AN ABSTRACT OF THE THESIS OF

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Title: INVESTIGATIONS ON CARDIAC GLYCOSIDES AND INTERACTIONS WITH Na^+,K^+-ATPase

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ABSTRACT

The roles of sugar structure in determining the ability of cardiac glycosides to bind to the sugar binding site of the Na^+,K^+-ATPase has been of wide interest and debate. To delineate the roles of glycosides' 4'-OH and 6'-OH groups, a series of three digitoxigenin 2',3',6'-trideoxyglucosides (sugars with a OH group only at C4') and four pairs of β-D-digitoxosides/β-D-6'-hydroxydigitoxosides were synthesized. The trideoxyglucosides were obtained by the glycal condensation of digitoxigenin with respective glycals (L-rhamnal and D-allal). The β-D-6-OH-digitoxosides were obtained by either Koenig Knorr or Boivin condensation methods. The genins needed were synthesized by the modification of the C17 side group of digitoxigenin. The β-D-digitoxosides were obtained by the partial hydrolysis of digitoxin, followed by the modification of the C17 side group.

I_{50} values for these glycosides were then determined with hog kidney Na^+,K^+-ATPase under type I binding conditions (Mg^{2+} + Na^+ + ATP). In contrast to the significant activity-decreasing role of 6'-OH groups with α-glycosides, with the four β-D glycoside pairs little effect was seen. The 6'-OH group sometimes increased or decreased activity slightly, or had no effect at all.

Compared with the corresponding glucosides or rhamnosides, trideoxyglucosides with a β linkage (D or L) showed a significant decrease in activity. The α-L analogue, however, showed no decrease in activity at all.

These studies provide the first evidence that the "5'-methyl binding site" and "4'-OH binding site" on Na^+,K^+-ATPase are not equally accessable by α- and β-sugars, or even by D and L sugars--within the range of energetically possible sugar conformations of these glycosides. The 4'-OH is the primary site for hydrogen binding regardless of sugar configuration. However, it appears that the 5'-methyl of β-D-sugars may not be able to reach the "5'-methyl binding site" as well as the 5'-methyl of α-L sugars.

In the second part of the research, high pressure liquid chromatography was studied to facilitate the analytical separation of unprotected α- and β-glycosides. No thin layer chromatographic technique has been found to achieve this goal, making purification of glycoside mixtures extemely difficult. Three different types of columns and mobile phases were tried. The best separations of four anomeric pairs of the glycosides investigated were obtained with a μ-Bondapak carbohydrate analysis column with 50 to 60% ethanol in isooctane as the mobile phase.

INVESTIGATIONS ON CARDIAC GLYCOSIDES AND INTERACTIONS WITH Na^+,K^+-ATPase

by

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Dean of College of Pharmacy

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INVESTIGATIONS ON CARDIAC GLYCOSIDES AND INTERACTIONS WITH Na^+,K^+-ATPase

PART I: AN INVESTIGATION OF THE ROLES OF SUGAR HYDROXY GROUPS IN THE ACTIVITIES OF CARDIAC GLYCOSIDES

CHAPTER ONE

CARDIAC STEROIDS

A. INTRODUCTION

One of the most important groups of steroids in modern drug therapy is the cardiac steroids. Plants and their extracts containing the cardiac steroids have been used throughout history. Squill, for example, was cited in the Ebers Papyrus of ancient Egypt in 1600 B.C. As a classic medicine, plants and their extracts have been used as emetics, diuretics, heart tonics, arrow poisons and even as aids for treating toothaches.[1,2]

Cardiac steroids are one of the most important medicines in the modern treatment of congestive heart failure to (1) increase the strength of myocardial contraction which will result in increased cardiac output; (2) decrease heart size, venous pressure and blood volume; and (3) promote diuresis and relief of edema. A fourth important action of cardiac steroids is to slow the ventricular rate in atrial fibrilation.

Cardiac steroids have a very small therapeutic index, consequently adverse effects are commonly seen. Up to 30% of hospitalized patients treated with cardiac steroids reportedly experienced some potentially serious toxicity.[5] (For further review, see references 1-7).

In spite of a great number of investigations, the mechanism of the cardiac steroids' therapeutic and toxic effects have yet to be completely characterized.

B. CHEMISTRY OF CARDIAC STEROIDS

Cardiac steroids consist of two groups of steroid derivatives - cardenolides, which have a butyrolactone ring at C17, and bufadienolides, which have a α-pyrone ring at C17. Both cardenolides and bufadienolides have a common feature with respect to the steroid backbone. The B/C rings are always trans and C/D rings are always cis. The A/B rings may have either a cis or trans arrangement, although the A/B trans configuration is rare.[1,4]

In nature, cardiac steroids are usually found in the corresponding 3-β-glycosides with one to four sugar residues, as in digoxin (Figure 1).

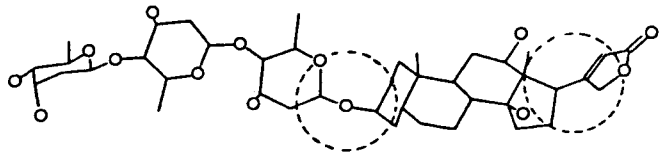


Figure 1. Digoxin

By far, the most important sources of cardiac steroids are two species of Digitalis - D. purpurea and D. lanata. There are hundreds of cardiac glycosides found in nature. However, only a few different cardenolide and bufadienolide aglycones have been found (see Table 1). The sugars are attached to each other via a 1-4 linkage, with each sugar in a chair conformation (for further review, see Fullerton[1], and Davis et al.[4])

	PLANT SOURCE	PRECURSOR GLYCOSIDE	SPLIT OFF BY ENZYMATIC AND MILD ALKALINE HYDROLYSIS *	GLYCOSIDE	SPLIT OFF BY ACID HYDROLYSIS *	-AGLYCONE, OR GENIN
DIGITALIS	*D. purpurea* (leaf)	Purpurea-glycoside A (deacetyldigilanid A)	Glucose	Digitoxin	Digitoxose (3)	Digitoxigenin
		Purpurea-glycoside B (deacetyldigilanid B)	Glucose	Gitoxin	Digitoxose (3)	Gitoxigenin
		———	———	Gitalin	Digitoxose (2)	Gitaligenin (gitoxigenin hydrate)
	D. lanata (leaf)	Lanatoside A (digilanid A)	Glucose + acetic acid	Digitoxin	Digitoxose (3)	Digitoxigenin
		Lanatoside B (digilanid B)	Glucose + acetic acid	Gitoxin	Digitoxose (3)	Gitoxigenin
		Lanatoside C (digilanid C; cedilanid)	Glucose + acetic acid	Digoxin	Digitoxose (3)	Digoxigenin
STROPHANTHUS	*S. Kombé* (seed)	K-strophanthoside	Glucose	K-strophanthin-β (strophanthin)	Glucose + cymarose	Strophanthidin
		K-strophanthoside	Glucose (2)	Cymarin	Cymarose	Strophanthidin
		K-strophanthin-β	Glucose	Cymarin	Cymarose	Strophanthidin
		———	———	Cymarol	Cymarose	Strophanthidol
	S. gratus (seed)	———	———	Ouabain (G-strophanthin)	Rhamnose	Ouabagenin (G-strophanthidin)
SCILLA (SQUILL)	*Urginea maritima* or *indica* (bulb)	Scillaren A	Glucose	Proscillaridin A	Rhamnose	Scillaridin A

* One mole of sugar or acetic acid is split off, unless the number of moles is otherwise indicated in parentheses.

Table 1. Botanical Sources and Major Chemical Components of Cardiac Glycosides of Clinical Importance. (Goodman and Gilman[2])

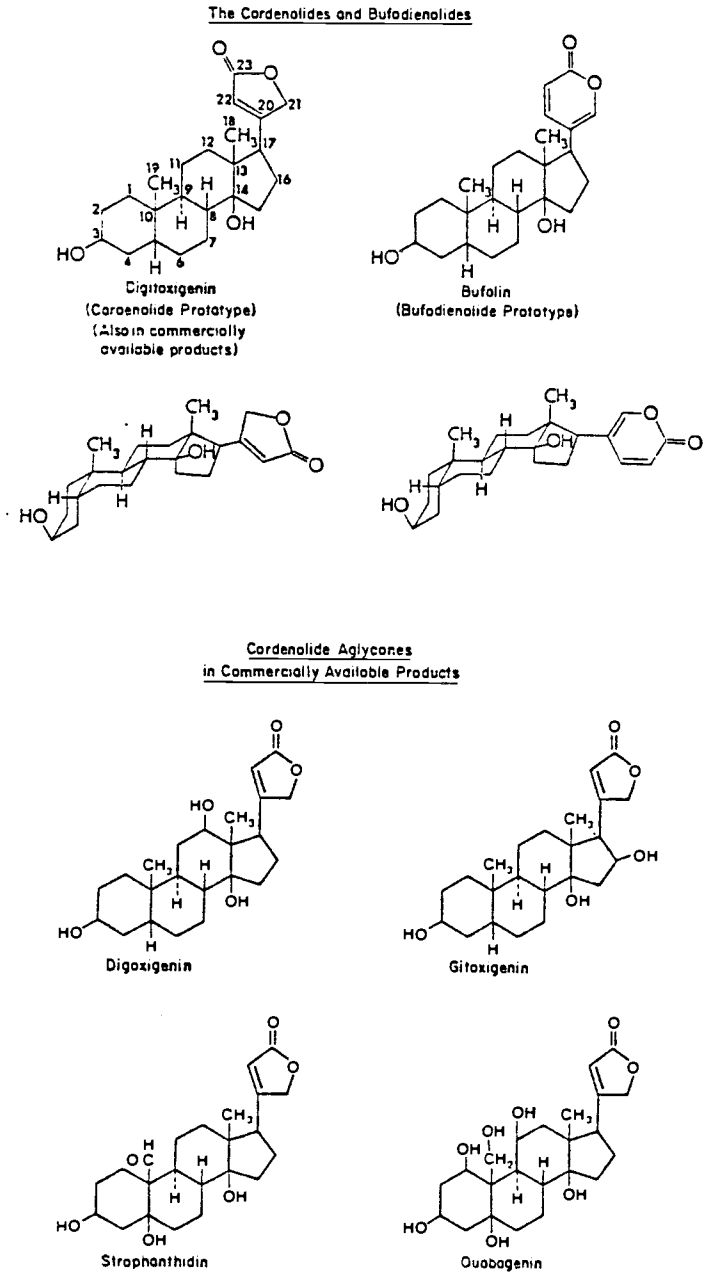


Figure 2. Structures of some naturally occuring cardiac aglycones (from Fullerton[1]).

C. Na^+,K^+-ATPase

RECEPTOR OF CARDIAC STEROIDS

Sodium potassium adenyltriphosphate phosphohydrolase, Na^+,K^+-ATPase, is an enzyme found in nearly all mammalian cells. It plays a very important role in regulating ion balance, heart contraction, kidney functions and nerve transmissions.[2,8] Many procedures have been used for isolating high activity and stable Na^+,K^+-ATPase (references 9-12 are recommended for a good review).

Na^+,K^+-ATPase consists of several subunits (Figure 3). The α subunit, a catalytic subunit with a molecular weight between 90,000-100,000, constitutes about 70% of the total protein by weight.[10] The second subunit, the β subunit, is a glycoprotein[13] with a molecular weight of about 45,000. This glycoprotein constitutes about 19% of the total protein.[10] Its functional role, however, is still unknown.[14] A third and possibly fourth subunit, $\gamma 1$ and $\gamma 2$, each with a molecular weight of about 12,000, have been reported.[15] The γ subunits are polypeptides and constitute about 8.5% of the total protein.[13] There is still an uncertainty about their functional role(s). However, a recent report indicates that the γ components are present in stoichiometric amount in the enzyme and are quite important in the activity of the Na^+,K^+-ATPase.[15]

The stoichiometric arrangement of the α and β subunit in the holoenzyme still remains an unsettled problem.[16] However, it is now generally accepted that the quarternary structure of Na^+,K^+-ATPase consists of at least a dimer of the α subunit. The quaternary structure of the holoenzyme has been suggested to be $\alpha_2\beta_1$,[17] $\alpha_2\beta_2$[10,16,17] and $\alpha_2\beta_4$.[18]

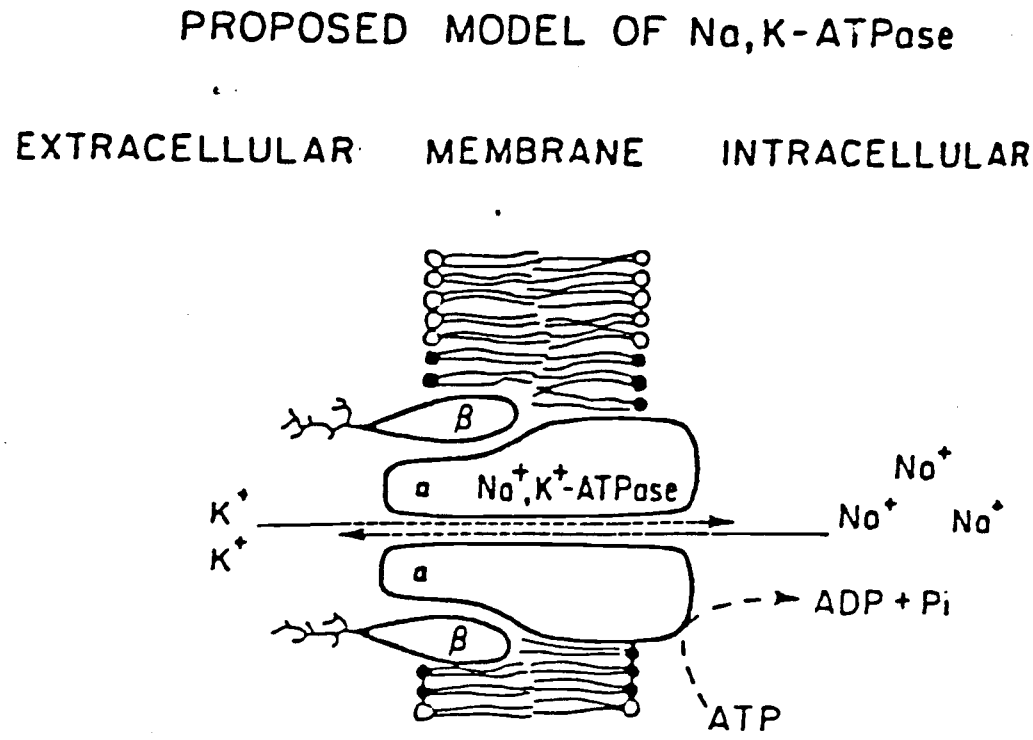


Figure 3. Diagrammatic model of membrane Na^+,K^+-ATPase representing protein subunits and ionic exchange. The enzyme is represented as a dimer consisting of one α- and β-subunit per functional unit. The α protein represents the Mr = 90,000-100,000 catalytic subunit, and the β-subunit represents the Mr = 45,000 glycoprotein. The α subunit is known to serve as the binding site for digitalis glycosides extracellulary and as the ATP hydrolysis site intracellulary. The stochiometry of ion exchange is probably 3 Na^+ transported extracellular space in exchange for 2 K^+ transported to the intracellular space (Schwartz and Adams[10]).

Apparently, in intact cells, the activity of Na^+,K^+-ATPase is regulated by the concentration of sodium inside the cell membrane $[Na^+]_i$ and its increase is believed to be closely related to the positive inotropic effect of cardiac steroids.[2]

In 1963, Repke[7] postulated that Na^+,K^+-ATPase was the cardiac steroid receptor, and was responsible for the positive inotropic effect. This hypothesis was then further investigated by others.[2,9,20,21] At present, there is a strong support for the concept that Na^+,K^+-ATPase inhibition is the cause of the inotropic effect.[9,10,13,20-24] However, there are still some arguments about the mechanism(s). The most widely accepted belief is that the increased sodium ion concentration inside cell membranes increases the intracellular calcium ion concentration either by competing for intracellular calcium sites or by sodium induced increase in calcium influx. This indirect increase in $[Ca^{2+}]_i$ then increases the myocardial contractility since more calcium is available for interaction with myofibril (Figure 4).[2,16] The hypothesis that inhibition of the Na^+,K^+-ATPase is essential for the inotropic effect of cardiac steroids is not, however, universally accepted (see Okita et. al.[25,26], Rhee[27], Godfrey and Burton[28], Lullman[29] and Erdmann[30]).

Until recently, the only therapeutically useful inotropic agents were the cardiac steroids, i.e., cardenolides, bufadienolides, cassaine (cardenolide-3-bromo-acetate).[1,2,31] However, in 1982, a new group of inotropic agents began to attract widespread interest. Included in this group are amrinone[32], milrinone[33], sulmazole[34], fenoximone[35], CI-914 and CI-930.[36] (Figure 5)

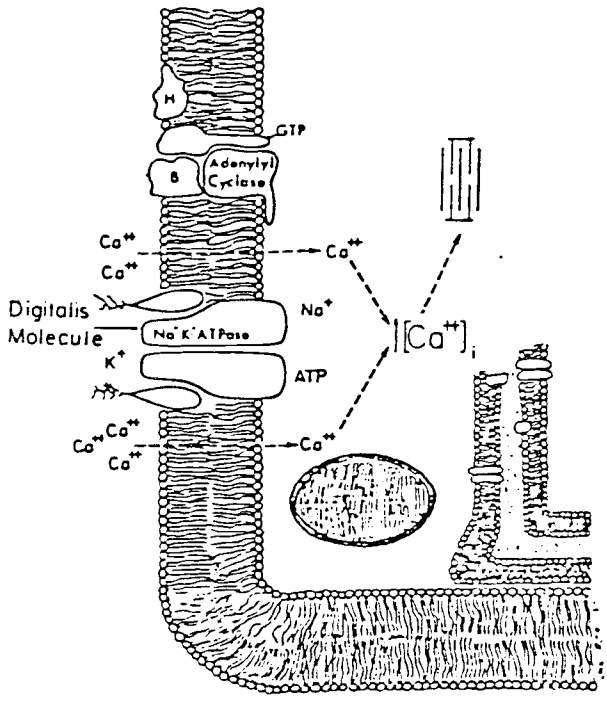


Figure 4. Proposed mechanism of digitalis positive inotropic action. See text for detail (Schwartz and Adams[10])

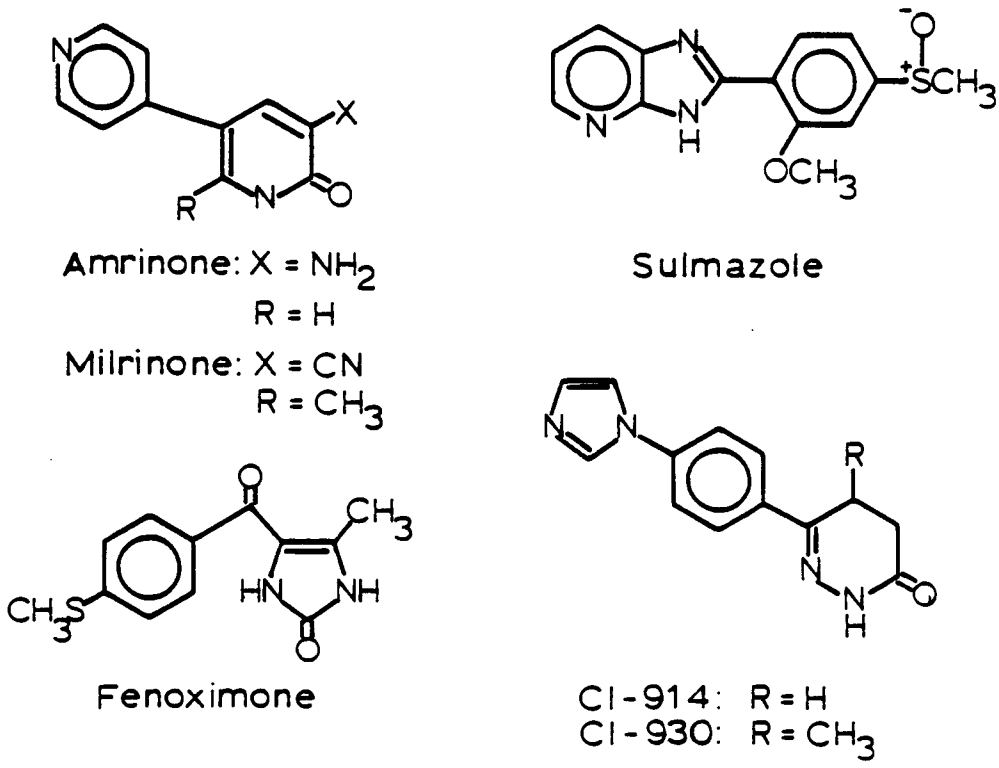


Figure 5. Structures of non Na^+,K^+-ATPase inhibiting (non cardiac steroid) positive inotropic agents.

