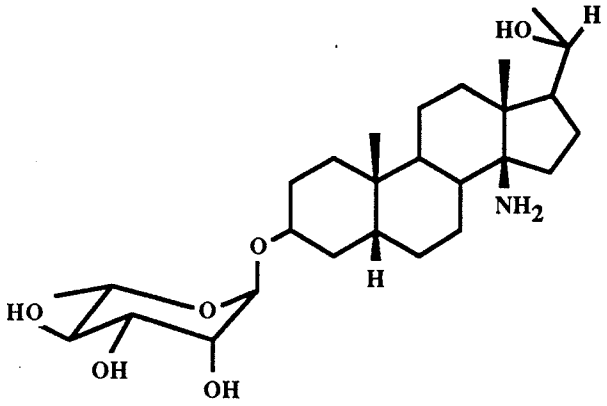
Digitalis and related steroid glycosides have a strong positive inotropic effect (an increase in the force of contraction of the heart muscle) on cardiac muscle which is the basis for their clinical use. However, as clinical agents they show a poor therapeutic index, producing harmful arrythmic side effects.



14ß-Hydroxyprogesterone



14ß-Hydroxyprogesterone 3ß-glucoside



14-Amino-5ß,14ß-pregnane-3ß,20ß-diol α-L-rhamnoside

LND 623 (Jarreau *et al.*, 1984)

Figure 28. Structure of 14-amino-5ß,14ß-pregnane-3ß,20ß-diol α-L-rhamnoside, 14ß-hydroxyprogesterone and 14ß-hydroxyprogesterone 3ß-glucoside.

It is generally accepted that Na^+, K^+-ATPase acts as the receptor for the cardiac glycoside. It is still a matter of debate whether the cardiotonic and cardiotoxic effects are independent from each other. According to one concept, therapeutic and toxic effects are only dependent on the degree of Na^+, K^+-ATPase inhibition. However, there is evidence that these effects can be separated possibly through a balance of cardiostimulant and cardiodepressant effects acting through different receptors. Although, the search for natural or synthetic substances with improved therapeutic properties has been pursued intensively, it has thus far not produced satisfactory results. Prerequisites for the success of such attempts include knowledge of the structure-activity relationships of such molecules, and separation of the therapeutic effects from the toxic effects. It is possible to separate therapeutic and toxic effects only if two different mechanisms or two different receptor sites are responsible.

This thesis focuses on the following SAR objectives:

(1) Generally it has been accepted that a C-17ß α,ß-unsaturated γ-lactone group is essential for high cardioactivity. However, it has been shown in these laboratories by us (Templeton *et al.*, 1991a,b) and others (Jarreau *et al.*, 1984) that the γ-lactone can be replaced by C-17ß acetyl derivatives and retain high potency. This research was directed towards further investigation of functional group requirement at the C-17 position. Furthermore synthesis of 21-norpregnane derivatives was carried out to

determine the effect of shortening the acetyl chain from a two carbon to a one carbon attachment.

(2) The nature of the glycoside is well known to affect the cardioactivity of the cardiac glycoside (Smith, 1989). It was therefore, important to determine the effect of changing the number of sugar units. Consequently the mono- and bis-digitoxoside derivatives as well as the trisdigitoxoside derivatives were synthesized.

(3) The more water soluble ouabain derivatives, potentially useful in pharmacological testing, were also synthesized.

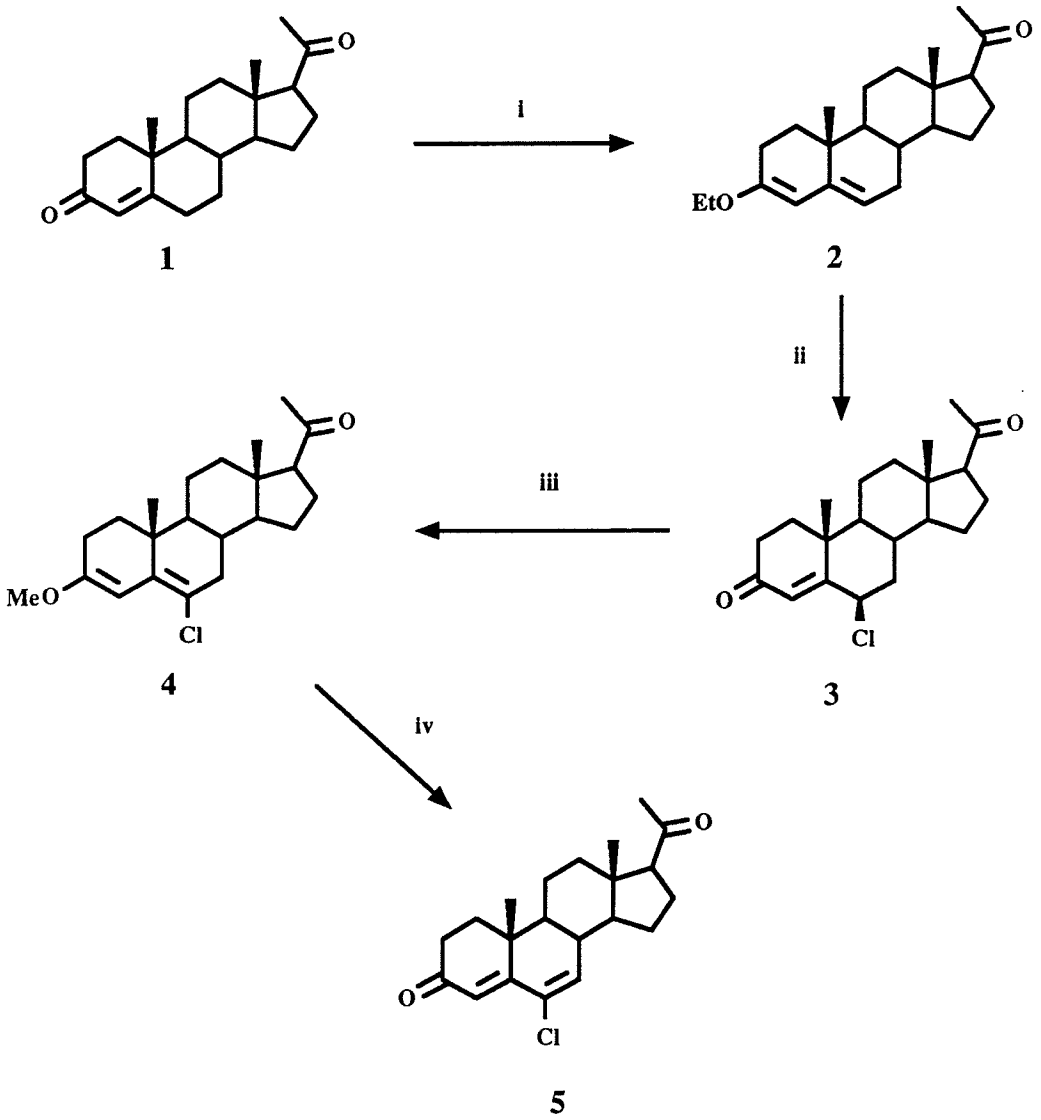
(4) It was planned to screen the compounds for both cardiac and non-cardiac pharmacological activities. This possibility arises because of the universal distribution of Na^+, K^+-ATPase in body tissues. Selective effects on nerve, brain, kidney, and smooth muscle, for example, may give rise to new therapeutic agents. Specifically some compounds were to be tested for their effect on the Na^+/K^+ urinary excretion ratio. Related compounds have been shown to cause K^+-sparing diuresis. These tests were to be carried out in collaboration with other workers, however, this work has not been completed sufficiently for their inclusion.

DISCUSSION

I. CHEMISTRY: SYNTHESIS OF 6-CHLORO SUBSTITUTED STEROIDAL HORMONES.

The chlorination of 3-oxo-4-ene steroids has been described in the literature using a number of different synthetic routes (Brückner *et al.*, 1961; Mori and Yamada, 1963; Yasuda, 1963; Cross *et al.*, 1963). The following synthetic route was employed (Yasuda, 1963). Treatment of pregn-4-ene-3,20-dione (**1**) (Scheme 1) with triethyl orthoformate and a catalytic amount of p-toluenesulfonic acid in dioxane gave the corresponding 3-ethyl enol ether (**2**) which was converted into a mixture of isomeric 6α-, 6ß-chloro derivatives through the attack of the electrophilic reagent, N-chlorosuccinimide (NClS), in aqueous acetone (Ercoli, 1963). It has been observed for α,ß-unsaturated ketones in ring-A that the 6ß-chloro isomer (**3**) is the major product with substitution taking place mainly from the ß side at C-6. The 6ß-Cl isomer was isolated as the pure isomer by crystallization. Further treatment of **3** with trimethylorthoformate and p-toluenesulfonic acid led to the 6-chloro enol ether (**4**). This compound was required for treatment with dichlorodicyanoquinone (DDQ) to give the 6-chloropregna-4,6-diene-3,20-dione (**5**) (Scheme 1).

Agnello and Laubach (1960) found that at elevated temperatures chloranil (tetrachlorobenzoquinone) converted the 4-en-3-one steroids into the corresponding



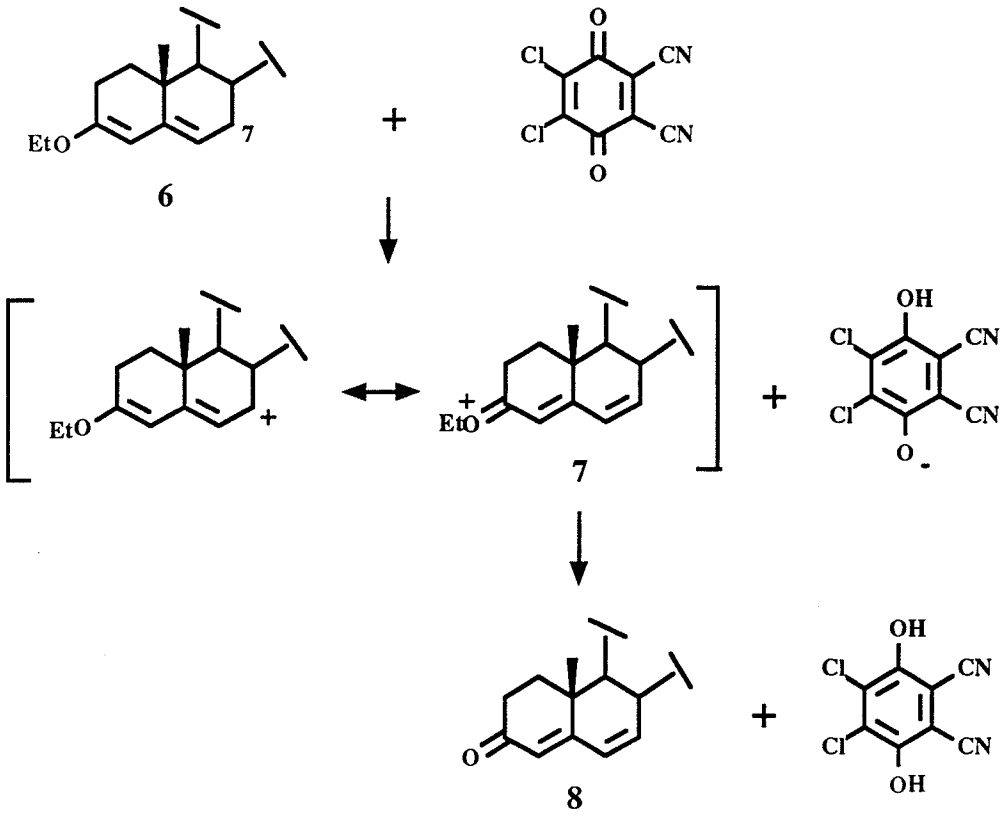
Reagents and conditions: i, $(EtO)_3CH$-p-TsOH . H_2O-dioxane, r.t., 3hrs; ii, NClS-NaOAc-95% aq. Me_2CO, *ca* 5 °C, 1hr; iii, $(MeO)_3CH$-p-TsOH . H_2O-dioxane, r.t., 10hrs; iv, DDQ-tert-BuOH, reflux, 3hrs.

Scheme 1. Synthesis of 6-chloropregna-4,6-diene-3,20-dione.

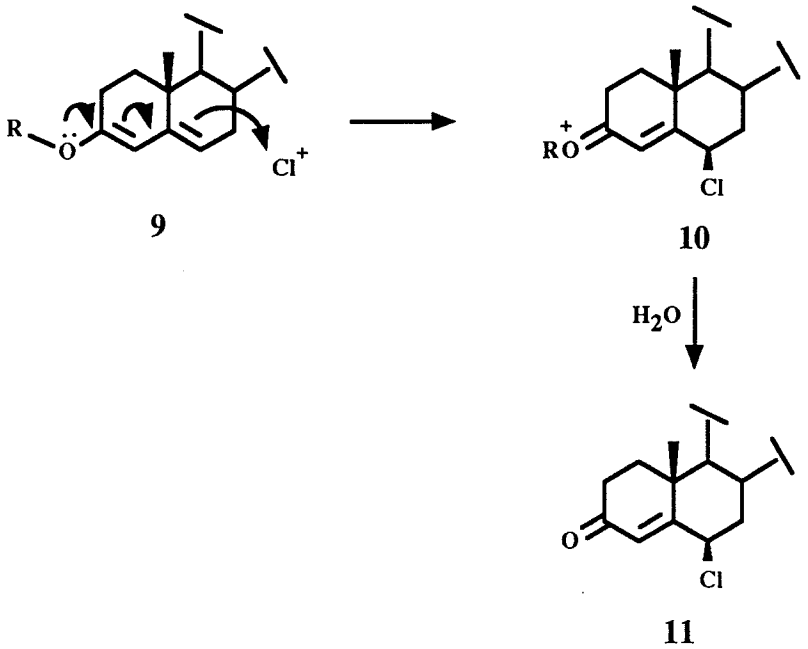
6-dehydrosteroids and proposed that the reaction proceeded via the 3,5-dienol ether followed by C-7α hydride ion abstraction. In support of the enol mechanism were the observations that a 5-ene-3-ketone and a 3,5-dienol ether underwent conversion by chloranil to the 4,6-dien-3-one at a much greater rate than did the 4-en-3-one. Later, Pradhan and Ringold (1964) demonstrated a similar reaction mechanism earlier proposed by Campbell and Babcock (1959) for dehydrogenation of the 3-ethyl enol ether (**6**) with DDQ in aqueous media (Scheme 2). This involves the loss of the axial 7α-hydrogen forming an oxonium species and the anion of the hydroquinone. Addition of the electrophilic chlorine to the double bond on ring B produces an electron sink which initiates oxonium ion formation (Scheme 3). Decomposition of the oxonium salt (**7**) by water from the aqueous solvent then leads to the conjugated ketone (**8**). Pradhan and Ringold (1964) have also observed that the analogous 7α-methyl-4-en-3-one derivative failed to undergo dehydrogenation while the 7ß-isomer does so readily. This indicates that specific removal of the 7α (axial) hydrogen probably occurs in the unsubstituted compound.

The observed preference for 6ß-chloro substitution can be rationalized as follows:

(**1**) 6ß-Proton loss during enolization. Loss of the 6ß-H was confirmed by Subrahmanyam *et al.* (1966), using androst-4-ene-3,7-dione. Its 6ß-deuterio derivative revealed that the 6ß-proton was lost fifty-three times more rapidly than the 6α-proton, whereas results for the 19-nor analogue showed a three



Scheme 2. Proposed mechanism for DDQ oxidation of a dienyl enol ether.



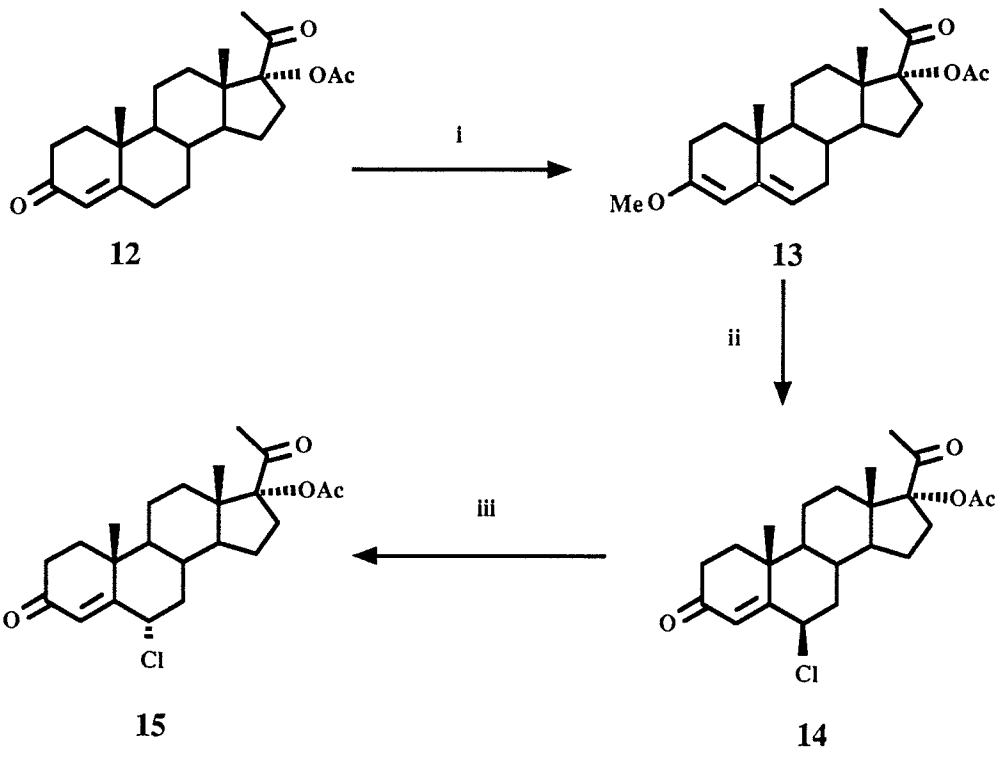
Scheme 3. Proposed mechanism for electrophilic addition to a dienyl enol ether.

hundred fold preference for axial proton removal in the absence of steric hindrance by the C-19 methyl group. The preferential exchange of the 6ß-hydrogen during the formation of the 3,5-dienol ether indicates that the attack of chlorine occurs also from the ß-side at C-6.

(2) The high reactivitie of the alkyl enol ether. Reactivity probably results from involvement of a lone-pair of electrons from the C-3 oxygen that is initiated by the electrophilic species and which leads to an oxonium ion (**10**) at C-3 (Scheme 3). The loss of the alkyl residue can result from attack by water to form a C-3 hemiketal as an intermediate in the formation of the 3-ketone (**11**).

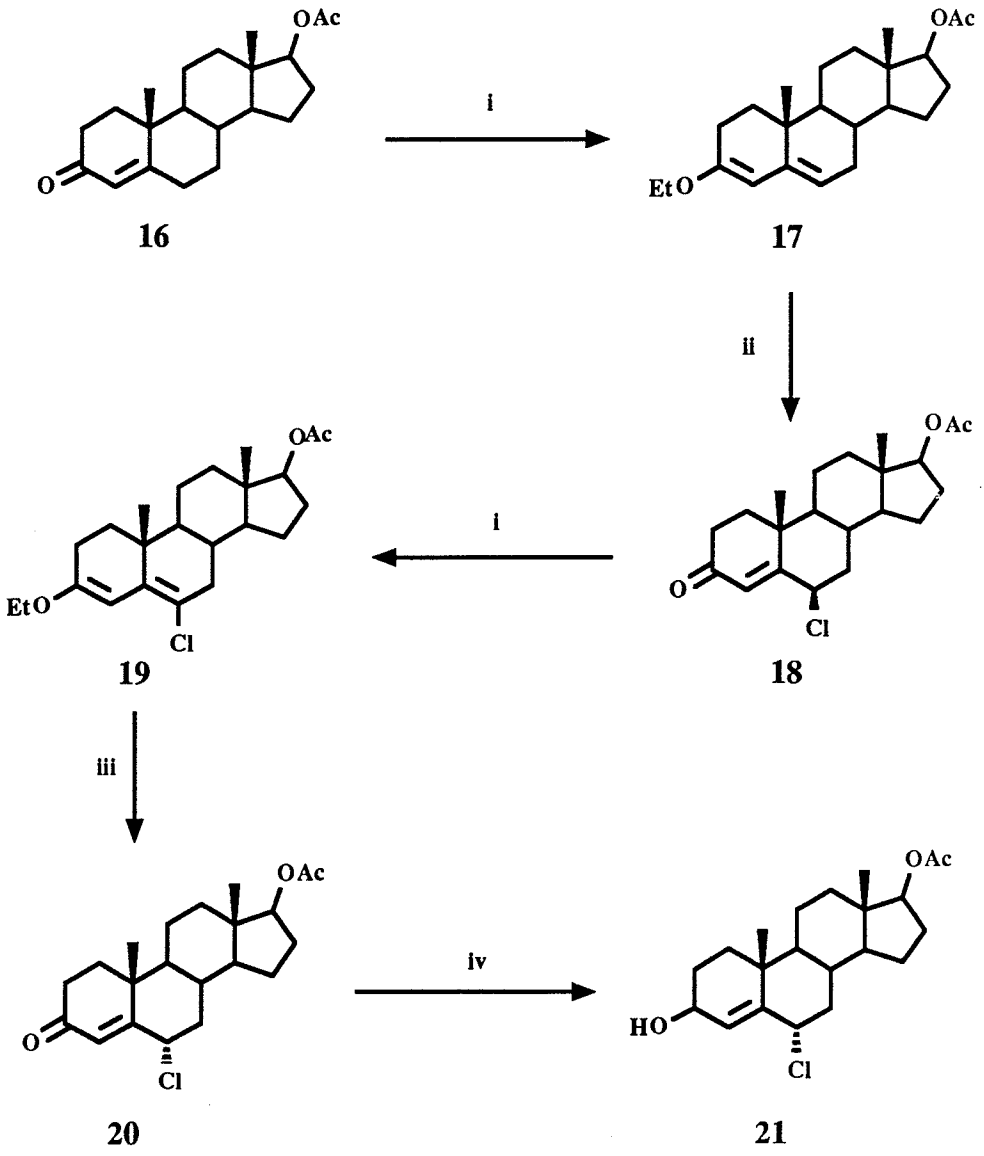
Introduction of a chlorine atom at C-6 in 17α-acetoxyprogesterone (**12**) (Scheme 4) or 17ß-acetoxytestosterone (**16**) (Scheme 5) to give exclusively the 6ß-epimer (**14** & **18**, in Scheme 4 & 5, respectively) was carried out by a similar synthetic pathway to that described above.

Epimerization of the 6ß-chloro to the 6α-chloro epimer took place under treatment with hydrogen chloride gas bubbling into acetic acid, as shown for compound **14,** or alternatively by reaction of the ethyl enol ether (**19**) (Scheme 5) with 20% hydrochloric acid in acetic acid. The epimerization of the 6ß-chloro substituent takes place more rapidly through its enol ether intermediate and with less strongly acidic conditions compared to epimerization of the 6ß-chloro-4-en-3-



Reagents and conditions: i, $(MeO)_3CH$-p-TsOH . H_2O-dioxane, r.t., 3hrs; ii, NClS-NaOAc-HOAc-95% aq. Me_2CO, r.t., 1hr; iii, HCl gas-HOAc, r.t., 3hr.

Scheme 4. Synthesis of 17α-acetoxy-6α-chloropregn-4-ene-3,20-dione.

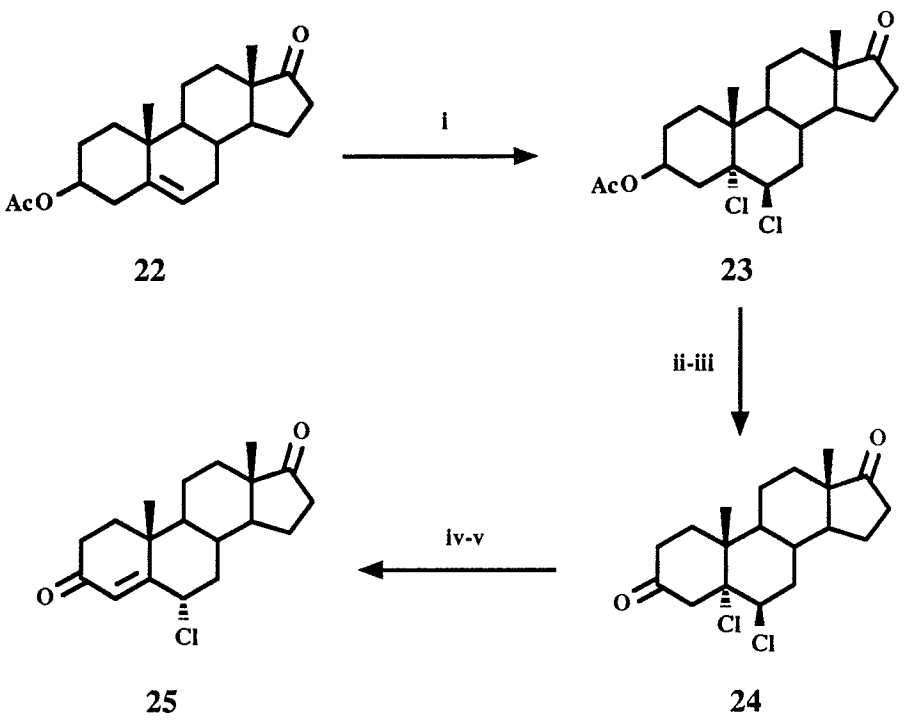


Reagents and conditions: i, $(EtO)_3CH$-p-TsOH . H_2O-dioxane, r.t., 3hrs; ii, NClS-NaOAc-HOAc, r.t., 1hr; iii, 20% HCl-HOAc, r.t., 1.5hrs.; iv, LTBAH-THF, r.t., 1hr.

Scheme 5. Synthesis of 17ß-acetoxy-6α-chloroandrost-4-en-3-one and 17ß-acetoxy-6α-chloro-3ß-hydroxyandrost-4-ene.

one derivative. This indicates that the enol ether intermediate must be formed before the configuration of this substituent can change to yield the stable equatorial 6α-isomer and that this requires more strongly acidic conditions and a longer reaction time. This would require a 6α-H, and removal of the axial (6ß-H) is more highly energetically favoured than removal of the equatorial (6α-H), as the 6ß position is substituted by the chlorine atom. Evidence for this stereochemistry of the chlorine was obtained from ^{1}H NMR data (Table 2). Taking the C-13 proton signal of 17ß-acetoxytestosterone (**16**) as a reference, the 6ß-chloro compound (**18**) (Scheme 5) showed a greater downfield shift for its C-10 methyl protons signal (1.47 ppm) compared to the 6α-chloro substituent (**20**) (1.22 ppm). Furthermore, the 6α-H in the 6ß-chloro compound shows only a small coupling constant (J 2.0,3.8 Hz) associated with an equatorial-equatorial or axial-equatorial coupling while the corresponding 6ß-H in the 6α-chloro compound shows a larger coupling (J 1.9,5.1,12.8 Hz) associated with an axial-axial coupling (Bhacca and William, 1964). Reduction of 17ß-acetoxy-6α-chloroandrost-4-en-3-one (**20**) (Scheme 5) with lithium tri-tert-butoxy-aluminohydride (LTBAH) gave the 3ß-alcohol (**21**) as the main product.

The chlorination of 3ß-acetoxyandrost-5-en-17-one (**22**) (Scheme 6) with sulfuryl chloride in pyridine as described by Mori and Yamada (1963) provided an alternative synthetic route for the introduction of a chlorine atom at C-6 via the 5α,6ß-dichloro compound (**23**). The assignment of the configurations at C-5 and



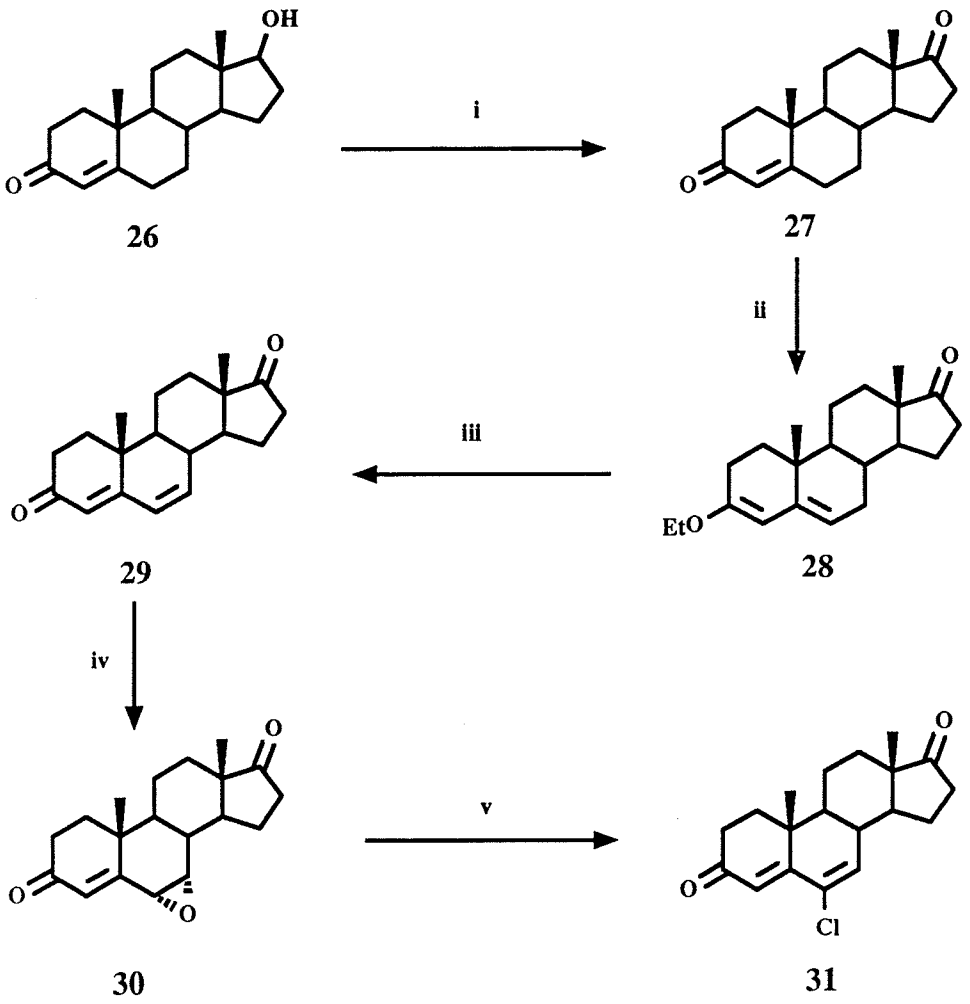
22 23 24 25

Reagents and conditions: i, SO_2Cl_2-pyridine, r.t., 3hrs; ii, 0.1 M KOH-MeOH, r.t., 18hrs; iii, CrO_3-H_2O-HOAc, r.t., 1hr; iv, NaOAc-MeOH, reflux, 3hrs v, 20% HCl-HOAc, r.t., 18hrs.

Scheme 6. Synthesis of 6α-chloroandrost-4-ene-3,17-dione.

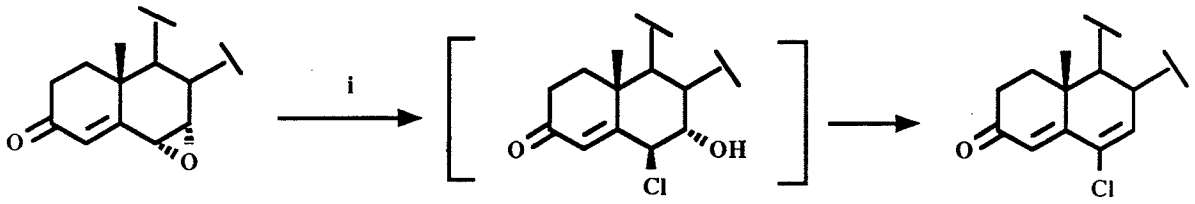
C-6 in **23** was made by Mori and Yamada (1963) as follows. First, chlorination of **22** with chlorine by this method is known to give 5α,6ß-dichloro compound (Cutler, 1959a,b) and the reaction with sulfuryl chloride in pyridine afforded the same dichloro compound (**23**). Second, the 6ß-configuration of the chlorine atom was fully established because the configuration of the halogen atom at C-6 was retained during dehydrohalogenation (Bowers, 1959). Base catalysed hydrolysis of the C-3 acetate (**23**), followed by oxidation with chromic anhydride in acetic acid gave the 3-oxo compound (**23**). This ketone (**23**) could be transformed into the 6α-chloro-4-en-3-one (**25**) by refluxing with sodium acetate in methanol to introduce the C-4 double bond by ß-elimination of chloride followed by treatment with 20% hydrochloric acid in acetic acid.

6-Chloroandrosta-4,6-diene-3,17-dione (**31**) was prepared as described by the method of Brückner *et al.* (1961) as follows (Scheme 7). Oxidation of testosterone (**26**) with chromic anhydride and acetic acid gave the diketone (**27**). This was converted to the ethyl enol ether (**28**) with triethylorthoformate and p-toluenesulfonic acid in dioxane. Subsequent dehydrogenation with dichloro-dicyanoquinone (DDQ) in aqueous acetone gave the corresponding conjugated ketone (**28**). Epoxidation with 3-chloroperoxybenzoic acid in chloroform afforded the 6α,7α-epoxide (**30**) accompanied by traces of by-products from which the epoxide was separated by flash chromatography on silica gel. The 6α,7α-epoxide was opened under acidic conditions using hydrogen chloride gas in acetic acid.



Reagents and conditions: i, CrO_3-H_2O-HOAc, r.t., 1hr.; ii, $(EtO)_3CH$-p-TsOH-dioxane, r.t., 3hrs; iii, DDQ-95% aq. Me_2CO, r.t., 30 min; iv, 3-chloroperoxybenzoic acid-$CHCl_3$, r.t., 24hrs; v, HCl gas-HOAc, r.t., 1hr.

Scheme 7. Synthesis of 6-chloroandrosta-4,6-diene-3,17-dione.



Reagent and conditions: i, HCl gas-HOAc, r.t., 1hr.

Scheme 8. Formation of 6-chlorovinylic derivative *via* chlorohydrin intermediate from 6α,7α-epoxide.

This protonates the epoxide oxygen, greatly enhancing the normal polarization of the C-O bonds and thereby aiding nucleophilic attack by an anion (Cl^-) with concerted breaking of the C-O bond to give a halohydrin intermediate. Acid catalyzed elimination resulted in vinylogous elimination of the 7α-hydroxyl group (Scheme 8) and formation of the 6-chlorodienone (**31**).

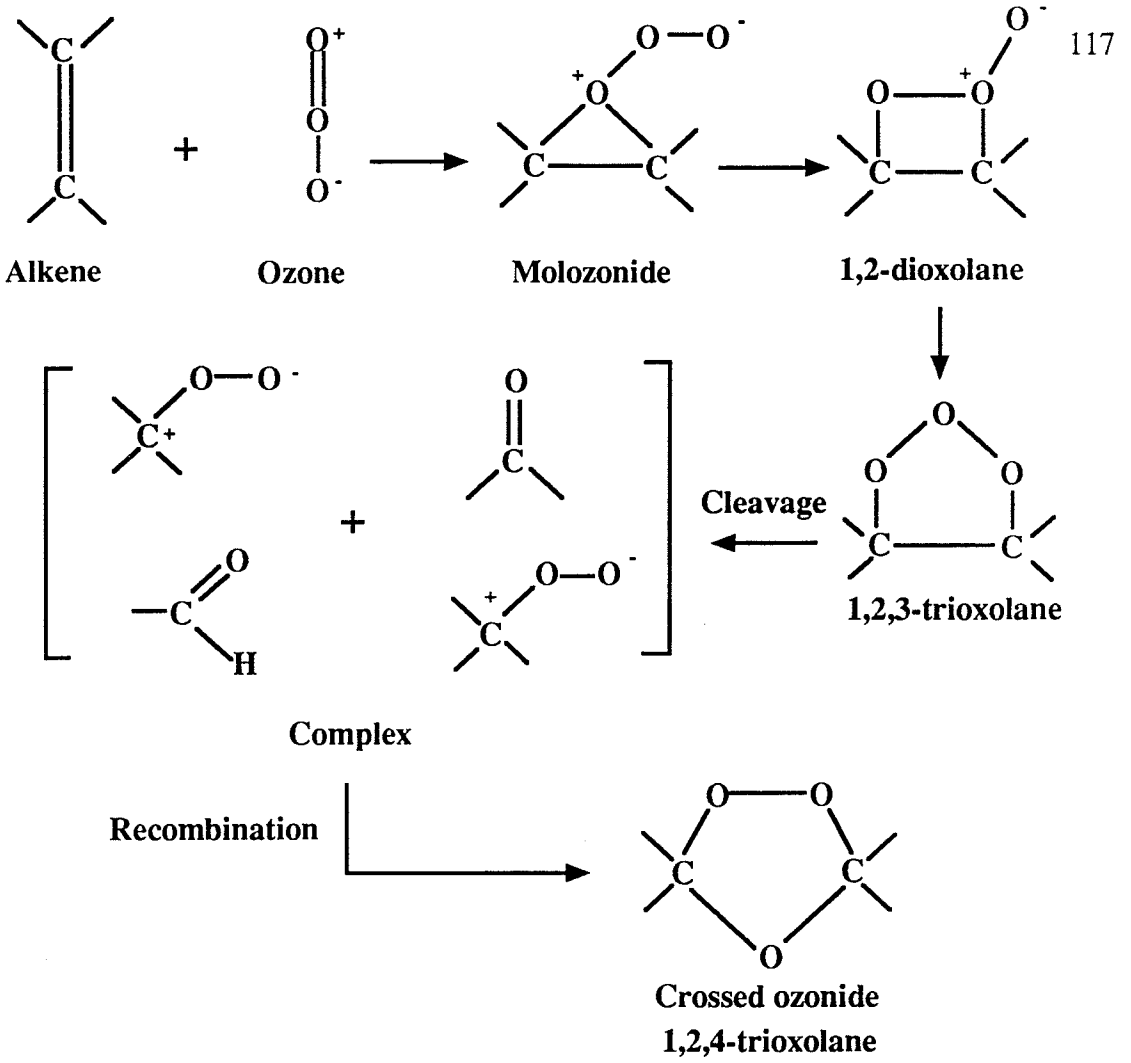
II. CHEMISTRY: SYNTHESIS OF PREGNANE DIGITOXOSIDE ANALOGUES FROM CARDENOLIDES (DIGITOXIN).

The reaction of ozone with alkenes in solution at low temperature constitutes an important method for cleavage of carbon-carbon double bonds to form ketones and/or aldehyde groups (Bailey, 1959; Murray, 1968; Story *et al.*, 1971; Razumovskii and Zaikov, 1984).

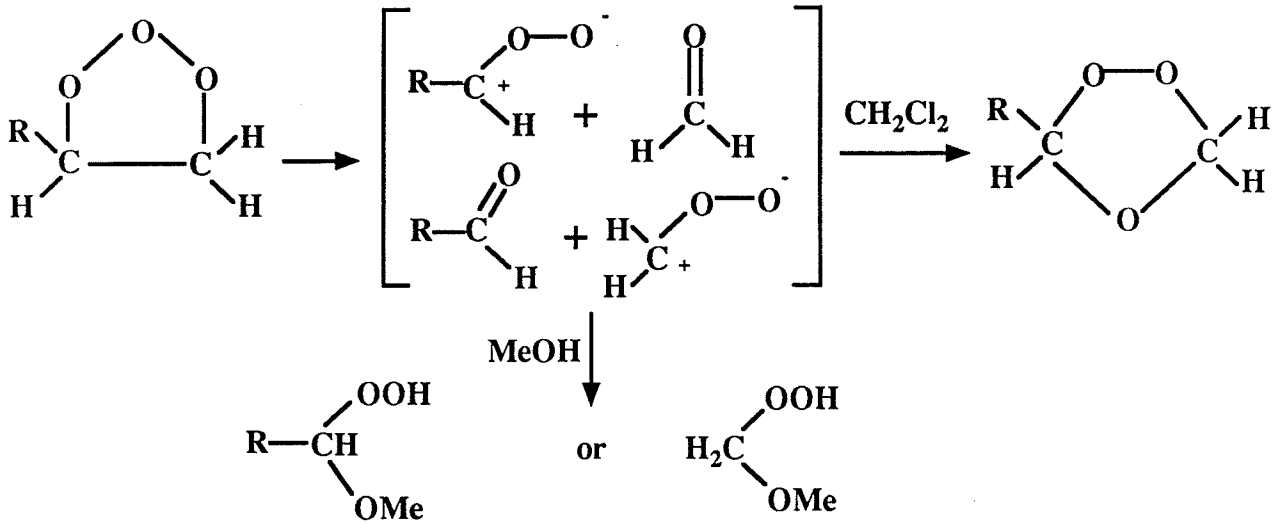
The general mechanistic aspects of this reaction discussed below are based on established mechanisms (Criegee, 1968). The first step in ozonolysis is an addition of ozone to the double bond and formation of alkene-ozone adduct which is called either the molozonide, the initial ozonide or the primary ozonide (Scheme 10). The cyclic adduct containing a three-membered ring (molozonide) is highly unstable and direct evidence for its existence is lacking. This species is believed to rearrange to one containing a four-membered ring and this rapidly converts into a 1,2,3-trioxolane to give the earliest intermediate which has been characterized spectrally (Bailey *et al.*, 1966; Hull *et al.*, 1972). A subsequent cleavage-recombination pathway is considered to be the major contributing route to the

crossed ozonide or 1,2,4-trioxolane, which is the final ozonide product formed. Such a product can exist if the zwitterionic intermediates are formed (Story *et al.*, 1971) in a non-reactive environment. When ozonolysis is performed in hydroxylic solvents *e.g.*, methanol, the zwitterionic cleavage intermediates are trapped as α-hydroperoxy ethers (Keaveney *et al.*, 1967) (Scheme 10). Recombination is then prevented and the carbonyl compound formed in the cleavage step can also be isolated under these conditions, whereas use of inert (nonparticipating) solvents results in the formation of ozonides and polymeric peroxides (Knowles and Thompson, 1960). It has been reported that if the carbonyl products are desired, it is advantageous to carry out ozonolysis in methanol. The resulting α-methoxyalkyl hydroperoxides can then be reduced with dimethyl sulphide, giving the carbonyl compounds in good yield (Pappas *et al.*, 1966). Other reducing agents have been also employed for the same purpose *e.g.*, zinc in acetic acid, sodium sulfide and trimethylphosphite (Knowles and Thompson, 1960; Challighan and Wilt, 1961).

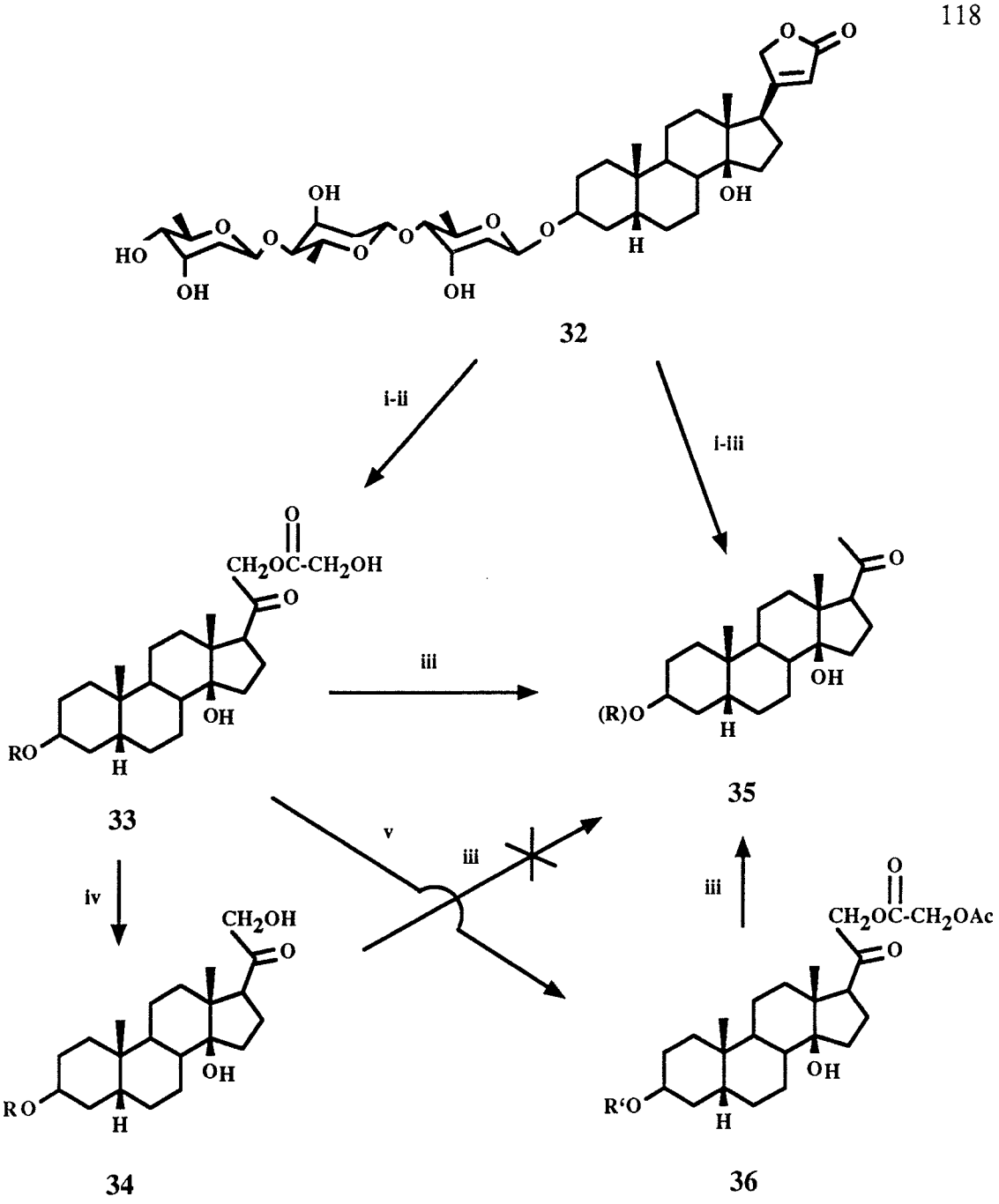
Despite the complexities in the mechanism of ozonolysis the reaction constitutes a high-yield method for cleavage of carbon-carbon double bonds. The conversion of the α, ß-unsaturated γ-lactone of digitoxin (**32**) into the acetyl side chain of **35** was achieved by a "one-pot" procedure using ozone and excess zinc in acetic acid (Scheme 11). The direct ozonolysis of digitoxin (**32**) in methanol at low temperature followed by reduction of the ozonide product with excess zinc and



Scheme 9. Mechanism of ozonide formation.



Scheme 10. Mechanism of ozonide decomposition.



R = ß-D-trisdigitoxose

R' = ß-D-trisdigitoxose tetraacetate

(R) = R or R'

Reagents and conditions: i, O_3-MeOH, -70 °C, 45 min; ii, Zn-AcOH,r.t., 5min; iii,Zn-AcOH, r.t., 20hrs; iv, 5% aq. $KHCO_3$-MeOH, r.t., 16hrs; v, $(Ac)_2O$, reflux, 1.5hrs.

Scheme 11. Ozonization products of digitoxin.

acetic acid for 5 min gave the 21-hydroxyester (**33**) as the major product from the intermediate 21-glyoxaldehyde ester (Zingg and Meyer, 1960; Ragab *et al.*, 1962) plus the 21-hydroxymethylketone (**34**) as a minor product . In this work it was found that longer treatment (20 hrs) resulted in further reduction of the 21-hydroxyester (**33**) to the methyl ketone (**35**) in good yield.

This novel method for the conversion of the α, ß-unsaturated γ-lactone to the acetyl group has three synthetic advantages:

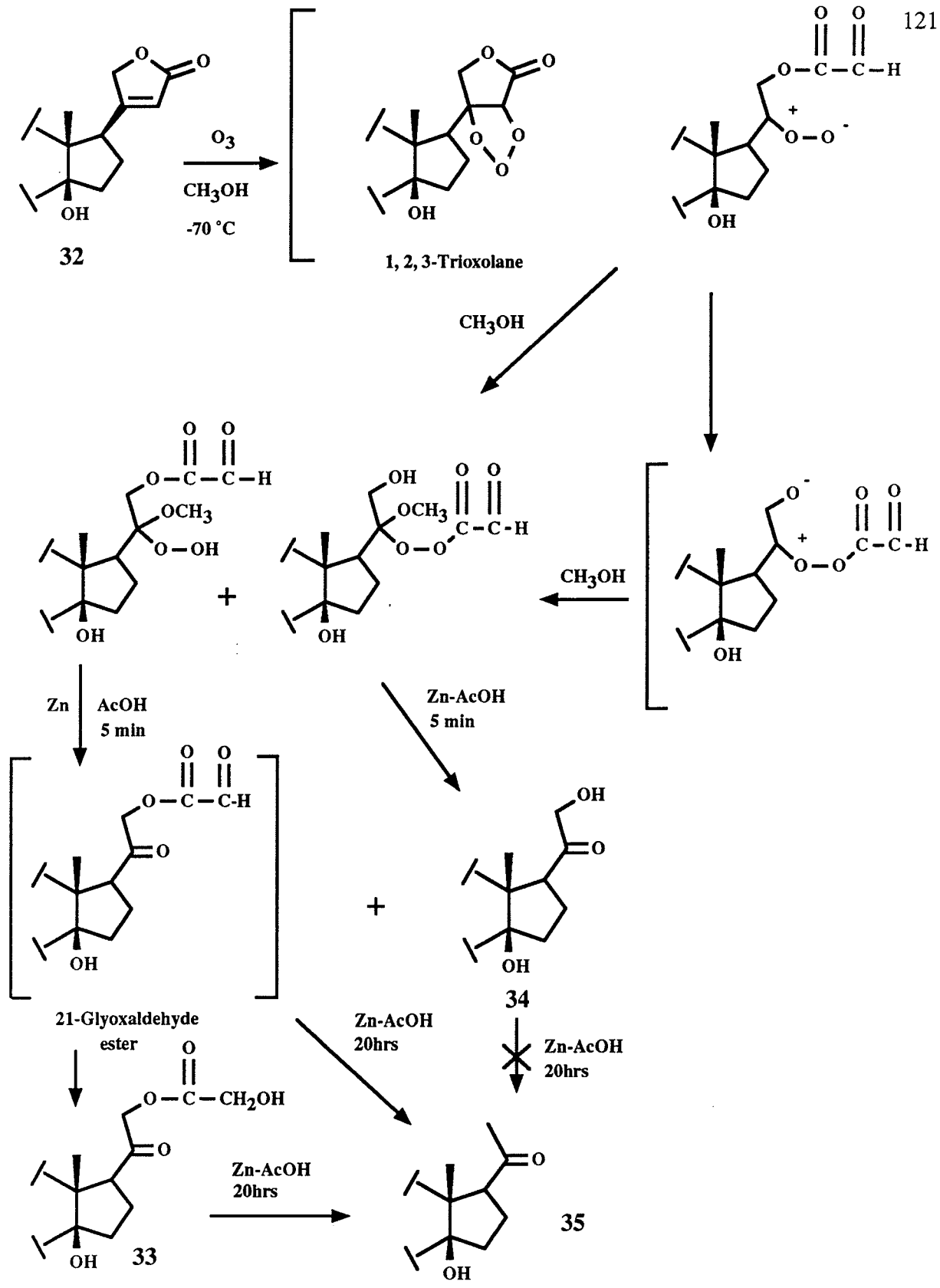
(1) Removal of C-21 oxygen function through the multistep sequence reported previously (Theil *et al.*, 1980; Lindig, 1983) by tosylation, iodide substitution and reduction with zinc in acetic acid (Reichstein and Fuchs, 1940; Baran J.J., 1964; Wolff *et al.*, 1970), or treatment with sodium hydrogen sulfide (Theil *et al.*, 1980) for the preparation of the C-17 acetyl substituted compound was considerably shortened and a better overall yield was obtained.

(2) The multistep procedure required protection of the glycoside alcohols. This is not necessary when using the excess zinc and acetic acid treatment of the ozonide for 20 hrs.

(3) This method also allowed an improved synthesis of 3ß,14-dihydroxy-5ß,14ß-pregnan-20-one by initial protection of the 3ß-alcohol by reaction with 2,2,2-trichloroethyl chloroformate in pyridine to form the 2,2,2-trichloroethyl carbonate derivative. This group was subsequently removed by zinc in

acetic acid at the time of the ozonide reduction (Templeton *et al.*, 1991b) and α-elimination.

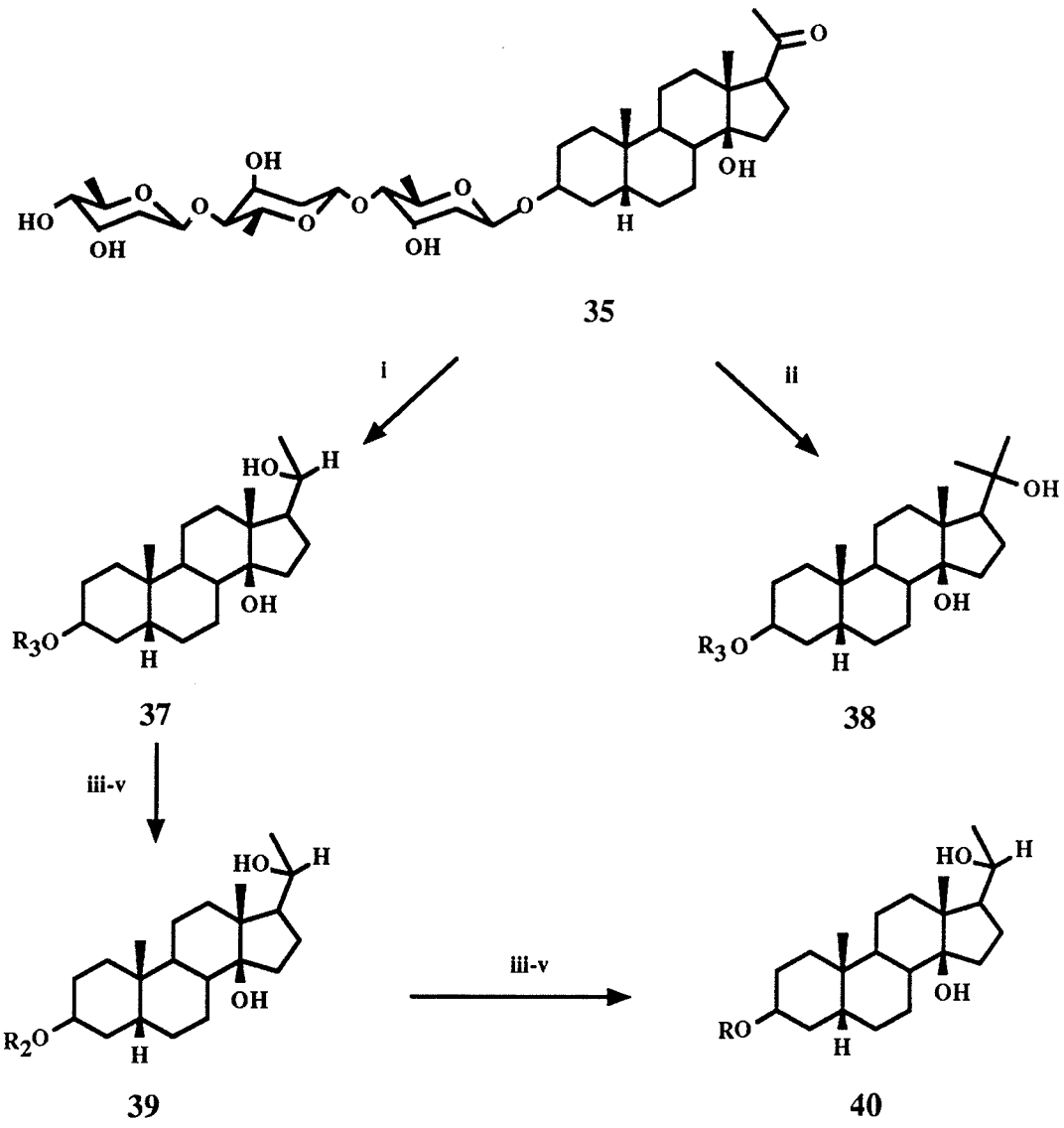
A proposed mechanism based on the earlier mechanistic studies accounted for this reaction (Scheme 12). The formation of the 21-glyoxaldehyde ester intermediate, which has not been adequately characterized by ^{1}H and/or ^{13}C NMR assignments or isolated in a pure form is rapidly reduced by zinc and acetic acid to the 21-hydroxyester (**33**). The minor product **34** was not reported previously from the ozonolysis reaction of digitoxin (**32**). Evidence for the formation of **33** and **34** can be obtained from the ^{1}H and ^{13}C NMR of the total crude product when compared with the individual NMR data for the pure compounds given in Tables 3 and 6, repectively. It has been reported (Zingg and Meyer, 1960; Ragab *et al.*, 1962) that the reductive work-up of the ozonide with zinc in acetic acid affords the 21-hydroxyester (**33**), which may results from reduction of the intermediate glyoxaldehyde (Scheme 12). Acetylation of **33** with acetic anhydride afforded the 21-acetoxyacetate (**36**) (Scheme 11), confirming the presence of a hydroxyl group. Longer treatment of the ozonide with excess zinc and acetic acid (20 hrs) gave the corresponding 21-methyl ketone (**35**) in 78% yield. Treatment of **33** under the same conditions also gave the same 21-methyl ketone (**35**) whereas on similar treatment the 21-hydroxyketone (**34**) was not significantly converted into the methyl ketone (**35**). It appears that the 21-hydroxyester (**33**) is the principal intermediate in the formation of the desired 21-methyl ketone (**35**). Mild alkaline



Scheme 12. Proposed mechanism of the ozonolysis in methanol and Zn-HOAc reduction of the C-17 α,ß-unsaturated γ-lactone ring of digitoxin.

hydrolysis of the 21-hydroxyester (**33**) gave the 21-hydroxyketone (**34**) as previously described (Reichstein and Fuchs, 1940; Hunziker and Reichstein, 1945; Meyer and Reichstein, 1947; Baran, 1964; Wolff *et al.*, 1970; Rabitzsch, 1971). It was necessary to perform this reaction under argon to exclude oxygen in order to avoid autoxidation of the hydroxyester to the glyoxaldehyde which further cyclizes to a hemiketal with the 14ß-hydroxyl group (Ragab *et al.*, 1962).

With the 21-methyl ketone (**35**) readily available, derivatization at C-20 was possible for SAR studies. Two derivatives obtained from the 20-methyl ketone (**35**) (Scheme 13) were the 20ß-alcohol (**37**), obtained by reduction with lithium tri-tert-butoxyaluminohydride in tetrahydrofuran, and the 20-methyl compound (**38**), obtained by addition of methylmagnesium iodide. The 20ß-OH compound was the predominant product from lithium tri-tert-butoxyaluminohydride reduction of **35**. Reduction of 3ß-acetoxy-14-hydroxy-5ß,14ß-pregn-20-one with sodium borohydride has been shown (Lindig, 1983) to give the 20ß-alcohol as the major product in a ratio of approximately 1:3 (α:ß). Reduction with lithium tri-tert-butoxy-aluminohydride gave a mixture of epimers in a ratio of 1:2 (α:ß), based on ^{1}H NMR comparison of the 20α- and 20ß-H for the corresponding genin (3ß-OAc) (Templeton and Ling, private communication). A similar ratio is inferred for the trisdigitoxosides where the 20α- and 20ß-H signals are obscured by the glycoside protons. Their structures (**37**, **38**) were confirmed by ^{1}H NMR and ^{13}C NMR spectra (Tables 3 & 6, repectively). The ^{1}H NMR of the 20ß-alcohol (**37**) shows



Reagents and conditions: i, LTBAH-THF, r.t., 1hr; ii, CH_3MgI-THF, r.t., 3hrs; iii, $NaIO_4$-EtOH, r.t., 1hr; iv, LTBAH-THF, r.t., 16hrs; v, 0.0065 HCl-MeOH, r.t., 2.5hrs.

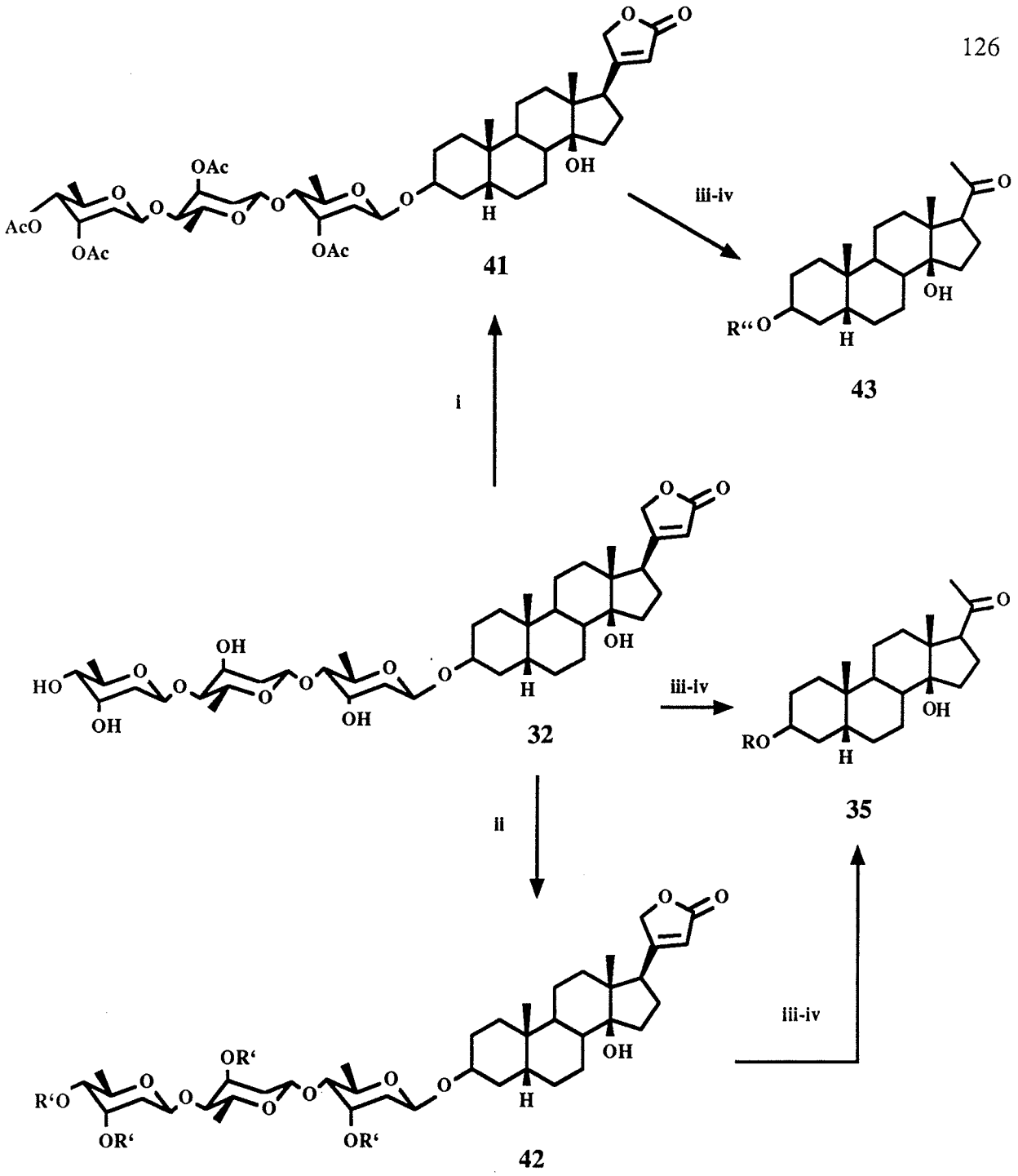
Scheme 13. Synthesis of mono-, bis-, and trisdigitoxosides of 3ß,14-dihydroxy-5ß,14ß-pregnan-20ß-ol.

the chemical shift for the 20-methyl group to be 1.25 ppm (a doublet) and the ^{13}C NMR shows the absence of the carbonyl group signal. The structural assignment for **38** was based on ^{13}C NMR data (Table 6) which showed the presence of new methyl and quaternary carbons and loss of the C-20 carbonyl.