Although there are still a lot of debates to relate the Na^+,K^+-ATPase inhibition and positive inotropic effect, investigators have generally agreed that Na^+,K^+-ATPase inhibition and the resultant depletion of intracellular potassium ion within the myocardium is the main cause of toxicity.[1,2] Chronic treatment with cardiac steroids may also disturb the nerve transmission process.[6,37] The great toxicity of cardiac steroids has been a major stimulus in studying their mechanism of action. If the toxicity and inotropic activity are caused by different mechanisms[38,39] - which we believe is unlikely - then it would be possible to design better and safer cardiac steroids. However, much attention has been given to glycosides such as actodigin (Figure 6) which have been claimed to have more easily controlled pharmacokinetic properties.[40]

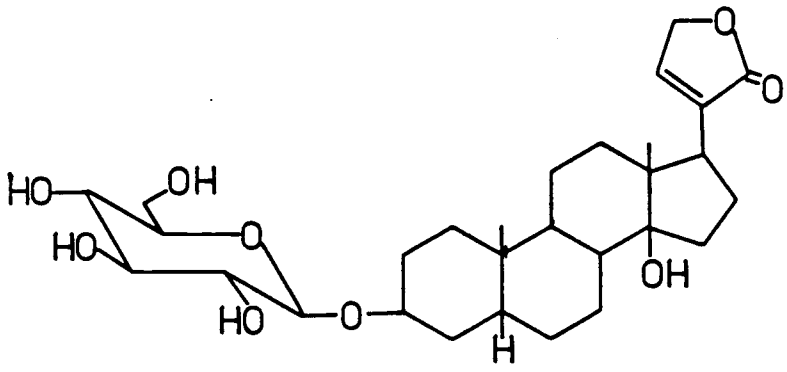


Figure 6. Structure of Actodigin

CHAPTER TWO

STRUCTURE ACTIVITY RELATIONSHIPS OF CARDIAC GLYCOSIDES

CHAPTER TWO

STRUCTURE ACTIVITY RELATIONSHIPS OF CARDIAC GLYCOSIDES

A. INTRODUCTION

Investigators generally agree that binding of the cardiac glycosides to Na^+,K^+-ATPase is closely related to positive inotropic activity. However, an uncertainty remains as to whether inotropic activity is indeed the result of the inhibition of the enzyme.

Interaction between a cardiac steroid and Na^+,K^+-ATPase appears to involve at least two binding sites - genin binding site and sugar binding site.[9,10,19] As well be discussed in this chapter, in spite of many investigations into the structure activity relationship (S.A.R.) of the genin and sugar portion of the cardiac glycosides, there is not full agreement about the factor(s) that regulate the positive inotropic effect of the cardiac glycosides.

As will be discussed further in this chapter, our group (Fullerton and coworkers)[41] in 1979 introduced the "carbonyl oxygen distance" principle which gave an excellent explanation to the S.A.R. of all but some genins which are more potent than digitoxigenin. However, Repke and coworkers[42] have argued vigorously against it. Another more complicated study centers on the biological role of the sugar portion of these glycosides. There are many arguments about the role of C3'-OH and C4'-OH group in the formation of the hydrogen bond with the Na^+,K^+-ATPase, which is believed to be responsible for enhancement of the inotropic potency of the genin portion. The second important

feature of the sugar moiety is the C5'-methyl group. Although there has been suggestions that the C5'-methyl group may bind to the enzyme through a hydrophobic bond, however, this has never been proven. This will be discussed further in Section D of this chapter.

B. ROLE OF THE GLYCOSIDE'S GENIN AND SUGAR PORTION IN INTERACTION WITH Na^+,K^+-ATPase

There is a great difference in the inotropic potency between cardiac genin and glycoside due to the differences in the nature of their binding to the Na^+,K^+-ATPase, the putative receptor of cardiac steroids. The glycoside-enzyme complexes are more stable than the genin-enzyme complexes.

This difference was studied by Yoda and Yoda[43-46] by measuring the association rate constant (K_a) and dissociation rate constant (K_d) of various glycosides both in type I and type II complexes. Type I complex is that of cardiac genin or glycosides and Na^+,K^+-ATPase in the presence of Mg^{2+} + Na^{2+} + ATP (type I condition), while the type II complex is obtained in the presence of Mg^{2+} + Pi (type II condition).

The results of the study showed that K_a is dependent only on the genin portion of the cardiac glycosides and K_d is dependent on both the genin and the sugar portion in type I complexes.[43] This study confirmed the presence of the sugar-specific binding site on the enzyme and provided a general picture of the role of the genin and sugar portion of the cardiac glycosides in binding with the enzyme.

Since the association rate constant (K_a) is dependent on the nature of the steroid moiety, the association between steroid moiety and steroid-specific site of the enzyme must precede the sugar binding or be the rate determining step in forming the cardiac glycoside-enzyme complexe. On the other hand, the dissociation rate constant (K_d) of type II complexes is dependent on the nature of the sugar moiety.

Therefore the dissociation of the bond(s) between the sugar moiety of the cardiac glycoside and the sugar-specific site of the enzyme must precede the steroid dissociation or be the rate determining step in the dissociation process, followed by dissociation of the steroid moiety from the steroid-specific site. As the result, Yoda[43] proposed a model to explain the mechanism of binding between the cardiac glycoside and Na^+,K^+-ATPase as follows:

1. Reaction step of steroid moiety and steroid-specific site:

$$E + \text{Steroid-sugar} \rightleftharpoons E - \text{Steroid-sugar}$$

2. Rearrangement of the enzyme, i.e., the activation of sugar-specific site:

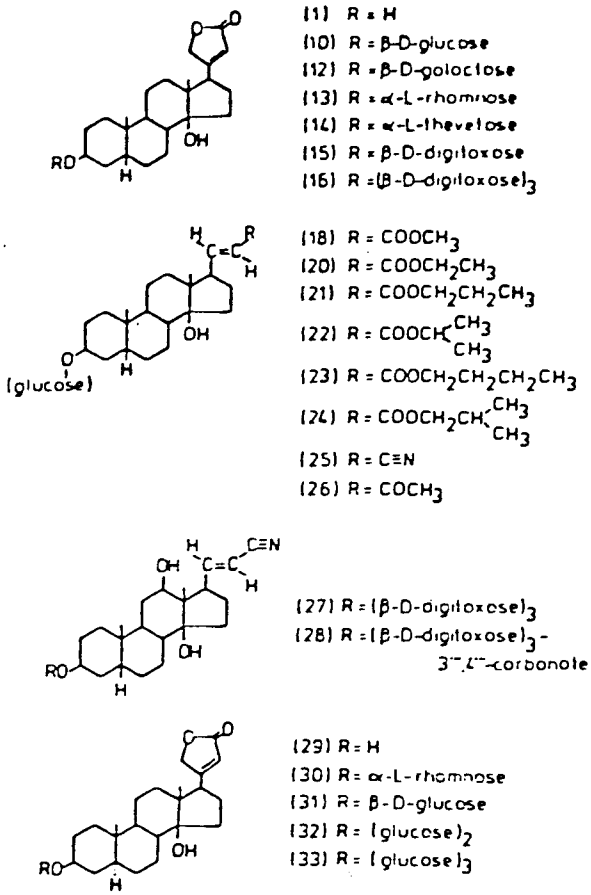
$$E - \text{Steroid-sugar} \rightleftharpoons E' - \text{Steroid-sugar}$$

3. Reaction step of sugar moiety and sugar-specific site:

$$E' - \text{steroid-sugar} \rightleftharpoons E' \begin{matrix} \text{Steroid} \\ | \\ \text{sugar} \end{matrix}$$

Brown and coworkers[17] reported that a linear relationship exists between the Kd and the positive inotropic potency of the cardiac glycosides. Thirty-three glycosides were used in the study, and the positive inotropic potencies were measured from pig left atrium (Figure 7). The inotropic potency is directly proportional to the stability of the drug-enzyme complex. The more stable the complex (the smaller the Kd value), the higher the inotropic potency of the cardiac glycoside.

Although the presence of the sugar moiety in a glycoside increases the inotropic or Na^+,K^+-ATPase inhibiting activity compared to the corresponding aglycone, in general the maximum enhancement is only about



Structural formulae of compounds used in this study.

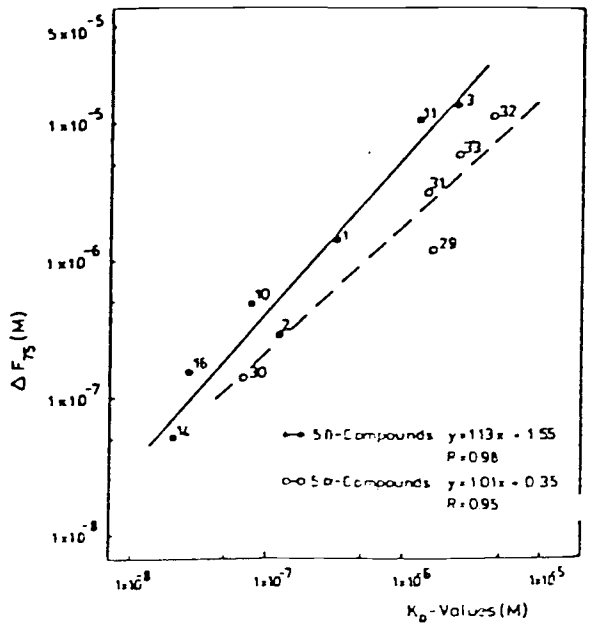
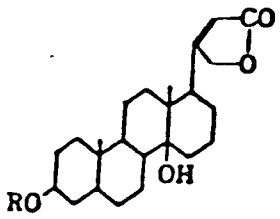


Figure 7. K_D values and inotropic potencies on guinea pig heart. The K_D values determined on guinea pig heart Na^+,K^+-ATPase are taken from Table 4. Inotropic potency on the isolated guinea pig left atria has been defined as the F_{75} value. The F_{75} value is the concentration, interpolated from at least ten cumulative dose-response curves, which increases the force of contraction by 75% (from Reference 42).

20 fold (ie. digitoxigenin α-L-rhamnoside) compared to digitoxigenin. Variation in the aglycone structure, however, can change the activity by several orders of magnitude. Thus, it is apparent that the steroid aglycone provides the major part of the binding to the receptor, but the sugar portion still plays a significant secondary role in the formation of the complex.[47,48] One report also suggests that the sugar portion, however, may play a major role in regulating the toxic potential of the cardenolide glycosides.[49] In this study, the drugs were introduced to mice intravenously, and the toxicity was measured in terms of LD_{50} value after a 24 hour period. The result showed that there was a parallelism between the LD_{50} value and the hydrophilicity of the cardiac glycoside (Figure 8).[49] Introducing one or more sugar residue(s) at the 3β position which increase(s) the polarity of the genin such as glucose, decreases the toxicity of the glycosides. On the other hand, a relatively nonpolar sugar such as digitoxose was found to increase the toxicity of the glycosides as well as enhancing positive inotropic activity.[49]



I : (digitoxigenin) R = H
II : R = β-D-Glu
III : R = β-D-Glu-(1→6)-β-D-Glu
IV: R = β-D-Glu-(1→6)-β-D-Glu-(1→6)-β-D-Glu
V : R = β-D-Dig
VI: R = β-D-Dig-(1→4)-β-D-Dig
VII: (digitoxin) R = β-D-Dig-(1→4)-β-D-Dig-(1→4)-β-D-Dig
Glu: glucopyranose
Dig: digitoxopyranose

TABLE I. Water–Oil Distribution of Digitoxin Analogues

Compound	Water/*n*-BuOH partition coefficient (37°)	Compound	Water/*n*-BuOH partition coefficient (37°)
I	0	V	2.61×10^{-3}
II	4.65×10^{-4}	VI	1.82×10^{-4}
III	25.2×10^{-5}	VII	1.37×10^{-3}
IV	128×10^{-5}		

Mice Intravenous LD_{50}'s of Digitoxin Analogues

Compound	LD_{50} (nanomole/10 g body weight)	Compound	LD_{50} (nanomole/10 g body weight)
I	36	V	208
II	>310	VI	126
III	>780	VII	85
IV	>730		

Figure 8. Water–oil distribution and mice intravenous LD_{50} data of digitoxin analogues, data of Takiura and coworkers.[49]

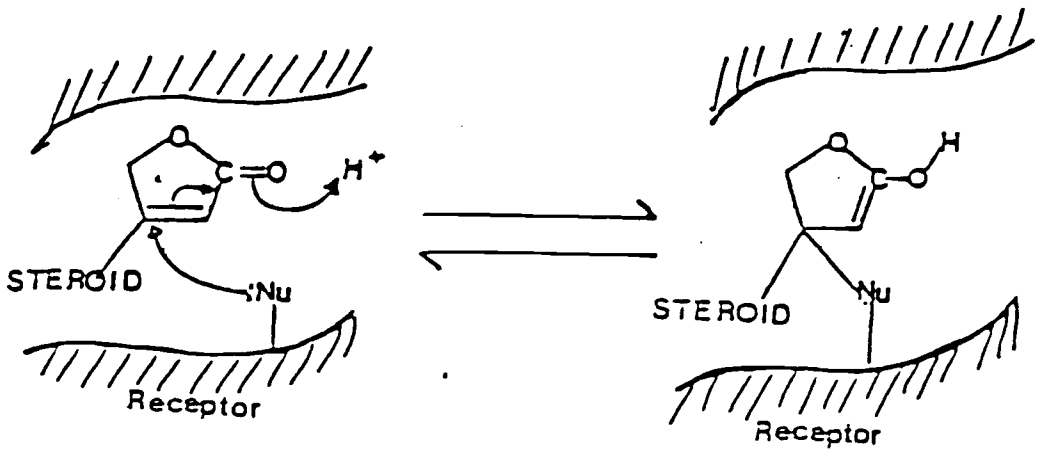
C. STRUCTURE ACTIVITY RELATIONSHIPS OF THE GENIN PORTION

It was previously believed that the capacity of a cardiac steroid genin to inhibit Na^+,K^+-ATPase was determined by the C17-side group, particularly C20-C22 double bond, and the C14-OH. Many models have been proposed which focused on the C17 side group. Examples include (Figure 9):

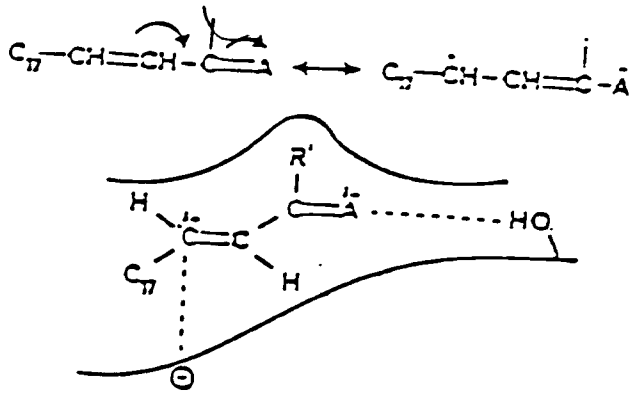
1. Michael attack by the Na^+,K^+-ATPase on the unsaturated lactone ring.[50]
2. Ionic bonding of the β carbon on the unsaturated lactone ring.[51]
3. Molecular dipole attraction.[52]

Fullerton, Rohrer, Ahmed, From and coworkers, however, found that those models could not give a clear explanation for the biological activity of a number of genins with a modified C17 side group.[53-59] In order to seek a better explanation for the wide variation in activity of these genins, Fullerton and coworkers have applied a multidisciplinary approach including:

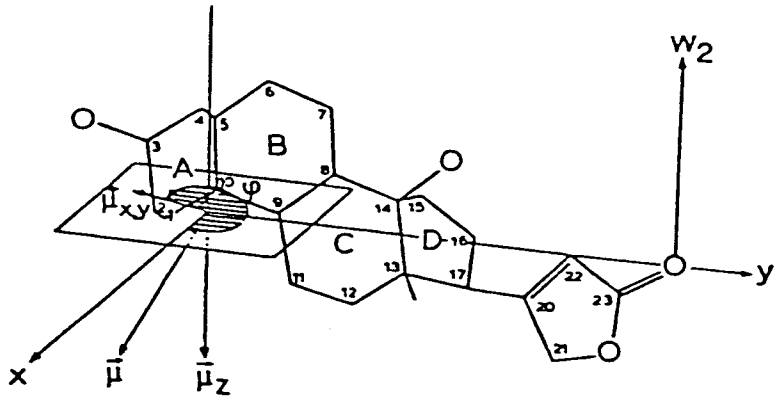
1. Synthesis of a variety of novel genins,
2. X-ray crystallography of these genins to determine exact atomic positions,
3. Generation of conformational energy diagrams for rotation of the C17-C20 bond using a version of the molecular mechanics program CAMSEQ[60] in conjunction with the NIH PROPHET computer system (Figure 10). These diagrams were then used to determine the more stable conformation of the C17 side group. These conformations were then used for computer graphic analysis and study,



Kupchan[50]



Thomas[51]



Repke[52]

Figure 9. Proposed models for the role of C17 lactone ring.

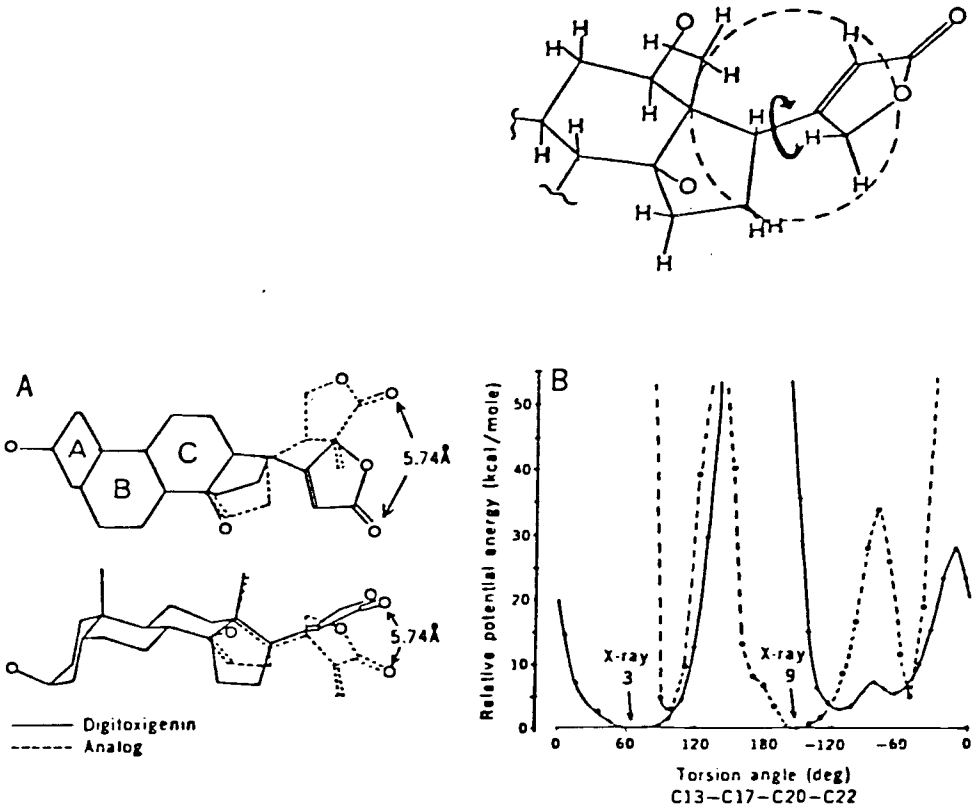


Figure 10. A. Measurement of the distance between carbonyl oxygens using the PROPHET computer system procedure FITMOL.

B. Conformational energy diagrams for rotation of C17-C20 bond of digitoxigenin and analog (from Fullerton et al[53]).

4. Superimposition of each genin in its preferred conformation with digitoxigenin as a standard using the NIH PROPHET computer program FITMOL[61], and then measurement of the distance between corresponding atoms (Figure 10),
5. Determination of the genin biological activity with Na^+,K^+-ATPase from different species and tissues under both type I and type II conditions.[55]

This novel approach led to the remarkable discovery that the Na^+,K^+-ATPase inhibitory and inotropic activities of the genins were a simple linear function of the C17 side group carbonyl oxygen distance relative to that of digitoxigenin (Figure 11).[55] For genins with equivalent C17-C20 conformational energy wells, only one conformation fits this correlation.[53]

The carbonyl oxygen distance relationship, however, does not provide an adequate explanation for the genins which are more active than digitoxigenin (i.e., having a negative carbonyl oxygen distance). As can be seen in Figure 12, genins having a negative carbonyl oxygen distance are not as close to the regression line as genins having a positive carbonyl oxygen distance (genins less active than digitoxigenin). Thus, to some extent the application of the carbonyl oxygen distance relationship is limited.