Synthesis of mono and bis ß-D-digitoxoside derivatives was carried out for comparison of their biological activities with other C-3 glycosides. Degradation of the sugars to the mono and bis derivatives was achieved by the method of Satoh and Aoyama, (1970) except that lithium tri-tert-butoxyaluminohydride was substituted for sodium borohydride in the dialdehyde reduction. Three steps are required for the stepwise hydrolysis of the sugar units *i.e.*, oxidation of the diol terminal sugar of the 20ß-alcohol (**37**) to the dialdehyde intermediate, followed by reduction with lithium tri-tert-butoxyaluminohydride and mild acid hydrolysis to give the bisdigitoxoside (**39**) (Scheme 13); the second digitoxose unit was removed in a similar manner to give the monodigitoxoside (**40**). The concentration of acid used here (0.0065 N HCl) did not effect complete sugar hydrolysis of the glycoside from the steroid molecule as the intact digitoxose units are relatively stable under this condition (Goldstein *et al.*, 1965). This method for selective degradation of the sugar units is an improvement on the method reported earlier by Kaiser *et al.*, (1957) for the partial hydrolysis of the trisdigitoxoside (digitoxin and gitoxin) to the corresponding bis and mono digitoxoside. Furthermore, the direct acid hydrolysis described by Kaiser *et al.* (1957) gave not only the bis and mono

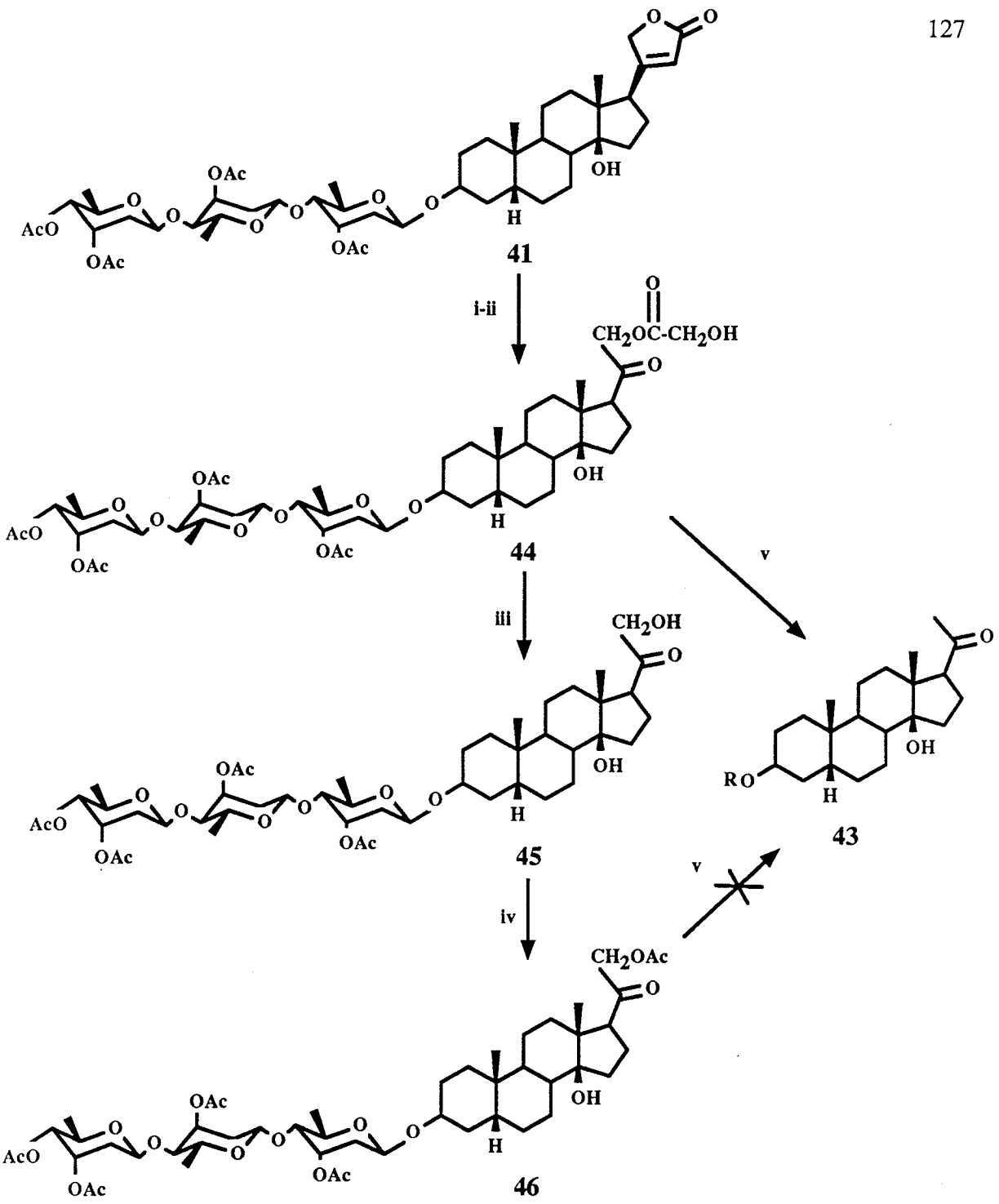
digitoxosides, but also genin and starting material. The resulting mixture of products made the isolation of the individual components more difficult. The controlled degradation by meta-periodate oxidation of polysaccarides containing vicinal alcohols, reduction and hydrolysis is well established (Goldstein *et al.*, 1965; Kubota and Hinoh, 1968). The relative ease of hydrolysis of the primary alcohol reduction product compared with the glycosides themselves requires explanation. Intra-molecular assistance of hydrolysis by one of the primary hydroxyl groups formed has been proposed to rationalize the relative hydrolysis rates (Satoh and Aoyama, 1970). The structures of the bis and monodigitoxoside derivatives have been established by their ^{1}H and ^{13}C NMR spectra given in Tables 3 and 6, respectively, and are consistent with published data (Habermehl *et al.*, 1985; Drakenberg *et al.*, 1990).

Acetylation of the trisdigitoxoside **32** (Scheme 14) by refluxing with acetic anhydride for 1.5 hrs afforded the digitoxin tetraacetate (**41**) as the main product (Rabitzsch, 1971), while treatment with 2,2,2-trichloroethyl chloroformate in pyridine gave digitoxin tetra-2,2,2-trichloroethyl carbonate (**42**). Ozonolysis and zinc in acetic acid treatment (20hrs) of **41** and **42** gave the corresponding methylketones **35** and **43**, respectively as the major products. Ozonolysis of compound **42** followed by shorter treatment with zinc in acetic acid gave the 21-hydroxyester (**45**) (Scheme 15). This was selectively hydrolysed at C-21 with potassium hydrogen carbonate in an inert atmosphere to avoid oxidation to the



Reagents and conditions: i, $(Ac)_2O$, reflux, 1.5hrs; ii, $CCl_3CH_2CO_2Cl$-pyridine, 0 °C, r.t., 1hr; iii, O_3-MeOH, -70 °C, 1hr; iv, Zn-AcOH, r.t., 20hrs.

Scheme 14. Synthesis of digitoxin tetraacetate and tetra-2,2,2-trichloroethyl carbonate, and their conversion to the 14-hydroxy-5ß,14ß-pregnan-20-one derivatives.



Reagents and conditions: i, O_3-MeOH, -70 °C, 1hr; ii, Zn-AcOH, 5 min; iii, $KHCO_3$-MeOH, r.t., 70 min; iv, $(Ac)_2O$-pyridine, r.t., 18hrs; v, Zn-AcOH, r.t., 20hrs.

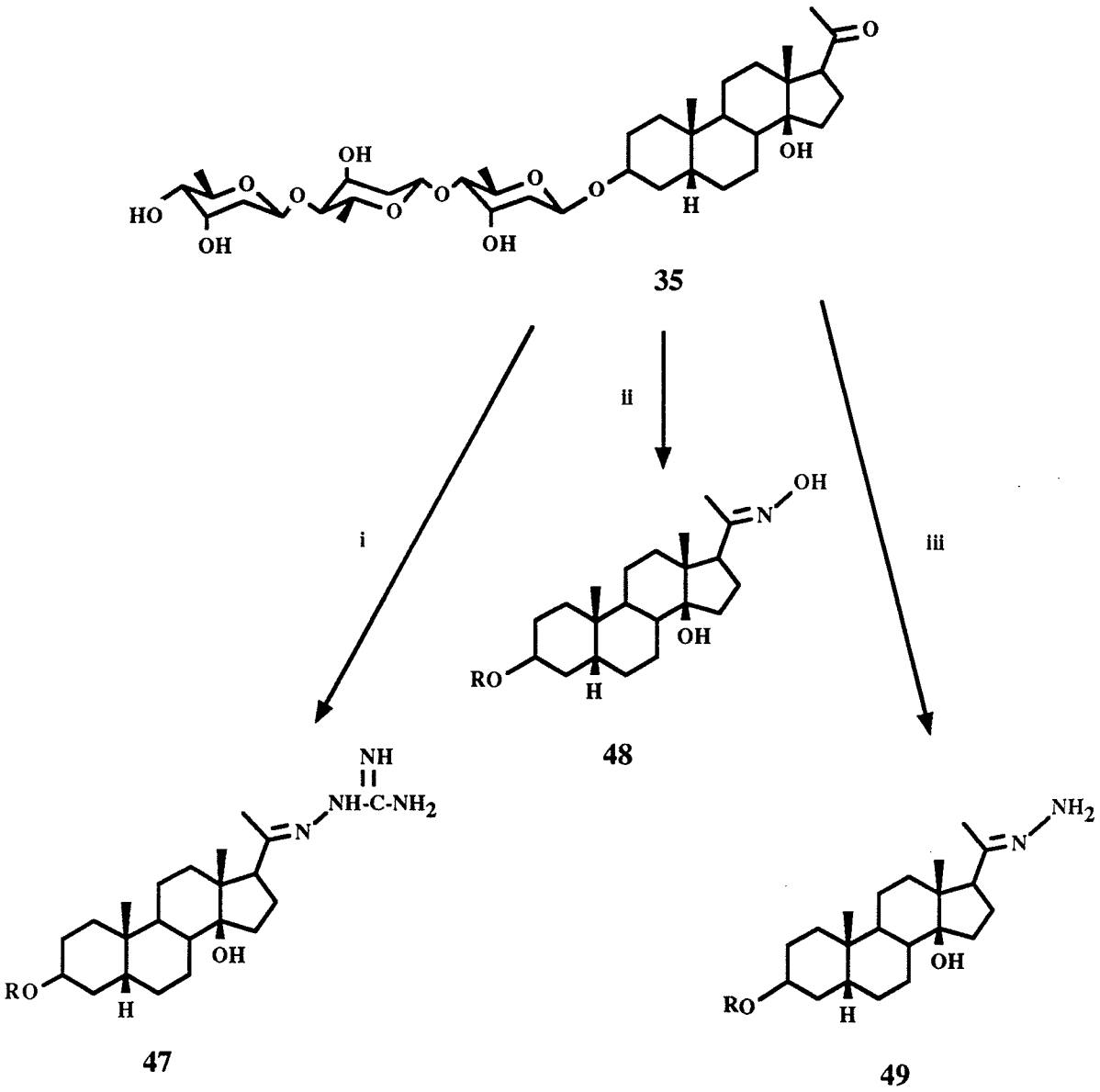
Scheme 15. Ozonolysis of digitoxin tetraacetate, and zinc and acetic acid reduction of C-21 oxygenated dervatives.

carboxylic acid as described in the literature (Rabitzsch, 1971). This gave the 21-hydroxyketone (**45**) which in turn, on acetylation, gave the 21-acetoxyketone (**46**). When the 21-hydroxyester (**44**) was treated with zinc and acetic acid for 20 hrs it was converted into the 21-methyl ketone (**43**) whereas similar treatment of the 21-acetoxyacetate gave **36** (Scheme 12) in about 70% yield together with 30% starting material as shown by the ^{1}H NMR of the total crude product. The 21-acetoxyketone (**46**) was unchanged after similar treatment with zinc and acetic acid. This indicates that the hydroxyacetoxy group at C-21 is a better leaving group than the hydroxy, acetoxy or acetoxy acetate substituents, possibly through interaction of the hydroxyl group on the zinc surface.

A previous report (Slates and Wendler, 1957) showed that 21-acetoxy-17α-hydroxypregn-4-ene-3,20-dione on treatment with zinc and acetic acid is also unchanged, even at elevated temperatures. However, this reaction was reported for the 14-αH series and does not necessarily agree with that found in the 14ß-hydroxy series. The large excess of zinc used for α-elimination was shown to be necessary, because with smaller amounts of this reagent no product was seen on TLC after vigorous stirring for 20 hrs at room temperature. A large excess of zinc, as is generally required in zinc reductions, may be necessary because the zinc has an uneven distribution of reactive surface area. Competition and/or activation of the zinc surface may also play a role in the reduction. Alternatively, a ten fold excess of 2% zinc-copper couple in ethanol or acetic acid proved to be ineffective in

formation of the 21-methyl ketone (**35**). When excess 2% zinc-copper couple was used in a manner similar to that used with zinc alone in acetic acid, the 21-methyl ketone was obtained. These results indicate that the couple was no more effective than the untreated zinc. Reactions with zinc-silver, zinc-amalgam or acid washed zinc gave similar results (Templeton and Ling unpublished results).

Derivatives made from the lactone ring at the C-17ß position of digitoxin (**32**) were synthesized from the key intermediate structure, the 21-methyl ketone (**35**). An outline of the synthetic route to the 20-guanylhydrazone (**47**), 20-oxime (**48**) and 20-hydrazone (**49**) is shown in Scheme 16. The reaction sequence for the synthesis is essentially that described by Boutagy and Thomas (1973) for the preparation of **47**. Accordingly, treatment of the 21-methyl ketone (**35**) with a slight excess of aminogaunidine bicarbonate and an equivalent amount of potassium hydroxide in 95% ethanol for 6 hrs under reflux gave the desired product. The ^{1}H NMR spectrum (Table 3) showed the chemical shift of the 21-methyl group at (1.99 ppm), *i.e.* an upfield shift compared to the 21-methyl group of the starting material (**35**) (2.26 ppm). The ^{13}C NMR (Table 6) displayed a signal at 164.84 ppm for the 20-C=N and at 159.03 ppm for =N-NHC. The 20-ketone (**35**) readily underwent condensation with hydroxylamine hydrochloride in a mixture of pyridine (Vogel, 1956), aqueous sodium acetate and 95% ethanol (Robinson *et al.*, 1966) giving the 20-oxime. The non-nucleophilic base (pyridine) was used in order to minimize isomerization at the C-17 position. The 20-hydrazone (**49**) was made by

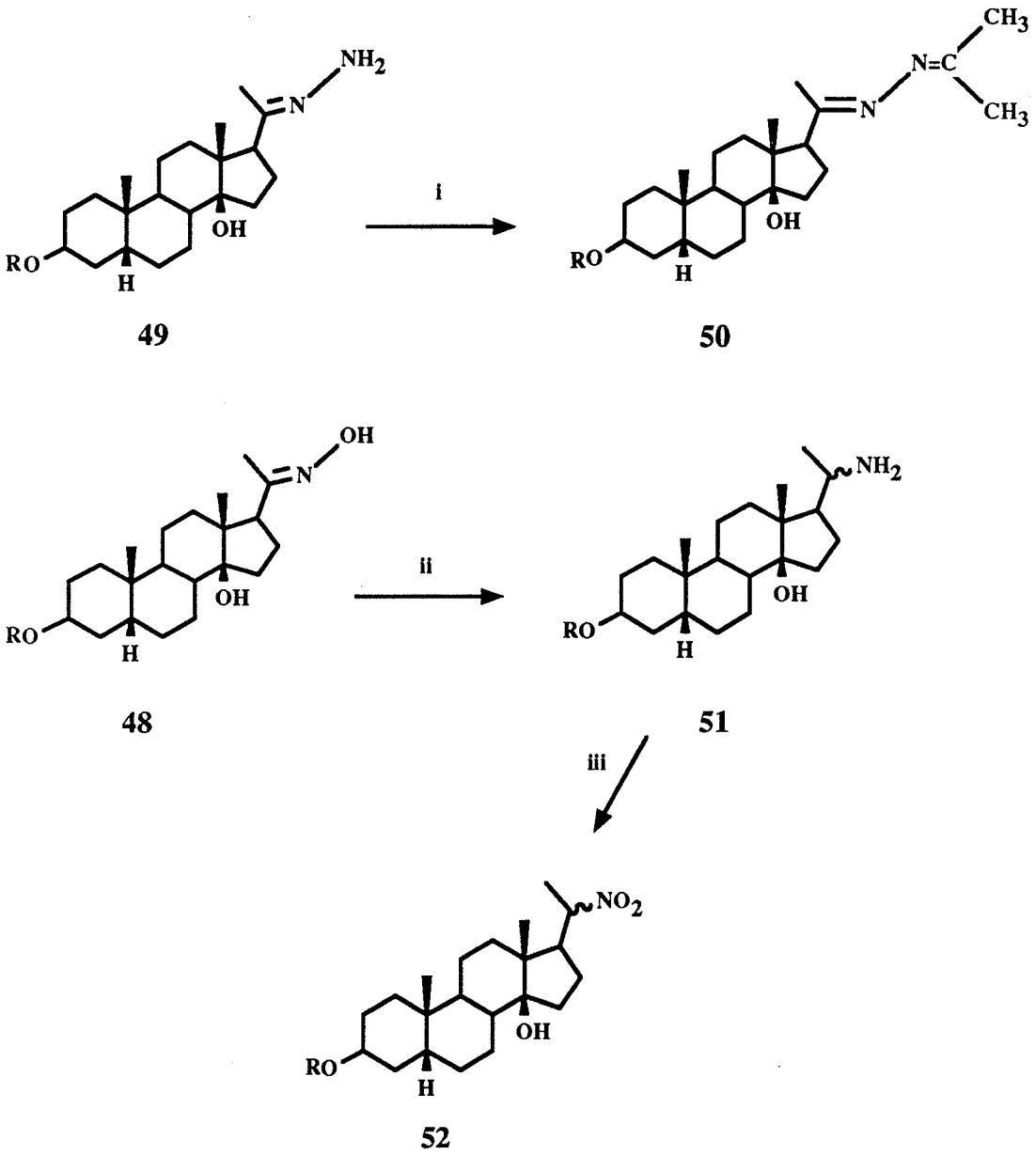


Reagents and conditions: i, $H_2NNHC(=NH)NH_2 . H_2CO_3$-KOH-95% EtOH, reflux, 6hrs;
ii, H_2NOH . HCl-aq. NaOAc-pyridine-95% EtOH. reflux, 2hrs;
iii, 85% NH_2NH_2-Et_3N-95% EtOH. reflux, 2hrs.

Scheme 16. Synthesis of the C-20 guanylhydrazone, oxime and hydrazone of 14-hydroxy-5ß,14ß-pregn-20-one trisdigitoxoside derivatives.

condensation of **36** with 85% hydrazine in the presence of a basic catalyst (Barton *et al.*, 1962) *e.g.*, triethylamine and 95% ethanol under reflux. The stereochemistry of these derivatives may be either *cis*(Z)- and/or *trans*(E) but direct evidence from their ^{1}H NMR spectra for a mixture was not observed. However, based on the observation of Oliveto (1972) regarding the stereochemistry observed in the Beckmann rearrangement of oximes, the products are expected to be only *trans*(E) and thus that stereochemistry has been tentatively assigned as such for **47**, **48** and **49**. The by-product **50** (Scheme 17) was conveniently obtained during the purification of the 20-hydrazone (**50**) by crystallization from acetone. The ^{1}H (Table 3) and ^{13}C NMR (Table 6) were consistent with the product shown. Two signals of approximately equal intensity appeared at 1.91 ppm and 1.95 ppm which integrate for three protons each. The signal for the 21-methyl appeared further downfield (2.07 ppm). The ^{13}C NMR also showed two new methyl signals at 24.02 ppm and 25.04 ppm. The quaternary carbon signal of the isopropylidine group was observed at 172.87 ppm.

Reduction of the oxime (**48**) with sodium in 1-propanol under reflux for 135 min (Robinson *et al.*, 1966) proved a satisfactory method for synthesizing the 20-amine (**51**). The product appeared to be a mixture of the epimeric 20-amino compounds by ^{1}H and ^{13}C NMR. The isomers (20α- and 20-ß-amine) could not be separated by chromatography. It is interesting to note that sodium-alcohol reduction of steroidal 20-oximes gives the 20-amine mixture in which the 20ß-



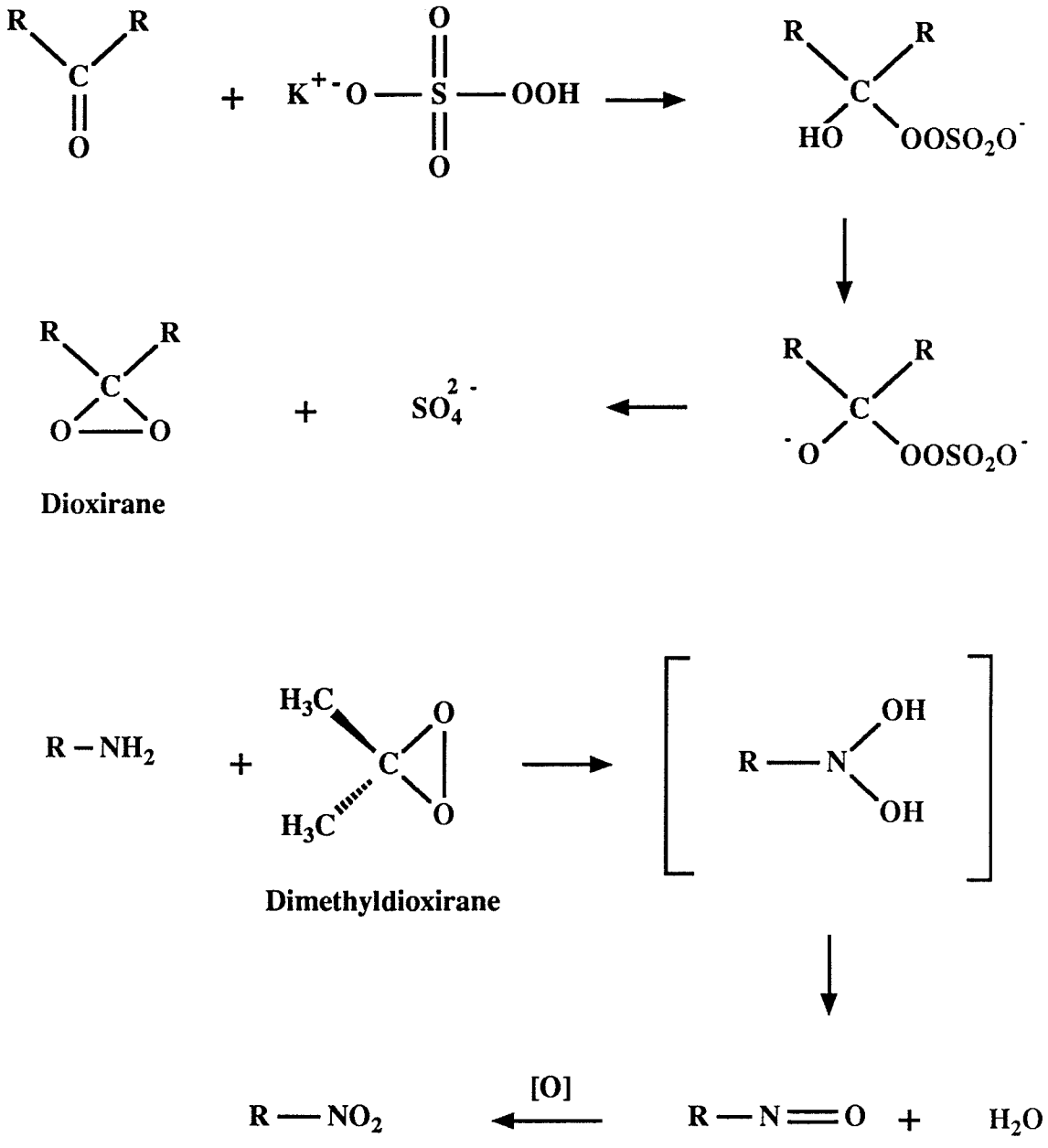
R = ß-D-trisdigitoxose

Reagents and conditions: i, Me_2CO, heat, 10 min; ii, Na-n-propanol, reflux, 2.25hrs; iii, 3-chloroperoxybenzoic acid-$CHCl_3$, r.t., 5hrs or dimethyl-dioxirane-CH_2Cl_2, r.t., 15 min.

Scheme 17. Synthesis of the C-20 isopropylidine, amine and nitro derivatives of 14-hydroxy-5ß,14ß-pregnane trisdigitoxoside.

isomer predominates in 14α-steroids (Chien *et al.*, 1964). A signal comparable to the C=N signal at 163.87 ppm in the ^{13}C NMR spectrum of the oxime (**48**) was absent from the spectrum of the product (**51**).

The conversion of the 20ξ-amine (**51**) to the 20ξ-nitro compound (**52**) (Scheme 17) could be achieved using either peracid, dioxirane or ozone in an inert solvent *e.g.*, dichloromethane. Robinson *et al.* (1966) used 3-chloroperoxybenzoic acid in chloroform as the oxidizing system. Their reaction was initially carried out at room temperature on **51**. The product isolated however, contained some nitroso dimers. Therefore, they modified the conditions were modified so that the amine was added slowly to a solution of the peracid in refluxing chloroform, and formation of the nitroso compound could thereby be prevented. These conditions for the oxidation of **51** were not employed because of the instability of both the C-14ß OH and the glycoside linkage. Consequently, the reaction was carried out at room temperature and the crude product was purified by chromatography (*ca.* 24% yield). Another oxidation procedure was carried out by bubbling ozone into a solution (Bailey *et al.*, 1972) of the amine. Keinan and Mazur (1977) reported that the oxidation of primary amines to nitro compounds could be effected by using dry ozone on silica gel at - 78 °C in better yield . However, this procedure may not be applicable to the steroids because ozone can also oxidize unreactive saturated hydrocarbons under such dry conditions (Price and Tumolo, 1964; Cohen *et al.*, 1975; Stary *et al.*, 1976).



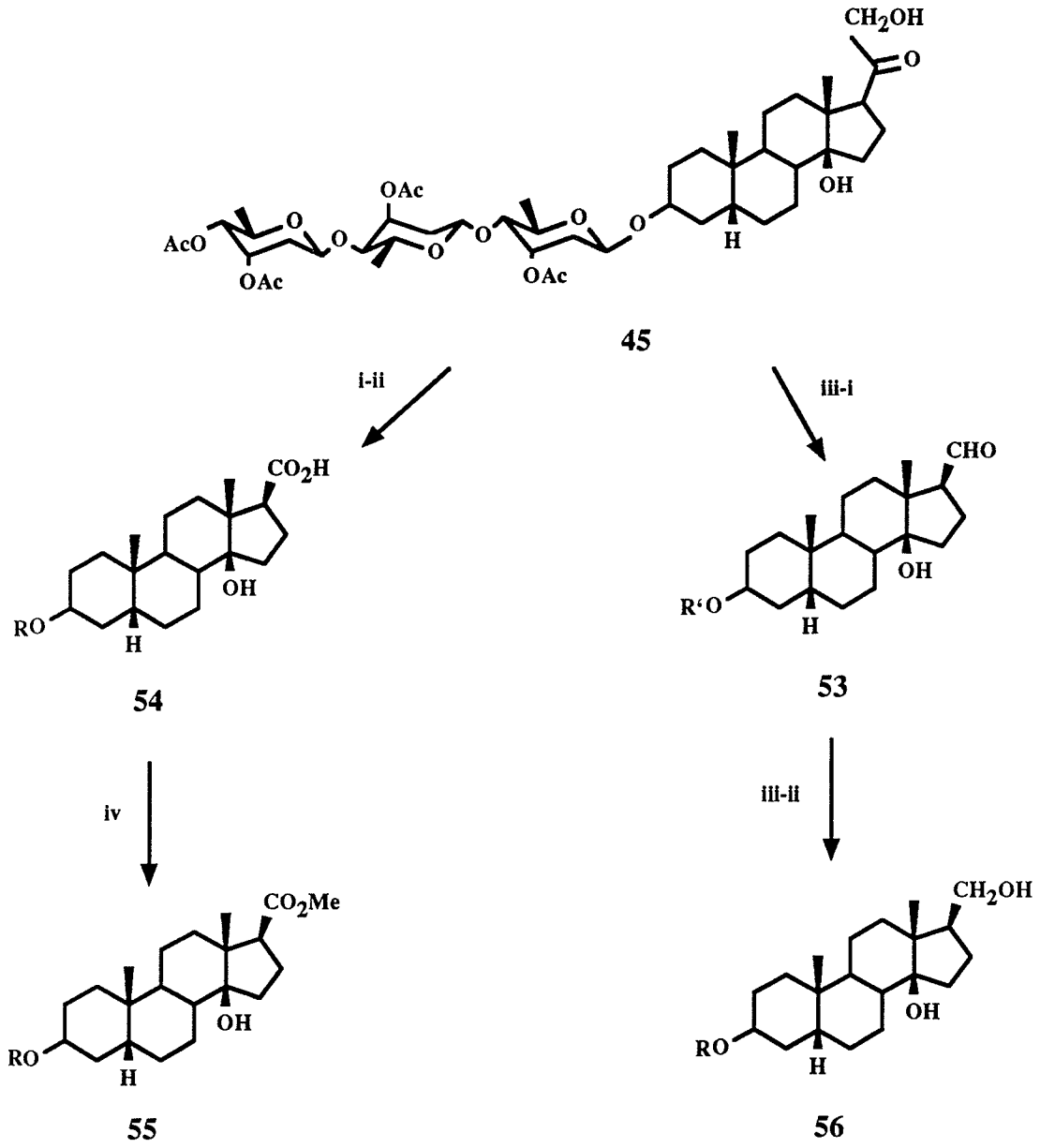
Scheme 18. Synthesis and application of the dioxirane reagent.

More recently, Murray *et al.* (1986) explored a simple method for the oxidation of nitrogen-containing compounds. In their original publication (Murray and Jeyaraman, 1985), they succeeded in isolating dimethyldioxirane in solution as a powerful oxygen atom donor. The events leading to the successful isolation of dioxiranes began with an observation by Montgomery (1974). Essentially, what Montgomery observed was that certain ketones enhance the rate of decomposition of monoperoxysulfuric acid. This led to the proposal that the monoperoxysulfate anion was adding to the ketone to give the intermediate shown in Scheme 19. Montgomery further proposed that the intermediate reacted further to give a dioxirane. The suggested reaction pathway is shown in Scheme 19. The advantage of this reaction is that it can be carried out under mild conditions, no workup is needed and it is suitable for more sensitive compounds which are unstable to acidic or basic conditions. Thus, the 20ξ-amine (**51**) was oxidized by this reagent into the nitro derivative (**52**) and the yield (43%) was greater than that obtained with peracid or ozone oxidation. Confirmation of the identity of the 20ξ-nitro compound (**52**) came from elemental analysis and ^{13}C NMR. Assignment of the C-20 carbon showed that it had shifted further downfield (87.65 ppm) compared with the starting material (49.65 ppm) (see Table 6).