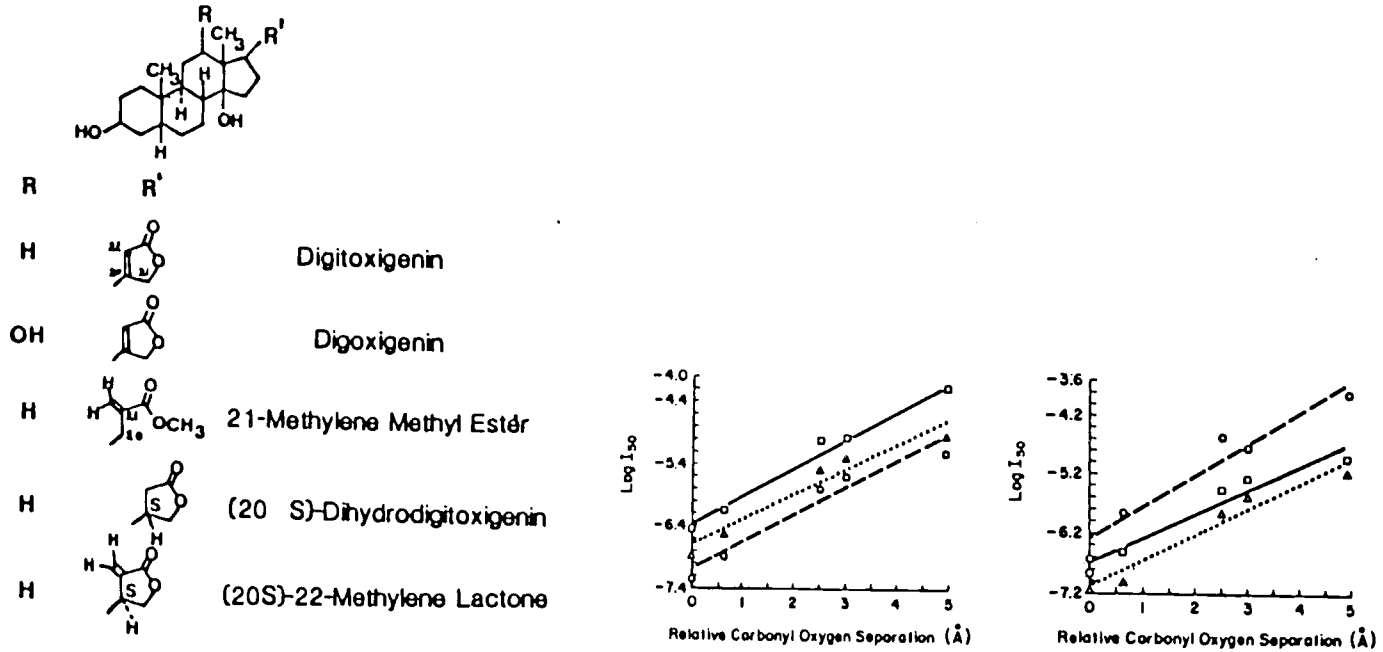


Figure 11. A (left). Structure of genins used in various experiments.

B (center). Correlation between relative carbonyl oxygen separation in five genins and their I_{50} values for Na^+,K^+-ATPase from different sources including rat brain (□——□), hog kidney (Δ····Δ), and cat heart (o - - - o).

C (right). Correlation between relative carbonyl oxygen separation in five genins and their I_{50} values for hog kidney enzyme under different binding and substrate conditions including type I (□——□), type II (Δ····Δ), and K^+-p-NPPase inhibition under type II binding condition (o - - - o). (From Ahmed et al[53])

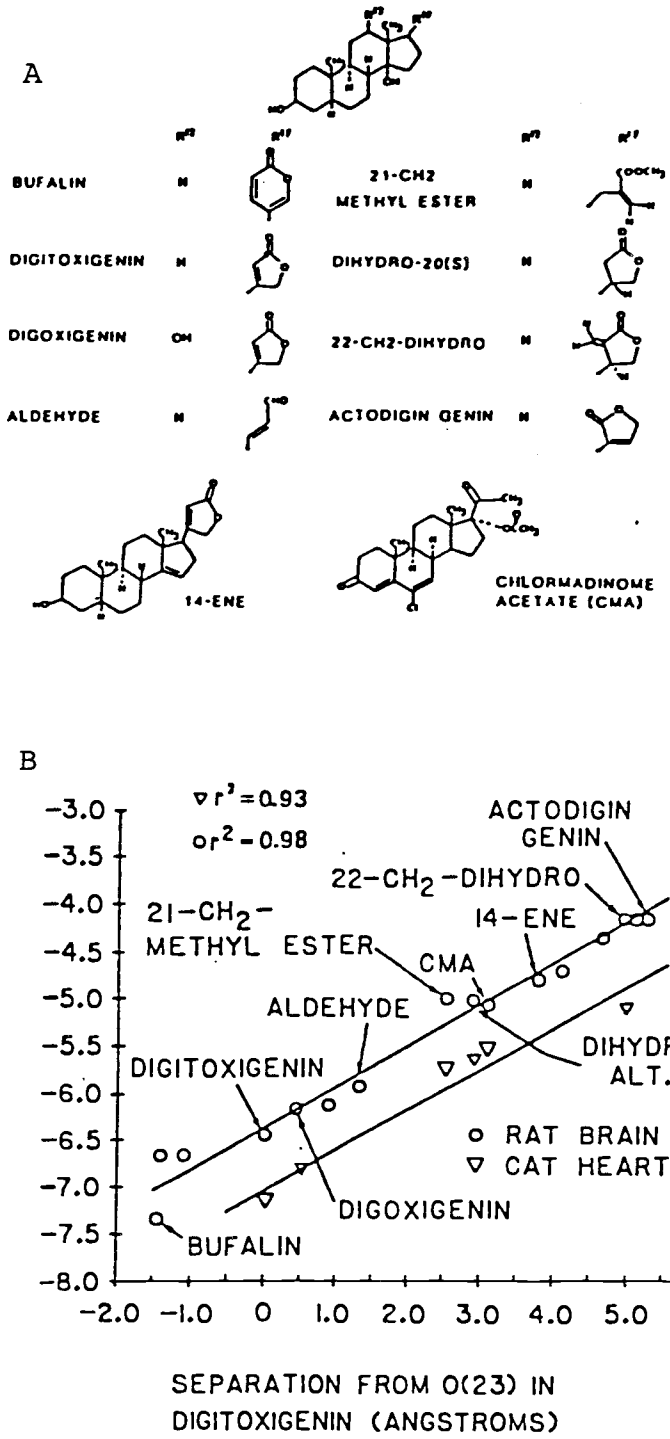


Figure 12. A. Examples of genins studied.

B. Correlation between relative carbonyl oxygen separation and log I_{50} values for Na^+,K^+-ATPase from rat brain and cat heart (from Fullerton et al[53]).

From the genin studies by Fullerton and coworkers, several tentative conclusions can be drawn about the role of the genins in biological activity:

1. A linear relationship exists between the activity and the carbonyl oxygen distance with digitoxigenin as standard. For each 2.2 Å displacement of carbonyl oxygen, the activity drops by one order of magnitude (Figure 11).[55]
2. The C14-β-OH group is not necessary for the activity since some genins without C14-β-OH were found to be more active than identical genins with C14-β-OH.[58]
3. The lactone ring is not essential for the activity. Some genins with an open ring C17 side group were found to be more active than genins with a C17 lactone ring,[55,58] for example: 21-methylene methyl ester vs (20S)-22-methylene lactone (Figure 11).[58]
4. The unsaturated bond on C17 side group plays only an indirect role by affecting the position of the carbonyl oxygen.[58]

D. STRUCTURE ACTIVITY RELATIONSHIPS OF SUGAR PORTION

ROLE OF C4'-OH and C6'-OH

Although many naturally occurring cardiac glycosides contain tri- or tetra- saccharide moieties, the first sugar attached to the steroid genin at the C3-OH has been shown to have the greatest influence on receptor binding and activity.[43,44] The first sugar is therefore of particular interest. Extensive studies on the biological role of the sugar portion was first conducted by Yoda and Yoda.[43-46]

By measuring the K_d values of various glycosides having the same genin moiety, Yoda found that the stability of the cardiac glycoside - Na^+,K^+-ATPase complexes under both type I and type II conditions increased in the order of: tetrahydropyranyl ether < digitalose < digitoxose < 6-deoxy glycose = fucose < 6-deoxy gulose < rhamnose.[43]

Yoda's results indicated that a C3' hydroxyl group either axial or equatorial and C5' α methyl group are responsible for the binding to the sugar-specific site of the enzyme. However, a C3' axial hydroxyl group had more affinity than the equatorial hydroxyl group. Yoda concluded that C3'-OH was the most likely binding site for the sugar portion to the sugar specific site of the enzyme through a hydrogen bond. Complexes having a C3'-methoxy group had less stability than those having a free C3'-OH group, as shown in the case of cymarin vs helveticoside and odoroside H vs digiproside (Figure 14).

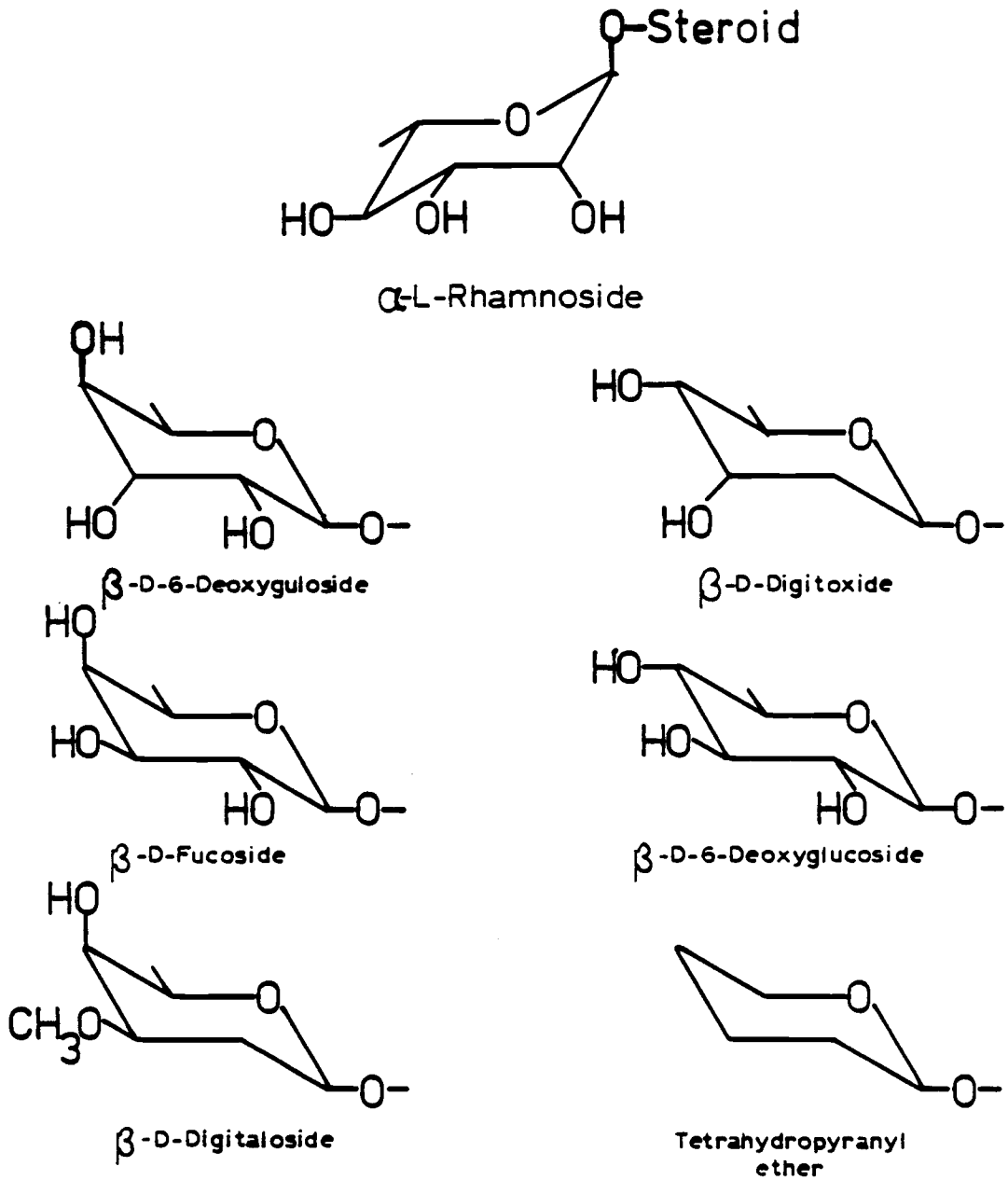
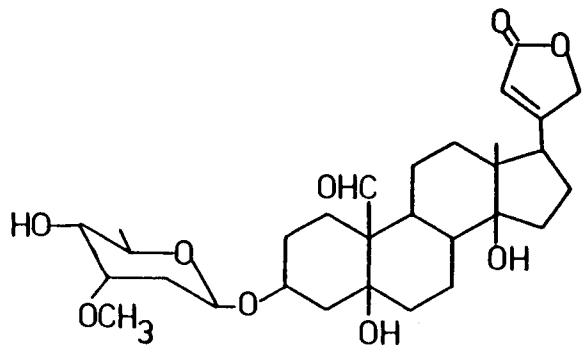
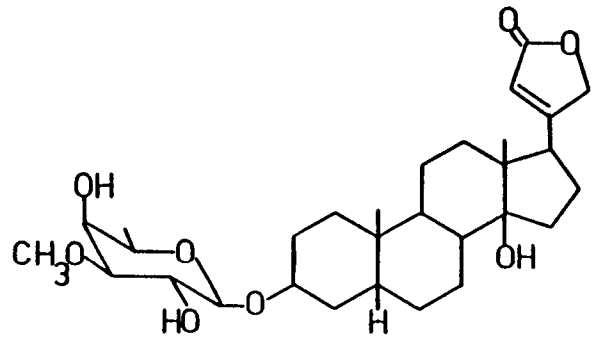


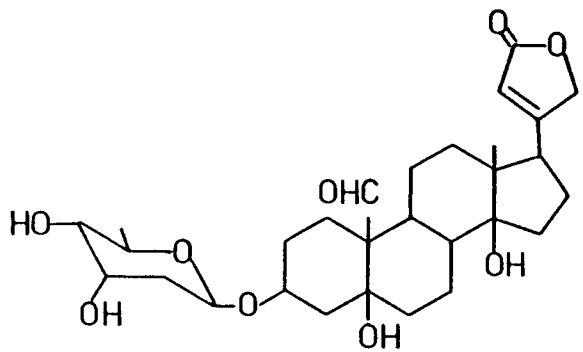
Figure 13. Structures of sugars which form sugar moieties of cardiac glycosides used in Yoda[43] study.



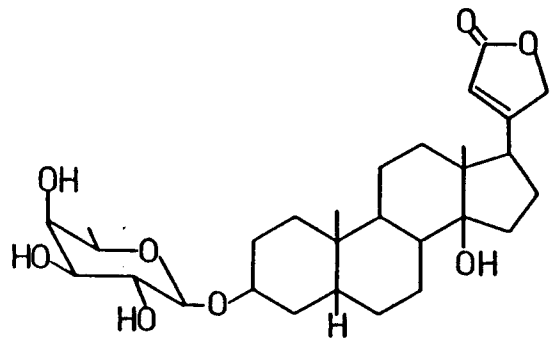
Cymarin



Odoroside H



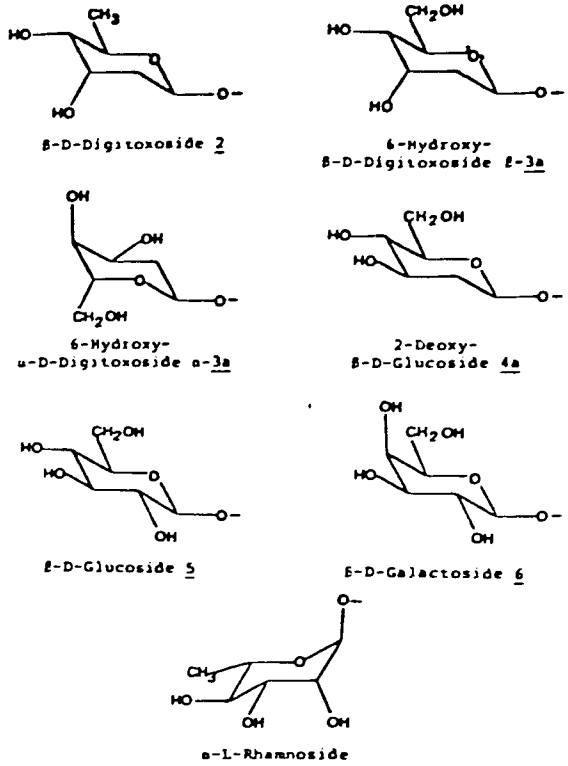
Helveticoside



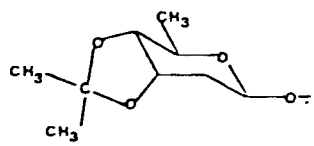
Digiproside

Figure 14. Structure of four cardiac glycosides used in Yoda[43] study.

Studies conducted by Fullerton[62,63] and Brown[64], however, reached a quite different conclusion. These authors have undertaken a systematic modification of the structure of the sugar portion and determination of the I_{50} value of the respective cardiac glycoside on isolated Na^+,K^+-ATPase. Both groups found that sugars having an equatorial C4'-OH group are the most effective in enhancing the biological potency of the genin. Changing the C4'-OH group from equatorial to axial will dramatically decrease the biological activity. For example, digitoxigenin β-D-glucoside having a equatorial C4'-OH has an I_{50} value of 1.17×10^{-8} M, while digitoxigenin β-D-galactoside having an axial C4'-OH has an I_{50} value of 6.45×10^{-8} M[63] (see Table 2). Thus, altering the stereochemistry of C4'-OH from equatorial to axial decreases the biological activity by 5 fold, while altering the stereochemistry of C3'-OH from axial to equatorial (6-hydroxy-β-D-digitoxoside to 2-deoxy-β-D-glucoside (Table 2) reduces biological activity only by a factor of 2. A direct inotropic activity measurement using the electrically driven left guinea pig atria by Brown and Thomas[64] affords a similar result. β-D-Glucose increases the positive inotropic activity of digitoxigenin by a factor of 2.8, whereas β-D-galactose is without effect. Similarly, digitoxigenin α-L-rhamnoside and α-L-thevetoside, (Figure 15) two of the most active glycosides studied by Brown and Thomas[64] also have an equatorial C4'-OH group. The equatorial C4'-OH group is thus common to all the potent β-D and α-L-glycosides studied. Addition of axial C3'-OH or α-L configuration may to some extent further increases the biological activity.[64]



steroid	I_{50}, M
1	1.17×10^{-7}
2	7.04×10^{-9}
2 acetonide	4.18×10^{-8}
β-3a	1.07×10^{-8}
α-3a	9.33×10^{-8}
4a	2.82×10^{-8}
5	1.17×10^{-8}
6	6.45×10^{-8}



2 acetonide

Table 2. Hog kidney Na^+,K^+-ATPase inhibiting activity (I_{50}) of various glycosides used in study by Kihara and Fullerton et al.[64]

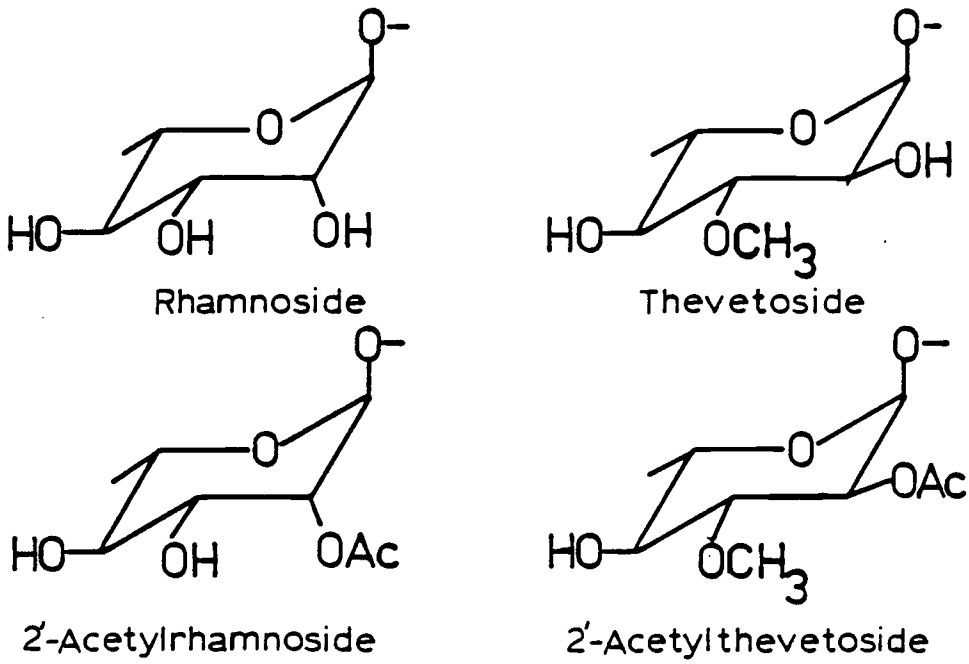
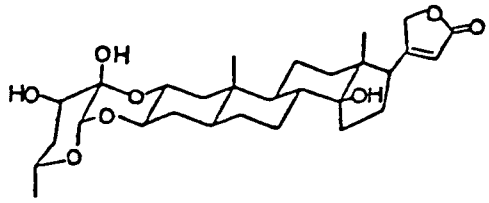


Figure 15. Structures of two pairs of cardiac glycosides used in Brown[47] study.

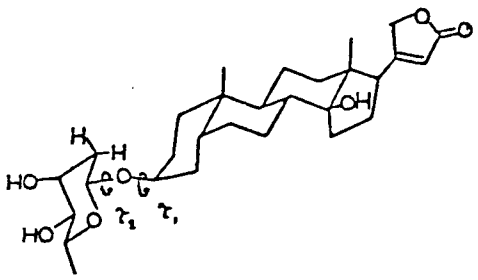
Rohrer, Fullerton and coworkers[56] in 1984 reported that the presence of the C3-sugar does not significantly alter the conformation of the C17 side group of the digitoxigenin. Based on this study, it follows that digitoxigenin β-D-glucoside and digitoxigenin β-D-galactoside would have the same degree of C17 side group carbonyl oxygen interaction with the steroid - specific binding site of the Na^+,K^+-ATPase. It is apparent that β-D-glucose has some degree of interaction with the sugar-specific binding site of the enzyme which results in an increase of the inotropic activity of the digitoxigenin, whereas in the case of β-D-galactose, there is no or a very small degree of interaction with the enzyme.

These results clearly demonstrate the importance of the biological role of C4'-OH group. Recently, a different approach has been used by Chiu and Thomas.[48] The focus of their work was the unusual glycoside-like natural product called gomphoside (Figure 16), a compound which has in effect a rigid sugar attached to C3. A computer program was used by these workers to generate a conformational energy diagram for rotation of the C3-O-C1' and to superimpose the cardiac glycoside used in the study with gomphoside as standard. They then measured the distance between C3'-OH and C4'-OH of the glycosides to the C3'-OH of the gomphoside (Figure 16). Chiu and Thomas[48] Found that glycosides with a preferred conformation (conformation at low energy level) similar to the conformation of gomphoside and a restricted rotational freedom around C3-O-C1' bond were more active relative to those with a wider rotational freedom around C3-O-C1' bond. Glycosides with a gomphoside-like

A



Gomphoside



β-D-Digitoxoside

B

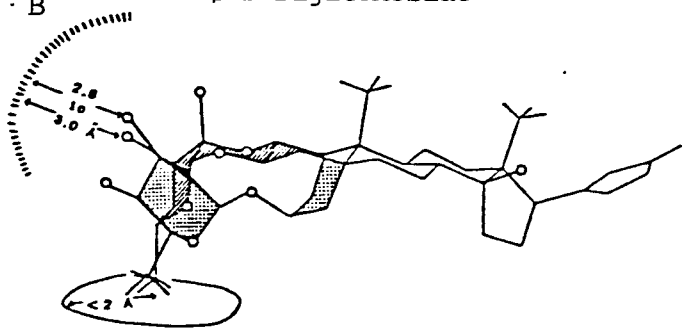


Figure 16. A. Structure of gomphoside and β-D-digitoxoside.

B. Superimposition of gomphoside and β-D-digitoxoside used in Chiu and Thomas[51] study.

conformation only at a high energy level were less active relative to those which could exist at that conformation at a lower energy level. However, their study still could not obtain a conclusive answer to solve the controversy over the role of C3'-OH and C4'-OH.

From the studies of Fullerton[62], Yoda[43] and Brown[64], there has been an unproven suggestion that there could be a second major binding site for the sugar portion. This presumably involves a hydrophobic bond between the C5'-methyl group of the 6-deoxy sugars and a specific site on the Na^+,K^+-ATPase[43-46,62,64] It was proposed that increasing the polarity of the C5'-methyl group by introducing a 6'-hydroxyl group would increase the polarity of the C5'-methyl, decrease the C5'-hydrophobic bond, and result in decreased biological activity.[43,62,64] Introducing a methoxy or acetyl group to the C5'-methyl also has been found to decrease biological activity - possibly due to a steric effect which inhibits the close interaction between C5'-methyl group and hydrophobic binding site on the enzyme.[43] The variation in biological activity obtained by altering the C5'-methyl group is not quite significant (Table 2). However, a parallel result was obtained by all investigators. It may suggest that this hydrophobic bond plays only a secondary role with respect to the hydrogen bond between C3'-OH or C4'-OH and the enzyme in stabilizing the cardenolide glycoside-enzyme complex.

The third important feature of the sugar moiety is the C2'-hydroxyl group. Yoda[43] reported in 1974 that the C2'-OH group did not play any role in type II binding condition. It seemed to form some degree of

hydrogen bond in the type I binding conditions and this weak hydrogen bond was postulated by Yoda to be responsible for the apparent difference in stability between type I and type II complexes. However, the evidence for this hydrogen bond is currently weak. Positive inotropic activity studies on the left atria conducted by Brown and coworkers[64] found that a C2'-OH group either axial or equatorial did not affect the observed biological activity. Further, the acetylation of the equatorial C2'-OH group (in thevetoside) (Figure 15) does not affect the activity. However, acetylation of the axial C2'-OH group (in rhamnoside) (Figure 15) may severely decrease the inotropic activity, in some cases even lower than the inotropic of the genin itself. This implies that the C2'-axial acetyl-oxy group inhibits the formation of the drug receptor complex and may also imply that axial C2'-OH group, when free, may contribute significantly to the receptor interaction. Acetylation of the C2'-OH thevetoside reduces activity by a factor of 2.6[64] which is almost in a complete agreement with the study conducted by Fullerton and coworkers.[62] Digitoxigenin β-D-glucoside has an I_{50} value of 1.15×10^{-8}M (measured on isolated hog kidney Na^+,K^+-ATPase), elimination of the C2'-OH group affords the digitoxigenin β-D-2'deoxy glucoside which has an I_{50} value of 2.82×10^{-8} (Table 2). The same degree of reduction in the inotropic activity is also obtained when stereochemistry of the C2'-OH group is altered from equatorial (β-D- and β-L glucoside) to axial (β-D and β-L mannoside).[65]

CHAPTER THREE

RESULTS AND DISCUSSION

CHAPTER THREE

RESULTS AND DISCUSSION

A. GOALS

As discussed in Chapter II, in spite of a number of investigations, there is still uncertainty about the biological roles of sugar C3'-OH and C4'-OH. Yoda and Yoda[42] concluded that C3'-OH is the primarily binding site, whereas Fullerton et al[63] and Brown et al[64] strongly suggested that C4'-OH should be the primarily binding site. In order to solve this controversy, we designed and synthesized a series of three trideoxyglucosides - 8, 11, and 22 - having the same genin moiety, but the sugar having only one hydroxy group at C4' (Figure 17), and then determined the biological activity in terms of I_{50} values on isolated hog kidney Na^+,K^+-ATPase under type I binding condition. If it is indeed the case that C4'-OH is the primarily binding site, trideoxyglucosides should have a comparable inotropic activity relative to the corresponding rhamnoside or glucoside, since elimination of the C2'-OH and C3'-OH should not have a great effect upon the biological activity.

As discussed in the previous chapter, there have been several suggestions about the presence of a second binding site involving the C5'-methyl group of 6'-deoxysugars.[43,62,64] However, a systematic investigation has never been reported. As shown in Table 2, introduction of a hydroxyl group at C5' in the 6'-hydroxy-β-D-digitoxoside decreases the biological activity (relative to the β-D-digitoxoside).[62]

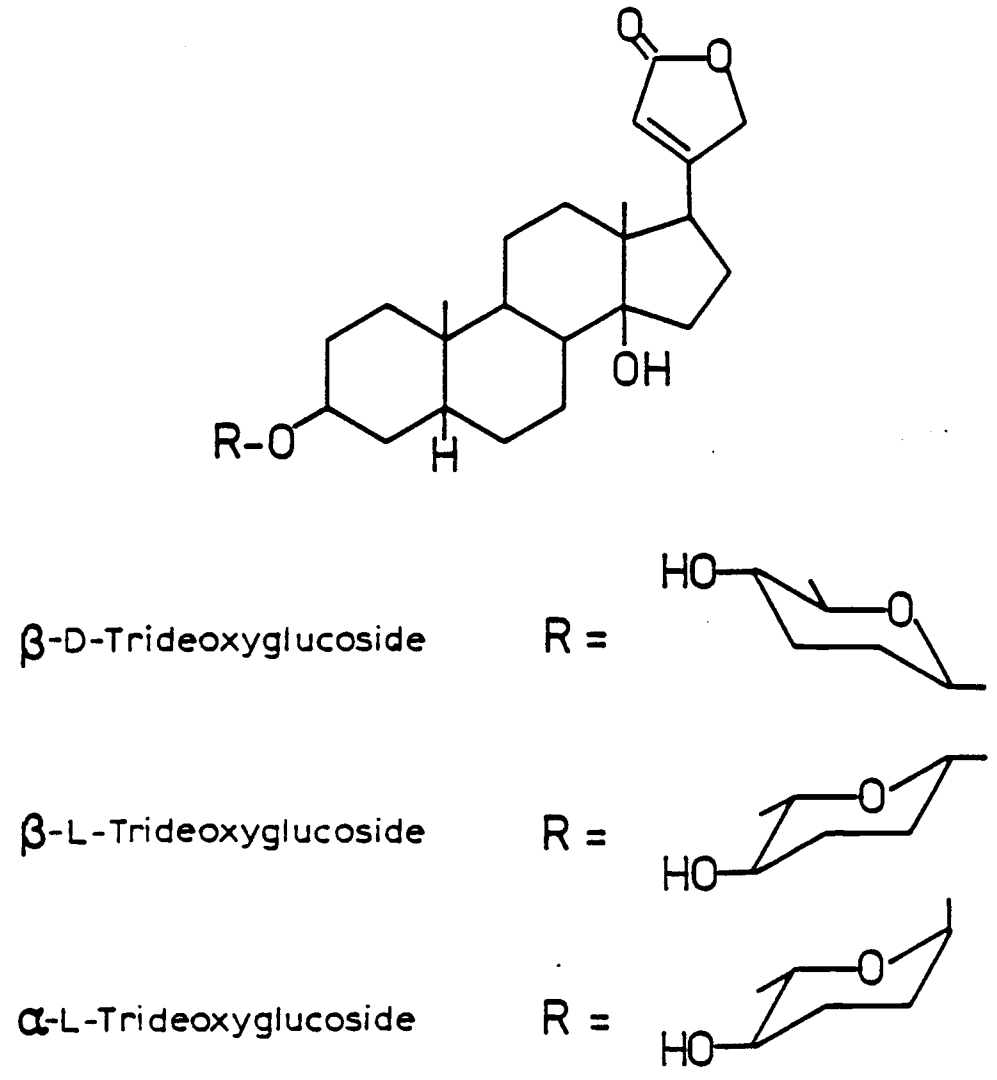
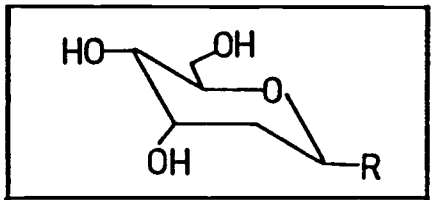
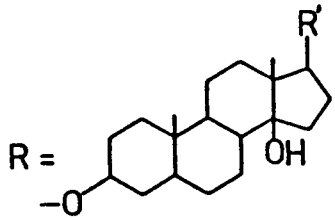


Figure 17. Structures of three trideoxyglucosides synthesized for the structure activity relationship study.

Yoda[43] and Brown et al[64] also suggested a role of the C5'-methyl group. However, Yoda's and Brown's conclusions were not based on a comparison of glycosides which were different only in the C5' moiety. In order to conclusively prove the biological role of the C5'-methyl group, we designed and synthesized a series of 6'-hydroxy-β-D-digitoxosides (Figure 18), and then compared their Na^+,K^+-ATPase inhibitory activity with a series of β-D-digitoxosides which differ only in the C5' moiety. It would be expected that if the earlier suggestions were true, introduction of the hydroxyl group at the C5'-methyl should decrease the biological activity of the β-D-digitoxosides by the same proportion for each glycoside pair.



β-D-6-OH-Digitoxide



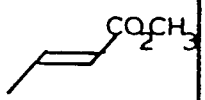
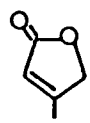
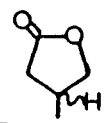
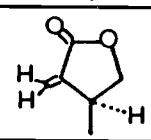
	R'
Methyl Ester	
Digitoxigenin	
Dihydro-R,S	
22-CH_2-Dihydro-R	

Figure 18. Structures of four β-D-OH-digitoxosides.

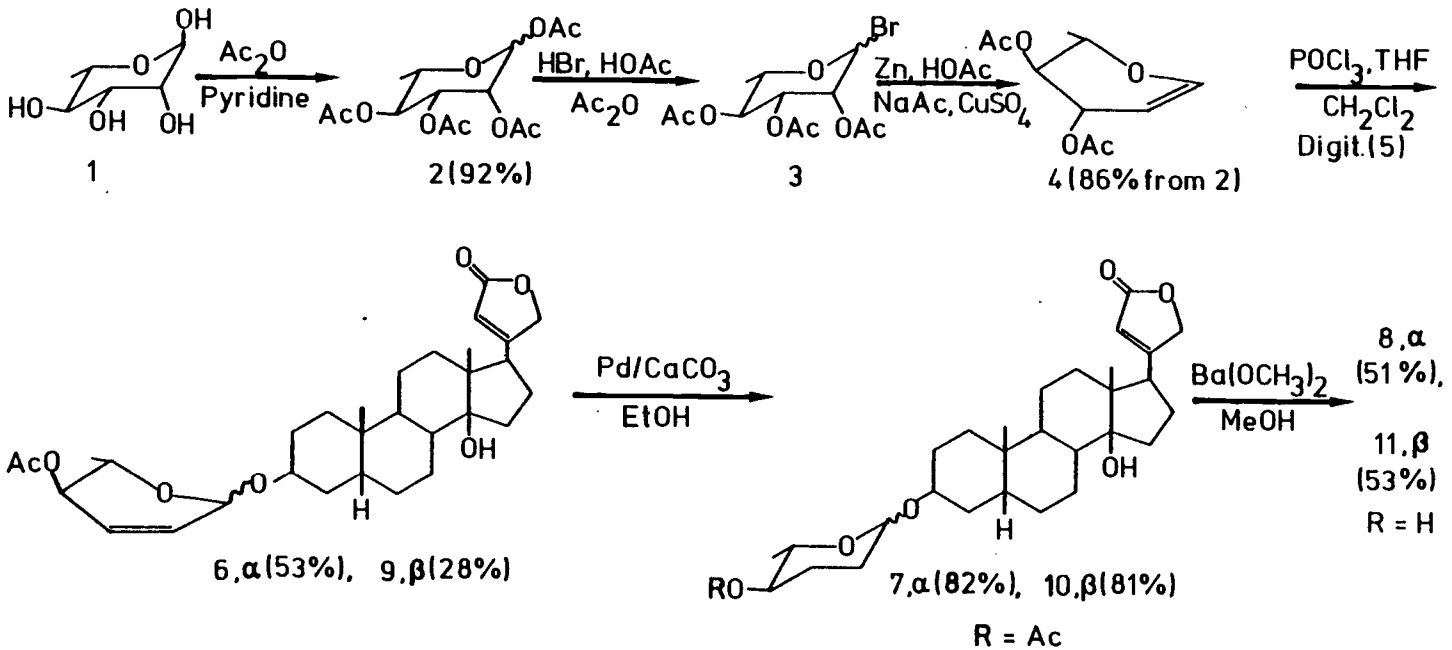
B. CHEMISTRY

1. SYNTHESES OF TRIDEOXYGLUCOSIDES

a. Digitoxigenin α-L-Trideoxyglucoside (8) and β-L-Trideoxyglucoside (8) and β-L-Trideoxyglucoside (11) (Scheme 1)

Trideoxyglucosides 8 and 11 were synthesized from α-L-rhamnose (1, Sigma) as shown in Scheme 1. α-L-Rhamnose was converted to 3,4-diacetyl rhamnal (4) following the procedure of Stache and coworkers.[66] Direct acetylation with acetic anhydride in dry pyridine for 12 hours afforded tetra-acetyl L-rhamnose (2) in 80% yield. ^{1}H NMR integration was correct for the presence of twelve acetate protons. Selective bromination of 2 at C1' was achieved with 31% of HBr in glacial acetic acid containing a small amount of acetic anhydride to trap any traces of water present. (The water would hydrolyze bromide 3 to triacetyl L-rhamnose.) Stirring for 2 hours at room temperature was found to be sufficient. Bromide 3 was not isolated nor characterized due to its extreme instability. After the two hours of stirring, the reaction mixture of 3 was added dropwise to a mixture of zinc dust and $CuSO_4$ in an acetate buffer (pH 8-9). (The Cu^{++} salt serves as a catalyst to activate the zinc powder, while the zinc actually removes the bromide ion.) The mixture was stirred for three hours to produce an 80% yield of 3,4-diacetyl rhamnal (4). The two vinyl protons appeared clearly in ^{1}H NMR [6.43 (1H, d, $J_{1,2}$ = 10 Hz, C1-H), 5.1 (1H, dd, $J_{1,2}$ = 10 Hz, $J_{2,3}$ = 1-2 Hz, C2-H)].

Finally, the 3,4-diacetyl rhamnal (4) was reacted with digitoxigenin (5). The digitoxigenin was obtained via the hydrolysis of


Ac2O
Pyridine
HBr, HOAc
Ac2O
Zn, HOAc
NaAc, CuSO4
POCl3, THF
CH2Cl2
Digit.(5)
1
2(92%)
3
4(86% from 2)
Pd/CaCO3
EtOH
Ba(OCH3)2
MeOH
8,α (51%),
11,β (53%)
R = H
6,α(53%), 9,β(28%)
7,α(82%), 10,β(81%)
R = Ac

Scheme 1

digitoxin (USP grade, Sandoz, Basle, Switzerland) in typically 76% yield. The hydrolysis conditions (a 1:1 mixture of 0.08 N H_2SO_4 and methanol, 2 hours steam bath and continuous neutralization of H^+ during the work up) minimized formation of digitoxigenin 14-ene, but always formed in small amounts nevertheless. Continuous monitoring by TLC was found to be essential.

Glycals have been utilized as versatile starting materials for the synthesis of carbohydrate derivatives and, in recent years, chiral natural products. Glycals generally react with a nucleophile in the presence of Lewis acids or transition metals. However, a glycal having a suitable leaving group at the allylic position such as acetyl, para toluensulfonyl or paramethanosulfonyl reacts with nucleophiles to give 2',3' unsaturated glycosides.[67]

Glycosidation of glycal 4 and digitoxigenin (5) was performed following the method reported by Stache and coworkers.[68] A nucleopilic attack at C1' of glycal 4 results in the formation of carbanion at C2', which then forms a double bond by eliminating the acetyl group at C3'. The glycosidation was complete in 18 hours at room temperature and the products separated by flash chromatography (eluted with methylene dichloride and diethyl ether (8:1)) to yield 53% of α-anomer 6 and 28% of β-anomer 9. $POCL_3$, thus, affords a very high yield without the formation of 14-enes, common by products and the major problem in conventional glycosidations with cardiac steroids. Other catalysts including $BF_3 \cdot Et_2O$[67,69-71] and Zn triflate (Zn^{2+} trifluoromethanosulfonate) were tried without success.

Trideoxyglucosides 8 and 11 were prepared by subsequent hydrogenation of 6 and 9 in 80% yield each, followed by barium methoxide deacetylation to 8 and 11 (51-53%). The reduction step of the 2',3'-unsaturated bond of 6 and 9 is very crucial. Several hydrogenation catalysts were tried including 5% Pd/C, 5% Rh/C and 5% $Pd/CaCO_3$. It was found that 5% $Pd/CaCO_3$ (5 mg 5% $Pd/CaCO_3$ used for 100 mg glycoside) affords the best selectivity, i.e., no loss of the C20-C22-ene nor hydrogenolysis of allylic acetate. In our laboratory, the C20-C22-ene has been proven to be resistant to 5% Pd/C even after 96 hours of hydrogenation. However, the hydrogenolysis of the allylic acetyl group precludes the usage of 5% Pd/C.

The progress of the hydrogenation was monitored carefully with TLC and NMR (^1H and ^{13}C), which showed the disappearance of peaks at δ 5.60 - 5.80 (^1H) and δ 129.14 and δ 123.70 (^{13}C). The resistance of the C20-C22-ene to the hydrogenation conditions was shown by the NMR and UV spectra of 7 and 10. The NMR spectra showed a singlet at δ 5.85 (C22-H). The UV spectrum was consistant with an intact conjugated system (λmax = 218 nm, log ε = 4.30).

The stereochemical assignment of 8 and 11 was shown unequivocally by ^1H NMR based on the coupling constant of the C1' and C2' protons (Figure 19). In α anomer 8, the dihedral angle of $H^{1'}\text{-}C^{1'}\text{-}C^{2'}\text{-}H^{2'}{}_a$ is almost equal to $H^{1'}C^{1'}\text{-}C^{2'}\text{-}H^{2'}{}_e$ (so $J_{1',2'a} = J_{1',2'e}$). Therefore, the anomeric proton of 8 appears as a broad singlet since $J_{1',2'a} = J_{1',2e'}$ is very small.