The approach used to synthesize the 17ß-etianaldehyde (**53**), a key intermediate structure for the synthesis of 21-nor derivatives, is given in Scheme 20 and is essentially that reported by Eberlein *et al.* (1972a) except that the reagent

used for the mild reduction step was lithium tri-tert-butoxyaluminohydride instead of sodium borohydride. The vicinal diol intermediate was oxidized with sodium metaperiodate to give the 17ß-etianaldehyde (**53**). The ^{1}H NMR spectral data (Table 4) were consistent with this structure in that the aldehyde proton appeared as a downfield signal at 9.72 ppm as a doublet with a coupling constant of J 3.8 Hz. ^{13}C NMR data (Table 7) showed one signal downfield at 206.63 ppm (CHO) and an absence of an upfield signal that could be assigned to the C-21 carbon. Reduction of **54** with lithium tri-tert-butoxyaluminohydride in tetrahydrofuran at room temperature gave the 21-nor-alcohol intermediate which was further treated with sodium in methanol under reflux to give the 21-nor-alcohol 3ß-trisdigitoxoside (**56**). The complete reduction and base catalysed hydrolysis of the tetraacetate on the sugar moiety of the molecule was confirmed by ^{1}H and ^{13}C NMR spectra. For instance, the tetraacetate signals usually appear at high intensity as singlets in the envelope region of the steroid molecule in the ^{1}H NMR spectrum between 1.98 and 2.09 ppm (for digitoxin tetraacetate (**41**)) and these are not seen after base hydrolysis.

Oxidative cleavage of the 21-hydroxyketone (**45**) with sodium metaperiodate gave the 17ß-carboxylic acid intermediate which is a direct synthetic route for this derivative, and better than a route through oxidation of **53** (House, 1972). Further treatment of the crude acid product with sodium methoxide gave the 17ß-carboxylic acid ß-trisdigitoxose (**54**) (Scheme 19). The observed ^{1}H and ^{13}C NMR are in



R' = ß-D-trisdigitoxose tetraacetate

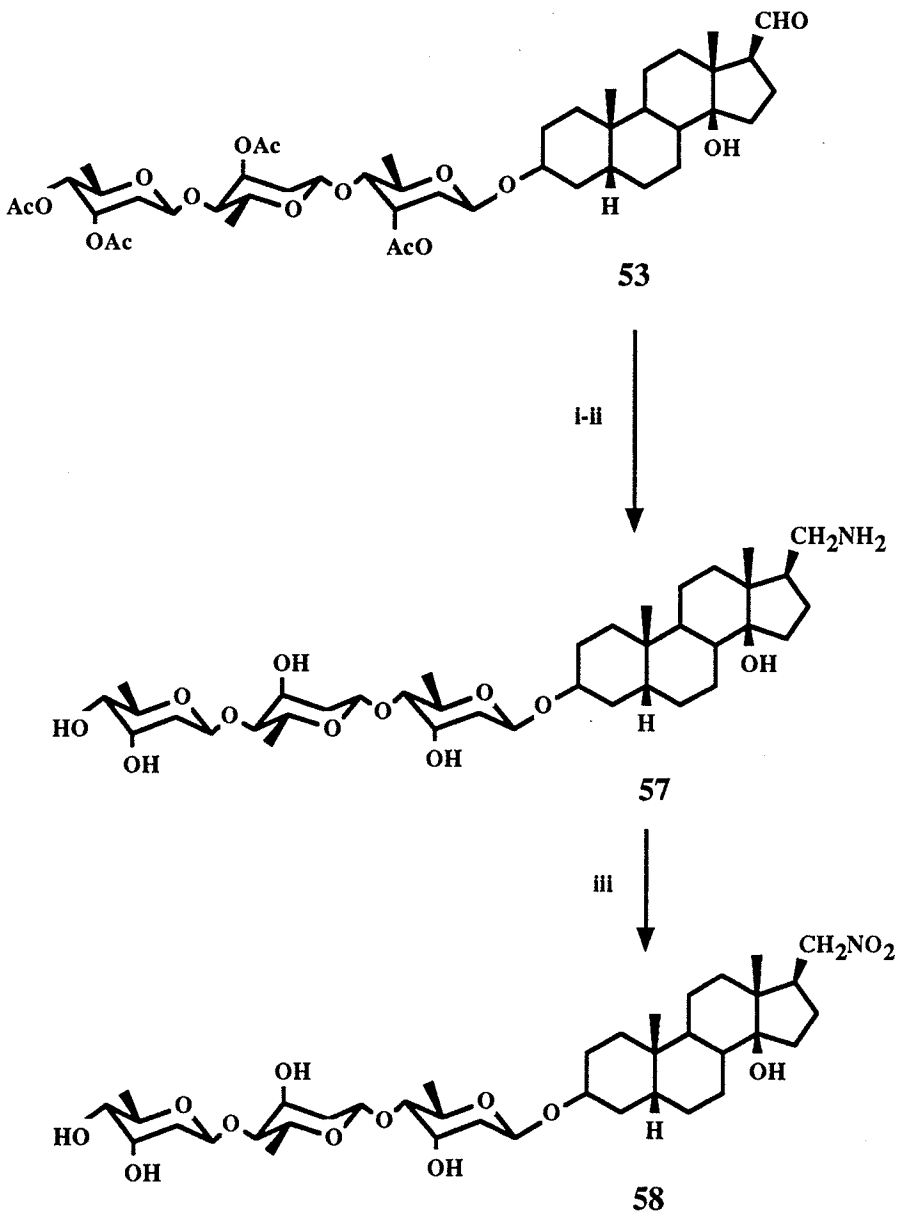
R = ß-D-trisdigitoxose

Reagents and conditions: i, $NaIO_4$-95% EtOH, r.t., 1hr; ii, CH_3ONa-MeOH, reflux, 1hr; iii, LTBAH-THF, r.t., 15 min.; iv, CH_2N_2-Et_2O-MeOH, r.t., 2 min.

Scheme 19. Synthesis of 14-hydroxy-21-nor-5ß,14ß-pregnane C-20 etianic acid, ester and alcohol derivatives.

agreement with the expected product structure. The tetraacetate signals were not observed in the ^{1}H NMR spectrum and the C-20 carbonyl group of the carboxylic acid now showed a chemical shift in the ^{13}C NMR at 181.12 ppm compared to the starting material **45** (217.20 ppm) (Table 7). Further confirmation was obtained from the synthesis of the 20-methyl ester (**55**) through a one step reaction of **54** with diazomethane in ether-methanol. Base hydrolysis of the tetraacetate with sodium methoxide under reflux gave the 20-carboxylic acid 3ß-trisdigitoxoside (**55**). The structural assignment was based on the ^{1}H NMR (Table 4) which showed a 20-methyl ester signal at 3.71 ppm as a singlet and the ^{13}C NMR which showed a new methyl signal at 52.35 ppm (Table 7).

The synthetic route to the C-20 amine (**57**) is outlined in Scheme 21. The key step in this sequence was the reduction of the oxime to the amine. Reaction of the 17ß-etianaldehyde (**53**) with hydroxylamine hydrochloride and a mixture of pyridine and aqueous sodium acetate in 95% ethanol under reflux gave a mixture of two products which were distinguishable on TLC and were presumed to be the *cis* and *trans* isomeric oximes. Dissolving metal reduction is one of the methods used for the reduction of oximes (Robinson *et al.*, 1966). Thus, the crude mixture was dissolved in 1-propanol and treated with sodium under reflux. Besides the reduction step, removal of the acetate groups also occurred. The only product obtained was the 21-nor-amine (**57**). Complete reduction was evident from the TLC and ^{1}H NMR spectrum (Table 4). The C-20 protons appeared to be



Reagents and conditions: i, NH_2OH . HCl-NaOAc-pyridine-95% EtOH, reflux, 2hrs; ii, Na-1-propanol, reflux, 2.5hrs; iii, O_3-CH_2Cl_2, -70 °C, 2.5hrs.

Scheme 20. Synthesis of 14-hydroxy-21-nor-5ß,14ß-pregnane trisdigitoxoside, C-20 amine and nitro derivatives.

equivalent and were obscured as two adjacent signals centred at 2.70 and 2.72 ppm of the AB part of an ABX system with coupling constant $J_{AX} = J_{BX} = 6.0$. Further support for the 21-nor-amine (**57**) was obtained from the ^{13}C NMR spectrum (Table 7) which showed a signal at 42.51 ppm assigned to the C-20 carbon.

As mentioned earlier, saturated primary amines undergo oxidation reactions with ozone in solution (Bailey *et al.*, 1972) resulting in the formation of the corresponding nitroalkanes. When the 21-nor-amine (**57**) (Scheme 20) was ozonized in dichloromethane, the resulting product was separated chromatographically to give the 21-nor-nitro derivative (**58**). The elemental analysis was consistent with the expected product. The ^{13}C NMR (Table 7) offered more information for the assignment of the structure. For example, the signal assigned to C-20 in **58** (81.25 ppm) was shifted further downfield as compared with **57** (42.51 ppm).

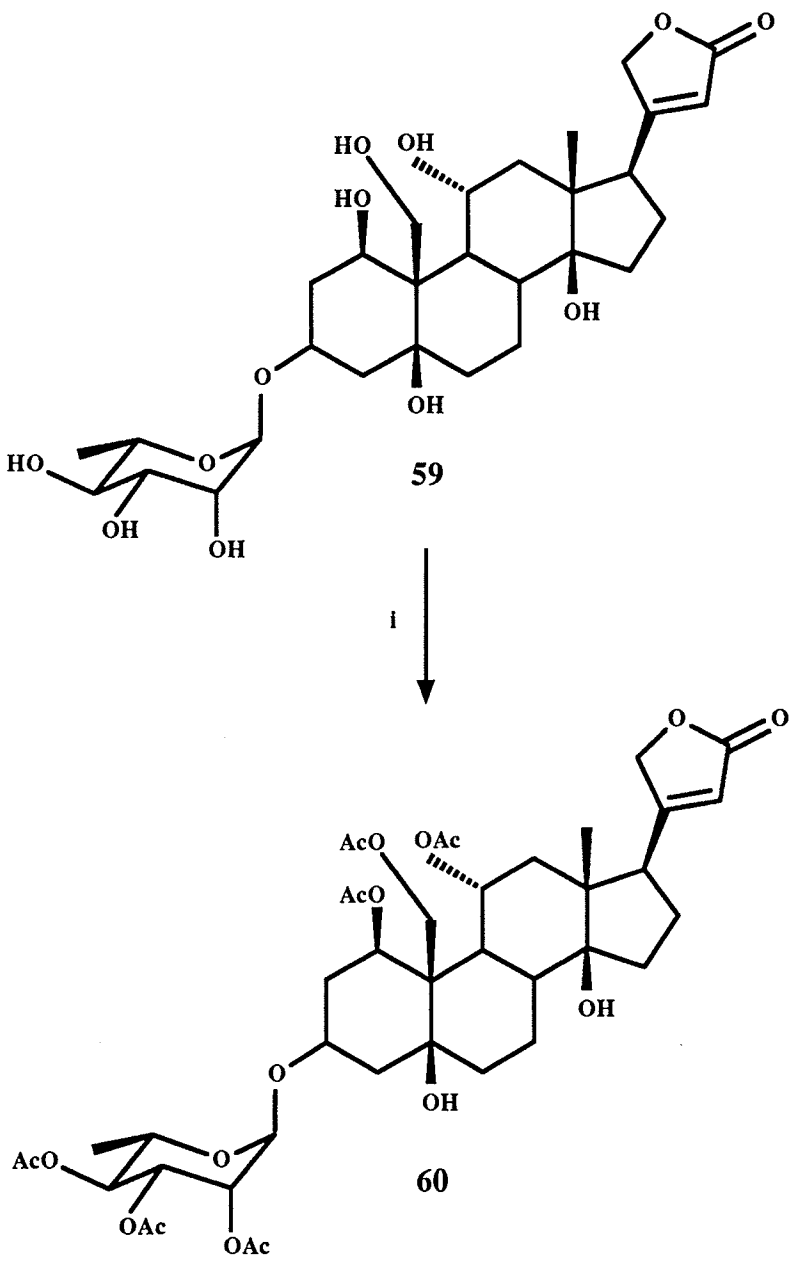
III CHEMISTRY: SYNTHESIS OF PREGNANE RHAMNOSIDE ANALOGUES FROM CARDENOLIDES (OUABAIN).

The synthetic strategy for the conversion of ouabain (**59**) into pregnane derivatives was different from that described for digitoxin (**32**) because of the presence of both primary and secondary hydroxyl groups in the molecule. For example, a direct attack of ozone on the γ-lactone of **59** in methanol can cause oxidation of certain hydroxyl groups in the molecule (*i.e.*, 1ß-, 11α- and 19-hydroxyl) to form carbonyl groups. We observed the ozone oxidation of the C-3ß-

alcohol of digitoxigenin to the C-3 ketone (Templeton *et al.*, 1991b). The reductive work-up of the ozonide, which requires zinc and acetic acid, makes it more difficult to isolate the crude product due to the hydrophilic nature of the molecule. Consequently, a protective group for the alcohols was necessary to carry out these transformations. Esters, being reasonably stable under ozonolysis and the acidic conditions of the reaction were employed to protect the hydroxyl groups.

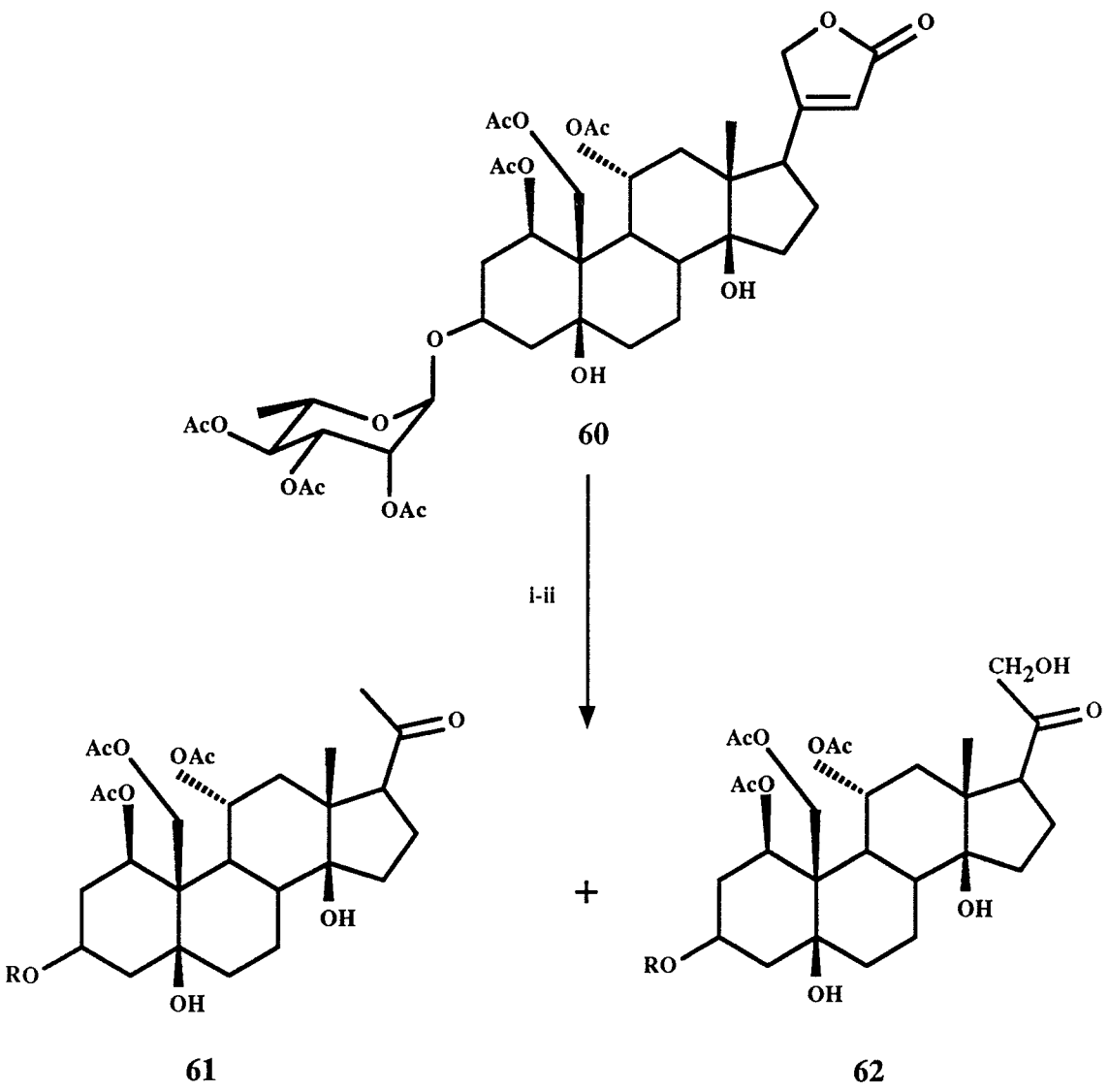
Acetylation of ouabain (**59**), by refluxing with acetic anhydride, yielded ouabain hexaacetate (**60**) (Scheme 21). The melting point was in agreement with that reported by Mannich and Siewert (1942) and the ^{1}H NMR spectrum displayed six signals for the hexaacetate methyl groups as singlet protons of equal intensity (1.93 to 2.15 ppm) (Table 5). The six signals for the carbonyl group of the hexaacetate are also seen further downfield in the ^{13}C NMR between 169.56 and 173.16 ppm (Table 8). Support for this assignment can be seen from the homonuclear shift correlation spectrum as this technique (Kirk *et al.*, 1990) will show the interrelationship between the various signals.

Ozone treatment of ouabain hexaacetate (**60**) in dichloromethane at -70 $^{\circ}C$, followed by excess zinc and acetic acid for 24 hrs at room temperature gave two products in about equal yield which were separated chromatographically (**61**, **62**) (Scheme 22). The ^{1}H NMR spectrum of **61** indicated the presence of 21-methyl protons as a singlet which integrated as three protons, and the absence of a signal in the downfield region compared to the starting material (Table 5), assigned to the



Reagent and condition: i, $(Ac)_2O$, reflux, 1.5hrs.

Scheme 21. Synthesis of ouabain hexaacetate.

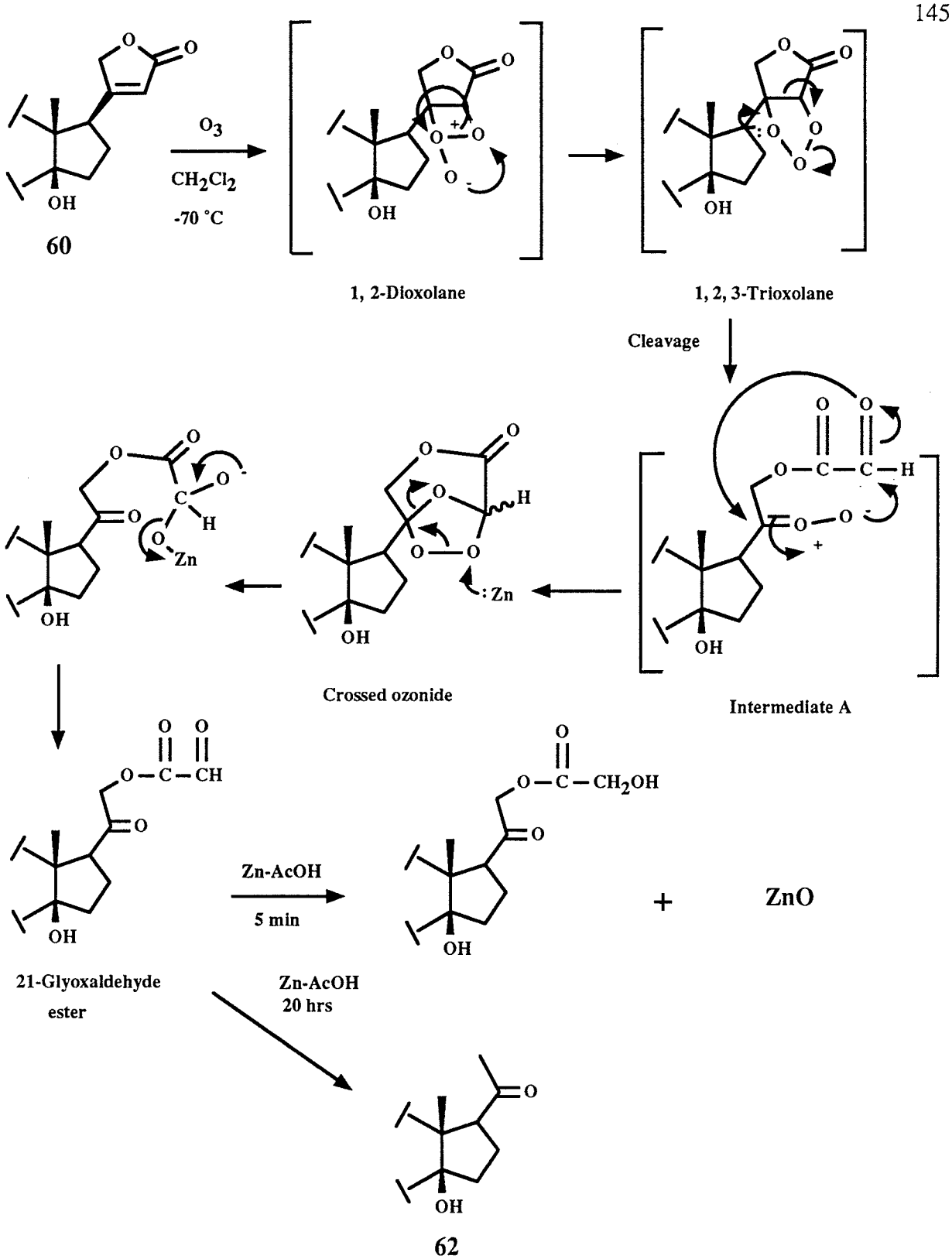


R = α-L-Rhamnose triacetate

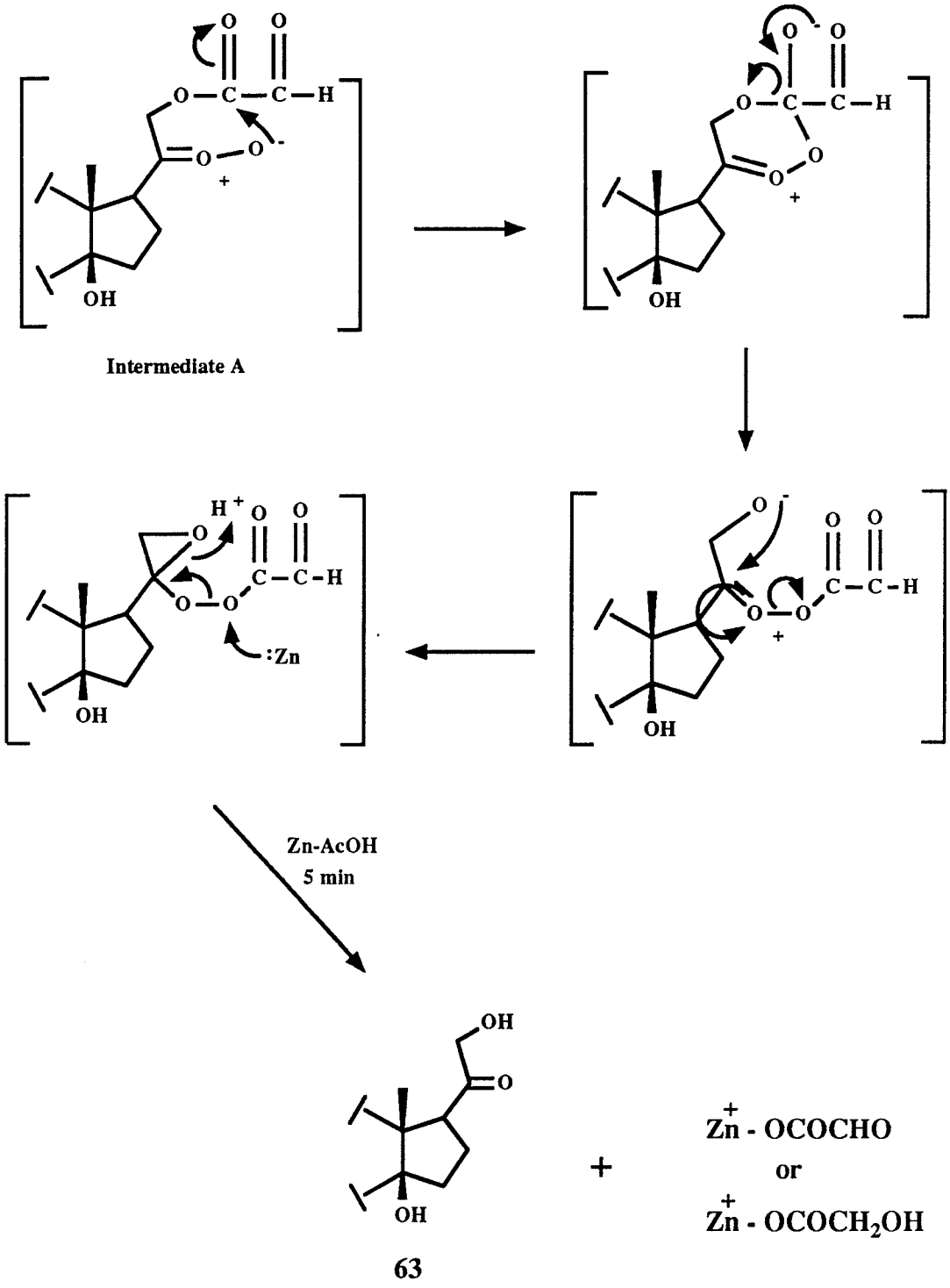
Reagents and conditions: i, O_3-CH_2Cl_2, -70 °C, 1hr; ii, Zn-AcOH, r.t., 24hrs.

Scheme 22. Synthesis of pregnane derivatives from ouabain hexaacetate.

22-H (5.87 ppm). The C-21 methyl signal was also present in the ^{13}C NMR spectrum (Table 8) at (33.33 ppm). The second product (**62**) was identified as the 21-hydroxy ketone at the C-17ß position, based on the data given in the ^{1}H and ^{13}C NMR spectra (Tables 5 and 8). The signal at 3.03 ppm appeared as a triplet (J 4.5 Hz) and was assigned to the 21-OH. A multiplet around 4.18-4.34 ppm was assigned to the 21-H_2 protons. These appeared together with the 3α-H and 5ß-OH proton signals. The methylene signal of the C-21 carbon in the ^{13}C NMR showed a large shift downfield (70.14 ppm) compared to the C-21 methyl carbon of **61** (33.33 ppm) which was now absent. From a comparison of the ozonation of ouabain hexaacetate (**60**) in dichloromethane to the results obtained from digitoxin (**32**) in methanol, the formation of the 21-hydroxyketone (**62**) in a similar amount with **61** was unexpected. Differences in the rates of the two breakdown processes of the ozonide ouabain hexaacetate (**60**) on the zinc surface can account for the different ratio of the products formed. The same product ratio of **61** to **62** was also observed when methanol was used as the ozonolysis solvent, suggesting that the difference in ozonide breakdown is related to the way in which the structures of digitoxin (**33**) and ouabain (**60**) affect the zinc surface interaction. The proposed mechanism in Scheme 24 shows the formation of the ozonolysis products (**62**) and (**63**) in dichloromethane. The end products of ozonation (Scheme 23), unlike in methanol, cannot react with dichloromethane (Razumovskii and Zaikov, 1984) but can form, through intermediate A, by rearrangement of the bipolar ion, the more



Scheme 23. Proposed mechanism of ozonolysis of the C-17 α,β-unsaturated γ-lactone ring of ouabain hexaacetate in dichloromethane.

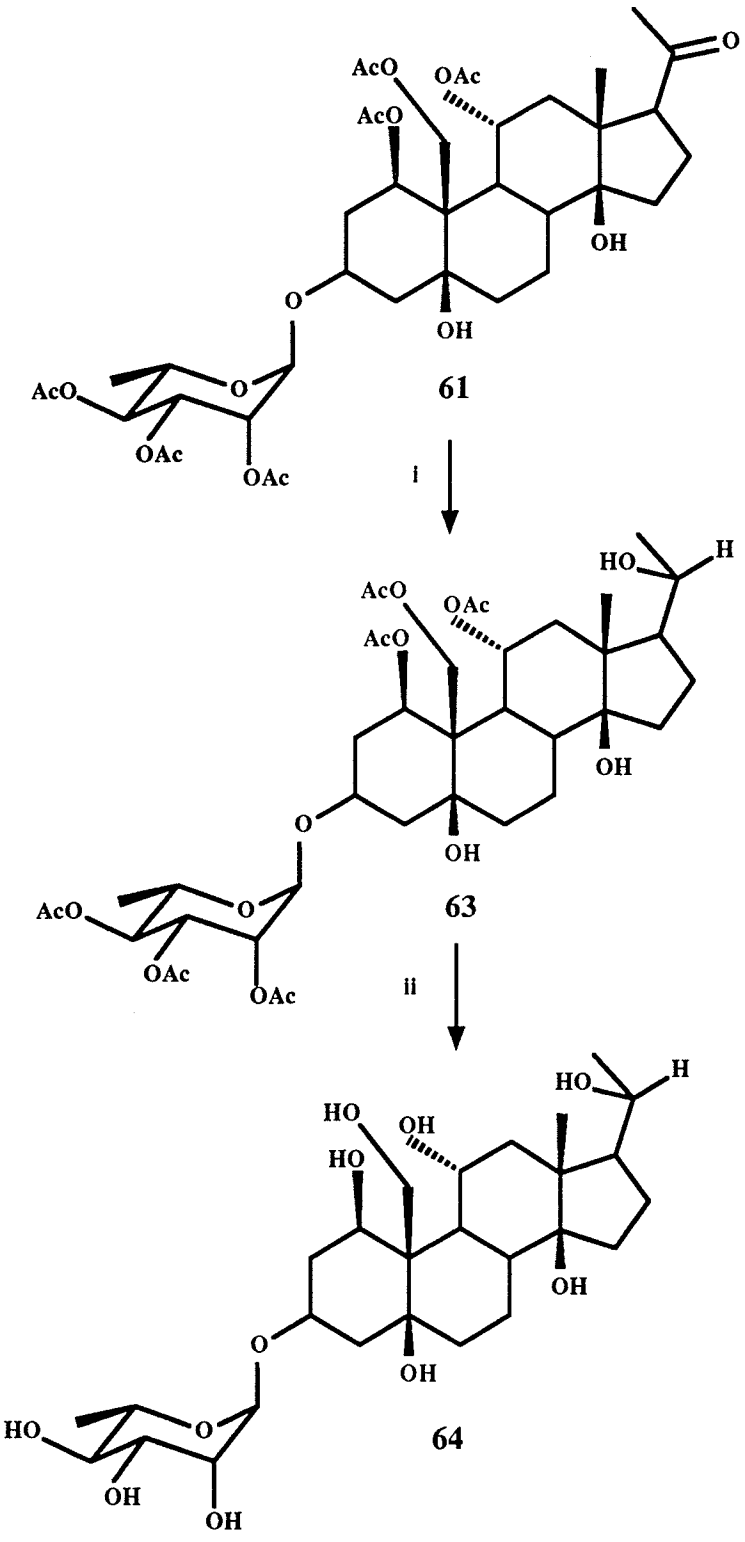


63

Scheme 23 (cont'd)

stable crossed ozonide intermediate (1,2,4-trioxolane). This ozonide forms the 21-glyoxaldehyde ester through reduction with zinc in acetic acid, which is rapidly reduced to the 21-hydroxyester. On longer treatment it yields the 21-methylketone (**61**). An alternative rearrangement of intermediate A can occur through attack on the ester carbonyl leading to a cyclic product which further rearranges to the epoxide. The epoxide on reaction with zinc yields the 21-hydroxymethylketone (**62**) (Scheme 23, cont'd). This mechanism would predict formation of the 21-hydroxymethylketone if the aldehyde carbonyl is reduced to the primary alcohol.