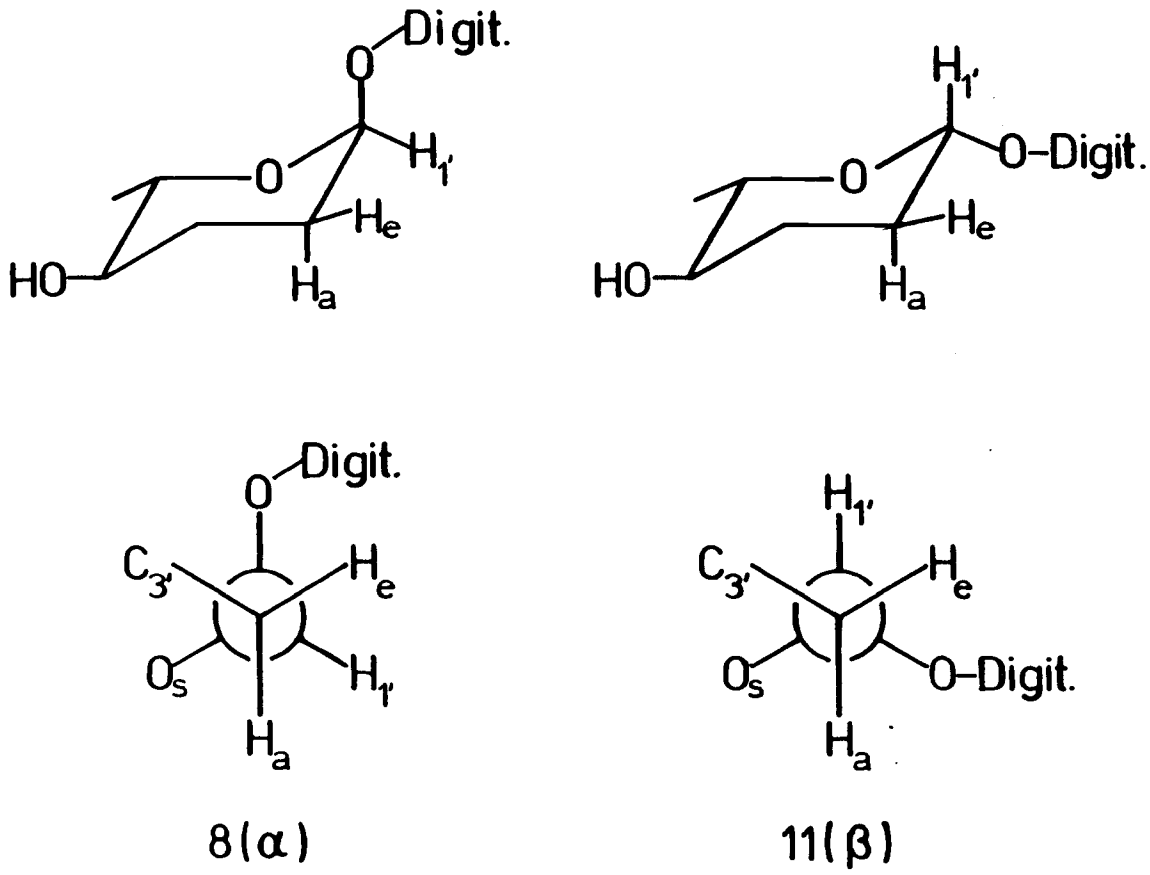
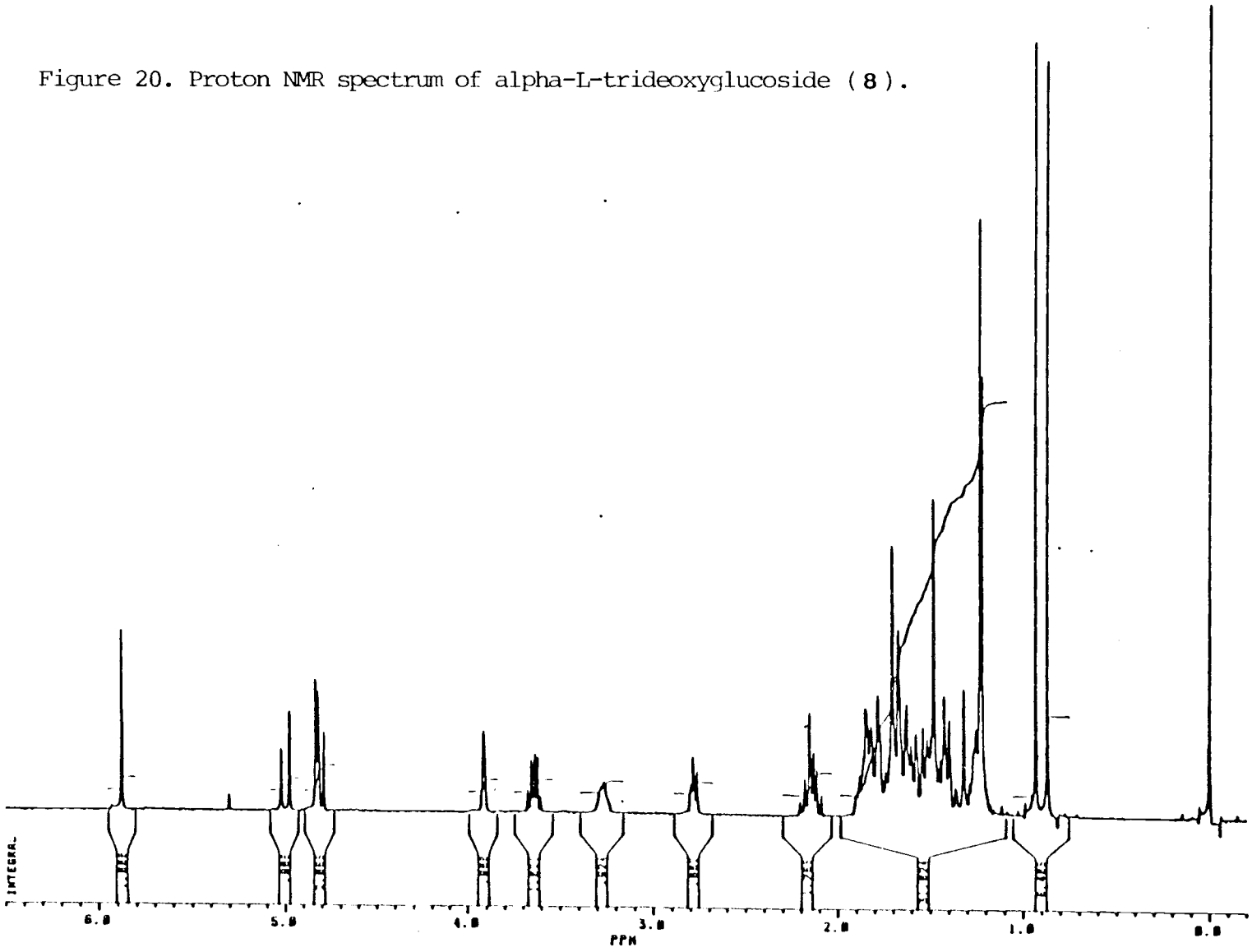


Figure 19. Dihedral angle between C1' and C2' protons of trideoxy-glucosides analogues.

CARBON ATOM NUMBER	8	11	23
1	30.48	31.43	30.15
2	30.26	31.33	27.69
3	72.29	75.82	72.34
4	33.25	33.12	33.15
5	36.44	36.28	30.70
6	26.48	26.43	26.66
7	21.14	21.17	21.19
8	41.72	41.81	41.86
9	35.66	35.68	35.65
10	35.21	35.18	35.21
11	21.33	21.29	21.34
12	39.99	40.04	40.05
13	49.68	49.65	49.63
14	85.56	85.55	85.60
15	33.05	32.05	32.15
16	26.89	26.89	26.89
17	50.94	50.97	50.95
18	15.80	15.79	15.79
19	23.71	23.63	23.76
20	174.69	174.75	174.65
21	73.53	73.55	73.51
22	117.58	117.56	117.63
23	174.86	174.95	174.75
1'	94.08	99.27	93.86
2'	29.83	29.99	30.24
3'	23.77	24.30	24.18
4'	69.60	71.47	69.54
5'	70.69	72.38	70.61
6'	17.93	18.13	17.91

Table 3. ^{13}C NMR data of α-L-trideoxyglucoside (**8**), β-L-trideoxyglucoside (**11**), and β-D-trideoxyglucoside (**23**).

Figure 20. Proton NMR spectrum of alpha-L-trideoxyglucoside (**8**).



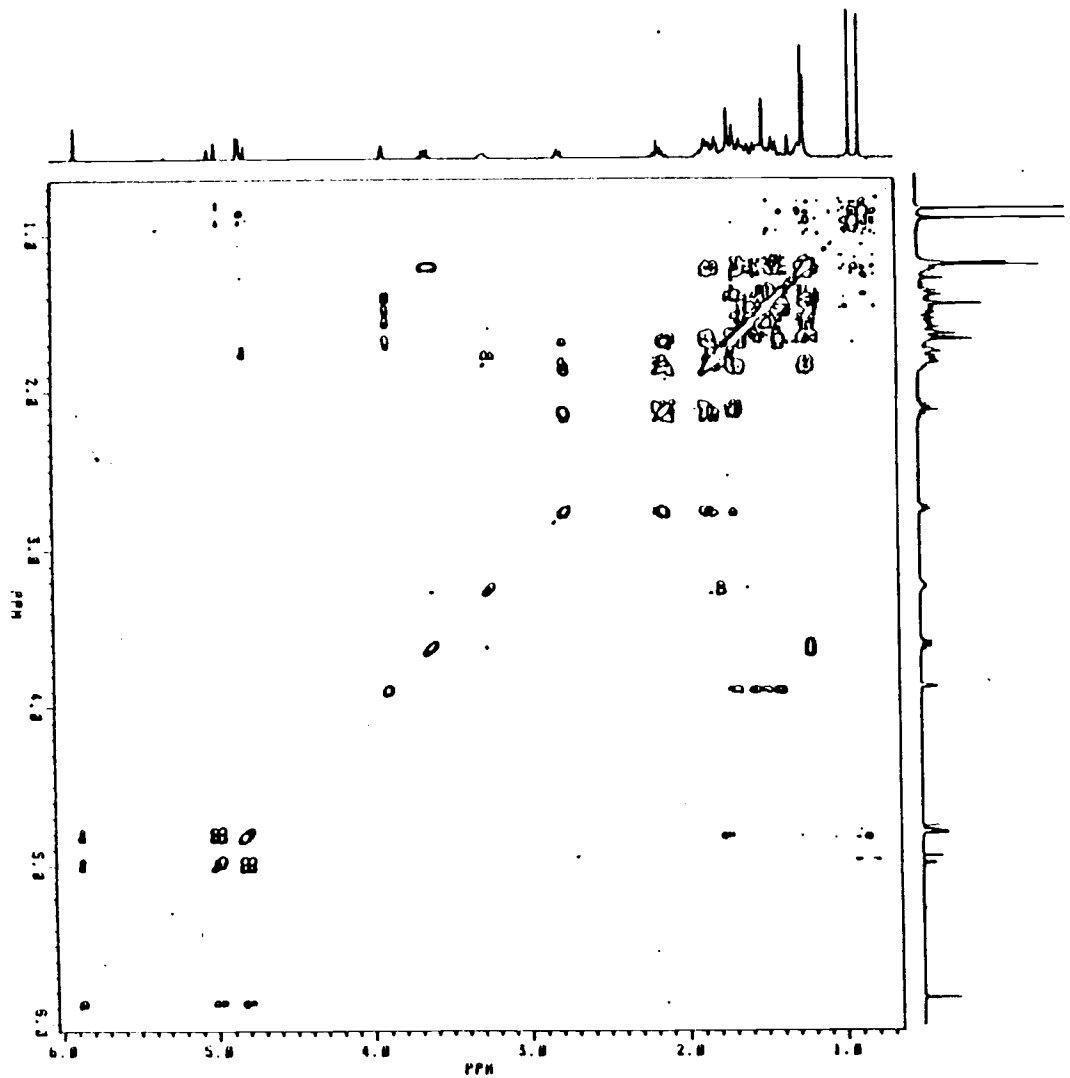


Figure 21. Proton-proton chemical-shift correlation spectrum of alpha-L-trideoxyglucoside (**8**).

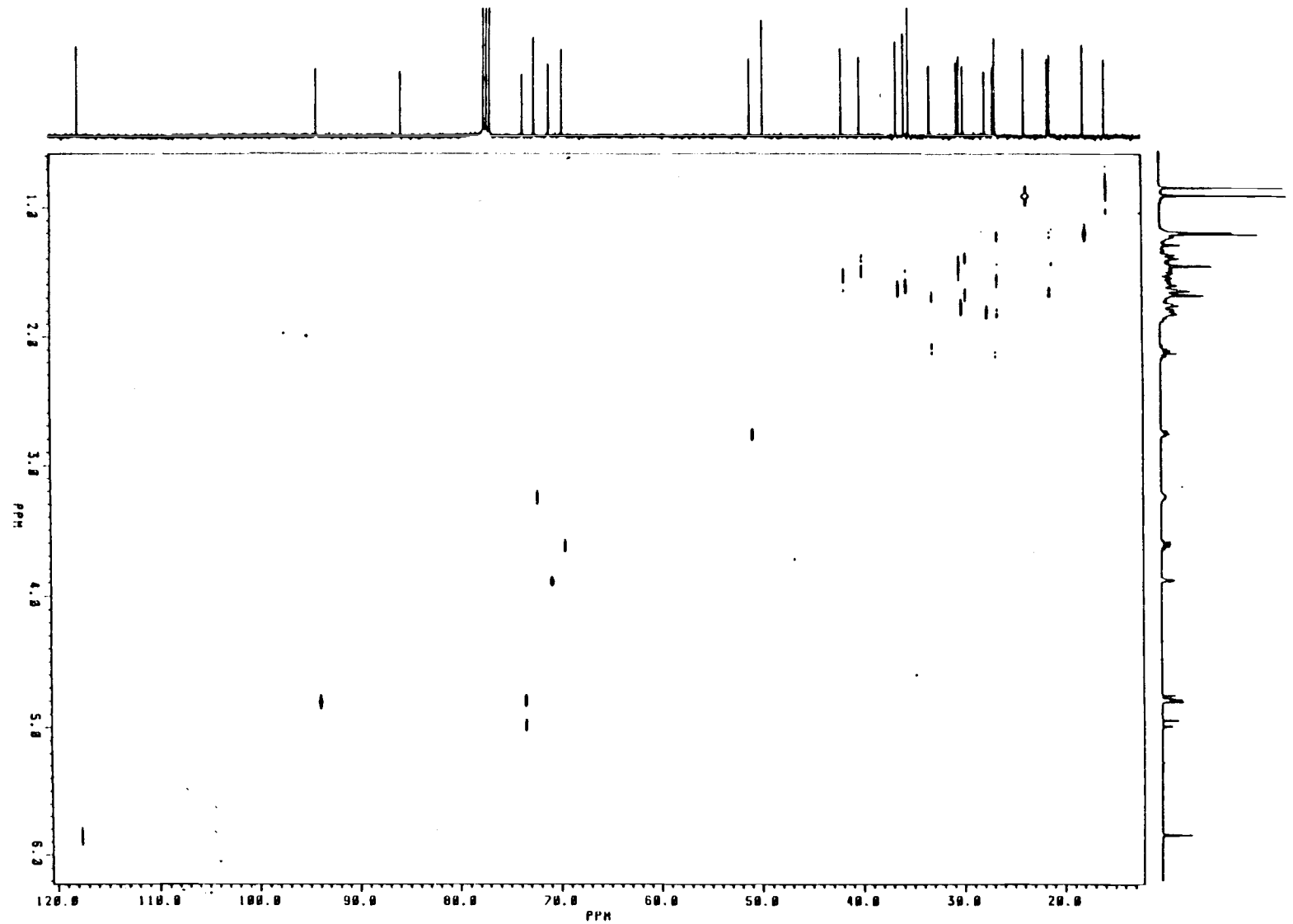
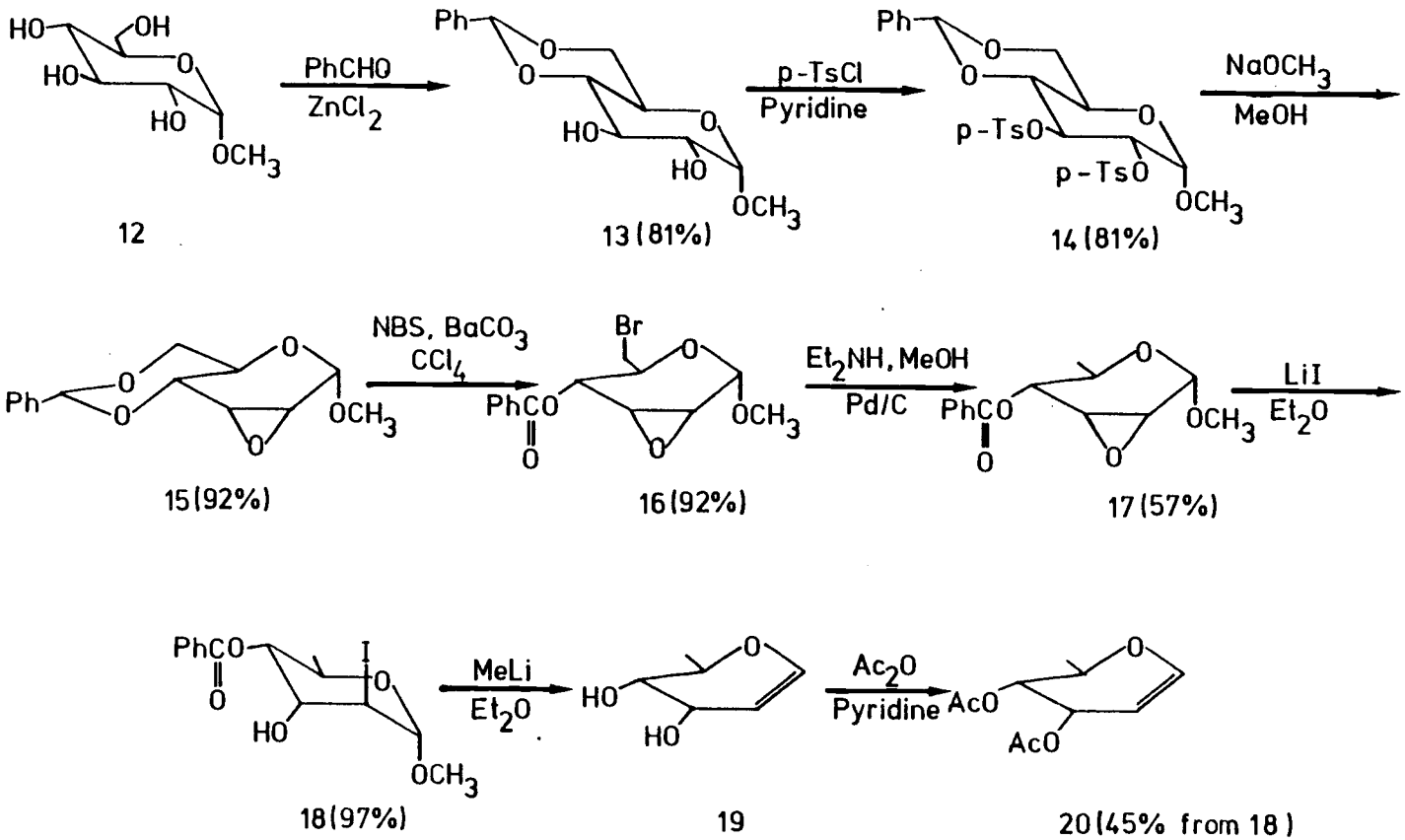


Figure 22. Proton-carbon chemical-shift correlation spectrum of alpha-L-trideoxy-glucoside (**8**).

The respective dihedral angles in β anomer 11, however, are very different. $H^{1'}-C^{1'}-C^{2'}-H^{2'}{}_a$ is almost 180° whereas $H^{1'}-C^{1'}-C^{2'}-H^{2'}{}_e$ is about 70° (estimated using a Dreiding model). Therefore, the anomeric proton of β anomer shows a doublet to doublet at δ 4.48 with $J_{1,2a}$ = 2Hz and $J_{1,2e}$ = 9.2Hz. The "Klyne rule"[72,73] (that β anomers of D-sugar cardiac glycosides have more negative molecular rotation $[M]_D$ than that of the corresponding α anomers) cannot be applied because these sugar analogues have lost many assymetric centers. α Anomer 8 has an $[M]_D$ value of -23.9 whereas β anomer 11 has a value of +47.4. These signs also appear for the corresponding trideoxy-D-glucoside 24 (Scheme 3). The β-D-anomer 24 has an $[M]_D$ value of +70.14. Thus, the determination of the anomeric structure of 8, 11, and 24 were based primarily on the coupling constant between the anomeric proton and equatorial and axial protons at C2' position.

b. Digitoxigenin β-D-trideoxyglucoside (23) (Scheme 2 and 3)

Trideoxyglucoside 23 was synthesized from methyl α-D-glucose and digitoxigenin (5) as shown in Scheme 2 and 3. Reacting methyl α-D-glucoside with benzaldehyde at the presence of $ZnCl_2$ room temperature for 48 hours afforded exclusively 4,6-benzylidene derivative 13, (methyl 4,6-O-benzylidene-α-D-glucopyranoside). The proximity of the 4 and 6 hydroxy groups permit the formation of benzylidene bond. In subsequent reaction with para-toluene sulfonyl chloride in dry pyridine, then sodium methoxide in dry methanol, 13 was converted to 15 in a high yield.


PhCHO
ZnCl2
p-TsCl
Pyridine
NaOCH3
MeOH
NBS, BaCO3
CCl4
Et2NH, MeOH
Pd/C
LiI
Et2O
MeLi
Et2O
Ac2O
Pyridine
12
13 (81%)
14 (81%)
15 (92%)
16 (92%)
17 (57%)
18 (97%)
19
20 (45% from 18)

Scheme 2

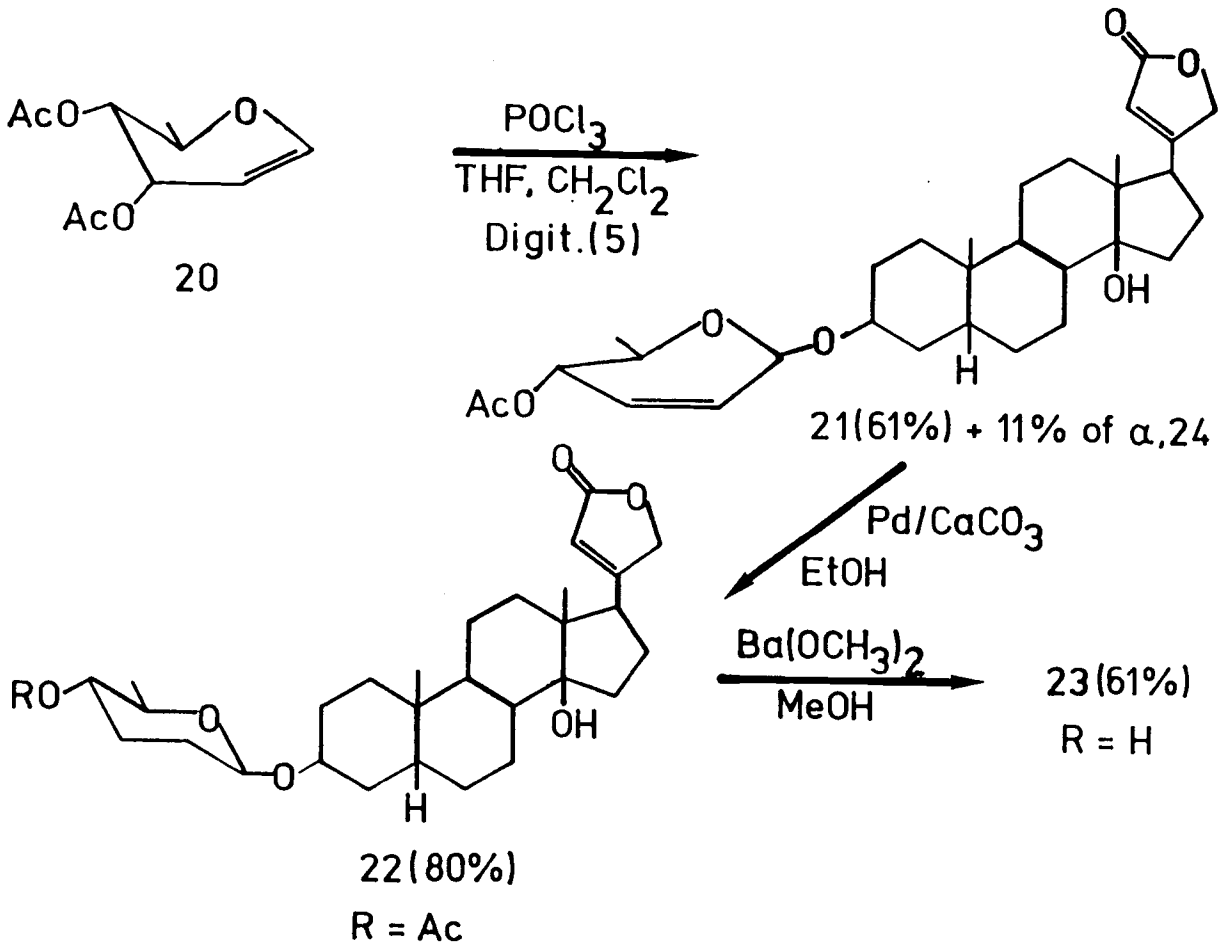
Selective bromination at C6 of 15 was achieved through a radical reaction following a method reported by Hanessian and coworkers.[74] A solution of 15 (Scheme 1), N-bromosuccinimide and barium carbonate in dry CCl_4 was refluxed for 2 hours. The formation of bromine radical can be detected easily with the appearance of red color within fifteen minutes of refluxing which then vanished after one hour. Compound 16 was obtained from 15 in 92.6% yield. Hanessian reported that 16 can be easily reduced to 17 by simple hydrogenation with 20% Pd/C and $BaCO_3$. The reaction was completed in six hours, affording an 82% yield of 17. $BaCO_3$ is used to neutralize the hydrobromic acid produced in the reaction, to protect the acid sensitive 1-O-methyl group. However, our attempts to use the Hanessian method[74] with several different stoichiometries were unsuccessful. Even when the hydrogenation was continued for one week, it afforded only a 15-20% yield. However, we found out that 16 could be easily converted to 17 by replacing the $BaCO_3$ with diethyl amine. The hydrogenation was completed in 5 hrs with 76% yield of 17 after purification by flash chromatography. The ^1H NMR spectrum was consistant with the assigned structure. δ = 1.22 (d, $J_{5,6}$ Hz, 3H, C6-CH_3)

Compound 17 was converted to methyl 4-O-benzoyl-2,6-dideoxy-2-iodo-α-D-altropyranoside (18) (Scheme 2) following a lithium iodide procedure reported by Feast, Overend and William.[75] Due to a steric effect, the iodine ion will selectively attack C2 and thus afford an almost quantitative yield of 18. Utilization of lithium iodide has an additional advantage over sodium iodide.[67,75] Lithium iodide in

contrast to sodium iodide, is capable of reacting with an epoxide under basic conditions to yield the lithium alkoxide of the iodohydrin. With sodium iodide, acid is required to neutralize the reaction condition. A nucleophilic attack by methyl lithium on the iodine atom of 18 leads to the elimination of the 1-O-methyl group, and formation of 1,2,6-trideoxy-D-ribo-1-hexenopyranose (19). In a similar reaction, Lemieux et al[76] reported of using a stoichiometry of 32 mmol of methyl lithium to 1 mmol of sugar. The reaction was complete after refluxing for eighteen hours. We tried the reaction with only a 5 to 1 stoichiometry, and found that the reaction was complete in two hours at room temperature or thirty minutes when refluxed.

Because of the basic conditions required for the reaction, the 4-O-benzoyl protecting group was simultaneously hydrolized. An attempt to isolate diol 19 failed due to the water solubility of 19. As a result, the reaction mixture of 19 was just evaporated to dryness and the residue immediately acetylated with an excess amount of acetyl anhydride in dry pyridine at 0-5°C for 12 hours to afford diacetate 20 in 45% yield from 18.

The glycosidation reaction of 20 and digitoxigenin (5) (Scheme 3), subsequent reduction of C2'-C3' double bond, and hydrolysis of the 4'-O-acetyl protective group to form glycoside 23 were carried out in the same manner as the corresponding L-sugar analogues 8 and 11 in Scheme 1.


AcO
AcO
O
20
POCl3
THF, CH2Cl2
Digit.(5)
O
O
O
OH
H
AcO
21(61%) + 11% of α,24
Pd/CaCO3
EtOH
Ba(OCH3)2
MeOH
23(61%)
R = H
RO
O
O
OH
H
22(80%)
R = Ac

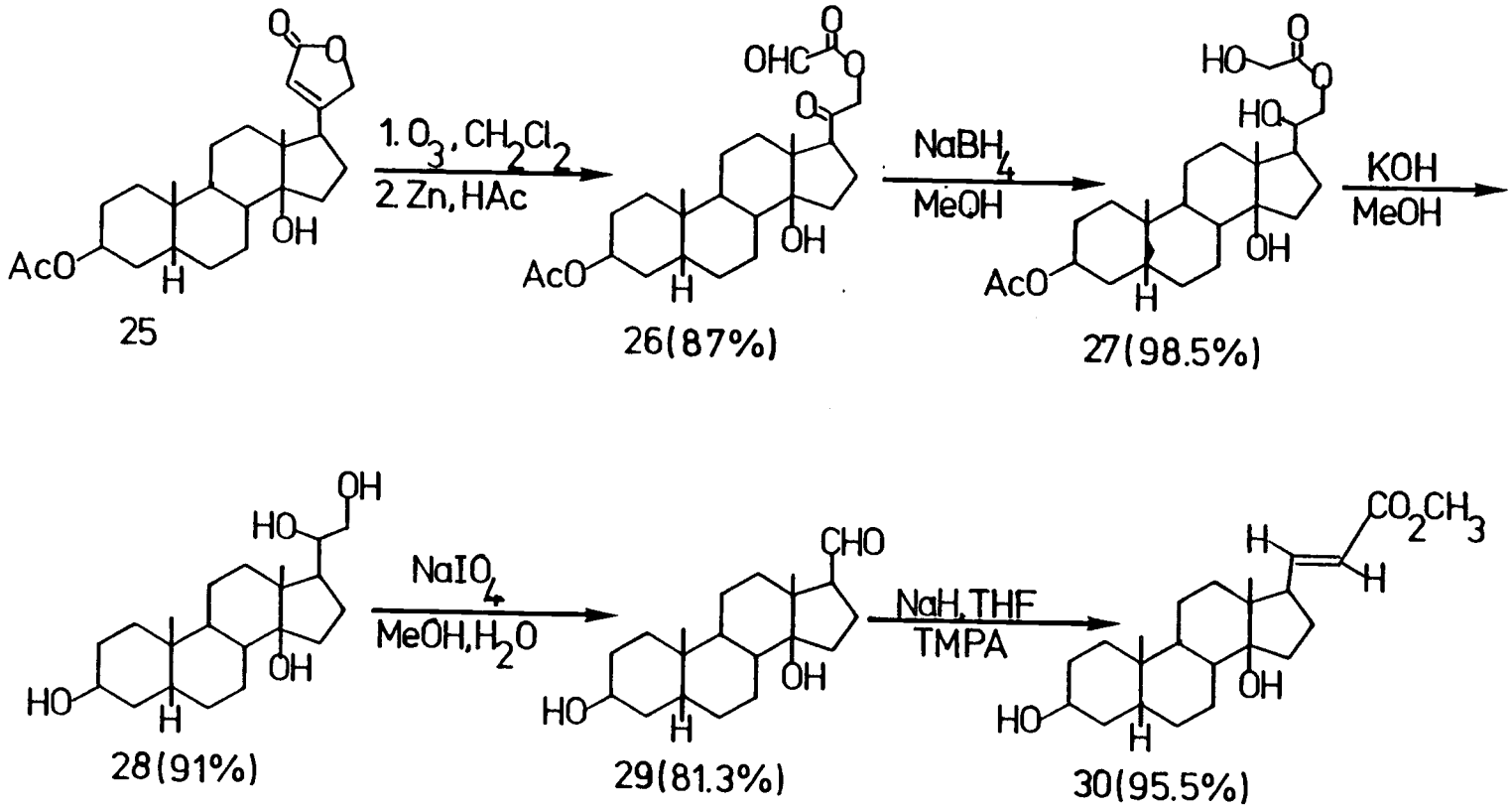
Scheme 3

2. SYNTHESIS OF 6-HYDROXYDIGITOXOSIDES

a. β-D- and α-D-Acyclic methyl esters 37 and 35. (Scheme 4-6)

Glycosides 35 and 37 were synthesized from methyl α-D-glucoside (12) and digitoxigenin (5).

The conversion of digitoxigenin (5) to the C17-β-aldehyde (39) (Scheme 4) was conducted using a modification of the procedure reported by Fullerton[56], Thomas[77], and coworkers. It was necessary to protect the C3 hydroxyl group of the digitoxigenin to avoid oxidation of the C3-hydroxyl during ozonolysis. A C3 acetyl group was used because it is resistant to ozonolysis, and it would be hydrolized automatically during the hydrolysis of 27 and 28 (Scheme 4). The C3-hydroxyl group, however, is resistant to the periodate oxidation in the conversion of 28 to 29. Ozonolysis was allowed to continue for five hours to assure a complete oxidation of the C20-C22 lactone ring double bond. (It is possible to reduce the reaction time by increasing the flow rate of the ozone. For reasons of safety, this was not done, since it would require a higher oxygen pressure and electric voltage). The saturation of the reaction mixture by ozone was checked by the blue purple color instead of the 5% potassium iodide solution in concentrated hydrochloric acid (a more sensitive method). Further, the ozonolysis was done in a highly concentrated solution (15 g of digitoxigenin acetate 25 in 100 ml of dry methylene dichloride), due to the size limitation of the available ozonolysis apparatus. The ozone formed was reduced with zinc dust/acetic acid, with subsequent steps shown in Scheme 4.



25

26(87%)

27(98.5%)

28(91%)

29(81.3%)

30(95.5%)

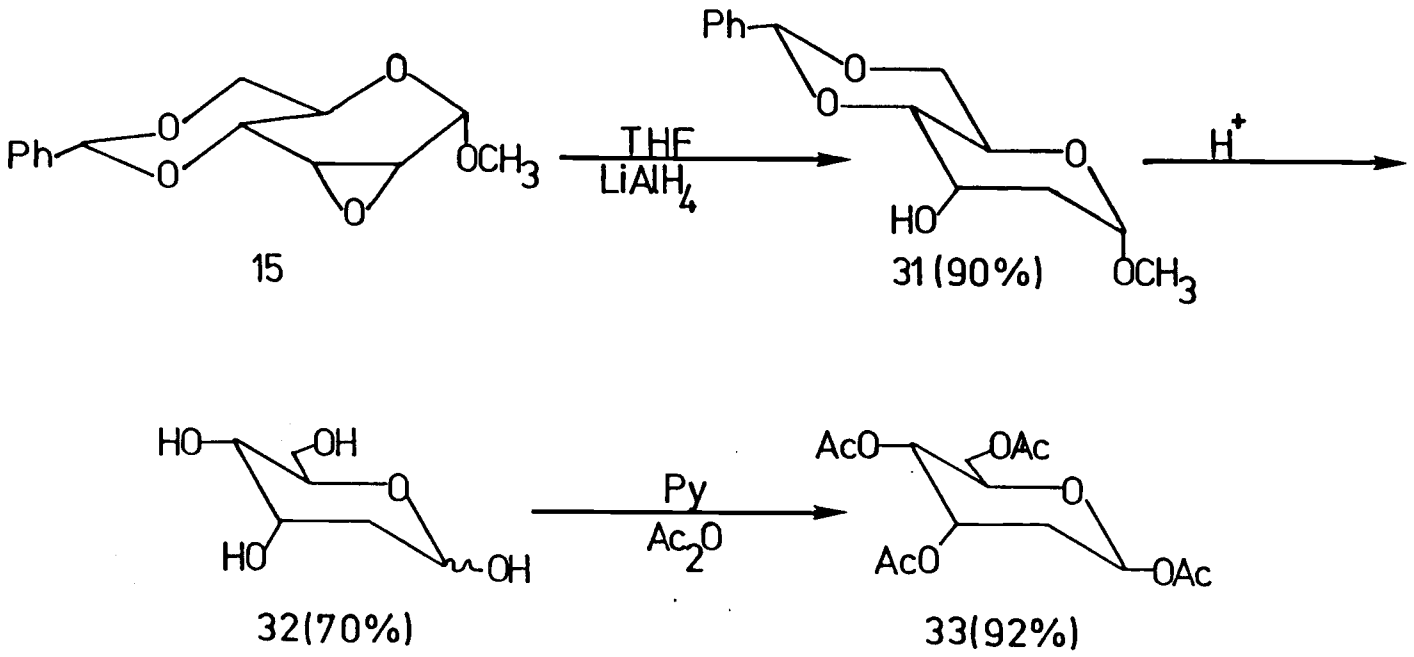
TMPA=$(CH_3O)_2P(O)CH_2CO_2CH_3$

Scheme 4

During periodate oxidation of diol 28 to 17-β-aldehyde 29, precaution must be taken to avoid the formation of the corresponding 17-α-aldehyde analogue. The oxidation was therefore monitored very carefully with TLC, and was found to be generally over within thirty minutes. The two isomers (17-α-CHO and 17-β-CHO) can be differentiated easily by TLC and by their melting points which are 170-174°C for 17-α-CHO and 75-80°C for the 17-β-CHO.[77] Furthermore, the chemical shift of the aldehyde proton is also different for those two products (δ = 9.8 for 17-α-CHO and δ-9.7 for 17-β-CHO).[77] In IR spectra, the C=O peak is shifted from 1725 (5) to 1705 (29). IR spectra also shows a small peak below 2800 cm^{-1} due to the absorption of the stretch C-H bond of the aldehyde group.

Aldehyde 29 was subjected to a phosphonate modified Wittig reaction with trimethyl phosphonoacetate. This method selectively affords the trans product 30 in a very good yield (95.5%). The assigned trans configuration of 30 was also established by NMR. Trans olefins usually have a coupling constant of 12-18 Hz, whereas cis olefins display 6-12 Hz. The coupling constant of the C20-H and C22-H of 30 was 15 Hz. It should be added that very similar reactions have also been used in our laboratory, always selectively producing the desired trans isomers.

The needed tetra-O-acetyl sugar 33 (Scheme 5) was synthesized from the benzylidene-protected epoxy sugar 15 as shown previously in Scheme 2.[62] Reacting 15 with lithium aluminium hydride in dry tetrahydrofuran selectively produced methyl 4,6-O-benzylidene-2-deoxy-α-D-ribo-hexopyranose (31) in excellent yield. The presence of the benzylidene



15

31 (90%)

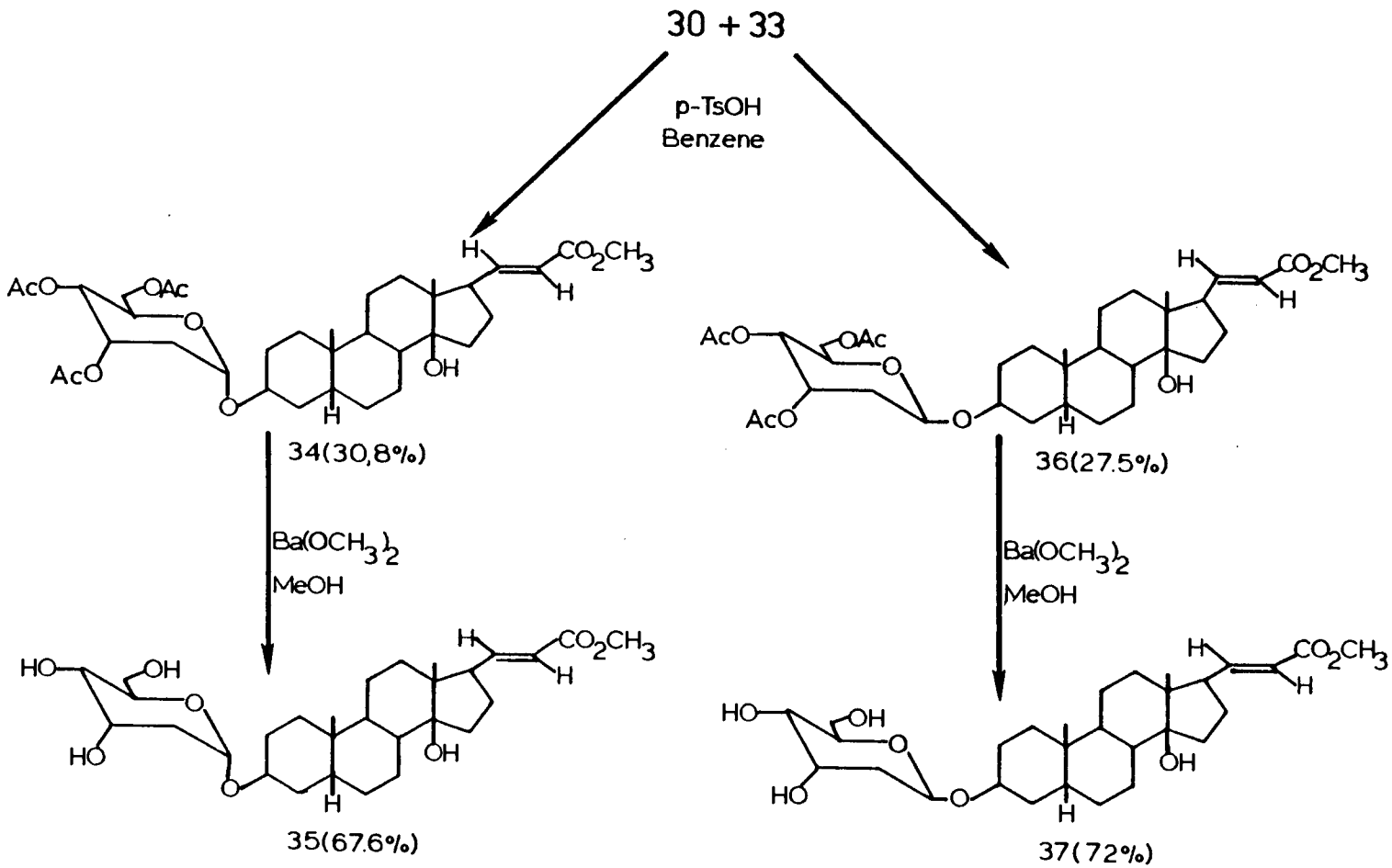
32 (70%)

33 (92%)

Scheme 5

protecting group at C4 and C6 positions creates steric hindrance and prevents hydride attack at C3. Thus, attack only occurs at C2 to afford 31 exclusively. The presence of two protons at C2 was confirmed by NMR showing multiplet at δ = 2.2 and δ = 2.0. Further, the IR and NMR spectra were identical with the unpublished spectra of 31 previously synthesized by Kihara and coworkers.[63] The benzylidene and methyl groups were removed by refluxing 31 with 0.1 N H_2SO_4 under N_2 for four hours. The nitrogen atmosphere was used to minimize oxidation of the tetraol product 32, found to be quite sensitive to oxygen at refluxing temperature (90°C). Under N_2, the reaction afforded a 70% yield, but without N_2 only a 48% yield of pure 32 would be obtained. The tetraol 32 was then acetylated in a usual manner to afford 92% of tetraacetyl sugar 33.