The next step in the sequence (Scheme 24) required reduction of the 20-keto function to the analogous alcohol. Reaction of the 21-methylketone (**61**) with lithium tri-tert-butoxyaluminohydride in tetrahydrofuran at room temperature for 15 min gave the 20ß-alcohol (**63**). The product was identified using ^{1}H NMR and ^{13}C NMR (Tables 5 and 8). The signal for the 21-methyl group appears at 1.28 ppm as a doublet (J 3.3 Hz) due to splitting by the C-20αH. The multiplet at 3.84 ppm was assigned to the 20α-H. One signal appearing at 3.98 ppm as a singlet and integrating as a single proton was assigned to the C-14ß OH (Table 5). Finally, the ^{13}C NMR spectrum showed that the C-20 keto function of **61** had been removed. Deacetylation of the hexaacetate with sodium methoxide in methanol under reflux afforded the hexol (**64**) (Scheme 24). In the present case, the usual work-up was found ineffective for isolating the product because the polyhydroxyl groups on the steroid molecule render it more soluble in the aqueous layer than in



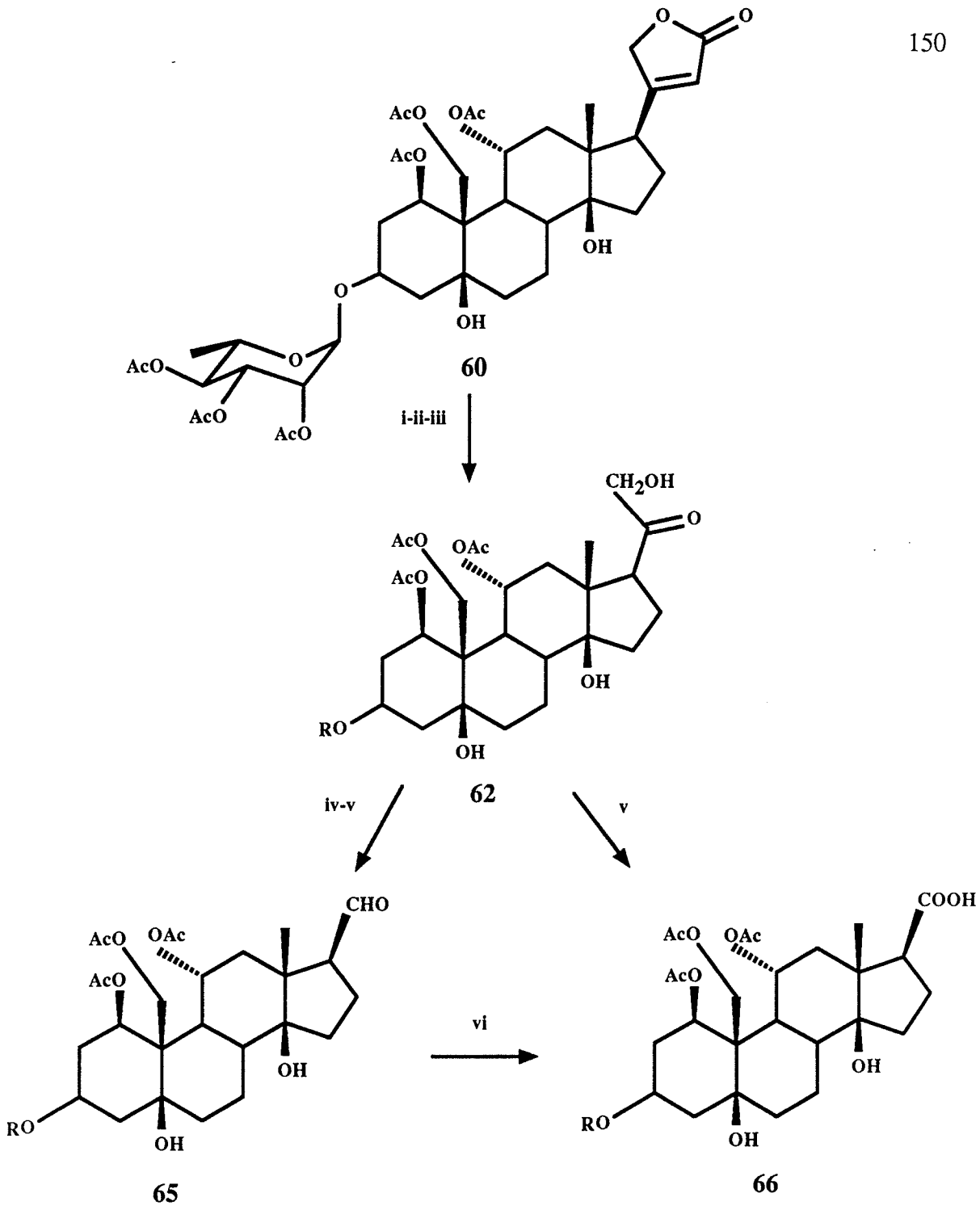
Reagents and conditions: i, LTBAH-THF, r.t., 15 min; ii, $NaOCH_3$-MeOH, reflux, 1.5hrs.

Scheme 24. Synthesis of ouabain derivatives .

the organic layer. Because extraction failed, a weakly acidic cation exchange-resin with carboxylic acid functionality was used for work-up. Use of a strongly acidic sulfonic acid resin led to dehydration of the 14ß-hydroxyl group to the corresponding Δ^{14} derivative and caused hydrolysis of the sugar to the genin. To avoid epimerization the reduction step was therefore carried out before basic hydrolysis of the hexaacetate in the 21-methyl ketone (**61**). The 20-keto function in a 14ß-hydroxy steroid, which exists in a thermodynamically unstable configuration, undergoes inversion to the more stable 17α-compound if enolization occurs under basic conditions (Boutagy and Thomas, 1971). The ^{1}H NMR spectrum of **64** shows no acetate signals.

The synthesis of the 21-hydroxyketone was carried out by either of the synthetic routes outlined in Scheme 22 or 25. Ozonolysis of ouabain hexaacetate (**60**) (Scheme 25) and a brief treatment with zinc and acetic acid followed by selective mild base hydrolysis (Tamm *et al.*, 1957; Eberlein *et al.*, 1972a,b) of the 21-hydroxyacetoxy group gave the C-17ß 21-hydroxymethylketone (**62**). The melting point, and ^{1}H and ^{13}C NMR spectra were in agreement with the product obtained from Scheme 23.

Two steps were required for the synthesis of the 21-nor-etianaldehyde (**65**). First, reduction of **62** with lithium tri-tert-butoxyaluminohydride in tetrahydrofuran at room temperature gave the diol intermediate which consists of the C-20 epimers. Second, oxidative cleavage of the diols with sodium metaperiodate in 95% ethanol



R = α-L-Rhamnose triacetate

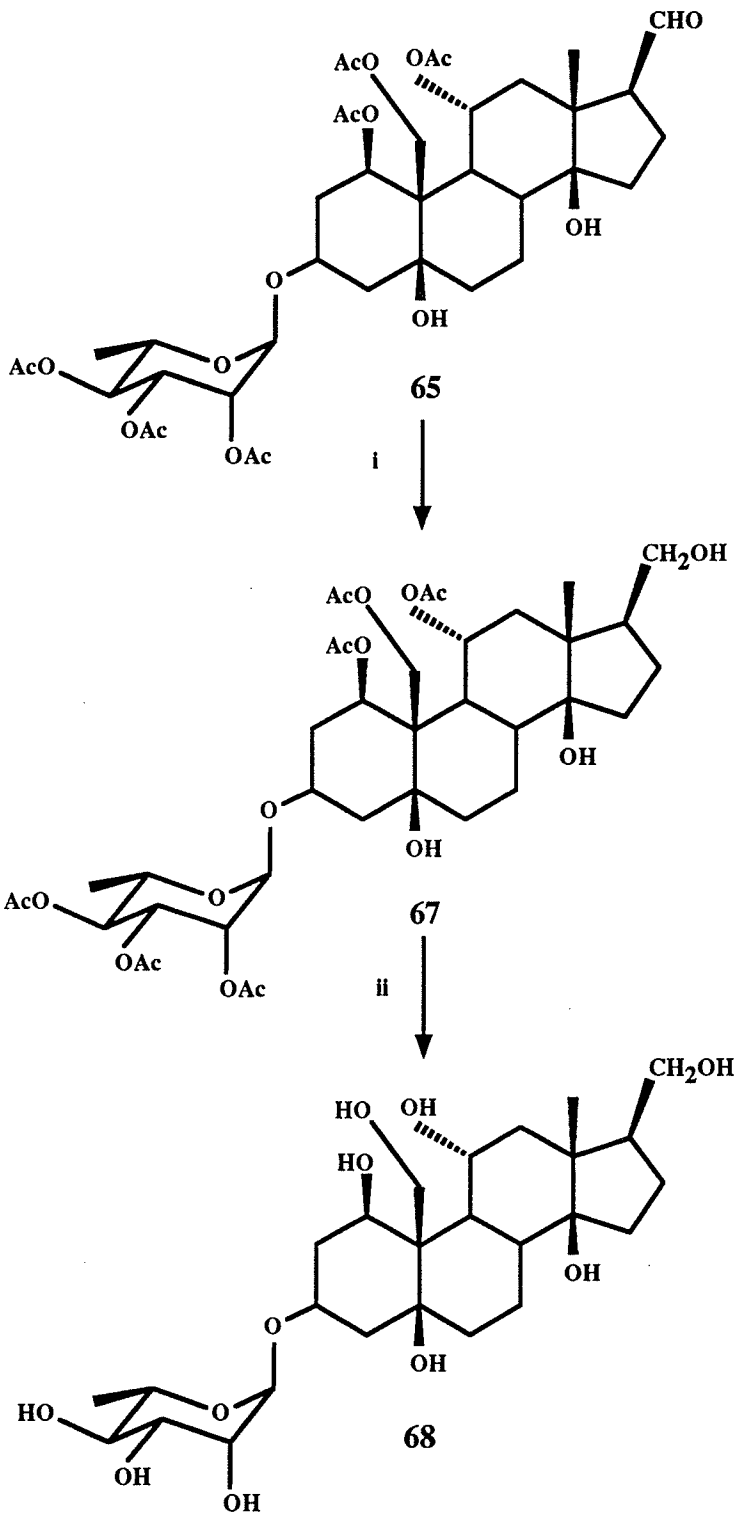
Reagents and conditions: i, O_3-CH_2Cl_2, -70 °C, 1hr; ii, Zn-AcOH, r.t., 5 min; iii, $KHCO_3$-MeOH, r.t., 4hrs; iv, LTBAH-THF, r.t., 15 min; v, $NaIO_4$-95% EtOH, r.t., 1hr; vi, CrO_3-AcOH, r.t., 1hr.

Scheme 25. Synthesis of oubain 17ß-etianaldehyde and 17ß-etianic acid.

gave the 21-nor-etianaldehyde (**65**) whose ^{1}H NMR spectrum displayed an aldehyde proton as a doublet at (9.73 ppm) with a coupling constant of J 3.1 Hz. The ^{13}C NMR also confirmed the formation of the aldehyde group by the signal observed at 205.29 ppm shift.

Direct oxidative cleavage of the 21-hydroxyketone **62** with sodium metaperiodate (Scheme 26) led to the formation of the 17ß-carboxylic acid (**66**) as did oxidation of **65** with chromic anhydride in acetic acid (Yasuda, 1963). The data reported in Tables 5 and 8 for ^{1}H and ^{13}C NMR, repectively, support the assignment of the structures shown. The signal assigned to the C-13 methyl protons was a singlet centred at 1.17 ppm and was shifted downfield as compared with that of the starting material (**63**) 1.00 ppm. The chemical shift of the C-20 carbonyl in the carboxylic acid was at 180.25 ppm in the ^{13}C NMR and was distinguishable from the chemical shift for the C-20 carbonyl group of **62** at 216.64 ppm and **65** at 205.29 ppm. Furthermore, the C-14 carbon signal which appears at 83.47 ppm and at 83.70 ppm for compounds **63** and **66**, respectively was shifted to 84.72 ppm for **66**.

A similar synthetic sequence to that used for the 20ß-alcohol (**64**) (Scheme 25) is outlined in Scheme 26 for synthesis of the 21-nor-alcohol (**67**). Reduction of the 21-nor-etianaldehyde (**65**) with lithium tri-tert-butoxyaluminohydride in tetrahydrofuran gave the 21-nor-alcohol hexaacetate (**67**). The ^{1}H NMR showed a signal appearing at 3.50 ppm as a doublet of doublets and integrated for one

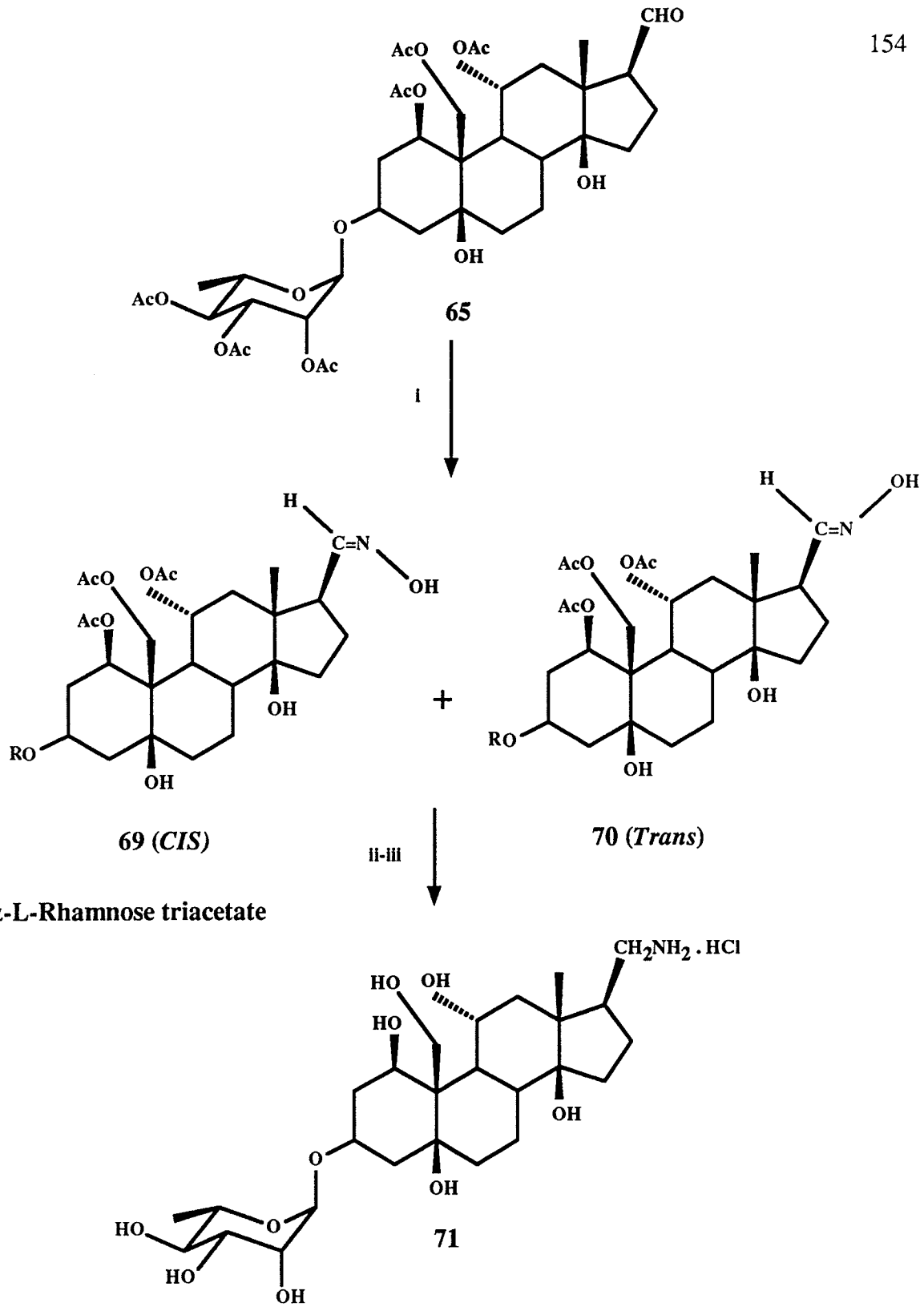


Reagents and conditions: i, LTBAH-THF, r.t., 15 min; ii, NaOMe-MeOH, reflux, 1.5hrs.

Scheme 26. Synthesis of 21-nor-ouabain derivatives from the 21-nor-ouabain etianaldehyde.

proton which was interpreted as the 20-H_A of the 20-methylene protons and showed a coupling constant of J 3.0 Hz. The multiplet pattern for H_B was centred at 3.75 ppm together with a 5'-H proton of the sugar. The ^{13}C NMR provided more information for assignment of the structure, for example, the C-20 carbon signal appeared at 62.23 ppm instead of 205.29 ppm for compound **65**, showing that a complete reduction of the starting material had taken place. Base-catalysed hydrolysis of **68** with a nucleophilic base, *e.g.*, sodium methoxide in methanol under reflux, afforded the hexol (**68**). The ^1H NMR of **68** showed the two signals of the 20-methylene protons at chemical shifts of 3.46 ppm and 3.64 ppm as two doublets for each, which exhibit an ABX pattern with coupling constants of J_{AB} 10.7 Hz and $J_{AX} = J_{BX}$ J 3.3 Hz. In addition, the hexaacetate signals were not observed in the upfield region.

The oximes **69** and **70** were made by reaction of the 21-nor-etianaldehyde (**65**) with hydroxylamine hydrochloride in a mixture of pyridine, sodium acetate, water and 95% ethanol under reflux, which gave two products, namely the *cis* and *trans* isomers (Scheme 27). These condition are similar to that described in Scheme 16. The *cis* and *trans* isomers were distinguishable on TLC and hence could be separated chromatographically. The ^1H NMR data shown in Table 5 were consistent with the *cis* and *trans* isomers of the oxime. The C-20H of the *cis* isomer showed a signal further downfield at 7.55 ppm as a doublet and integrated for one proton whereas in the *trans* isomers it had a chemical shift of 6.82 ppm



Reagents and conditions: i, H_2NOH . HCl-NaOAc-pyridine-95% EtOH, reflux, 1.5hrs; ii, NaOMe-MeOH, reflux, 1.5hrs; iii, H_2-PtO_2-MeOH, r.t., 24hrs.

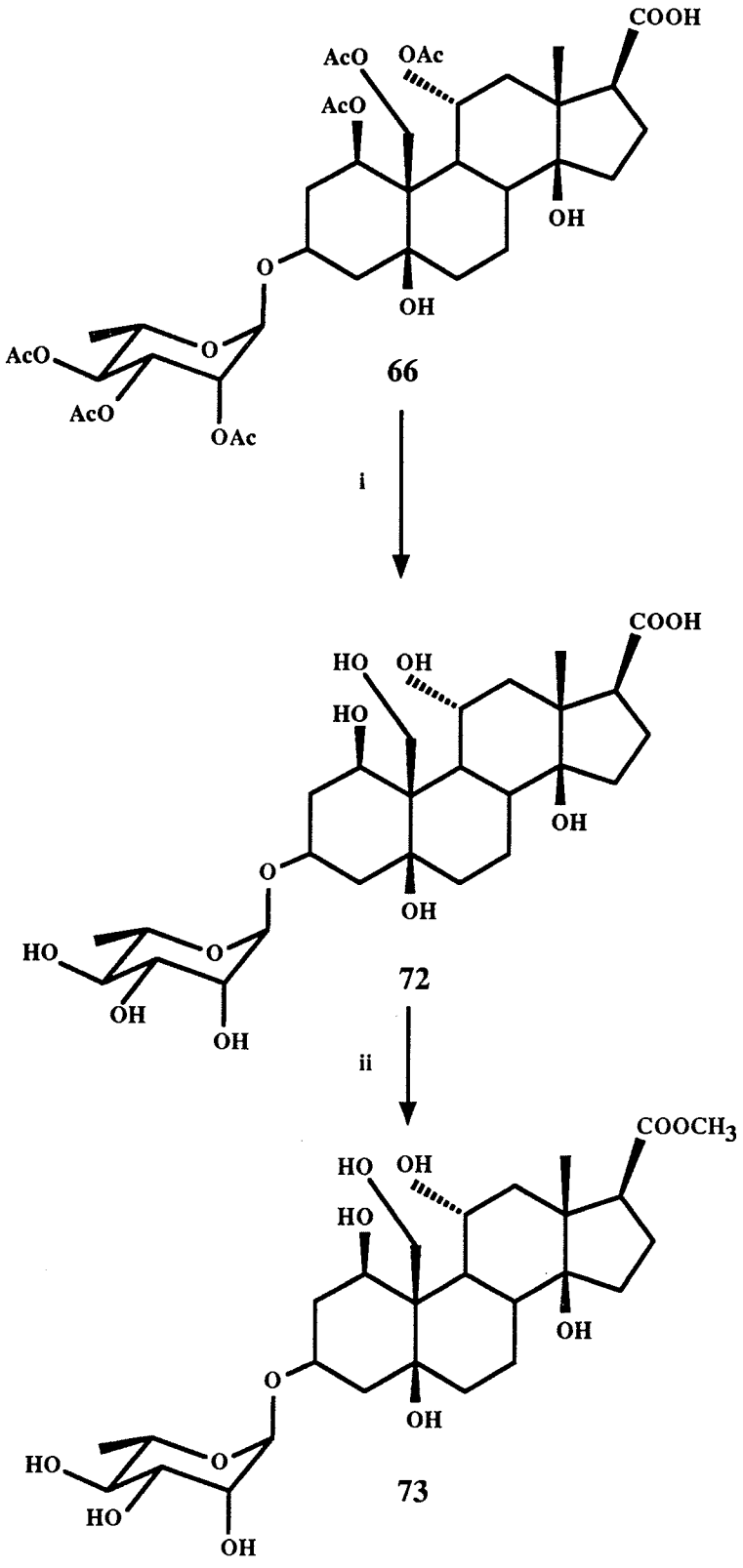
Scheme 27. Synthesis of 21-nor-oxime isomers and the amine hydrochloride from the 21-nor-ouabain etianaldehyde.

and also integrated for one proton. The coupling constants for C-20H with C-17αH was dependent on the position of the hydroxyl group; for the *cis* isomer, the coupling constant was J 8.6 Hz and for the *trans* isomer J 8.0 Hz. Further confirmation of the structures was obtained from the ^{13}C NMR for the C-18 carbon chemical shift; the *cis* isomer showed a singlet at 15.85 ppm and the *trans* isomer at 14.94 ppm. In addition, both isomers showed further downfield signals at 156.36 ppm and 156.14 ppm respectively, assigned to the C-20 carbon (Table 8).

Deacetylation of the hexaacetate (**69** and **70**) with sodium methoxide and methanol was carried out under reflux, and the crude mixture of both isomers was reduced using platinum oxide in methanol containing a trace of chloroform under hydrogen (Secrist and Logue, 1972). The crude amine hydrochloride (**71**) obtained from this reaction (shown in Scheme 27) could not be crystallized. Chloroform was used in the reaction to generate hydrogen chloride in trace amounts to facilitate the reduction. A mechanistic test has been used by others to investigate this possibilty (Secrist and Logue, 1972). A blank solution of absolute ethanol, chloroform and catalyst was hydrogenated for 1 hr. The resulting solution gave a positive silver nitrate test, indicating the presence of ionic chloride whereas pretested chloroform gave a negative silver nitrate test. Based on this result, they concluded that the reaction occurred by hydrogenolysis of the chloroform to produce the hydrogen chloride which combined immediately with the amine. The ^{1}H NMR of the crude product supports the structural assignment. The signal for

the C-20 H_A proton of the methylene protons appeared at 3.02 ppm as a doublet and showed a coupling constant of J 5.8 Hz. The H_B proton overlapped with other signals. Furthermore, no signal corresponding to the vinylic proton (C-20) was present which indicated that reduction was complete. On the other hand, the dissolving metal reduction method (sodium in 1-propanol) (Robinson *et al.*, 1966) was thought to be a shorter route for the synthesis of the 21-nor-amine (**71**). This method can reduce the oxime as well as remove the acetate groups and the resulting product is more water soluble, therefore, use of a weakly acidic ion-exchange resin for isolation of the product failed due to protonation of the amine by the acid function in the resin (-COOH).

Two steps were required for the synthesis of the 21-nor-methyl ester (**73**) from the protected 21-nor-carboxylic acid (**66**). The synthetic route is outlined in Scheme 29. First, alkaline cleavage of the hexaacetate (**66**) with sodium methoxide and methanol under reflux gave **72**. This product was isolated through ion-exchange chromatography and the structural assignment was supported by the ^1H NMR data shown in Table 5. Treatment of **72** with diazomethane in methanol gave the 21-nor-methyl ester (**73**). The ^1H NMR spectrum indicated the presence of a signal at a chemical shift of 3.72 ppm as a singlet which integrated for three protons and was assigned to the 21-nor-methyl ester (**73**).



Reagents and conditions: i, NaOMe-HOMe, reflux, 1.5hrs; ii, CH_2N_2-Et_2O-MeOH, r.t.

Scheme 28. Synthesis of 14-hydroxy-21-nor-5ß,14ß-pregnane derivatives from 21-nor-ouabain etianic acid.

Table 2. ^{1}H NMR Chemical shifts (J in Hz)[a]: 14α-steroidal hormone derivatives.

Compound	13-Me	10-Me	20-Me	17α-OAc	17ß-OAc	Other
1	0.67s	1.20s	2.13s			2.55t (J 9.0) (17α-H), 5.73s (4-H).
3	0.73s	1.47s	2.13s			4.75dd (J 2.2,3.9) (6α-H), 5.88s (4-H).
5	0.72s	1.14s	2.13s			6.31s (4- and 7-H).
12	0.68s	1.19s	2.11s	2.05s		5.75s (4-H).
15	0.68s	1.23s	2.11s	2.05s		6.40d (J 1.8) (4-H), 2.94m (16ß-H), 4.68ddd (J 1.9,5.1,12.8) (6ß-H)[b].
16	0.84s	1.23s			2.05s	4.60dd (J 7.7,9.1) (17α-H), 2.95m(16ß-H), 5.73d (J 1.8) (4-H).
18	0.90s	1.47s			2.05s	4.62dd (J 7.8,9.1) (17α-H), 4.75dd (J 2.0, 3.8) (6αH) 5.88s (4-H).
19	0.82s	1.00s			2.05s	4.62dd (J 7.8,9.2) (17α-H), 5.61d (J 1.6) (4-H), 1.33t (J 4.5) ($C\underline{H}_3CH_2O$), 3.86m ($CH_3C\underline{H}_2O$).
20	0.84s	1.22s			2.04s	4.61dd (J 7.8,9.2) (17α-H), 6.38d (J 1.7) (4-H), 4.70ddd (J 1.9,5.1,12.9) (6ß-H)[b].
21	0.80s	1.08s			2.03s	4.22m (3α-H), 5.95dd (J 1.7,3.4) (4-H), 4.57m (6ß- and 17α-H).
25	0.91s	1.24s				6.39d (J 1.8) (4-H), 4.70ddd (J 1.9,5.1,12.8) (6ß-H)[b]
31	0.96s	1.17s				6.33s (4-H), 6.38d (J 2.3) (7-H).

[a]Values (δ, ppm) for solutions in $CDCl_3$ (Me_4Si internal standard). [b]dd and allylic coupling.

Table 3 ^{1}H NMR Chemical shifts (J in Hz)[a]: 14-Hydroxy-5ß,14ß-pregnane-3ß-D-digitoxoside derivatives.

Compound	10-Me	13-Me	3-H[b]	17-H	Other
32	0.85s	0.91s	3.99m	2.78m	4.85d, 5.02d (J_{AB} 18.3) (21-H_2), 5.84s (22-H).
33[e]	0.95s	0.96s		2.87dd (J 4.4,9.4)	4.26m (3', 3''-H, $\underline{C}H_2OH$), 4.91m (1'-,1''-, 1'''-H, 21-H_2).
34[e]	0.93s	0.95s	4.04m	2.85dd (J 3.9,9.8)	4.27d, 4.34d (J_{AB} 19.6) (21-H_2).
35[e]	0.95s	0.96s	4.03m	2.95dd (J 4.3,9.4)	2.26s (20-Me).
36[f]	0.92s	0.94s	4.00m	2.79dd (J 4.3,9.3)	2.16s (21-$CH_2OCOCH_2OCO\underline{C}H_3$), 4.73s (21-$CH_2OCO\underline{C}H_2OAc$), 4.73d, 4.83d ($J_{AB}$ 17.4) (21-H_2).
37[e]	0.95s	1.16s	4.04m		1.25m (20-Me), 3.80m (20-αH).
38[e]	0.96s	1.18s	4.04m		1.28s, 1.38s (20 and 20'-Me).
39[e]	0.95s	1.16s	4.04m		1.25m (20-Me, 6'-, 6''-H), 3.80m (20-H), 4.91m (1'-, 1''-H), 3.19dd (J 3.1,9.6) (4'-H), 3.24dd (J 2.9,9.4) (4''-H), 3.83 (5'-, 5''-H).

Table 3 (cont'd).

40[e]	0.95s	1.16s	4.04m		1.26d (J 6.5) (20-Me), 3.76m (20-H), 4.91dd (J 1.8,9.6) (1'-H), 4.91dd (J 1.8,9.6) (2'-H), 4.01m (3'-H), 3.20dd (J 3.1,9.5) (4'-H), 3.76m (5'-H), 1.26d (J 6.5) (6'-H)
41[f]	0.92s	0.86s	3.99m	2.76m	4.79d, 4.98d (J_{AB} 18.1) (21-H), 5.86s (22-H).
42[f]	0.91s	0.87s		2.78m	1.24m (6'-,6''-,6'''-H), 3.34dd (J 3.0,9.5) (4'-, 4''-H), 3.88m, 4.00m (5'-, 5''-, 5'''-, 3-H), 4.44dd (J 3.0,9.9) (4'''-H), 4.78m (3'-, 3''-, 3'''-H, 21-H_2, CCl_3CH_2O), 5.38m, 5.44m (1',1'',1'''-H), 5.87m (22-H).
43[f]	0.92s	0.95s	4.00m	2.89dd (J 4.3,9.1)	2.22s (20-Me).
44[f]	0.92s	0.96s	4.00m	2.78dd (J 4.4,9.4)	4.32s ($OCOCH_2OH$), 4.77d, 4.88d (J_{AB} 17.5) (21-H_2).
45[f]	0.90s	0.92s	4.00m	2.72dd (J 3.8,9.5)	3.07s (21-OH), 4.06s (14-OH), 4.28m (21-H_2).
46[f]	0.92s	0.95s	4.00m	2.78dd (J 4.2,9.3)	2.17s (21-OAc), 4.03 (14-OH), 4.64d, 4.76d (J_{AB} 17.6) (21-H_2).
47[e]	0.95s	0.94s	4.04m	2.52dd (J 4.5,9.0)	1.99s (20-Me).
48[e]	0.94s	0.93s	4.04m	2.45dd (J 5.0,9.0)	1.93s (20-Me).

Table 3. (cont'd)

49[e]	0.94s	0.89s	4.04m	2.47dd (J 5.0,9.0)	1.86s (20-Me)
50[e]	0.95s	0.99s	4.04m	2.57m	1.91s, 1.95s ($(CH_3)_2C$=N-), 2.07s (20-Me)
51[c,e]	0.95s	0.95s	4.04m		1.25m (20-Me)[d]
52A[e]	0.95s	1.03s			1.58d (J 6.4) (20-Me)
52B[e]	0.93s	0.87s			1.49d (J 6.7) (20-Me), 2.39dd (J 9.0,18.0) (16 ß-H), 4.75m (20-H)

[a]Values (δ, ppm) for solutions in $CDCl_3$-CD_3OD (1:1), except **36**, **41**, **43**, **43**, **44**, **45** and **46**, which are in $CDCl_3$ ($SiMe_4$ internal standard). [b]The 3α-H and the 3'''-H each appear as overlapping broad signal. [c]Major isomer. [d]Obscured by 6', 6'', 6'''-Me signals.

[e]Trisdigitoxoside spectra **33**, **34**, **35**, **37**, **38**, **39**, **40**, **47**, **48**, **49**, **50**, **51**, **52A**, and **52B** are in agreement with digitoxin **32**; 1.23d, 1.24d, 1.27d (J 6.2, 6'-, 6''-, 6'''-Me), 3.17m (4'-, 4''-, 4'''-H), 3.78m (5'-, 5''-, 5'''-H), 4.00m (3'''-H), 4.21m (3'-,3''-H), 4.88m (1'-, 1''-, 1'''-H). [f]Trisdigitoxide tetraacetate spectra (**36**, **41**, **42**, **43**, **44**, **45** and **47**) are in agreement with digitoxin tetraacetate **41**; 1.98s, 2.09s (3'-, 3''-, 3'''-, 4'''-OAc), 1.13d (J 6.2), 1.20d (J 6.4), 1.23d (J 6.5, 6'-, 6''-, 6'''-Me), 3.27dd (J 3.1,9.8), 3.30dd (J 3.1,9.8) (4'-, 4''-H), 3.83m (5'-, 5''-, 5'''-H), 4.51dd (J 2.9,9.9) (4'''-H), 4.69dd (J 1.8,11.0), 4.72dd (J 1.8,10.9), 4.78dd (J 2.1,9.4) (3'-, 3''-, 3'''-H), 5.38m (1'-, 1''-, 1'''-H).

Table 4. ^{1}H NMR Chemical shifts[a]: 14-Hydroxy-21-nor-14ß,5ß-pregnane-3ß-D-digitoxoside derivatives.

Compound	10-Me	13-Me	3-H	17-H	Other
53[b]	0.93s	1.03s	4.00m	2.30m	9.72d (J 3.8) (20-CHO)
54[c]	0.96s	1.07s	4.04m	2.61t (J 4.9)	
55[c]	0.94s	0.97s	4.03m	2.62t (J 6.7)	3.71s (CO_2Me)
56[c]	0.95s	0.99s	4.04m		3.43dd, 3.70dd (J_{AB} 10.8, J_{AX} 1.9, J_{BX} 2.9) (20-H_2)
57[c]	0.94s	0.95s	4.03m		2.70s, 2.72s ($J_{AX} = J_{BX}$ = 6.0) (20-H_2)
58[c]	0.94s	0.95s	4.04m		2.44m (16ß-H or 17α-H), 4.56s, 4.59s ($J_{AX} = J_{BX}$ = 8.6) (20-H_2)

[a]Values (δ, ppm) for solutions in $CDCl_3$-CD_3OD (1:1), except **54**, which is in $CDCl_3$ ($SiMe_4$ internal standard).
[b]Trisdigitoxoside tetraacetate spectrum (**54**) is in agreement with digitoxin tetraacetate **36** (Table 2); 1.98s, 2.09s (3‘-, 3‘‘-, 3‘‘‘-, 4‘‘‘-OAc), 1.13d (J 6.2), 1.20d (J 6.4), 1.23d (J 6.5, 6‘-, 6‘‘-, 6‘‘‘-Me), 3.27dd (J 3.1,9.8), 3.30dd (J 3.1,9.8) (4‘-, 4‘‘-H), 3.83m (5‘-, 5‘‘-, 5‘‘‘-H), 4.51dd (J 2.9,9.9) (4‘‘‘-H), 4.69dd (J 1.8,11.0), 4.72dd (J 1.8,1.1), 4.78dd (J 2.1,9.4) (3‘-, 3‘‘-, 3‘‘‘-H), 5.38m (1‘-, 1‘‘-, 1‘‘‘-H). [c]Trisdigitoxoside spectra **55**, **56**, **57**, **58**, and **59** are in agreement with digitoxin **33** (Table 2); 1.23d, 1.24d, 1.27d (J 6.2) (6‘-, 6‘‘-, 6‘‘‘-Me), 3.22m (4‘-, 4‘‘-, 4‘‘‘-H), 3.83m (5‘-, 5‘‘-, 5‘‘‘-H), 4.05m (3‘‘‘-H), 4.26m (3‘-,3‘‘-H), 4.91m (1‘-, 1‘‘-, 1‘‘‘-H).

Table 5. ^{1}H NMR Chemical shifts[a]: Ouabain derivatives.

Compound	13-Me	3-H	17-H	Other
59	0.94s	4.23s	2.91dd (J 5.5,8.6)	4.12d, 4.39d (J_{AB} 11.7) (19-H_2), 4.96dd, 5.01dd (J_{AB} 18.6) (20-H_2), 5.91s (22-H)
60	0.97s	4.25s	2.74dd (J 5.0, 8.9)	4.0s (14ß-OH), 4.38d, 4.99d (J_{AB} 12.0) (19-H_2), 4.76dd, 4.91dd (J_{AB} 18.1) (20-H_2), 5.87s (22-H), 6.03m (1α-H)
61[b]	1.06s	4.25s	2.89m	1.67s (20-Me), 3.97s (14ß-OH), 4.39d, 5.00d (J_{AB} 12.0) (19-H_2), 6.04m (1α-H)
62[b]	1.00s	4.25s	2.73m	3.03t (J 4.5) (21-OH), 4.18-4.34m (3α-H, 5ß-OH, 21-H_2), 4.38d, 4.99d (J_{AB} 12.0) (19-H_2)
63[b]	1.25s	4.24s		1.28d (J 3.3) (20-Me), 3.84m (20α-H), 3.98s (14ß-OH), 4.44d, 4.97d (J_{AB} 12.1) (19-H_2)
64[c]	1.21s	4.11m[b]		1.25d (J 6.2) (20-Me), 4.12[b], 4.40d (J_{AB} 11.3) (19-H_2, 1α-H)
65[b]	1.17s	4.26s	3.10dt (J 3.1, 3.1, 8.9)	4.01s (14ß-OH), 4.42d, 4.97d (J_{AB} 12.2) (19-H_2), 6.03m (1α-H), 9.73d (J 3.1) (20-CHO)

Table 5 (cont'd).

67[b]	1.16s	4.26s	2.64m	4.05m (14ß-OH), 4.40d, 5.03d (J_{AB} 12.0, 12.3) (19-H_2), 6.04s (1α-H)
68[b]	1.00s	4.24s		3.50dd (J_A 2.99) (20-H_A), 3.75m (20-H_B, 5'-H), 3.99s (14ß-OH), 4.43d, 4.98d (J_{AB} 12.0) (19-H_2), 6.07m (1α-H)
69[c]	1.06s	4.13m		3.46dd, 3.64dd (J_{AB} 10.7, $J_{AX} = J_{BX}$ 3.3) (20-H_2), 3.65-3.75m (1α-H), 4.13d[b], 4.40d (J_{AB} 11.6) (19-H_2, 3α-H, 11ß-H)
70[b]	1.03s	4.26m		4.00s (14ß-OH), 4.42d, 4.96d (J_{AB} 12.25) (19-H_2), 6.04m (1α-H), 7.55d (J 8.6) (20-H)
71[b]	1.08s	4.26m	3.28m	4.00s (14ß-OH), 4.42d, 4.95d (J_{AB} 12.0) (19-H_2), 6.05m (1α-H), 6.82d (J 8.0) (20-H)
72[c]	1.04s	4.13m		3.02d (J_A 5.8)[c] (20-H_A), 4.39d (J_{AB} 11.6) (19-H_2)
73[c]	1.14s	4.12m	2.47t (J 6.2)	4.14d, 4.40d (J_{AB} 11.7) (19-H_2)
74[c]	1.07s	4.12m	2.70m	3.72s (20-CO_2Me), 4.16d, 4.43d (J_{AB} 11.8, 11.7) (19-H)

[a]For solutions in $CDCl_3$, except **59**, **64**, **68**, **71**, **72** and **73** which are in CD_3OD ($SiMe_4$ internal standard).
[b] Rhamnoside triacetate spectra **61**, **62**, **63**, **65**, **66**, **67**, **69** and **70** are in agreement with ouabain hexaacetate **61**; 1.93s, 1.95s, 2.03s, 2.13, 2.14s and 2.15s (11α-, 19ß-, 1ß-, 2'-, 3'- and 4'-OAc), 1.19d (J 6.25) (6'-Me), 3.74m (5'-H), 4.94s (1'-H), 5.13m (2', 3', 4'-H and 11ß-H).
[c]Rhamnoside spectra **64**, **68**, **71**, **72** and **73** are in agreement with ouabain anhydrous **59**; 1.25d (J 6.3) (6'-Me), 3.37t (J 9.5) (4'-H), 3.74m (2'-, 3'-, 5'-H and 11ß-H), 4.85m (1'-H).

Table 6. ^{13}C NMR Chemical shifts[a]: 14-Hydroxy-5ß,14ß-pregnane 3ß-D-trisdigitoxoside derivatives.

Compound	(32)	(33)[b]	(34)[b]	(35)[b]	(36)[c]	(37)[b,g]	(38)[b]	(39)[e]	(40)[f]	(41)	(42)[d]	(43)[c]	(44)[c]
Carbon													
1	30.17	30.31	30.33	30.34	30.06	30.63	30.40	30.31	30.40	30.15	29.99	30.05	30.13
2	27.04	26.98	26.98	26.66	27.02	26.32	26.99	26.99	27.06	26.63	26.61	26.69	26.72
3	73.15	73.57	73.49	73.61	72.32	73.61	73.69	73.65	73.70	73.00	73.02	72.97	73.00
4	30.57	30.72	30.72	30.72	30.24	31.08	30.81	30.75	30.83	30.15	30.14	30.25	30.31
5	36.70	36.98	36.98	36.98	36.37	37.40	37.14	37.07	37.19	36.25	36.29	36.41	36.43
6	27.31	27.09	27.09	27.09	27.70	26.99	27.28	26.66	26.69	26.90	26.89	26.69	26.78
7	21.57	21.30	21.30	21.30	21.35	21.36	21.98	21.03	21.13	21.15	21.15	21.56	20.93
8	41.80	40.69	40.60	40.59	40.08	40.20	40.19	40.86	40.98	41.85	41.85	39.97	40.16
9	36.08	35.86	35.88	35.86	35.25	36.01	40.69	36.01	36.25	35.75	35.75	35.19	35.34
10	35.56	35.66	35.64	35.70	35.19	36.49	35.99	35.69	35.76	35.16	35.16	35.20	35.26
11	21.76	22.05	22.07	22.06	21.57	22.22	21.00	21.89	21.98	21.36	21.39	20.86	21.65
12	40.36	39.65	39.52	39.75	39.32	42.31	42.18	41.97	42.08	40.05	40.05	39.35	39.45

Table 6 (cont'd).

Compound	(32)	(33)[b]	(34)[b]	(35)[b]	(36)[c]	(37)[b,g]	(38)[b]	(39)[e]	(40)[f]	(41)	(42)[d]	(43)[c]	(44)[c]
Carbon													
13	50.32	50.32	49.98	~50	49.89	~50	~50	~50	~50	49.60	49.58	49.32	50.00
14	85.53	86.03	86.12	86.22	84.90	86.27	85.80	85.93	86.02	85.56	85.57	84.97	84.99
15	32.84	34.25	34.33	34.29	33.97	32.55	31.90	32.23	32.29	33.10	33.16	33.92	34.03
16	26.85	25.21	25.23	25.39	24.93	27.57	27.28	27.24	27.34	26.62	26.61	24.92	25.00
17	51.46	58.49	27.19	63.00	57.75	57.13	60.29	56.97	57.12	50.90	50.91	62.38	57.97
18	16.00	15.42	15.42	15.55	15.29	16.86	19.89	16.58	16.16	15.77	15.77	15.34	15.37
19	23.82	23.98	23.98	24.03	23.71	24.32	23.99	24.01	24.06	23.61	23.64	23.72	23.78
20	177.15	212.20	212.88	219.43	210.36	72.13	73.32	71.83	24.06	174.47	174.42	217.75	210.29
21	73.43	69.69	70.24	33.16	60.33	23.53	31.38	23.22	71.83	73.41	73.41	33.34	69.71
22	117.26								117.63	117.65			
23	176.37								174.47	174.42			
$O\underline{C}OCH_2$-		172.68			170.13								
$OCO\underline{C}H_2$-		60.29			69.49								
$OCOCH_2O\underline{Ac}$					167.08								

Table 6 (cont'd).

Compound	(45)[b]	(46)[c]	(47)[b]	(48)[b]	(49)[b]	(50)[b]	(51)[b]	(52A)[b]	(52B)[b]
Carbon									
1	30.14	30.07	30.34	30.41	30.27	30.32	30.19	30.54	30.15
2	26.73	26.67	27.01	27.10	26.96	26.99	27.14	26.82	26.82
3	73.02	72.96	73.29	73.73	73.22	73.25	73.15	73.15	73.15
4	30.30	30.25	30.76	30.82	30.70	30.47	30.62	30.54	30.15
5	36.44	36.39	37.08	37.13	37.02	37.03	36.95	36.90	36.90
6	26.73	26.71	27.17	27.24	27.14	27.14	26.88	27.04	27.04
7	20.93	20.88	21.92	22.00	21.88	21.54	21.80	20.95	21.07
8	40.14	40.07	40.54	41.19	40.83	40.69	40.10	41.25	41.26
9	35.27	35.26	35.87	36.10	35.88	35.89	35.75	36.07	35.95
10	35.27	35.19	35.68	35.76	35.61	35.66	35.50	35.54	35.54
11	21.66	21.59	21.54	21.67	21.52	21.97	22.37	20.58	21.68
12	39.34	39.35	40.19	40.53	40.22	40.37	42.51	41.24	40.74
13	49.89	49.76	~50	~50	~50	~50	50.14	47.77	47.67
14	85.05	84.86	86.70	86.30	86.20	86.13	85.57	86.28	86.35
15	34.02	34.00	34.37	34.08	34.09	34.38	32.18	31.45	31.21
16	24.99	25.01	27.85	27.24	27.36	28.05	29.98	25.45	24.75
17	57.95	57.82	59.32	57.39	59.56	59.43	56.73	54.62	52.55
18	15.35	15.26	15.86	15.85	15.68	15.81	16.61	16.30	14.03
19	23.77	23.72	24.02	24.13	24.00	24.02	23.89	23.78	23.78
20	217.20	217.27	164.84	163.87	159.94	163.87	49.65	88.56	87.35
21	70.21	69.20	20.77	16.46	17.22	18.24	10.67	21.28	20.34

Table 6 (cont'd).

Compound	**(45)**[b]	**(46)**[c]	**(47)**[b]	**(48)**[b]	**(49)**[b]	**(50)**[b]	**(51)**[b]	**(52A)**[b]	**(52B)**[b]
Carbon									
$OCO\underline{C}H_3$		20.44							
$O\underline{C}OCH_3$		169.98							
$=N\text{-}NH\underline{C}$			159.03						
$N=\underline{C}(CH_3)_2$						172.87			
$N=C(\underline{C}H_3)_2$						24.02, 25.04			

[a]For solutions in $CDCl_3$-CD_3OD (1:1), except **36**, **41**, **42**, **43**, **44**, **45** and **46**, which are in $CDCl_3$.
[b]Trisdigitoxoside spectra **33**, **34**, **35**, **37**, **38**, **47**, **48**, **49**, **50**, **51**, **52A** and **52B** are on agreement with digitoxin **32**: 95.91 (1'), 99.39, 99.56 (1'', 1'''), 37.40, 37.76, 38.47 (2', 2'', 2'''), 67.07,61.21, 68.15 (3', 3'', 3'''), 82.73, 83.02 (4', 4''), 74.45 (4'''), 68.60, 68.73 (5', 5''), 70.20 (5'''), 18.11, 18.18, 18.23 (6', 6'', 6''').
[c]Trisdigitoxoside tetraacetate spectra **36**, **43**, **44**, **45** and **46** are in agreement with digitoxin tetraacetate **41**: 95.85 (1'), 98.72, 98.83 (1'', 1'''), 35.75, 36.31 (2', 2'', 2'''), 67.38, 67.86 (3', 3''), 68.94 (3'''), 79.54 (4', 4''), 72.32 (4'''), 69.01, 69.62 (5', 5''), 70.03 (5'''), 17.65, 17.99, 18.21 (6', 6'', 6'''), 169.85, 169.88, 170.17, 170.17 ($O\underline{C}OCH_3$).
[d]95.28 (1'), 98.72, 98.83 (1'', 1'''), 35.57, 35.70, 36.19 (2', 2'', 2'''), 67.35, 68.70, 68.76 (3', 3'', 3'''), 79.22, 79.28 (4', 4''), 72.16 (4'''), 75.24, 75.74, 76.84 (5', 5'', 5'''), 17.72, 18.04, 18.09 (6', 6'', 6'''), 76.57, 76.70, 77.00, 77.06 ($OCO\underline{C}H_2CCl_3$), 153.03, 153.20, 153.27, 153.59 ($O\underline{C}OCH_2CCl_3$), 94.05, 94.20, 94.59, 94.72 ($OCOCH_2\underline{C}Cl_3$).

[c]95.94, 95.52 (1', 1''), 37.86, 38.62 (2', 2''), 67.26, 68.27 (3', 3''), 83.20, 73.29 (4', 4''), 68.74, 70.23 (5', 5''), 18.24, 18.27 (6', 6''). [f]96.14 (1'), 39.08 (2'), 68.60 (3'), 73.61 (4'), 70.12 (5'), 18.29 (6'). [g]30.49 (20-$\underline{C}H_3$)

Table 7. ^{13}C NMR Chemical shifts[a]: 14-Hydroxy-21-nor-14ß,5ß-pregnane-3ß-D-trisdigitoxoside derivatives.

Compound	**(53)**[c]	**(54)**[b]	**(55)**[c]	**(56)**[c]	**(57)**[c]	**(58)**[c]
Carbon						
1	30.20	30.37	30.39	30.76	30.89	30.86
2	26.63	27.00	27.16	27.99	27.12	27.10
3	73.06	73.35	73.67	73.27	73.83	73.51
4	30.20	30.76	30.77	30.31	30.46	30.49
5	36.36	37.05	37.09	37.06	37.28	37.28
6	26.63	27.09	27.03	27.16	27.36	27.26
7	21.42	21.33	21.46	21.40	21.54	21.27
8	41.09	40.58	40.73	40.94	41.29	41.58
9	35.76	35.92	35.86	36.11	36.18	36.30
10	35.18	35.71	35.72	35.64	35.77	35.83
11	20.23	21.97	22.04	22.47	21.02	21.76
12	38.11	38.85	39.25	37.51	40.41	38.91
13	~50	~50	~50	~50	~50	~50
14	87.17	87.20	86.48	84.89	84.76	86.26
15	33.03	33.75	34.09	32.77	33.11	32.02
16	20.30	25.06	24.77	30.11	23.95	25.55
17	61.88	57.72	56.35	51.80	52.37	54.13

Table 7 (cont'd).

Compound	(53)[c]	(54)[b]	(55)[c]	(56)[c]	(57)[c]	(58)[c]
Carbon						
18	14.96	15.29	15.47	15.00	15.33	14.65
19	23.65	23.97	24.03	24.03	24.12	24.05
20	206.63	181.12	180.80	62.59	42.51	81.25
$CO_2\underline{C}H_3$			52.35			

[a]For solutions in $CDCl_3$-CD_3OD (1:1), except **53**, which is in $CDCl_3$.
[b]Trisdigitoxoside tetraacetate spectrum **53** is in agreement with digitoxin tetraacetate **41** (Table 6): 95.85 (1'), 98.72, 98.83 (1'', 1'''), 35.75, 36.31 (2', 2'', 2'''), 67.38, 67.86 (3', 3''), 68.94 (3'''), 79.54 (4', 4''), 72.32 (4'''), 69.01, 69.62 (5', 5''), 70.03 (5'''), 17.65, 17.99, 18.21 (6', 6'', 6'''), 169.85, 169.88, 170.17, 170.17 ($O\underline{C}OCH_3$).
[c]Trisdigitoxoside spectra **54**, **55**, **56**, **57** and **58** are in agreement with digitoxin **32** (Table 5): 95.91 (1'), 99.39, 99.56 (1'', 1'''), 37.46, 37.81, 38.53 (2', 2'', 2'''), 67.07,67.27, 68.22 (3', 3'', 3'''), 82.82, 83.12 (4', 4''), 73,52 (4'''), 68.70, 68.83 (5', 5''), 70.20 (5'''), 18.23, 18.30, 18.34 (6', 6'', 6''').

Table 8. ^{13}C NMR Chemical shifts[a]: Ouabain derivatives.

Compound[b]	(60)	(61)	(62)	(63)	(65)	(66)	(67)	(69)	(70)
Carbon									
1	70.10	70.14	70.07	70.26	70.04	70.09	70.32	70.11	70.12
2	29.73	29.77	29.76	29.78	29.76	29.77	29.83	29.77	29.76
3	69.54	69.57	69.57	69.60	69.51	69.44	69.59	67.50	69.52
4	33.43	33.44	33.44	33.45	33.28	33.42	33.49	33.44	33.45
5	72.81	72.90	72.87	72.96	72.88	73.04	72.99	72.90	72.92
6	34.48	34.21	34.22	34.72	33.47	34.50	34.70	34.54	34.55
7	23.04	23.13	23.16	23.15	23.08	23.12	23.14	22.94	23.05
8	43.80	43.46	43.42	43.68	43.86	43.51	43.71	43.88	43.98
9	40.10	38.89	38.94	39.29	39.41	38.90	39.52	40.06	40.07
10	47.13	47.21	47.20	47.16	47.19	47.19	47.21	47.16	47.14
11	68.90	68.93	68.92	68.97	68.94	68.97	69.03	68.96	68.96
12	44.79	44.19	44.04	46.57	43.33	43.51	45.13	43.52	43.41
13	49.43	48.92	49.31	48.10	50.14	48.75	47.70	48.87	48.85
14	84.16	83.44	83.47	83.56	83.70	84.72	82.59	84.32	84.47
15	33.17	34.65	34.61	32.47	33.51	33.85	33.06	32.98	32.80
16	26.69	24.62	24.66	26.12	19.81	24.18	21.60	24.57	24.86
17	50.05	61.42	56.91	55.48	61.08	77.20	50.51	49.56	43.98
18	16.09	15.58	15.51	16.75	15.38	15.41	15.20	15.85	14.94
19	60.99	61.16	61.16	61.19	60.84	61.16	61.25	61.10	61.13
20	173.16	216.96	216.64	71.88	205.29	180.25	62.23	156.36	156.14
21	73.35	33.33	70.14	23.38					

Table 8 (cont'd).

Compound[b]	(60)	(61)	(62)	(63)	(65)	(66)	(67)	(69)	(70)
Carbon									
22	118.19								
23	174.16								

[a]For solutions in $CDCl_3$, ($SiMe_4$ internal standard).
[b]All rhamnoside triacetate spectra are in agreement with the rhamnoside triacetate of ouabain hexaacetate **61;** 94.62 (1'), 69.54 (2'), 71.10 (3'), 70.62 (4'), 66.95 (5'), 17.41 (6'), 169.56, 169.76, 169.76, ($\underline{C}OCH_3$), 20.58, 20.66, 20.73 ($CO\underline{C}H_3$); 170.43, 170.84, 169.83 (11ß-, 19-, 1ß-$\underline{C}OCH_3$), 20.85, 21.11, 21.35 (11ß-, 19-, 1ß-$CO\underline{C}H_3$).

PHARMACOLOGICAL EVALUATION OF PREGNANE ANALOGUES OF CARDIAC GLYCOSIDES

I. 14α-Steroidal hormones.

While digitalis and related steroid glycosides have a strong positive inotropic effect on cardiac muscle and is the basis for their clinical use as clinical agents they show a poor therapeutic index and thus synthesis of more selective agents would be desirable. Structure activity relationships (SAR) of the cardiac glycosides, subjects of ongoing investigation, require major revisions of earlier conclusions which were based mainly on comparisons among naturally occurring substances (Kim *et al.*, 1980; Smith *et al.*, 1982; Schönfeld *et al.*, 1985, 1987; Schönfeld and Repke, 1988; Wiesner and Tsai, 1986; Templeton *et al.*, 1987a,b; Repke and Weiland, 1988). SAR studies have mainly revolved around altering the sugar moiety and replacing the α,ß-unsaturated γ-lactone with a structurally equivalent group. These modifications, as discussed previously (see SAR section pages 38-101), have led to compounds showing improved cardiotonic properties. LaBella and co-workers (1987), as part of their attempts to isolate an endogenous digitalis-like substance from mammalian tissues, screened a number of natural and synthetic steroids by using a radioligand binding assay (RBA) for relative receptor affinities. They measured the relative affinities of these compounds for the cardiac glycoside binding site, *i.e.* the steroid recognition site on Na^+, K^+-ATPase. Evidence for the lipid nature of an endogenous digitalis-like substance (LaBella *et al.*, 1985; Weiland *et al.*, 1987) together with the potent cardiac glycoside steroid structure was the basis for choosing steroids as the potential endogenous substances. LaBella *et al.* (1985) found

that the synthetic progesterone derivative, chlormadinone acetate (CMA) was capable of displacing [^{3}H] ouabain in RBA (IC_{50} 0.3 μM) and is the most potent 14α-steroid identified so far. CMA interacts at the ouabain site on Na^+, K^+-ATPase, inhibiting the enzyme in the same rank order of species sensitivity as do the cardiac glycosides and also inhibiting the sodium pump *in vitro* in guinea-pig atrium. However, CMA primarily exerts cardiodepressant effects in accordance with the frequently reported similar action of progesterone (Tanz, 1963). Negative inotropy may be mediated other than by Na^+, K^+-ATPase because progesterone itself has no significant cardiac-like actions. Positive inotropy by cardiac glycosides, cardiodepression by CMA and progesterone or low concentrations of cardiac glycosides, may reflect different affinities of the compounds for sites that mediate positive inotropy (Na^+, K^+-ATPase/pump inhibition) and negative inotropy. Consequently, progesterone derivatives related to CMA appear to be likely candidates for endogenous digitalis-like hormones. In accordance with these results, LaBella *et al.* (1985) suggested that body tissues may possess enzymes for conversion of progesterone to derivatives related even more closely than the semisynthetic CMA to the cardiac glycoside configuration.

A part of this study was to determine SAR and to find more potent 14α-derivatives. The addition of a C-6α-substituent to 17α-acetoxyprogesterone (**12**) (Scheme 4) in the cases examined increases RBA potency (see Table 9) while the C-6ß-isomer activity is weaker. This suggested a particular receptor interaction associated with the C-6 substituted carbon. Electronic effects may be interpreted as operating through an alteration in hydrogen-bonding induced in the C-3 carbonyl through the C-4 double bond

by C-6 substituents. That the electronic effect of the C-6α substituent is not a major factor affecting binding, is shown by differences in active congeners reported by Templeton *et al.* (1987a). For example, the C-6α-CH_3 (IC_{50} 12 μM) and C-6α-Br (IC_{50} 2 μM) derivatives of 17α-acetoxyprogesterone are sterically similar, but opposite in their inductive effect, yet both have similar RBA activity. Steric factors appear to be of major importance in establishing C-6 interaction, because the highest displacement potency occurs with the C-6α-Cl, -Br and -CH_3 substituents whereas 6α-OEt, show much weaker binding. Pregnane series steroid with a C-6α-Cl substituent are also more potent than the androstane-type steroids (Table 9). The effectiveness of the C-17 substituent in binding with the Na^+, K^+-ATPase receptor at the active site indicates that the C-17 ketone or the 17ß-acetoxy function can be equally effective in forming a possible hydrogen bonding than the acetate functional group since 6α-chloroandrost-4-ene-3,17-dione (**25**) showed (IC_{50} 3.2 μM) a similar displacement potency than the C-17ß-acetate (**20**) (IC_{50} 4 μM). In the case of the only two compounds tested with a 6-chloro vinylic group (**5** and **31** structural modifications of progesterone and androst-4-ene-3,17-dione, respectively) there was a large difference in the RBA potency (Table 9), suggesting a specific interaction with the receptor due to the presence of the 6-chloro vinylic group in this position. Reduction of the C-3 carbonyl group of 17ß-acetoxy-6α-chloroandrost-4-en-3-one (**20**) to the 3ß-alcohol (**21**) (Scheme 5), gives a compound with a weak RBA result with displacement activity (IC_{50} 153 μM), indicating that the ß-hydroxy is less effective than the carbonyl in forming strong interaction at the receptor site.

II. 14ß-Hydroxypregnane glycosides.

Studies by Templeton *et al.* (1989) have shown that both 14,20ß-dihydroxy-5ß,14ß-pregnane 3ß-ß-D-glucoside and 3ß-α-L-rhamnoside bind with high affinity to the cardiac glycoside receptor (IC_{50} 0.4 and 0.07 μM, respectively) in dog heart muscle as determined in a RBA. In the present work the trisdigitoxoside analogue in 14,20ß-dihydroxy-5ß,14ß-pregnane 3-ß-D-trisdigitoxoside (**37**) was also found to have high activity (IC_{50} 0.6 μM) in this assay, however, the order of RBA activity in this series was: α-L-rhamnose > ß-D-glucose > ß-D-trisdigitoxose. However, the 20ß-alcohol (**37**) was more active than the corresponding 20-ketone (**35**) (IC_{50} 1 μM). In addition, the receptor binding affinity of 14,20-dihydroxy-5ß,14ß-pregnane trisdigitoxoside (**37**) is not altered significantly by the progressive removal of the sugars since the potencies of the bis- (**39**) and monodigitoxosides (**40**) (Scheme 13) were found to be the same as **37** (Table 10). However, removal of the final sugar would be expected to decrease binding. This is in agreement with conclusions of Yoda and Yoda (1975) made on the basis of dissociation rate constant measurements with Na^+, K^+-ATPase enzyme complexes with digitoxin and its bis- and monodigitoxosides. They found that the dissocaition rate constants were similar for the three compounds. However, it has been shown (Templeton *et al.*, 1991a) that the α-L-rhamnoside binds more strongly in RBA than the mono-digitoxoside (**40**). On other hand, the decrease in binding for the ß-D-glucose and α-L-rhamnose is similar but is somewhat greater than for the ß-D-trisdigitoxoside. Again the α-L-rhamnoside appears to bind more strongly (Templeton *et al.*, 1991a). The binding affinity was increased by a factor of two upon reduction of the 20-ketone (**35**) to the 20ß-

alcohol (**37**), although binding is still much less than for the α,ß-unsaturated γ-lactone. However, unexpected results were found when comparison was made between the receptor binding of the corresponding 21-norpregnane derivative, *i.e.*, 14,20-dihydroxy-21-nor5ß,14ß-pregnane 3ß-D-trisdigitoxoside (**56**) (IC_{50} 0.5 μM) and its C-20 alcohol 3-α-L-rhamnoside (IC_{50} 0.35 μM) (Templeton, unpublished data). A possible explanation for the retention of activity in **56** may be attributed to the effect of the sugar moiety causing an approximate positioning of the steroid molecule within the receptor site, whereas in the 14,20-dihydroxy-21-nor-5ß,14ß-pregnane 3α-L-rhamnoside the sugar configuration is considerably different from the trisdigitoxoside. Thus attachment of the C-20 hydroxyl group to the active site of the enzyme is favoured because of formation of a hydrogen bond.

A number of C-20 derivatives containing a trisdigitoxoside group have been tested in the RBA (Table 10). The addition of another methyl group at the C-20 carbon (**38**) causes a decrease in RBA binding (IC_{50} 2 μM). Rotation of the polar function around the C-17 to C-20 bond will be restricted in **37** and it appears that either the ß-configuration is the optimum geometry for maximum receptor interaction or steric hindrance caused by the addition of the methyl group is too great.

Nitrogen derivatives containing a 3ß-trisdigitoxoside group have been tested in RBA (Table 10) because of the association a C-20 guanylhydrazone with cardiotonic properties (Thomas *et al.*, 1974a,b; Repke *et al.*, 1990). The 20ξ-amine (**51**) isomeric mixture (IC_{50} 0.3 μM) is more potent than either the 20-*trans*-oxime (**48**) (IC_{50} 1.3 μM), the 20-*trans*-hydrazone (**49**) (IC_{50} 5.2 μM) or the 20-isopropylidenyl derivative (**50**) (IC_{50}

5.0 μM). Steric factors may play an important role in determining the affinity of these compounds to the digitalis receptor site (Na^+, K^+-ATPase), although these effects are not the major factor in binding since the 20-*trans*-hydrazone (**49**) while sterically similar, yet has lower RBA activity than the larger guanylhydrazone. The extended nitrogen substituent appears to be of major importance in establishing C-20 interactions because the highest displacement activity occurs with the 20-amine substituent (**51**). However, with the 20-guanylhydrazone the binding activity appears to be even higher (IC_{50} 0.2 μM). This was rationalized on the basis of a previous suggestion (Thomas *et al.*, 1974a,b) that this latter group is able to ionize at physiological pH so that the enhancement in the formation of hydrogen bonds is greater. Comparison with nitrogen derivatives of 14-hydroxy-21-nor-5ß,14ß-pregnane shows the activity is not decreased for the 20-amine (**57**) (IC_{50} 0.4 μM). However, for other substituents *e.g.*, 20-carboxylic acid (**54**) and its methyl ester (**55**), the activity is much weaker (Table 10). The methyl ester analogue shows somewhat increased potency (IC_{50} 12.6 μM) over the acid.