The glycosidation of 30 and 33 was conducted using the method reported by Boivin and coworkers.[78] An equimolar amount of paratoluensulfonic acid with 30 was used as a catalyst, and the reaction afforded both α anomer 34 and β anomer 36 in a 1 to 1 ratio (total yield 56%). The assignment of the stereochemistry of the products was based on the ^1H and ^{13}C NMR. The optical rotation $[M]_D$ values were also consistent with the "Klyne rule"[73] which had been proven to be a powerful tool to determine the stereochemistry of this series of products.[62] The hydrolysis of the acetyl protective groups of 34 and 36 was conducted with barium methoxide in dry methanol and it afforded 35 and 37 in about 65% yield.


30 + 33
p-TsOH
Benzene
34(30,8%)
36(27.5%)
Ba(OCH3)2
MeOH
Ba(OCH3)2
MeOH
35(67.6%)
37(72%)

Scheme 6

The unique characteristic of this glycosidation method is that for still unknown reasons, the reaction can proceed only with benzene as reaction medium. Several solvent systems were tried including methylene dichloride, tetrahydrofuran and mixture of both methylene dichloride and tetrahydrofuran, but without success. Surprisingly, the rate of the reaction seems to also depend on the type of genin. Reaction with unsaturated methyl ester 30 was complete in three hours, whereas digitoxigenin (5) required 48 hours. We also wanted to synthesize the 6-hydroxydigitoxose analogue of the genins shown in Figure 23, but no reaction could be observed by TLC even after 96 hours of refluxing. There is no obvious explanation for their lack of reactivity.

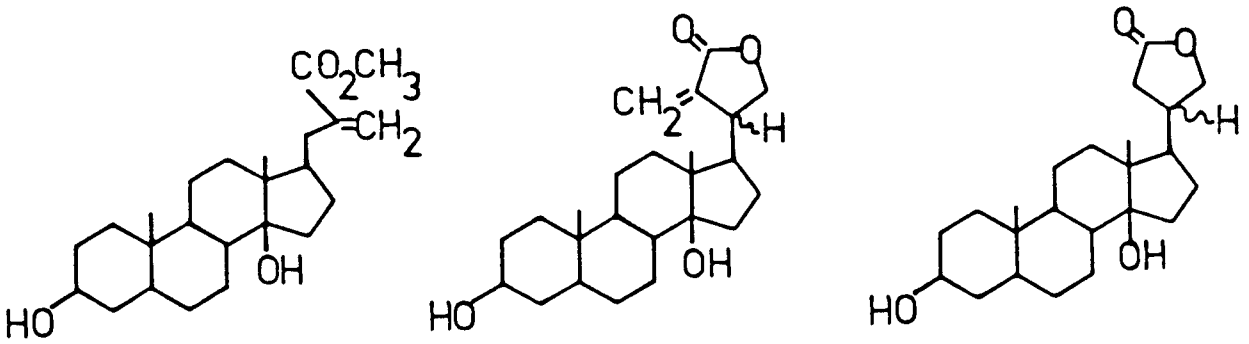
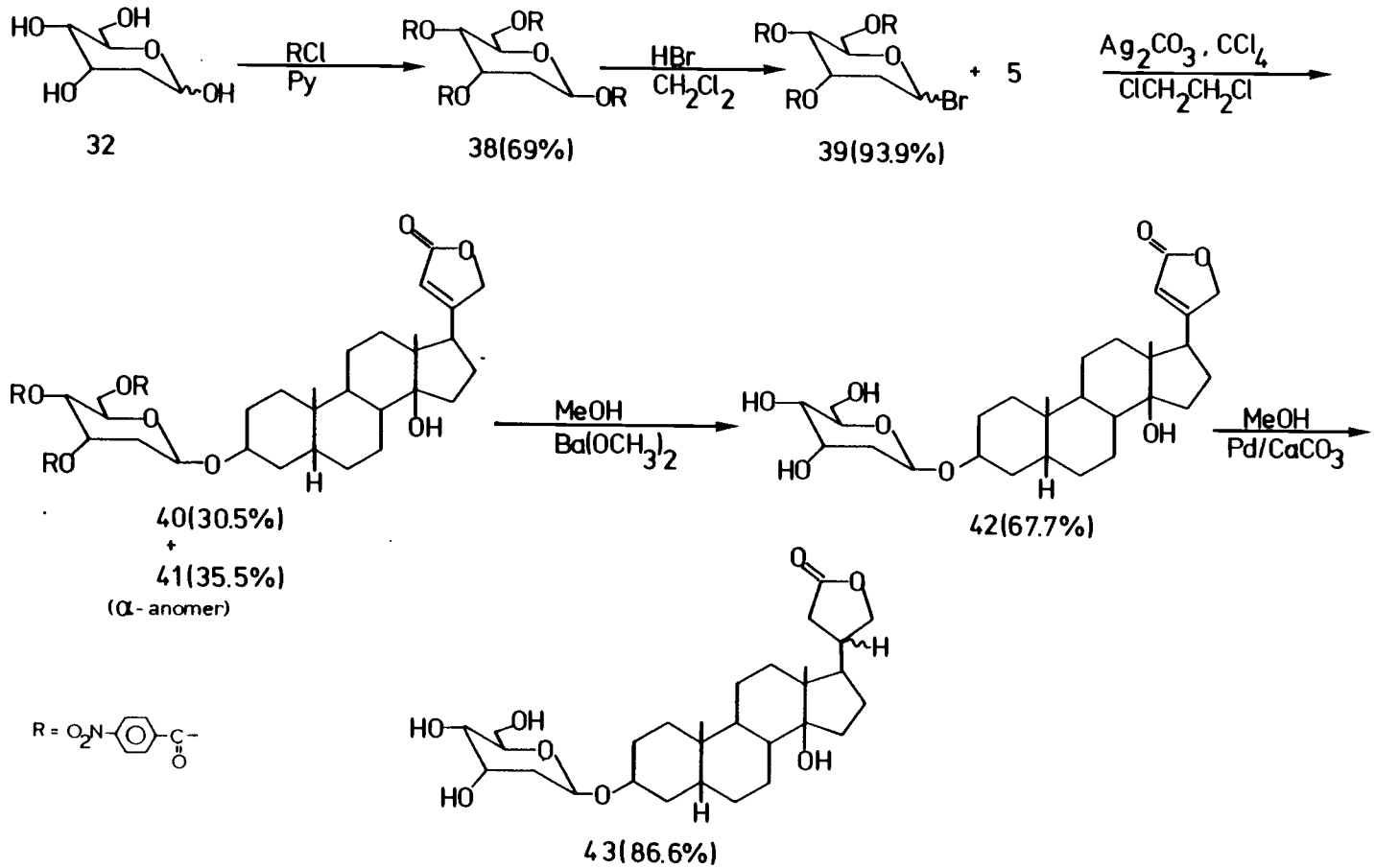


Figure 23. Structure of three genin tried for glycosidation reaction.

b. β-D-Dihydrodigitoxigenin 43 (Scheme 7).

Glycoside 43 was synthesized from 6-OH-digitoxose (32) and digitoxogenin (5) by a modified Koenig Knorr method[70,80] as shown in Scheme 7.


RCl
Py
HBr
CH_2Cl_2
+ 5
Ag_2CO_3, CCl_4
$ClCH_2CH_2Cl$
32
38(69%)
39(93.9%)
MeOH
$Ba(OCH_3)_2$
MeOH
$Pd/CaCO_3$
40(30.5%)
+
41(35.5%)
(α-anomer)
42(67.7%)
43(86.6%)
R = O_2N-C6H4-C(=O)-

Scheme 7

The needed tris-O-(paranitrobenzoyl)-protected sugar halide (39) was synthesized from methyl α-D-glucoside as shown in Schemes 2,5, and 7. Benzene as a reaction medium has been proven to be excellent and widely used for Koenig Knorr glycosidation method. However, it was precluded in this reaction due to the insolubility of this particular sugar halide (39) in benzene. Thus, a mixture of ethylene dichloride and carbon tetrachloride was used instead. The solution of the sugar halide 39 in this solvent mixture was added dropwise to the reacton under constant distillation to azeotropically remove the water produced during the reaction. The reaction was complete in six hours at 80-85°C.

The products were isolated by flash chromatography to yield β-anomer 40 (30.5%) and α-anomer 41 (35.5%). The p-nitrobenzoyl protective groups were hydrolized in barium methoxide condition to afford 43 (67.7%), followed by reduction of the C20-C22 double bond by 5% $Pd/CaCO_3$ to yield 43 (86.6%).

The assignment of the stereochemistry of the products 40 and 41 were performed in the same manner as the corresponding methyl ester analogues 35 and 37. We also tried to employ this glycosidation method to make other 6-hydroxydigitoxose analogues with other genins. This glycosidation method afforded a good yield with digitoxogenin (5) and methyl ester (30). However, for unknown reasons, we found that this method gave only a poor yield (10-15%) with genins shown in Figure 23.

C. BIOLOGICAL RESULTS

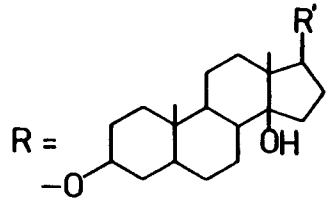
Biological experiments were conducted Dr. Khalil Ahmed, Dr. Arthur H.L. From, and Mr. Michael Barone at the University of Minnesota.

Hog kidney Na^+,K^+-ATPase, purified as described by Ahmed et al,[55] was used to determine the inhibitory activity of the glycosides. The inhibition of the Na^+,K^+-ATPase was measured under equilibrium type I binding condition (i.e., with Mg^{2+}, Na^+, and ATP as the binding ligand). The enzyme was reacted with the glycosides for a period of 2 hours. This results in maximum binding of the drug to the Na^+,K^+-ATPase as evidenced by maximum inhibition after this period of incubation as reported by Fullerton et al.[62] After the two hours incubation, an appropriate amount of KCl was added (to achieve final concentration of 10 mM) to initiate the ATP hydrolysis by Na^+,K^+-ATPase. The hydrolysis was terminated by adding cold trichloroacetic acid (10% w/v). Under these conditions, a linear hydrolysis rate can be measured in the presence or absence of the drugs. The ATPase inhibitor was calculated as the activity in the presence of Mg^{2+}, Na^+ and K^+ minus that in the presence of Mg^{2+} and Na^+. All the drugs were added as ethanolic solution and was restricted to 20 µl per-2 ml reaction. This amount has been proved not to have significant effect on the ATPase activity. The same amount of ethanol was also added deliberately to the control to compensate any effect which might occur. (Further details of the biological assay have been given[55,62]).

I_{50} values (concentration required for 50% inhibition of the Na^+,K^+-ATPase activity) were calculated as described by Fullerton et al.[53] These results are shown in Tables 4 and 5. Each determination was confirmed at least three times and the variation in I_{50} values should not exceed 5% value. The I_{50} vs carbonyl oxygen distance was shown in Figure 24.

COMPOUNDS	I_{50}	RELATIVE POTENCY
Digitoxigenin	1.20×10^{-7}	1.00
Digitoxigenin β-D-trideoxyglucoside (23)	4.47×10^{-8}	2.68
*Digitoxigenin β-D-glucoside	1.05×10^{-8}	11.43
Digitoxigenin β-L-trideoxyglucoside (11)	3.98×10^{-8}	3.02
*Digitoxigenin β-L-glucoside	1.60×10^{-8}	7.50
*Digitoxigenin β-L-rhamnoside	4.79×10^{-9}	25.05
*Digitoxigenin β-L-mannoside	1.78×10^{-8}	6.74
Digitoxigenin α-L-trideoxyglucoside (8)	7.24×10^{-9}	16.58
*Digitoxigenin α-L-glucoside	6.03×10^{-8}	1.99
*Digitoxigenin α-L-rhamnoside	6.76×10^{-9}	17.75
*Digitoxigenin α-L-mannoside	1.05×10^{-7}	1.14

Table 4. Hog kidney Na^+,K^+-ATPase inhibiting activity (I_{50}) of various cardiac glycosides used in this study. *Obtained from previous study by Rathore and Fullerton et al.[65]



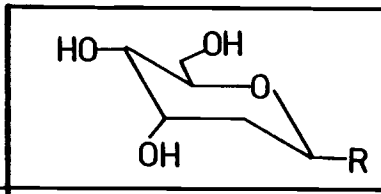
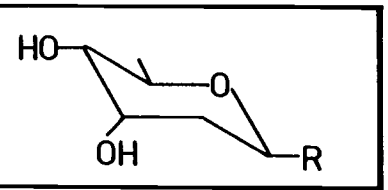
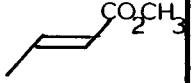
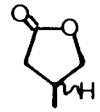
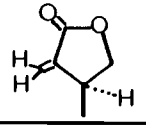
R	R'	Relative carbonyl oxygen separation	 β-D-6-OH-Digitoxide		 β-D-Digitoxide	
			I_{50}	log I_{50}	I_{50}	log I_{50}
Methyl Ester		-1.42	2.14×10^{-8}	-7.67	2.98×10^{-8}	-7.53
Digitoxigenin		0	1.07×10^{-8}	-7.97	7.0×10^{-9}	-8.15
Dihydro-R,S		4.11	1.58×10^{-7}	-6.80	3.75×10^{-7}	-6.43
22-CH_2-Dihydro-R		5.08	1.48×10^{-6}	-5.83	2.80×10^{-6}	-5.55

Table 5. Hog kidney Na^+,K^+-ATPase inhibiting activity (I_{50}) data of various cardiac glycosides used in this study. I_{50} data for genins and β-D-digitoxides were obtained from Kihara and Fullerton et al.[63]

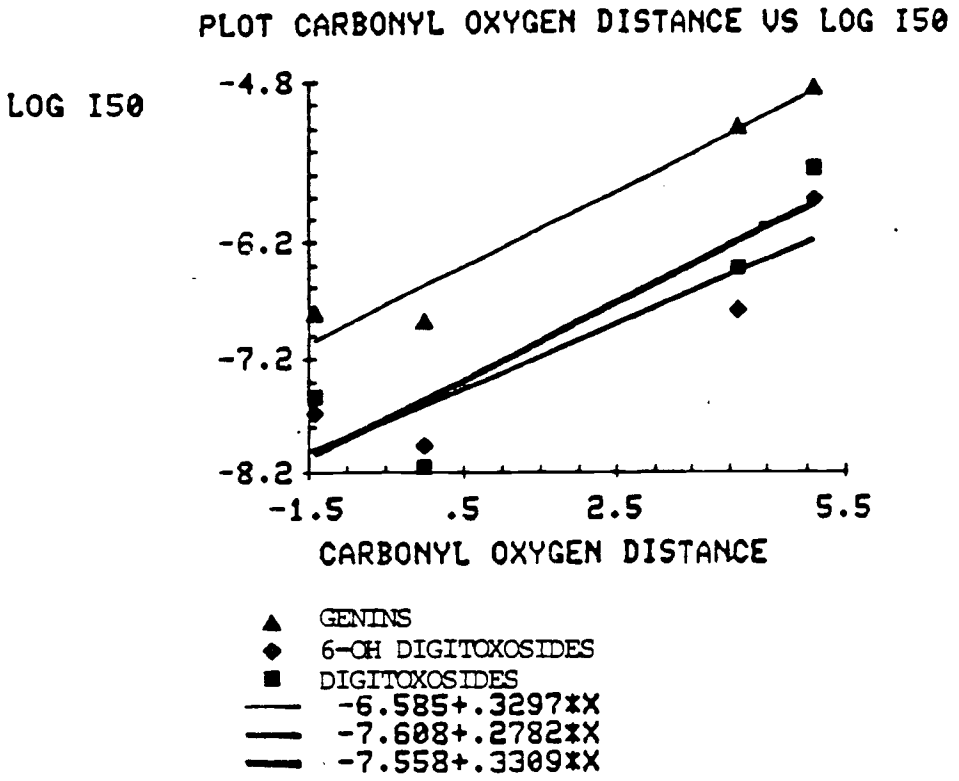


Figure 24. Correlation between relative carbonyl oxygens separations and log I_{50} values of four genins, four 6-OH-digitoxosides, and four digitoxosides. I_{50} values were measured from hog kidney Na^+,K^+-ATPase. The regressions were obtained using PROPHET computer FITLINE program.

D. DISCUSSION

As discussed in Chapter II, Yoda and Yoda[43] have shown that under type I binding conditions, the association rate of cardiac glycosides with Na^+,K^+-ATPase is dependent upon the steroid portion, whereas the dissociation rate is dependent on both the sugar and the steroid. Variation in glycoside structure, as with the glycosides synthesized in these studies, largely reflect changes in dissociation rate constants for these compounds.

The focus of our work was on the roles of the 4'-OH and 6'-OH groups (or 5'-methyl). However, the observed biological activities reflect multiple factors. For example, the trideoxy glycosides have only a 4'-OH, but relative to glucose they have also lost the 2', 3', and 6'-hydroxyls. Relative to rhamnose, they have lost the 2', and 3'-hydroxyls. For clarity, each group will be discussed individually, with the discussion based on several compounds.

a. The C4'-Hydroxyl Binding Site

As shown in Table 4, all three trideoxyglucosides 8, 11 and 23 are more active than their genin, digitoxigenin. The importance of the '4'-OH binding site' is especially supported by the very high activity of α-L-trideoxyglucoside 8, which is as active as the most active cardiac glycoside ever reported. Yet, it is clear that the 4'-OH groups of 8, 11, and 23 are not all equally able to interact with the binding site in their energetically allowed conformations. The β-L-trideoxy-glycoside 11, for example, is 5.5 times less active than 8. (As noted

earlier, the α-L-trideoxyglucoside 8 has previously been reported by Stache and coworkers.[66] In isolated guinea pig heart experiments, they also noted the remarkable activity of this compound.

Correct positioning of 4'-OH groups for optimal binding at the 4'-hydroxyl binding site is also shown by the studies of Kihara, Fullerton and coworkers[63] and by Brown and coworkers.[64] Digitoxigenin β-D-glucoside (with 4'-OH equatorial) was found to be 6 times more active than the β-D-galactoside (with 4'-OH axial) (Table 2). In an unreported study of a variety of β-D-glucosides and β-D-galactosides with varying C17 side groups, Fullerton and coworkers found the same stereochemical dependance to be consistantly observed.

The activity of digitoxigenin α-L-rhamnoside (synthesized in our laboratory by Hargovind Rathore[65]) is consistant with a major role for the 4'-OH binding site. Elimination of the 2'-OH and 3'-OH groups to produce α-L-trideoxyglucoside 8 does not significantly decrease biological activity at all.

b. The C5'-Methyl Binding Site

As reviewed in Chapter II, Kihara, Fullerton and coworkers reported that there was a 1.5 times decrease in activity in the conversion of digitoxigenin β-D-digitoxoside to β-D-6-OH-digitoxoside (Table 2). Similarly, in studies with digitoxigenin α-L-rhamnoside and β-D-glucoside, Yoda[43], Brown, and Thomas[64] concluded that there could be a hydrophobic bond between a C5'-methyl and a specific site on the Na^+,K^+-ATPase. This suggestion was supported by the work of Rathore et al[65] in comparing the activities of digitoxigenin α-L and β-L

rhamnosides with the corresponding α-L and β-L mannosides (Table 4). (Rhamnose is 6'-deoxymannose.) A 4 fold increase in activity was found for the β-L-rhamnosides and 15 fold for the α-L-rhamnoside compared with the mannosides of the same stereochemistry.

However, our more extensive studies with β-D-6'-hydroxy/5'-methyl pairs (Figure 5) show quite different results. There is no consistant pattern in change of Na^+,K^+-ATPase inhibition among the four pairs, and in no case is the change in activity large. In three out of four of the pairs, activity increased slightly. Clearly, the 5'-methyls of β-D-sugars, in their energetically allowed conformations, are unable to make the same close contact with the putative 5'-methyl binding site as the α-L and β-L sugars.