The most potent derivative identified in this work is the digitoxin cardiac glycoside analogue 20-nitro-14-hydroxy-3ß-trisdigitoxosyloxy-21-nor-5ß,14ß-pregnane (**58**) (Scheme 20). This derivative does not possess the lactone group of the potent digitalis glycosides yet it has a very low an IC_{50} value (0.07 μM), in the [^{3}H]-ouabain radioligand binding assay. Our understanding of the receptor interaction based on the hypothesis of Thomas discussed earlier can simply be described as involvement of a hydrogen bond between the lactone carbonyl group and a hydrogen donor from a receptor macromolecule (Glynn, 1957; Hoffman, 1966; Repke *et al.*, 1974). Computer based attempts have been

made to explain differences in cardiac activity (Fullerton *et al.*, 1979; Bohl *et al.*, 1984; Fullerton *et al.*, 1986). However, the conclusion made by Fullerton *et al.* (1986), that "the position of the side chain carbonyl oxygen is the major determinant of genin activity" cannot be correct in the light of the observed activity for compounds lacking a carbonyl or even an unsaturated group (*e.g.*, 20ß-alcohol) near C-17. Electronic effects may be interpreted as operating through the inductive effect of this group ($-NO_2$) on the formation of hydrogen bonding to produce optimum activity. The isomeric mixture of 20ξ-nitro derivative (**51**) shows less binding (IC_{50} 0.45 µM) compared with the above analogue. This difference in activity needs further study, especially a study of the pure isomers, as binding activity is expected to be better for the 20ß-nitro isomer.

The IC_{50} value of the 14,20ß-dihydroxy-5ß,14ß-pregnane derivative of ouabain (**64**, IC_{50} 3.3) (Table 10) was found to be about three times lower than that of the 20-alcohol of the 21-nor-ouabain derivative (**68**, IC_{50} 10) which may be because for drug-receptor interaction, there is little need for free rotation of the C-20 substituent around the C-17 to C-20 bond. Also the presence of a number of polar hydroxyl groups on the steroidal molecule may not be a factor in lowering the biological activity of these compounds in RBA displacement because ouabain itself is highly active. Interestingly, Templeton (unpublished data) found that the 20ß-alcohol of 14-hydroxy-5ß,14ß-pregnane 3α-L-rhamnoside give an IC_{50} value of 0.07 µM, whereas the 20-alcohol of 14-hydroxy-21-nor-5ß,14ß-pregnane 3α-L-rhamnoside was less active (IC_{50} 0.35 µM). This difference may result from hydrogen bonding, elsewhere than at the active site of the Na^+, K^+-ATPase enzyme.

Similarly low potencies are seen with other derivatives of 21-nor-ouabain, for example, the 20-carboxylic acid (**72**) and its methyl ester (**73**) (Table 10). Low receptor binding in both compounds shows that the functional groups (*e.g.* -COOH and -COOMe) in question are less favourable for optimum displacement in the RBA.

The 20-amine hydrochloride (**71**) substituent in 21-nor-ouabain showed very low displacement activity (IC_{50} 65 μM) compared with the other compounds in the series (Table 10). This may indicate that certain other factors interfere with receptor binding and/or that the flexibility of this group, allowing free rotation around the C-17 to C-20 bond may permit interact ion with other acidic functions rather than binding to the active site of the enzyme.

Table 9. IC_{50} values of cardiotonic 14α-pregnane derivatives.

Compound	Substituents			IC_{50}[a]
	C-3	C-6	C-17ß	
1[b]		H	Ac	60
5[c]		vinylic chloride	Ac	25
12[b]		H	Ac & α-AcO	84
15[b]		α-Cl	Ac & α-AcO	3
20[d]		α-Cl	AcO	4
21[d]	OH	α-Cl	AcO	153
25[d]		α-Cl	=O	3.2
31[d]		vinylic chloride	=O	2.6

[a]IC_{50} values (in μM) represent the concentration that inhibits binding of [^{3}H]-ouabain by 50% in a radioligand binding assay (RBA) (Templeton *et al.*, 1987a). IC_{50} values (> 1-10 μM) were usually not repeated, whereas the more potent substances (< 1 μM) were repeated 1-5 times. Reproducebility among the more potent substances was approximately ±50%. Differences of one order were considered to be highly significant with respect to SAR. RBA were carried out by Mr. D. Stein in the laboratory of Dr. F. S. Labella, Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba.
[b]IC_{50} data from Templeton *et al.* (1987a).
[c]Templeton, J.F. and Kumar, V.P.S., unpublished synthesis and IC_{50} data.
[d]prepared as described in the experimental.

Table 10. IC_{50} values of cardiotonic 14-hydroxy-5ß,14ß-pregnane and 14-hydroxy-21-nor-5ß,14ß-pregnane derivatives.

Compound	Substituents		IC_{50}[a]
	C-3	C-17	
33[b]	tris-ß-D-digitoxoside	$-COCH_2OCOCH_2OH$	0.4
34	"	$-COCH_2OH$	1.9
35	"	$-COCH_3$	1.0
37	"	$-CH(ß\text{-}OH)CH_3$	0.6
38	"	$-COH(CH_3)_2$	2.0
39	bis-ß-D-digitoxoside	$-CH(ß\text{-}OH)CH_3$	0.6
40	mono-ß-D-digitoxoside	$-CH(ß\text{-}OH)CH_3$	0.6
47	tris-ß-D-digitoxoside	$-C(CH_3)=N-NH-C(=NH)NH_2$	0.2
48	"	$-C(CH_3)=N-OH$	1.3
49	"	$-C(CH_3)=N-NH_2$	5.2
50	"	$-C(CH_3)=N-N=CMe_2$	5.0
51	"	$-CH(\xi\text{-}NH_2)CH_3$	0.3
52	"	$-CH(\xi\text{-}NO_2)CH_3$	0.45
54	"	$-CH_2COOH$	29
55	"	$-CH_2COOMe$	12.6
56	"	$-CH_2OH$	0.5
57[c]	"	$-CH_2NH_2$	0.4
58[d]	"	$-CH_2NO_2$	0.07

Table 10. (cont'd).

64[e]	α-L-rhamnoside	-CH(ß-OH)CH_3	3.3
68[e]	"	-CH_2OH	10
71[e]	"	-CH_2NH_2.HCl	65
72[e]	"	-CH_2COOH	32
73[e]	"	-CH_2COOMe	9.2

[a]IC_{50} values (in µM) represent the concentration that inhibits binding of [^{3}H]-ouabain by 50% in a radioligand binding assay (RBA) (Templeton *et al.*, 1987a).. IC_{50} values (> 1-10 µM) were usually not repeated, whereas the more potent substances (< 1 µM) were repeated 1-5 times. Reproducebility among the more potent substances was approximately ±50%. Differences of one order were considered to be highly significant with respect to SAR. RBA were carried out by Mr. D. Stein in the laboratory of Dr. F. S. Labella, Department of Pharmacology and Therapeutics, Faculty of Medicine, University of Manitoba.
[b]Digitoxin IC_{50} 0.008 µM.
57[d] Average of 0.4, 0.3, 0.35 µM.
58[d] Average of 0.054, 0.067, 0.085 µM.
[e]Ouabain derivatives (Ouabain IC_{50} 0.01 µM).

CONCLUSIONS

A number of potentially cardioactive derivatives of 14-hydroxy-5ß,14ß-pregnane and 14-hydroxy-21-nor-5ß,14ß-pregnane 3ß-D-trisdigitoxosides with different functional groups at C-20 have been synthesized. Among these the 20-nitro-14-hydroxy-3ß-trisdigitoxosyloxy-21-nor-5ß,14ß-pregnane (**58**) showed the highest binding affinity (IC_{50} 0.07 µM). This shows that the lactone ring can be replaced by the primary nitro group and retain high receptor binding potency. Similarly the analogues primary amine derivative shows high potency (IC_{50} 0.4 µM). The relatively high binding of both the amino and nitro derivatives is surprising in that the type of electronic interaction expected is very different for these two derivatives. This suggests that part of the receptor site where interaction occurs the structural requirements for the interaction are not rigid and clearly do not depend upon a conjugated system as was previously thought.

Surprisingly, the more water soluble ouabain analogues showed greatly decreased receptor potency. Pregnane derivatives of the cardiac glycoside have been shown (Templeton *et al.*, 1988) to increase Na^+ excretion while retaining K^+ (K^+-sparing diuresis), unlike the natural cardiac glycoside. This is a positive property with respect to their potential use as therapeutic agents. Synthesis of the mono- and bisdigitoxosides when compared with the trisdigitoxoside showed no change in receptor binding potency. Therefore, it is concluded that the two terminal sugars do not play a significant role in receptor attachment for the pregnane derivatives in agreement with similar relationships previously found among the cardiac glycosides themselves.

EXPERIMENTAL

Instruments

Melting points (mp) were determined on a microscope equipped Koffler hot stage and are uncorrected. Proton magnetic resonance (^{1}H NMR) spectra were recorded using deuteriochloroform ($CDCl_3$), deuteriomethanol (CD_3OD) or a mixture of deuteriochloroform and deuteriomethanol in a ratio 1:1 as solvent and tetramethylsilane (TMS) as the internal standard using a Bruker AM300 MHz instrument for all synthetic compounds and were recorded at the Department of Chemistry, University of Manitoba. The following designations are used in characterizing ^{1}H NMR signals: singlet (s), doublets (d), triplet (t), doublet of doublet (dd), multiplet (m). Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were also recorded on the Bruker AM300 instrument using the polarization transfer spectroscopy technique (Doddrell & Pegg, 1980; Burum & Ernst, 1980) to identify primary, secondary, tertiary and quaternary atoms, in $CDCl_3$ (77.1 ppm peak as the internal standard) or $CDCl_3$:CD_3OD in a ratio 1:1. ^{1}H and ^{13}C NMR data are reported in Tables 2-4 and 6-8, respectively.

Elemental analyses were performed by Mr. W. Baldeo at the Microanalytical Laboratory, School of Pharmacy, University of London, England.

Materials

Thin-layer chromatography (TLC) was carried out on Analtech 25 precoated silica gel GHLF plates of 0.25 mm thickness using one of the following liquid phases: (a) 25% ethyl acetate-petroleum ether bp 30 to 60 °C for most genins and (b) 4.5-20% methanol-

dichloromethane for all glycosides, as indicated. The TLC plates were viewed under an ultraviolet (UV) source and/or by dipping in 8% v/v aqueous concentrated sulfuric acid followed by heating several minutes on a hot plate set to produce a characteristic colour. Flash (Still *et al.*, 1978) or dry column (Kihara *et al.*, 1984; Harwood, 1985) chromatography was carried out with silica gel (for flash and dry chromatography type, pH 7.1, moisture content 7.5%, particle size distribution "20-45 microns", Terochem Laboratories Ltd. was used) or alumina (for ordinary column chromatography, Activity II-III). Ion exchange resin Amberlite IRC 50 (Rohm and Haas Philadelphia. PA. 19105) with a weakly acidic cation exchanger-carboxylic functionality (methacrylic acid-DVB in hydrogen form) was washed with 5% aqueous acetic acid, water, methanol and dried at 110 °C for three days before use. The dry resin was used in batch operations and/or in column operations.

GENERAL WORK-UP REAGENTS

For work-up the following reagents were used:

(i) 1 M Hydrochloric acid.

(ii) Saturated aqueous solution of sodium hydrogen carbonate (10% w/v).

(iii) Saturated solution of sodium chloride (brine).

(iv) Anhydrous sodium sulfate.

The source of steroid materials are given in Table 11.

Table 11. Source of steroid materials.

Compound	Source
Hormones:	
Progesterone (**1**) & 17α-Acetoxy-progesterone (**12**)	Sigma Chemical Co., St. Louis, Mo.
17α-Acetoxy-6-chloropregna-4,6-dien-3,20-dione (Chlor-madinone, CMA)	Lilly Research Lab. Indianapolis, N.
Testosterone-17ß-acetate (**16**) & Testosterone (**26**)	Biosynth OSS Holland
Cardiac glycosides:	
Digitoxin (**32**) & Ouabain octahydrate (**59**).	Sigma Chemical Co., St Louis.

METHODS

I. SYNTHESIS OF HORMONES (14α-STEROID) DERIVATIVES.

6-Chloropregna-4,6-diene-3,20-dione (5) (Scheme 1).

To progesterone (**1**) (8 g) in freshly distilled dioxane (75 mL) was added p-toluenesulfonic acid monohydrate (250 mg) followed by triethylorthoformate (15 mL) and the mixture allowed to sit at room temperature with stirring for 3 hrs. Pyridine (2 mL) was added and the reaction solution was evaporated to dryness. The residue was diluted with water and extracted with dichloromethane, washed with hydrochloric acid, aqueous

sodium hydrogen carbonate, water, brine and dried and evaporated to give a crude product (**2**) which was dissolved in acetone (400 mL). To this solution was added 10% aqueous sodium acetate solution (40 mL) followed by N-chlorosuccinimide (6.72 g) and acetic acid (4 mL) at *ca.* 5 °C. The ice-water bath was removed and stirring continued. After 1 hr the reaction solution was evaporated under reduced pressure and the crude product was filtered, washed with water and dried and crystallized from dichloromethane-acetone to give the 6ß-chloro derivative (**3**) (3.5 g, 39%), mp 184-187 °C (lit., mp 183-184 °C, Mori and Yamada, 1963). Treatment of 6ß-chloropregn-4-ene-3,20-dione (**3**) (2.94 g) with trimethylorthoformate (6 mL) and p-toluenesulfonic acid monohydrate (100 mg) in freshly distilled dioxane (35 mL) for 10 hrs at room temperature gave on work up the 6-chloro-3-methyl enol ether (**4**). To the crude product (**4**) (1.6 g, 50%) in tert-butanol (35 mL) was added dichlorodicyanobenzoquinone (DDQ) (1.6 g) and the mixture refluxed for 3 hrs. The reaction solution was cooled and evaporated under reduced pressure to give a crude product which was chromatographed on alumina (20 g). Elution with benzene removed the product from the reagent to give an eluate (900 mg) which was dissolved in a benzene-ethyl acetate mixture (50 mL : 50 mL). The organic layer was washed thoroughly with 1 M potassium hydroxide solution followed by water and brine and dried to give a crude gum which was purified by crystallization to yield (**5**) (104 mg, 7%), mp 143-145 °C from dichloromethane-ethyl acetate (lit., mp 137-138 °C, Merck, 1964). Conversion of **1** to **5** was carried out by the method of Yasuda (1963).

17α-Acetoxy-6α-chloropregn-4-ene-3,20-dione (15) (Scheme 4).

Treatment of 17α-acetoxyprogesterone (**12**) (5 g) with trimethyl orthoformate (10

mL) and p-toluenesulfonic acid monohydrate (250 mg) in freshly distilled dioxane (65 mL) for 3 hrs at room temperature gave after work-up as described for the synthesis of 3-ethyl enol ether (**2**) (Scheme 1) (**13**) (2.9 g, 54%) mp 165-168 °C. This was dissolved in 95% aqueous acetone (160 mL) and treated with N-chlorosuccinimide (1.22 g), 10% aqueous sodium acetate (16 mL) and glacial acetic acid (1.6 mL) at *ca.* 5 °C. The ice-water bath was then removed and stirring continued for 75 min at room temperature. The reaction solution was poured into cold water and filtered. The remaining crude product was dissolved in dichloromethane, washed thoroughly with water, brine, dried and evaporated to yield an isomeric mixture of the 6α, 6ß-chloro derivative (**14**) (2.51 g, 85%). This mixture was dissolved in acetic acid (60 mL), cooled in an ice-water bath at *ca.* 5 °C and hydrogen chloride gas passed through the solution for 1.5 hrs, and then allowed to stand for an additional 1.5 hrs at room temperature. The reaction was poured into cold water, filtered and the crude product was washed with water and dissolved in dichloromethane which was washed with water, brine, dried and evaporated to give a crude residue (2.5 g). Three crystallizations gave the 6α-chloro derivative (**15**) (0.93 g, 32%), mp 210-211 °C from dichloromethane-acetone (lit., mp 215-216 °C, Ringold *et al.*, 1959). The method of Yasuda (1963) was employed to prepare **15** from **12**.

17ß-Acetoxy-6α-chloroandrost-4-en-3-one (20) (Scheme 5).

17ß-Acetoxyandrost-4-en-3-one (**16**) (5 g) was treated with triethyl orthoformate (10 mL) and p-toluenesulfonic acid monohydrate (240 mg) and freshly distilled dioxane (50 mL) for 3 hrs at room temperature and worked up (as described for the synthesis of

3-ethyl enol ether (**2**) (Scheme 1)) to give the 3-ethyl enol ether (**17**) (3.28 g, 60%). The 3-ethyl enol ether was dissolved in 95% aqueous acetone (200 mL) and treated with 10% aqueous sodium acetate (2.5 mL) and N-chlorosuccinimide (2.5 g) followed by acetic acid (3 mL) to give a crude product consisting of an isomeric mixture of 6α-and 6ß-chloro derivatives which was crystallized to give the 6ß isomer (**18**). This product (**18**) was dissolved in dioxane (freshly distilled, 125 mL) and treated with p-toluenesulfonic acid monohydrate (180 mg) and triethyl orthoformate (7 mL) for 10 hrs. Work-up as above (for the synthesis of 3-ethyl enol ether **2** (Scheme 1) gave the 6-chloro ethyl enol ether (**19**) (2.04 g, 57%), mp 144-145°C from dichloromethane-acetone (lit., mp 143-144 °C, Cross *et al.*, 1963). Treatment of **19** (0.5 g) with 20% hydrochloric acid in acetic acid (15 mL) at room temperature for 1.5 hrs and work-up as described above (for the synthesis of 6α-chloro-3-oxo-4-ene (**15**) (Scheme 4) gave the 6α-isomer (**20**) (132 mg, 28%), mp 158-160 °C from diethyl ether-acetone (lit., mp 157-158 °C, Cross *et al.*, 1963). The above procedure was that described by Cross *et al.* (1963).

17ß-Acetoxy-3ß-hydroxy-6α-chloroandrost-4-ene (21) (Scheme 5).

To 17ß-acetoxy-6α-chloroandrost-4-en-3-one (**31**) (400 mg) in freshly distilled tetrahydrofuran (50 mL) was added lithium tri-tert-butoxyaluminohydride (620 mg) and the solution stirred at room temperature for 1 hr under argon. TLC showed no starting material (25% ethyl acetate-hexane). The solution was poured into aqueous sodium hydrogen carbonate and extracted with diethyl ether to give the 3ß-alcohol (**32**) (316 mg, 79%), mp 157-159 °C from dichloro- methane-acetone.

Anal. C, 68.53; H, 8.51; Cl, 9.66, $C_{21}H_{31}ClO_3$ requires C, 68.74; H, 8.52; Cl, 9.66 %.

6α-Chloroandrost-4-ene-3,17-dione (25) (Scheme 6).

3ß-Acetoxyandrost-5-en-17-one (**22**) (4 g) (Scheme 6) was treated with sulfuryl chloride (2 mL) in pyridine (40 mL) at *ca.* 0 °C with stirring. After stirring for 3 hrs at room temperature, the mixture was poured into water, the crude product was extracted with dichloromethane, washed with hydrochloric acid, aqueous sodium hydrogen carbonate, water, and brine, dried and evaporated to gave the dichloro compound (**22**) (2.2 g, 43%), mp 217-219 °C from diethyl ether-acetone (lit. mp 217-218 °C, Mori and Yamada, 1963). The 5α, 6ß-dichloro compound (**23**) (2 g) was dissolved in 0.1 M methanolic potassium hydroxide solution (70 mL) and stirred for 18 hrs. The reaction mixture was poured into water and the product extracted with dichloromethane, washed with water, brine and dried and evaporated to give the 3ß-hydroxy intermediate (1.8 g). The crude product was oxidized with chromic anhydride (1.4 g) in water (4 mL) and acetic acid (20 mL) at room temperature for 1 hr, and the solution poured into water and extracted with dichloromethane which was washed with water, brine, dried and evaporated to yield (**24**) (1.65 g, 92%). Treatment of **24** (1 g) with sodium acetate (3 g) in methanol (20 mL) under reflux for 2 hrs followed by evaporation under reduced pressure gave a residue which was diluted with water and extracted with diethyl ether. The diethyl ether was washed with water, brine, dried and evaporated to give a crude product which was dissolved in glacial acetic acid (50 mL). Concentrated hydrochloric acid (2.5 mL) was added and after standing at room temperature for 18 hrs, the mixture was poured into water, extracted with dichloromethane which was washed with water, aqueous sodium hydrogen carbonate, water, brine, dried and evaporated to give the 6α-chloro-3-oxo-4-ene

(**25**) (440 mg, 32%), mp 221-225 °C from diethyl ether-acetone (lit., mp 215-217 °C, Mori and Yamada, 1963). Conversion of **22** to **25** was carried out as described by Mori and Yamada (1963).

6-Chloroandrosta-4,6-diene-3,17-dione (31) (Scheme 7).

To testosterone (**26**) (5 g) in acetic acid (20 mL) was added chromic anhydride (1 g) in acetic acid (5 mL) at room temperature. After 1 hr the reaction mixture was worked up as described for the synthesis of 5α, 6ß-dichloro-3-one (**24**) (Scheme 6) and the crude product dissolved in dioxane (freshly distilled, 50 mL). p-Toluenesulfonic acid monohydrate (240 mg) and triethyl orthoformate (5 mL) were added and after 3 hrs the mixture was worked up as described for the ethyl enol ether (**2**) (Scheme 1) to give the ethyl enol ether (**28**) (4.3 g) which was dissolved in 95% aqueous acetone (100 mL) and a solution of dichlorodicyanobenzoquinone (DDQ) (3.27 g) added. After 0.5 hr the reaction mixture was evaporated under reduced pressure. The residue was diluted with water and extracted with diethyl ether. The organic layer was washed thoroughly with a solution of sodium hydrogen carbonate, water, brine, dried and evaporated to give the diene-dione (**29**) (3.5 g, 71%), mp 169-172 °C from ether-acetone (lit., mp 170-172 °C, Alston *et al.*, 1976). After treatment of the diene-dione (**29**) (2 g) with 3-chloroperoxybenzoic acid (1.83 g) in chloroform (50 mL) for 18 hrs at room temperature the reaction mixture was washed with 10% aqueous sodium sulfite, aqueous sodium hydrogen carbonate, water, brine, dried and evaporated to give the crude epoxide which was purified on silica gel (flash chromatography). Elution with 30% ether-hexane gave

pure fractions of the 6α, 7α-epoxide (**30**) (522 mg 25%) mp 218-221 °C from acetone (lit., 216-222 °C, Alston *et al.*, 1976). Treatment of **30** (450 mg) with saturated hydrogen chloride gas in acetic acid (40 mL) at *ca.* 5 °C in an ice-water bath for 1 hr was followed by dilution with cold water and extraction with dichloromethane. The organic layer was washed with water, aqueous sodium hydrogen carbonate, water, brine, dried and evaporated to give the 6-chlorodiene-dione (**31**) (190 mg, 48%), mp 188-191 °C from diethyl ether-acetone (lit., 193-194 °C, Bruckner *et al.*, 1961). The method of Alston *et al.* (1976) was employed for the preparation of **30** from **27**. Conversion of **30** to **31** was carried out by the method of Bruckner *et al.* (1961).

II. SYNTHESIS OF DIGITOXIN ANALOGUES.

(i) 14-Hydroxy-5ß,14ß-pregnane 3ß-D-trisdigitoxside Derivatives.

Ozonolysis of digitoxin (32) (Scheme 11).

Digitoxin (**32**) (1 g) in methanol (100 mL) was cooled to -70 °C in a dry-ice acetone bath. A stream of ozone was passed into the solution to produce a dark blue colour and the reaction was completed after 45 min when TLC showed no starting material (10% methanol-dichloromethane). The excess ozone was removed by a stream of nitrogen. The solvent was removed completely at reduced pressure at less than 40 °C. The ozonide product was reduced as follows:

(1) Zinc-acetic acid treatment for 5 min.

To the ozonide product from digitoxin (**32**) (1 g) in acetic acid (300 mL) was added zinc powder (7.2 g) and the mixture stirred vigorously for 5 min. The reaction

mixture was filtered and the zinc washed thoroughly with methanol (*ca.* 50 mL) and the filtrate washed with water and saturated aqueous sodium hydrogen carbonate to give a residue which on flash chromatography over silica and elution with 3% methanol-dichloromethane gave the 21-hydroxyacetoxymethylketone (**33**) (644 mg, 62%), mp 217-219 °C from chloroform-acetone. Further recrystallization gave mp 223-225 °C.

Anal. C, 60.74; H, 8.32, $C_{41}H_{66}O_{15}.1/2\ H_2O$ requires C, 60.95; H, 8.48%.

(2) Zinc-acetic acid treatment for 20 hrs.

The ozonide product from digitoxin (**33**) (1 g) was dissolved in acetic acid (300 mL) followed by zinc powder (72 g) and then stirred vigorously for 20 hrs at which time TLC showed no starting material (10% methanol-dichloromethane). The reaction was worked up as above to give the 21-methyl ketone (**35**) (750 mg, 78%), mp 225-228 °C from chloroform-acetone. Further recrystallization gave a product mp 233-235 °C.

Anal. C, 64.39; H, 8.99, $C_{39}H_{64}O_{12}$ requires C, 64.62; H, 8.89%.

(3) Zinc-95% ethanol treatment for 20 hrs.

The ozonide product from digitoxin (**32**) (100 mg) was dissolved in 95% ethanol (20 mL) followed by the addition of zinc powder (1 g) and the mixture stirred vigorously for 20 hrs. TLC showed change when compared with an authentic sample of the 21-methylketone (**35**).

(4) 2% Zinc-copper couple-acetic acid treatment for 20 hrs.

(a) The ozonide product of digitoxin (**33**) (100 mg) was dissolved in acetic acid (30 mL) and freshly prepared 2% zinc-copper couple (1.3 g) (Templeton and Wie, 1975) added. After 20 hrs TLC indicated no significant amount of product

formed.

(**b**) 2% Zinc-copper couple (15 g) was added to the 21-hydroxyacetoxymethylketone (**33**) (200 mg) in acetic acid (150 mL) and the mixture stirred vigorously for 20 hrs. TLC (10% methanol-dichloromethane) showed that the 21-methylketone (**36**) was formed. The reaction mixture was worked-up as described previously to give **35** (120 mg, 66%), mp 228-232 °C from chloroform-acetone.

14,21-Dihydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnan-20-one (34).

The 21-hydroxyacetoxymethylketone (**33**) (100 mg) was dissolved in methanol at room temperature. After stirring for 16 hrs the solution was neutralized with acetic acid and extracted with dichloromethane. The organic layer was washed thoroughly with water and evaporated to give the 21-hydroxymethylketone (**34**) (68 mg, 73%), mp 225-228 °C from chloroform-acetone.

Anal. C, 63.26; H, 8.68, $C_{39}H_{64}O_{13}$ requires C, 63.22; H, 8.71%.

Acetic anhydride treatment of 21-hydroxyacetoxymethylketone (33).

A mixture of 21-hydroxyacetoxymethylketone (**33**) (255 mg) in acetic anhydride (10 mL) was heated to reflux. After 1.5 hrs TLC (4.5% methanol-dichloromethane) indicated no starting material. The reaction mixture was then cooled and evaporated under reduced pressure at *ca.* 80 °C. The residue was diluted with methanol and poured into aqueous sodium hydrogen carbonate. The crude product was extracted with dichloromethane, washed with water, brine, dried and evaporated to give the pentaacetate

(**36**) (190 mg, 59%), mp 147-151 °C.

Anal. C, 60.76; H, 7.44, $C_{51}H_{76}O_{20}$ requires C, 60.70; H, 7.59%.

Zinc-acid treatment of 14,21-dihydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnan-20-one (34) (Scheme 11).

The 21-hydroxyketone (**34**) (43 mg) was treated with zinc (3.2 g) in acetic acid (15 mL) and stirred for 20 hrs. TLC showed mainly starting material (**34**). The ^{1}H and ^{13}C NMR spectra showed that the crude product contained principally the starting material.

14-Hydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnan-20-one (35) from (36).

The 21-hydroxyacetoxymethyl ketone (**36**) (168 mg) was dissolved in acetic acid (150 mL) and stirred vigorously for 20 hrs with zinc powder (15 g) at room temperature. The reaction mixture was filtered and the remaining zinc washed with methanol (15 mL) and the filtrate diluted with dichloromethane. The organic layer was washed thoroughly with water and aqueous sodium hydrogen carbonate, then with water, brine, dried and evaporated. The residue was purified by flash chromatography to give, on elution with 3% methanol-dichloromethane, fractions of the 21-methyl ketone (**35**) (111 mg, 73%) which gave (76 mg), mp 234-235.5 °C from chloroform-acetone as shown by the ^{1}H and ^{13}C NMR spectra.

3ß-Trisdigitoxosyloxy-14,20ß-dihydoxy-5ß,14ß-pregnane (37).

A solution of the 21-methyl ketone trisdigitoxoside (**35**) (500 mg) and lithium tri-

tert-butoxyaluminohydride (2 g) in tetrahydrofuran (50 mL) was allowed to stir at room temperature under argon for one hour until reduction was complete (as shown by TLC, 10% methanol-dichloromethane). Sodium hydrogen carbonate was added and the mixture extracted with dichloromethane, washed thoroughly with water, brine, dried and evaporated to give the 20ß-alcohol 3ß-trisdigitoxoside (**37**) (405 mg, 81%) mp 232-234 °C from chloroform-acetone; three further crystallizations gave mp 238-241 °C.

Anal. C, 64.58; H, 9.16, $C_{39}H_{66}O_{12}$ requires C, 64.44; H, 9.15%.

3ß-Trisdigitoxosyloxy-14,20-dihydroxy-20-methyl-5ß,14ß-pregnane (38).

To a stirred suspension of magnesium powder (250 mg) in anhydrous diethyl ether (5 mL) was added, dropwise, methyl iodide (0.5 mL) followed by a solution of 21-methyl ketone (**35**) (100 mg) in freshly distilled tetrahydrofuran (10 mL) under argon at room temperature. After 3 hrs the reaction mixture was poured into 25% aqueous ammonium chloride and the product was extracted with dichloromethane, washed with aqueous sodium hydrogen carbonate, water, brine, dried and evaporated to give the 20-hydroxydimethyl 3ß-trisdigitoxoside (**38**) (40 mg, 39%), mp 237-238 °C, from acetone-dichloromethane.

Anal. C, 64.58; H, 9.14, $C_{40}H_{68}O_{12}$ requires C, 64.84; H, 9.25%.

Stepwise degradation of 3ß-trisdigitoxosyl sugar moiety (Scheme 13).

3ß-Bisdigitoxosyloxy-14,20ß-dihydroxy-5ß,14ß-pregnane (39).

20ß-Alcohol 3ß-trisdigitoxoside (**37**) was treated by the degradation method of

Satoh and Aoyama (1970) in three steps (oxidation, reduction and hydrolysis) as follows:

(a) Oxidation of the terminal sugar of (37) with sodium metaperiodate.

To the 20ß-alcohol 3ß-tridigitoxoside (**37**) (512 mg) in ethanol (95%, 45 mL) was added a solution of sodium metaperiodate (512 mg) in water (5 mL). The reaction mixture was left for 1 hr at room temperature when TLC (10% methanol-dichloromethane) showed no starting material. The precipitate of sodium iodate was removed by filtration and washed with dichloromethane and the combined filtrates were washed thoroughly with water, brine, dried and evaporated. The resultant residue (dialdehyde) was treated with lithium tri-tert-butoxyaluminohydride as described in (**b**).

(b) Reduction of the dialdehyde with lithium tri-tert-butoxyaluminohydride.

To the dialdehyde residue from (**a**) in freshly distilled tetrahydrofuran (125 mL) was added lithium tri-tert-butoxyaluminohydride (2.05g) and the reaction mixture stirred under argon at room temperature for 16 hrs when it was poured into cold aqueous sodium hydrogen carbonate and extracted with dichloromethane. This was washed with water, brine, dried and evaporated. The residue (dimethylol) was used directly for the next step.

(c) Acid-catalyzed hydrolysis of dimethylol.

To the dimethylol from (**b**) in methanol (57 mL) was added, with stirring at room temperature, hydrochloric acid (8.5 mL of 0.05 M). After 2.5 hrs TLC (10% methanol-dichloromethane) showed no starting material. After work-up as in step (**b**) the reaction mixture yielded the 20ß-alcohol 3ß-bisdigitoxoside (**40**) (372 mg, 93%), mp 135-139 °C from diethyl ether-hexane.

Anal. C, 65.26; H, 9.51, $C_{33}H_{56}O_9.1/2\ H_2O$ requires C, 65.43; H, 9.48%.

3ß-Digitoxosyloxy-14,20ß-dihydroxy-5ß,14ß-pregnane (40).

The 20ß-alcohol 3ß-bisdigitoxoside (**39**) (422 mg) was treated as described above in steps (**a**), (**b**), and (**c**). Oxidation with sodium metaperiodate (422 mg) in water (5 mL) to the corresponding dialdehyde which on reduction with lithium tri-tert-butoxyaluminohydride (1.69g) in tetrahydrofuran (125 mL) and subsequent acid hydrolysis with hydrochloric acid (8.5 mL of 0.05 M) in methanol (57 mL) gave the 20ß-alcohol 3ß-mono-digitoxoside (**41**) (150 mg, 45%), mp 118-121 °C from methanol-ether.

Anal. C, 68.28; H, 9.97, $C_{27}H_{46}O_6.1/2\ H_2O$ requires C, 68.18; H 9.96%.

Ozonolysis of digitoxin tetraacetate (41) (Scheme 14).

Digitoxin (**32**) (1.3 g) was acetylated by refluxing in acetic anhydride (140 mL) for 1.5 hr as described by Rabitzsch (1971) to give a non-crystalline product (1.4 g) (**42**) which was taken up in methanol (70 mL), cooled to -70 °C in dry-ice acetone bath and treated with ozone for 1 hr and worked-up as described for digitoxin (**32**). The resulting crude ozonide product was then reduced as follows:

(1) Zinc-acetic acid treatment for 5 min.

To the crude ozonide (1.4 g) in acetic acid (300 mL) was added one-tenth the quantity of zinc used in the reduction of digitoxin ozonolysis (7.2 g) and worked-up as described above for (**33**) (Scheme 11). The product was purified by flash chromatography on silica gel with 1.5% methanol-dichloromethane to give the 21-hydroxyacetoxymethylketone (**44**) (423 mg, 30%), mp 133-135 °C from diethyl ether-

hexane.

Anal. C, 60.59; H, 7.75, $C_{49}H_{74}O_{19}$ requires C, 60.86; H, 7.71%.

(2) Zinc-acetic acid treatment for 20 hrs.

The ozonide (500 mg) was mixed with zinc (36 g) and acetic acid (150 mL). The mixture was allowed to stir vigorously at room temperature for 20 hrs as described for digitoxin to give the 21-methyl ketone 3ß-tetraacetoxytrisdigitoxoside (**43**) (350 mg, 75%), mp 155-159 °C from diethyl ether-acetone.

Anal. C, 62.5; H, 8.3, $C_{47}H_{54}O_{16}.1/2\ H_2O$ requires C, 62.6; H, 8.2%.

Digitoxin tetra-2,2,2-trichloroethyl carbonate (42) (Scheme 14).

To digitoxin (**32**) (0.5 g) in pyridine (10 mL), cooled in an ice-bath, was added dropwise with stirring, 2,2,2-trichloroethyl chloroformate (0.9 mL). After standing at room temperature for 1 hr the reaction mixture was diluted with ice-water and extracted with dichloromethane. The organic layer was washed with water, 0.5 M hydrochloric acid, aqueous sodium hydrogen carbonate, brine, dried and evaporated to give a crude product which was chromatographed on silica gel. Elution with hexane removed a less polar by-product and elution with acetone gave the tetrakis-2,2,2-trichloroethylcarbonate (**42**) (857 mg, 94%), mp 155-159 °C from dichloromethane-methanol.

Anal. C, 43.57; H, 4.78; Cl, 28.76, $C_{53}H_{68}O_{21}Cl_{12}$ requires C, 43.41; H, 4.67; Cl, 29.01%.

Ozonolysis and zinc-acetic acid treatment of digitoxin tetra-2,2,2-trichloroethyl carbonate (42).

The tetra-2,2,2-trichloroethyl ester (**43**) (747 mg) was dissolved in dichloromethane

(10 mL) and methanol (60 mL) and treated with ozone in a dry-ice acetone bath at -70 °C for 1 hr. The excess ozone was removed by nitrogen and the colourless solution was brought to room temperature, and evaporated to dryness. The crude ozonide was dissolved in acetic acid (300 mL) and zinc powder (72 g) added. After vigorous stirring at room temperature for 20 hrs TLC (10% methanol-dichloromethane) showed no starting material. The reaction mixture was filtered and the remaining zinc washed with methanol (30 mL). The filtrate was diluted with dichloromethane which was washed thoroughly with water, brine, dried and evaporated to give the 21-methyl ketone (**35**) (Scheme 14) (240 mg, 62%), mp 224-227 °C from chloroform-acetone. ^{1}H and ^{13}C NMR were identical to the **35** obtained from digitoxin (**32**).

14,21-Dihydroxy-3ß-tetraacetoxytrisdigitoxosyloxy-5ß,14ß-pregnan-20-one (45).

To a solution of the 21-hydroxyacetoxymethyl ketone (**44**) (100 mg) in methanol (10 mL) was added under a nitrogen atmosphere, 6.75% aqueous potassium hydrogen carbonate (0.2 mL). After stirring for 70 min the reaction mixture was neutralized with acetic acid and extracted with dichloromethane, which was washed thoroughly with water, brine, dried and evaporated to give the 21-hydroxymethyl ketone (**45**) (78 mg, 84%), mp 131-133 °C from dichloromethane-methanol (lit., mp 137-140 °C, Rabitzsch, 1971).

21-Acetoxy-14-hydroxy-3ß-tetraacetoxydigitoxosyloxy-5ß,14ß-pregnan-20-one (46).

To the 21-hydroxymethyl ketone (**45**) (100 mg) in pyridine (10 mL) was added acetic anhydride (5 mL). The reaction mixture was allowed to sit at room temperature for

18 hrs, when it was poured into water and extracted with dichloromethane, which was washed with hydrochloric acid, water, brine, dried and evaporated to give the 21-acetoxymethylketone (**46**) (42 mg, 40%), mp 152-154 °C and 187-190 °C (lit., mp 154-157 °C and 189-189.5 °C, Rabitzsch, 1971).

Treatment of the 21-acetoxyketone (46) with zinc/acetic acid for 20hrs.

To a solution of the 21-acetoxyketone (100 mg) in acetic acid (30 mL) was added zinc (24 g) and the mixture stirred vigorously for 20 hrs. After filtration and work-up, as described for digitoxin (**32**), the residue gave the starting material (**46**) (60 mg recovery), mp 152-154 °C. ^{1}H NMR spectroscopy of the total reaction product showed the presence of only the 21-acetoxyketone (**46**).

14-Hydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane 20-guanylhydrazone (47) (Scheme 16).

The 21-methyl ketone (**35**) (100 mg) and aminoguanidine bicarbonate (100 mg) in 95% ethanol were heated to reflux with potassium hydroxide (30 mg) under argon for 6 hrs. TLC (10% methanol-dichloromethane) indicated no starting material. The reaction mixture was cooled to room temperature and diluted with dichloromethane. The organic layer was washed thoroughly with water, brine, dried and evaporated. Two crystallizations of the residue gave the 20-guanylhydrazone (**47**) (38 mg, 37%), mp 248.5-252 °C from diethylether-methanol.

Anal. C, 59.99; H, 8.72; N, 6.93, $C_{40}H_{68}N_4O_{11}.H_2O$ C, 60.21; H, 8.59; N, 7.02%.

14-Hydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane *trans* 20-oxime (48) (Scheme 16).

To a stirred solution of 21-methyl ketone (**35**) (200 mg) in a mixture of 95% ethanol (20 mL) and pyridine (5 mL) was added hydroxylamine hydrochloride (400 mg) and sodium acetate (286 mg) in water (5 mL). After 2 hrs under reflux the mixture was cooled and diluted with dichloromethane. The organic layer was washed with hydrochloric acid, water, brine, dried and evaporated to give the 20-oxime (**48**) (142 mg, 70%), mp 252-255 °C from chloroform-acetone.

Anal. C, 63.51; H, 8.92; N, 1.99, $C_{39}H_{65}NO_{12}$ C, 63.31; H, 8.85; N, 1.89%.

14-Hydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane 20-hydrazone (49).

The 21-methyl ketone (**35**) (250 mg) was refluxed with 85% hydrazine (1 mL) and triethylamine (freshly distilled, 6.6 mL) in 95% ethanol (20 mL). After 2 hrs, the reaction mixture was cooled and evaporated to dryness under reduced pressure. Crystallization several times from ether gave the hydrazone (**49**) (120 mg, 48%), mp 220-242 °C with decomposition.

Anal. C, 64.19; H, 9.52; N, 3.89, $C_{39}H_{70}N_2O_{10}$ requires C, 64.43; H, 9.70; N, 3.85%.

14-Hydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane 20-isoproplidinylhydrazone (50).

A mixture of 21-methyl ketone (**35**) (160 mg), 85% hydrazine (1 mL), triethylamine (freshly distilled, 4.5 mL) and 95% ethanol (15 mL) was heated to reflux for 2 hrs. On cooling, the reaction was evaporated completely. Crystallization from methanol-acetone gave the 20-isoprpylidinylhydrazone (**50**) (104 mg, 62%), mp 234-237 °C.

Anal. C, 65.07; H, 9.67; N, 3.54, $C_{41}H_{74}N_2O_{10}$ requires C, 65.22; H, 9.88; N, 3.71%

14-Hydroxy-20ξ-amino-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane (51).

A solution of the oxime (**48**) (275 mg) in 1-propanol (20 mL) was brought to reflux under argon and sodium (1.02 g) was added in small pieces over a peroid of 2 1/4 hrs. The solution was then cooled and diluted with dichloromethane. The organic layer was washed thoroughly with water, brine, dried and evaporated to give an isomeric mixture of the 20ξ-aminopregnane 3ß-D-trisdigitoxside (**51**) (115 mg, 43%), mp 205-209 °C from diethyl ether.

Anal. C, 64.38; H, 9.43; N, 1.76 $C_{39}H_{67}NO_{11}$ requires C, 64.53; H, 9.30; N, 1.93%.

14-Hydroxy-20ξ-nitro-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane (52).

(a) Oxidation with 3-chloroperoxybenzoic acid.

The 20ξ-aminopregnane (**51**) (700 mg) in chloroform (17.5 mL) was added dropwise with stirring to m-chloroperbenzoic acid (2.8 g in chloroform 17.5 mL) over a period of 20 min at room temperature. After 5 hrs the reaction mixture was washed twice with 10% aqueous sodium sulfite, aqueous sodium hydrogen carbonate, water, brine, dried and evaporated. Flash chromatography of the crude product on silica gel (3% methanol-dichloromethane as eluent) gave pure fractions of **52** (173 mg, 24%), mp 173-186 °C from chloroform-acetone.

(b) Oxidation with dimethyldioxirane.

The dimethyldioxirane was prepared as described by Adam *et al.*, (1987) by dissolving sodium hydrogen carbonate (12 g) in water (20 mL) followed by acetone (13 mL). To this mixture was added potassium peroxymonosulfate (25 g) and the reagent collected by distillation (the collection flash was cooled in a

dry-ice acetone bath) under reduced pressure *ca.* 150 Torr. A second portion of sodium hydrogen carbonate (12 g) was added and the reaction mixture left for 1 hr longer. The total volume of this distillate was about 26 mL. To the 20ξ-aminopregnane (**52**) (350 mg) in dichloromethane (25 mL) was added the dimethyldioxirane solution (26 mL) dropwise with stirring at room temperature. After 15 min TLC (10% methanol-dichloromethane) showed no starting material. The solution was evaporated at *ca.* 40 °C on a rotary evaporator to give a residue which was purified by flash chromatography on silica gel. Elution with 3% methanol-dichloromethane gave fractions of the 20ξ-nitro-pregnane (**53**) (157 mg, 43%), mp 215-223 °C from ether.

Anal. C, 61.98; H, 8.67; N, 1.81, $C_{39}H_{65}NO_{13}$ requires C, 61.97; H, 8.67; N, 1.85%.

(**ii**) 21-Nor-14-hydroxy-5ß,14ß-pregnane 3ß-D-tristoxoside drivatives.

14-Hydroxy-3ß-tetraacetoxytrisdigitoxosyl-5ß,14ß-pregnane 20-etianaldehyde (53) (Scheme 19).

To the 21-hydroxymethyl ketone (**45**) (600 mg) in freshly distilled tetrahydrofuran (100 mL) was added lithium tri-tert-butoxyaluminohydride (1.36 g). The reaction mixture was allowed to stir under argon at room temperature until reduction was complete (15 min) as indicated by TLC (10% methanol-dichloromethane). The reaction solution was evaporated and the residue diluted with aqueous sodium hydroxide solution and extracted with dichloromethane, which was washed thoroughly with water, brine, dried and evaporated to dryness to give a residue which dissolved in 95% ethanol (30 mL) and

aqueous sodium metaperiodate (600 mg in water 5 mL) was added with stirring. After sitting at room temperature for 1 hr TLC (2.5% methanol-dichloromethane) showed no starting material. The reaction solution was evaporated under reduced pressure and the residue diluted with water and extracted with dichloromethane, which was washed with water, brine, dried and evaporated to give a residue which was purified on silica gel (flash chromatography) to give the non-crystalline etianaldehyde (**53**) (140 mg, 24%). This product was used directly in the next step. The above procedure was that described by Eberlein et al. (1972a).

14-Hydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane 17ß-etianic acid (54).

To the 21-hydroxymethyl Ketone (**45**) (515 mg) in 95% ethanol (20 mL) was added an aqueous solution of sodium metaperiodate (1.06 g) in water (8 mL) at room temperature. After 1 hr TLC (4.5% methanol-dichloromethane) showed no starting material. The reaction mixture was evaporated under reduced pressure and acetic acid (20 mL) was added to the residue. The clear solution was poured into water and the crude product extracted with dichloromethane, which was washed thoroughly with water, brine, dried and evaporated. The residue was dissolved in a solution of sodium (160 mg) in methanol (20 mL) and heated to reflux for 1 hr, cooled and evaporated. The crude product was acidified with acetic acid and poured into water. Extraction with dichloromethane followed by washing thoroughly with water and brine gave on evaporation the 17ß-etianic acid trisdigitoxoside (**54**) (228 mg, 55%) mp 235-237 °C from chloroform-acetone.

Anal. C, 62.59; H, 8.75, $C_{38}H_{62}O_{13}$ requires C, 62.79; H, 8.59%.

14-Hydroxy-3ß-trisdigitoxosyloxy-5ß,14ß-pregnane 20ß-etianic acid methyl ester (55).

To a stirred solution of 17ß-etianic acid (**54**) (183 mg) in methanol (20 mL) was added dropwise, diazomethane in cooled diethyl ether at room temperature until a yellow colour persisted. The reaction was complete, as indicated by TLC (10% methanol-dichloromethane). After leaving at room temperature, the clear solution was evaporated to give the methyl ester (**55**) (153 mg, 82%), mp 254-257 °C.

Anal. C, 63.31; H, 8.76, $C_{39}H_{64}O_{13}$ requires C, 63.22; H, 8.71%.

14,20-Dihydroxy-3ß-trisdigitoxosyloxy-21-nor-5ß,14ß-pregnane (56).

To the 17ß-etianaldehyde (**53**) (140 mg) in tetrahydrofuran (freshly distilled, 20 mL) was added lithium tri-tert-butoxyaluminohydride (318 mg). The reaction mixture was allowed to stir at room temperature for 15 min under argon at which time the TLC (4.5% methanol-dichloromethane) showed no starting material. The solution was poured into cold sodium hydrogen carbonate and the product was extracted with dichloromethane, washed with water, brine, dried and evaporated. The crystalline residue was dissolved in a solution of sodium (40 mg) in methanol (20 mL) and the mixture was allowed to reflux. After 1 hr no starting material was observable by TLC (4.5% methanol-dichloromethane). Work-up as described for the synthesis of (**54**) (Scheme 19) gave the 21-nor alcohol trisdigitoxoside (**56**) (65 mg, 59%), mp 247-249 °C.

Anal. C, 64.01; H, 9.03, $C_{38}H_{64}O_{12}$ requires C, 64.02; H, 9.05%.

20-Amino-14-hydroxy-3ß-trisdigitoxosyloxy-21-nor-5ß,14ß-pregnane (57).

The 17ß-etainaldehyde (**53**) (300 mg) dissolved in a mixture of 95% ethanol (20 mL) to which hydroxylamine hydrochloride (600 mg) and a solution of sodium acetate (430 mg) in water (5 mL) and pyridine (7.5 mL) had been added. After 2 hrs reflux the TLC (4.5% methanol-dichloromethane) indicated two products had formed and no starting material remained. The reaction mixture was cooled and diluted with dichloromethane, which was washed thoroughly with water, hydrochloric acid, water, brine, dried and evaporated to give the crude product shown by TLC (2.5% methanol-dichloromethane) to be a mixture (presumably *cis* and *trans* isomers). The mixture was dissolved in 1-propanol (20 mL) and brought to reflux under argon. Sodium (1.22 g) was added in small pieces over a peroid of 2.5 hrs. After this time TLC (10% methanol-dichloromethane) showed only one component and no starting material. The reaction mixture was then cooled and diluted with dichloromethane, washed thoroughly with water, brine, dried and evaporated. Two crystallizations gave the 20-amine (**58**) (86 mg, 37%), mp 239-243 °C from methanol-ether.

Anal. C, 64.02; H, 9.35; N, 1.94, $C_{38}H_{65}NO_{11}$ requires C, 64.11; H, 9.20; N, 1.97%.

20-Nitro-14-hydroxy-3ß-trisdigitoxosyloxy-21-nor-5ß,14ß-pregnane (58).

The 20-nor-amine (**57**) (142 mg) in dichloromethane (50 mL) was cooled to -78 °C in a dry-ice acetone bath and ozone passed through the solution for 2.5 hrs. The mixture was then brought to room temperature and nitrogen was passed through the solution to remove excess ozone. Dichloromethane was evaporated to give a residue which was purified on silica (flash chromatography). Elution with 3% methanol-

dichloromethane gave pure fractions of the 20-nitro derivative (**59**) (50 mg, 34%), mp 246-249 °C. from diethyl ether.

Anal. C, 61.40; H, 8.61; N, 1.65, $C_{38}H_{63}NO_{13}$ requires C, 61.52; H, 8.56; N, 1.89%.

III. SYNTHESIS OF OUABAIN ANALOGUES.

(i) 14-Hydroxy-5ß,14ß-pregnane 3ß-α-L-rhamnoside Derivatives.

Ouabain hexaacetate (60) (Scheme 21).

Ouabain octahydrate (**59**) (4 g) was heated to reflux in acetic anhydride (140 mL) for 90 min at which time no starting material was detectable by TLC (4.5% methanol-dichloromethane). The reaction mixture was cooled to room temperature and evaporated completely under reduced pressure at *ca.* 80 °C to give the hexaacetate (**60**) (3.6 g, 78%), mp 304-307 °C from dichloromethane-methanol (lit., mp 302-304 °C, Raffauf and Reichstein, 1948).

Anal C, 58.59; H, 6.82, $C_{41}H_{56}O_{18}$ requires C, 58.84; H, 6.74%.

Ozonolysis of ouabain hexaacetate (60) followed by zinc-acetic acid teatment for 24 hrs.

Ouabain hexaacetate (**60**) (690 mg) in dichloromethane (40 mL) was treated with ozone at -70 °C for 1 hr (no starting material remained as indicated by TLC 4.5% methanol-dichloromethane). Excess ozone was removed with a stream of nitrogen and the solution allowed to come to room temperature over 1 hr at which time the solvent was evaporated to give the ozonide product. To the residue was added zinc powder (50 g) and acetic acid (100 mL). After vigorous stirring for 24 hrs, TLC (4.5% methanol-dichloromethane) indicated two major products and no starting material. The mixture was

filtered, washed with methanol and the filtrate diluted with water and extracted with dichloromethane, which was washed with water, brine, dried and evaporated to give a product which was separated by flash chromatography over silica gel. Elution with 1.6% methanol-dichloromethane gave fractions (245 mg) which yielded the 21-methyl ketone (**61**) (222 mg, 34%), mp 235-238 °C from dichloromethane-hexane.

Anal. C, 57.45; H, 6.87, $C_{39}H_{56}O_{18}$ requires C, 57.63; H, 6.94%.

Further elution gave fractions (242 mg) which gave the 21-hydroxymethylketone (**62**) (198 mg, 26%), mp 177-180 °C from dichloromethane-hexane, further recrstallization gave mp 179-183 °C.

Anal. C, 57.45; H, 6.87, $C_{39}H_{56}O_{18}$ requires C, 57.63; H, 6.94%.

3ß-(2',3',4'-Triacetyl-α-L-rhamnopyranosyl)oxy-5ß,14ß-pregnan-20-one-1ß,5,11α,14ß,19ß, 21-hexol-20-one 1,11,19-triacetate (62) (Scheme 25).

Ouabain hexaacetate (**60**) (750 mg) was treated with ozone as described above and the crude product treated with zinc (7.2 g) and acetic acid (40 mL) for 5 min. After filtration the product was dissolved in methanol (75 mL) under argon and potassium hydrogen carbonate (102 mg in water, 24 mL) added and stirred for 4 hrs. The mixture was neutralized with acetic acid and extracted with dichloromethane to give, after flash chromatography on silica (elution 1.4% methanol-dichloromethane), the 21-hydroxyketone (**62**) (226 mg, 31%), mp 178-180 °C.

3ß-(2',3',4'-Triacetyl-α-L-rhamnopyranosyl)oxy-5ß,14ß-pregnane-1ß,5,11α,14,19,20ß-hexol 1,11,19-triacetate (64).

The 21-methyl ketone (**61**) (128 mg) and lithium tri-tert-butoxy-aluminohydride (245 mg) were dissolved in dry tetrahydrofuran (freshly distilled, 40 mL) and stirred at room temperature for 15 min at which time no starting material remained by TLC (4.5% methanol-dichloromethane). The solution was poured into aqueous sodium hydrogen carbonate and extracted with dichloromethane to give the 20ß-alcohol (**63**) (110 mg, 86%), mp 242-246 °C from dichloromethane-hexane.

Anal. C, 58.33; H, 7.35, $C_{39}H_{58}O_{17}$ requires C, 58.64; H, 7.32%.

3ß-(α-L-Rhamnopyranosyl)oxy-5ß,14ß-pregnane-1ß,5,11α,14,19,20ß-hexol (64).

The hexaacetate (**63**) (100 mg) was refluxed in dry methanol (30 mL) with sodium (44 mg) for 1.5 hrs. After cooling Amberlite IRC 50 was added portionwise with stirring until the solution was neutral (batch technique) and filtered. The filtrate gave fractions (48 mg) from flash chromatography on silica gel on elution with 14% methanol-dichloromethane which on recrystallization yielded the hexol (**64**) (30 mg, 44%), mp 185-189 °C from ethanol-water.

Anal. C, 57.34; H, 8.55, $C_{27}H_{46}O_{11}.H_2O$ requires C, 59.27; H, 8.48%.

(**ii**) 14ß-Hydroxy-21-nor-5ß,14ß-pregnane 3ß-α-L-rhamnoside Derivatives.

3ß-(2',3'4'-Triacetyl-α-L-rhamnopyranosyl)oxy-21-nor-5ß,14ß-pregnan-20-one 1ß,5,11α,14,19-pentol 1,11,19-triacetate (65).

To the 21-hydroxyketone (**62**) (340 mg) in freshly distilled tetrahydrofuran (50

mL) was added lithium tri-tert-butoxyaluminohydride (638 mg). After 15 min aqueous sodium hydrogen carbonate was added and the mixture extracted with dichloromethane which was washed with water, brine, dried and evaporated. The crude product was dissolved in 95% ethanol (25 mL) and sodium metaperiodate (395 mg) in water (4 mL) added. After stirring for 1 hr, the reaction mixture was concentrated and the residue diluted with water and extracted with dichloromethane, which was washed thoroughly with water, brine, dried and evaporated to give the etianaldehyde (**65**) (204 mg, 62%), mp 160-170 °C from diethyl ether.

Anal. C, 57.83; H, 7.07, $C_{38}H_{54}O_{17}.1/2H_2O$ requires C, 57.64; H, 7.00%.

3ß-(2',3',4'-Triacetyl-α-L-rhamnopyranosyl)oxy-21-nor-5ß,14ß-pregnan-20-etianic acid 1ß,5,11α,14,19-pentol 1,11,19-triacetate (66).

(a) From (**62**) (Scheme 25):

To the 21-hydroxymethyl ketone hexaacetate (**62**) (100 mg) in 95% ethanol was added sodium metaperiodate (120 mg) in water (4 mL) and the solution stirred for 1 hr. Work-up as described for **62** (Scheme 25) gave (**66**) (83 mg, 84%), mp 279-283 °C from dichloromethane-hexane.

(b) From (**65**) (Scheme 25):

To the etianaldehyde (**65**) (100 mg) in glacial acetic acid (0.4 mL) was added chromic anhydride (50 mg) in acetic acid (0.4) and water (0.15 mL). After 1 hr the reaction mixture was poured into water and extracted with dichloromethane, thoroughly washed with water, brine, dried and evaporated to give the etainic acid (**66**) (57 mg, 56%), mp 279-281 °C from dichloromethane-hexane.

Anal. C, 57.25; H, 6.82, $C_{38}H_{54}O_{18}$ requires C, 57.13; H, 6.81%.

3ß-(2',3',4'-Triacetyl-α-L-rhamnopyranosyl)oxy-21-nor-5ß,14ß-pregnane 1ß,5,11α,14,19,20-hexol 1,11,19-triacetate (67).

A solution of **65** (137 mg) and lithium tri-tert-butoxyaluminohydride (262 mg) in tetrahydrofuran (freshly distilled, 40 mL) was stirred for 15 min, poured into aqeuous sodium hydrogen carbonate, and extracted with dichloromethane, washed with water, brine, dried and evaporated to give the 20ß-alcohol hexaacetate (**67**) (90 mg, 66%), mp 252-254 °C from dichloromethane-hexane.

Anal. C, 57.89; H, 7.23, $C_{38}H_{56}O_{17}$ requires C, 58.15; H, 7.23%.

3ß-(α-L-Rhamnopyranosyl)oxy-21-nor-5ß,14ß-pregnane-1ß,5,11α,14,19,20-hexol (68).

The 21-nor hexaacetate (**67**) (165 mg) was dissolved in a solution of sodium (72 mg) in methanol (30 mL) and heated to reflux for 1.5 hrs and the product treated with Amberlite IRC 50 as described for **65** (Scheme 25). The filtrate was chromatographed on silica gel; elution with 14% methanol-dichloromethane gave the 21-nor hexol (**68**) (84 mg, 75%), mp 198-202 °C from ethanol-diethyl ether.

Anal. C, 58.63; H, 8.33, $C_{26}H_{44}O_{11}$ requires C, 58.61; H, 8.45%.

3ß-(2',3',4'-Triacetyl-α-L-rhamnopyranosyl)oxy-21-nor-5ß,14ß-pregnan-20-aldehyde 1ß,5,11α,14,19-pentol 1,11,19-triacetate *cis*- (69) and *trans*-20-oxime (70) (Scheme 27).

To a stirred solution of the aldehyde hexaacetate (**65**) (200 mg) in 95% ethanol (20 mL) and pyridine (4 mL) was added hydroxylamine hydrochloride (400 mg) and aqueous sodium acetate (290 mg) in water (5 mL). After 1.5 hrs reflux, the reaction mixture was cooled and evaporated under reduced pressure. The residue was diluted with

water and extracted with dichloromethane, which was washed with hydrochloric acid, water, brine, dried and evaporated. The product was purified by flash chromatography on silica gel to give, on elution with 1.4% methanol-dichloromethane, the non-crystalline *cis*-isomer (**69**) (95 mg, 46%) and the *trans*-isomer (**70**) (52 mg, 25%), mp 172-176 °C from diethyl ether.

Anal. C, 56.39; H, 7.13; N, 1.69, $C_{38}H_{55}NO_{17}$. $1/2H_2O$ requires C, 56.51; H, 6.94; N, 1.72%.

3ß-(α-L-Rhamnopyranosyl)oxy-21-nor-5ß,14ß-pregnane-1ß,5,11α,14,19-pentol-20-amine hydrochloride (71).

To the 20ξ-oxime hexaacetate (**69,70**) (100 mg) in dry methanol (20 mL) was added sodium (48 mg) and the mixture heated to reflux for 1.5 hrs. After cooling the mixture was evaporated, diluted with water and eluted on an Amberlite IRC 50 column and the resulting filtrate evaporated. The residue was dissolved in methanol (10 mL) and platinum(IV) oxide (50 mg) was added followed by chloroform (0.25 mL). The mixture was stirred vigorously at room temperature under 1 atmosphere of hydrogen. After 24 hrs TLC (20% methanol-dichloromethane) indicated no starting material. Pyridine (3 drops) was added and the mixture filtered and evaporated. Elution (80% methanol-dichloromethane; containing 1% triethylamine) on silica gel (flash chromatography) gave the 20-amine hexol (**71**) (45 mg, 44%) as a non-crystalline material. ^{1}H and ^{13}C NMR were in agreement with the expected product (**71**).

3ß-(α-L-Rhamnopyranosyl)oxy-21-nor-5ß,14ß-pregnane-1ß,5,11α,14,19-pentol-20-etianic acid (72) (Scheme 28).

The 20-etianic acid hexaacetate (**66**) (150 mg) was added to a solution of methanol (30 mL) containing dissolved sodium (65 mg). After 1.5 hrs reflux, the mixture was cooled and evaporated under reduced pressure. The residue was diluted with water and eluted on a column of Amberlite IRC 50. The filtrate was evaporated and the product chromatographed on silica (flash chromatography); elution with 60% methanol-dichloromethane gave the etianic acid (**72**) (55 mg, 54%), mp 234-237 °C from methanol-diethyl ether.

Anal. C, 50.42; H, 8.00, $C_{26}H_{42}O_{12}$. 4 H_2O requires C, 50.48; H, 8.15%

3ß-(α-L-Rhamnosyl)oxy-21-nor-5ß,14ß-pregnane 1ß,5,11α,14,19-pentol-20-etianic acid methyl ester (73).

To the etianic acid rhamnoside (**72**) (140 mg) in methanol (20 mL), diazomethane on cooled diazomethane at room temperature. After the addition was complete a permanent yellow colour persisted and the TLC (20% methanol-dichloromethane) indicated no starting material was present. After leaving at room temperature, the clear solution was evaporated and the residue chromatographed on silica gel (flash chromatography); elution with 15% methanol in dichloromethane gave the methyl ester (**73**) (72 mg, 50%), mp 180-184 °C from methanol-diethyl ether.

Anal. C, 56.29; H, 7.95, $C_{27}H_{44}O_{12}$ requires C, 56.04; H, 8.01%.

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