In the 6'-OH L sugars, two factors may contribute to the significantly decreased activity relative to their 5'-methyl analogues. One possibility is that the 5'-methyl binding site is indeed hydrophobic as the earlier investigators have proposed. A steric factor is another possibility. For example, introduction of a C6'-acetyl or C6'-methoxy also decreases biological activity in the D-glucoside studied.[43] Steric interference with the all-important 4'-OH binding is possible, but less likely.

c. Roles of the 2'-OH and 3'-OH

Kihara, Fullerton and coworkers[63] observed a 2.5 fold decrease in activity between digitoxigenin β-D-glucoside and its 2'-deoxy analogue. Similar results were observed by Brown and coworkers.[64] Acetylation of the equatorial C2'-OH (thevetoside, see Figure 15) decreases the

activity by 2.5 fold. The Fullerton group further concluded that the C3'-OH was not nearly as important in binding of glycosides as C4'-OH.

However we observed that elimination of both the C2'-OH and 3'-OH groups of α-L-rhamnose to α-L-trideoxyglucoside (Table 4) does not significantly change activity. With the analogous β-L pair of glycosides (Table 4), however, there is an eight-fold decrease in activity. Thus, our tentative conclusion is that if binding to the 4'-hydroxyl binding site is optimal, 2'-OH and 3'-OH binding is not significant. However, if 4'-hydroxyl binding is less than ideal, 2'-OH and 3'-OH binding can be a help in overall stability of the glycoside/Na^+,K^+-ATPase complex.

Chiu and Thomas[48] recently reported the role of the C3'-OH of gomphoside. It is important to note, however, that two major differences can be observed between gomphoside and 'conventional' cardiac glycosides which may cause major changes in the overall glycoside conformation. Gomphoside has a 5α genin configuration and a two points attachment between genin and sugar portion (see Figure 16). This two points attachment eliminates the freedom of rotation around C3-O-C1'. As we demonstrated earlier, changes in the genin-sugar conformation may dramatically change the role of the sugar hydroxyl groups. Thus, it may be true that in gomphoside, the C3'-OH is the primary binding site. If there is freedom of rotation around C3-O-C1' bond, however, the C4'-OH may take over the role and be the primary binding site. In conjunction with an α-L-configuration, the C4'-OH assumes a more dominant role and is the only hydroxyl group which is responsible for the hydrogen bond formation with the Na^+,K^+-ATPase.

d. C17 side group carbonyl oxygen distance.

Table 5 contains the hog kidney Na^+,K^+-ATPase I_{50} data for the three series of genins, β-D-6-OH-digitoxosides, and β-D-digitoxosides. Plotting these I_{50} data against the relative carbonyl oxygen distance (Figure 24), the same linear relationship found with the genins was observed for each series of glycosides.

Linear regression analysis of the β-D-6-OH-digitoxoside and β-D-digitoxoside data showed a relatively uniform contribution of each sugar:

Genins: $\log (I_{50}) = 0.33\ D - 6.59$

$r^2 = 0.96,\ s = 0.27$

β-D-6-OH-digitoxosides: $\log (I_{50}) = 0.28\ D - 7.61$

$r^2 = 0.83,\ s = 0.49$

β-D-Digitoxosides: $\log (I_{50}) = 0.33\ D - 7.56$

$r^2 = 0.81,\ s = 0.61$

The regression fit lines for the three types of derivatives are relatively parallel with the slopes of 0.3, 0.28, and 0.33, which is consistent with the results of the previous study conducted in our laboratory that the sugars increase genin activity by a constant factor. Figure 24 shows that addition of the β-D-6-OH-digitoxose increases genin activity by an average of 10.5 times, while addition of β-D-digitoxose increases the activity by an average of 9 times. Introduction of hydroxyl group to C5'-methyl group, however, increases the activity of β-D-digitoxosides by an average of only 1.1 times, which is well within the experimental error. This fit line (regression) study once again supports our observations that in D-glycosides, C5'-methyl group does

not contribute anything to the Na^+,K^+-ATPase inhibiting activiyt of the cardiac glycosides.

e. Conclusion

As our laboratory has shown in previous studies[61], cardiac glycoside sugars have limited rotation about the C3-O and O-C1' bonds. Thus, it is not surprising that we have now observed a major stereochemical dependance on the effect of the 4'-OH, 3'-OH, and 2'-OH groups. In short, in glycosides in their energetically possible conformations, a particular hydroxyl may be able to bind well; but in other glycosides, the best conformations leave it at a distance not optimal for best binding. In D-glycosides, the enzyme's hydrogen bonding site must be somewhere between the C3'-OH and C4'-OH, thus C3'-OH may contribute significantly to the Na^+,K^+-ATPase inhibiting activity of the D-glycosides. The trideoxyglucoside study also demonstrates the importance of the α-L configuration. In α-L-glycosides, with the absence of the C6'-OH group ie: in α-L-rhamnoside, the C4'-OH plays a dominant role by being the only hydroxyl group to form hydrogen bond with the enzyme. Thus, eliminations of the C2'-OH and C3'-OH will not result in a significant change in biological activity. Further conformational and computer graphics studies are needed, but they must await x-ray crystallographic data (whose atomic positions are the basis of our molecular mechanics calculations).

The β-D-6-OH-digitoxoside study has lead to a remarkable discovery. Contrary to previous suggestions, there is no significant role of the C5'-methyl group in D-glycosides. C5'-Methyl group may play a

significant role in L-glycosides, however, further study is needed. This study also once again supports the work of Fullerton et al[61] that the sugar portion increases the Na^+,K^+-ATPase inhibiting activity of the genin by a constant factor and the biological activity of the cardiac glycosides is also a linear function of the relative carbonyl oxygen distance to that of digitoxigenin.

PART II: HIGH PERFORMANCE LIQUID CHROMATOGRAPHY SEPARATION OF CARDIAC GLYCOSIDE STEREOISOMERS

CHAPTER ONE

A. INTRODUCTION

Chromatography is a universal technique as it is equally applicable in every field related to chemical analysis. Chromatography in its various forms is also a very versatile technique. It can be carried out in a column or on a plate, and the sample may equally be a solid, a liquid, or a gas. At the present time, high performance liquid chromatography (H.P.L.C.) using a chemically bonded stationary phase is probably the most popular chromatographic technique. This is due to its high efficiency, and wide range of application either in analytical or preparative scale.

Lack of appropriate chromatography systems to quantitatively separate α and β-glycoside mixtures has been a persistent problem in our laboratories. Glycosidation reactions have typically produced mixtures of α- and β-anomers which could be separated by thin layer chromatography, albeit not preparatively. Typically, three flash chromatographies of benzyl or acetyl protected glycoside mixtures have been needed to achieve full preparative separation. Free (ie unprotected) glycoside mixtures have generally resisted all attempts even at non-preparative tlc separation.

The speed and high resolution of high performance liquid chromatography were therefore quite attractive. It was our goal to determine if HPLC conditions could be found to quantitatively separate α- and β-mixtures of unprotected glycosides. If base line separation could be achieved, we would scale up to preparative conditions using larger columns. A quantitative separation would be a very useful tool

in monitoring the pruification of glycosides, and would significantly improve our purification efficiency. A preparative separation technique would replace multiple flash chromatographies as our standard purification technique.

HPLC separation of cardiac glycoside mixtures have been reported, but not the separation of stereo-mixtures. For example, Yoshino et al[81] reported the separation of digoxin, β-methyldigoxin and three dimethyldigoxin analogues using a MicroPak NH_2 column (25 cm x 2 mm) with a mobile phase of 25% ethanol in isooctane at a flow rate of 40 ml/h. Many other papers showing separation of structurally different cardiac glycosides using either normal (NH_2, CN) or reverse phase column (C_{18}) have also been reported.[81-84] Typically used solvent systems include water-methanol, acetonitrile-water, methanol in methylene chloride saturated with water, methanol-water-acetonitrile, ethanol-isooctane.

B. HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

High performance liquid chromatography, first developed in 1967, has become a mainstay in the separation of organic small molecules, and even peptides.[84] The versitility of the method is largely attributable to the wide variety of stationary phases now available (including normal and reverse phase packing materials, ion exchange resins, and gel filtration polymers). The pH and ionic strength of solvents has been varied to great chromatographic advantage. Modern instruments also permit the gradual and programmable mixing of two or more solvents before they enter the stationary phase. The progress in HPLC applications and techniques has been truly remarkable.

Although a variety of HPLC techniques exist (including adsorption, ion exchange and exclusion), only the adsorption methods are applicable to steroid natural products which lack a charged functional group. Four intermolecular forces are responsible for the resolution in the adsorption chromatography:

1. Dispersion (London) forces;
2. dipole (orientation, induction) forces;
3. hydrogen bonds;
4. weak covalent bonding (i.e., acid-base interaction and complex formation).

The relationship between resolution and adsorption process is best described in terms of adsorption isotherm, plot of the equilibrium concentration of adsorbed solute, (X)a, versus the concentration of nonsorbed solute, (X)n, for solute X, at a given temperature. There are three possible isotherms for small sample size: linear, convex, and concave (Figure 25).

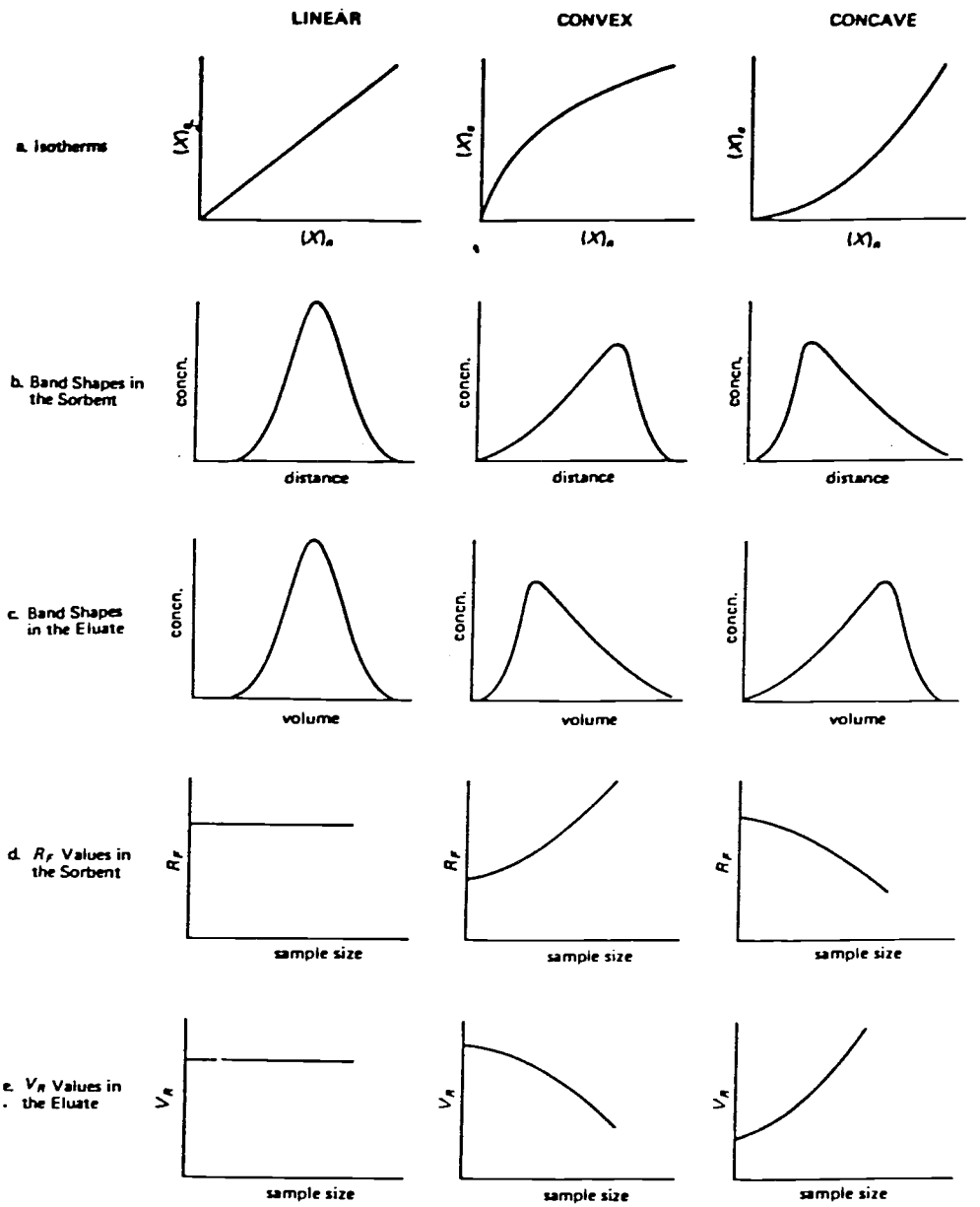


Figure 25. Shapes of isotherm and some of their chromatographic consequences (from reference 85).

Every isotherm becomes linear at a sufficiently low solute concentration, while at a high solute concentration, the adsorption isotherm is almost invariably convex. Band shape, either in the sorbent or in the eluate, as well as variation in RF values and retention volumes, V_R, are characteristically dependent upon the isotherm' shape (Figure 25). When the isotherm is linear, the slope of the isotherm is equal to the distribution coefficient, K, where $K = {}^{(X)a}/_{(X)n}$ (in ml of solvent/g of sorbent). K values define the migration rate of a sample band through a column, and is dependent upon the type of adsorbent, eluent, molecular structure of the sample, and temperature.

The role of the adsorbent in determining the value of K is determined by several factors including chemical composition of its surface, water content, surface area, and the geometrical arrangement of surface atoms or groups.[85] During the last decade, there has been development of the chemically bonded/modified adsorbents (bonded phase). Packings possessing polar functional groups have been slowly replacing the classical adsorbents such as silica gel or alumina in normal phase operation. The surface of these adsorbents are generally 'milder' due to the elimination of the reactive surface hydroxyl groups responsible for strong adsorption problems. They also give rise to fewer chemisorption, tailing, and catalytic activity problems.[86] Another advantage of bonded phases over conventional adsorbent is their rapid response to changes in the mobile phase composition, which reduces dramatically the time needed to reach a new equilibrium state.[86,87] Typical commercially available reverse- and normal-bonded phase are outlined in Table 6 and 7.[86] In normal phase chromatography solute retention is decreased with an increase in eluent polarity.[85]

Polarity (based on functionality)	Name[a]	Form[c]	Functionality	Base Material[b]	d_p (μm)	L, cm	Diameter (mm)	Comment
Weak	HiEff Micropart-ester	C	Ester	HiEff Micropart	5	25	5	Beware of hydrolysis of ester bond
	Nucleosil-NMe_2	B or C	Dimethylamino	Nucleosil 100 Å	5, 10	25	4.6	Sil60 NMe_2 is I-shaped; weak anion exchanger also; 6 μeq/m^2 coverage
	LiChrosorb DIOL	B or C	Diol	LiChrosorb	10	25	3.2, 4.6	Weakly polar, tetracycline analysis
	Chromegabond DIOL	C	Diol	LiChrosorb	10	30	4.6	Weakly polar
	Fe-Sil-X-1	B or C	Fluoroether	Sil-X-1	13 ± 5	50	3	Carbonyl compounds
Medium	Nucleosil-NO_2	B or C	Nitro	Nucleosil 100 Å	5, 10	25	4.6	Sil60 NO_2 is I-shaped; 6 μeq/m^2 coverage
	Cyano-Sil-X-1	B or C	Alkyl nitrile	Sil-X-1	13 ± 5	50	3	Weaker polarity than silica
	HiEff Micropart-CN	B or C	Nitrile	HiEff Micropart	5	25	5	Prepacked columns
	MicroPak-CN		Nitrile	LiChrosorb	10	25, 30	2.2, 4.0	Prep. columns available, monolayer
	μBondapak CN	C	Nitrile	μPorasil	10	30	3.9	9% Loading; can be used in reverse phase
	Nucleosil-CN	B or C	Nitrile	Nucleosil 100 Å	5, 10	25	4.6	Sil60 CN is I-shaped; 6 μeq/m^2 coverage.
	Partisil 10 PAC	C	Nitrile	Partisil	10	25	4.6	Packing density 0.45 g/ml, bulk density 0.70 g/ml; T atability to 70°C; monolayer
	Spherisorb CN	B or C	Nitrile	Spherisorb	5	10, 20, 25	4.6	Pore diameter 80 Å
	Vydac Polar TP	B or C	Nitrile	Vydac TP ads.	10	25	3.2, 4.6	Formerly an ether phase
	Zorbax-CN	C	Nitrile	Zorbax	6–8	25	2.4, 4.6	Also for reverse phase; less polar than silica
	Chromex HEMA	B	Polyhydroxyethyl-methacrylate	Same as functionality	11 ± 1	—	—	Has both hydrophilic and hydrophobic sites; also useful in reverse phase; 20% cross-linking
High	Amino Sil-X-1	B or C	Alkylamine	Sil-X-1	13 ± 5	50	3	Selective for nitro and aromatica
	Chromegabond-NH_2	C	Amino	LiChrosorb	10	30	4.6	Useful for peptides, carbohydrates; diamine also available
	Hypersil-APS	B	Aminopropyl	Hypersil	~7	—	—	Also for reverse phase
	LiChrosorb NH_2	B or C	Amino	LiChrosorb	10	25	3.2, 4.6	For sugars and peptidea
	MicroPak-NH_2		Aminopropyl	LiChrosorb	10	25, 30	2.2, 4.0	Prep. columns available, carbohydrates, nucleotides, monolayer
	μBondapak NH_2	C	Amino	μPorasil	10	30	3.9	9% Loading
	Nucleosil-NH_2	B or C	Amino	Nucleosil 100 Å	5, 10	25	4.6	Sil60 NH_2 is I-shaped; 6 μeq/m^2 coverage

[a] From Majors (29). [b] HiEff Micropart (Applied Science Laboratories); Nucleosil (Mackerey Nagel); LiChrosorb (E. Merck); Chromegabond (ES Industries); Sil-X (Perkin Elmer); MicroPak (Varian); μBondapak (Waters); Partisil (Whatman); Spherisorb (Phase Separations); Vydac (Separations Group); Zorbax (DuPont). [c] B, Bulk; C, packed columns.

Table 6. BPC Microparticulate packings for normal phase column (from reference 82).

Chain length	Name[b]	Form[c]	Functionality	Base material	Particle size (μm)	L (cm)	id (mm)	Comment
Long	MicroPak CH	B or C	Octadecylsilane	LiChrosorb Si	10	25, 30	2.2, 4	Polymeric layer, 22% loading
	Partisil ODS-2	C	Octadecylsilane	Partisil	10	25	4.6	16% Loading
	Zorbax-ODS	C	Octadecylsilane	Zorbax	6–8	25	2.4, 4.6	15% Loading
	Nucleosil C-18	B or C	Octadecylsilane	Nucleosil 100 A	5, 10	25	2.4, 4.6	Capacity twice C-8
	HiEff Micropart C-18	B or C	Octadecylsilane	HiEff Micropart	5	25	5	Pretested columns
	Hypersil-ODS	B	Octadecylsilane	Hypersil	~7	—	—	8% Carbon
	MicroPak MCH	B or C	Octadecylsilane	LiChrosorb Si	10	25, 30	2.2, 4	Monomeric layer 8% loading
	μBondaPak C_{18}	C	Octadecylsilane	μPorasil	10	30	3.9	10% Loading
	Vydac RP-TP	C	Octadecylsilane	Vydac TP Silica	10	25	3.2	10% Loading
	LiChrosorb RP-18	B or C	Octadecylsilane	LiChrosorb	5, 10	25	3	Monolayer, stable pH 1–9, 22% loading
	Spherisorb ODS	B or C	Octadecylsilane	Spherisorb	5, 10	25	3	Spherical, maximum pH 8
	Partisil ODS-1	C	Octadecylsilane	Partisil	10	25	4.6	5% Loading
	ODS-Sil-X-1	B or C	Octadecylsilane	Sil-X-1	13 ± 5	50	3	Irregular shape
Intermediate	LiChrosorb RP-8	B or C	Octylsilane	LiChrosorb	5, 10	25	3	Monolayer, stable pH 1–9, 13–14% loading
	Nucleosil C-8	B or C	Octylsilane	Nucleosil 100 A	5, 10	25, 30	4.6	General-purpose reverse phase
	Zorbax-C_8	C	Octylsilane	Zorbax	6–8	25	2.4, 4.6	For polar compounds; lower loading than ODS
	Chromegabond C_8	C	Octylsilane	LiChrosorb Si	10	30	4.6	C_{18}, C_2, C_1 also available
	Chromegabond-cyclohexane	C	Cyclohexane	LiChrosorb Si	10	30	4.6	Recommended for phenols; low polarity
	μBondaPak Phenyl	C	Phenyl	μPorasil	10	30	3.9	16% Loading
Short	Hypersil-SAS	B	Short alkyl chain	Hypersil	~7	—	—	For reverse-phase ion-pair and soap chromatography
	LiChrosorb RP-2	B or C	Dimethylsilane	LiChrosorb	5, 10	25	3	Formerly Si60 silanized, recommended for polar compounds

[a] From Majors. (29).
[b] MicroPak (Varian); Partisil (Whatman); Zorbax (DuPont); Nucleosil (Macherey-Nagel); HiEff Micropart (Applied Science Laboratories); Hypersil (Shandon Southern); μBondapak (Waters); Vydac (Separations Group); LiChrosorb (E. Merck); Spherisorb (Phase Separations); Chromegabond (ES Industries).
[c] B, Bulk; C, packed columns.

Table 7. BPC Microparticulate packings for reverse phase and ion partition columns (from reference 82).

Adsorbents having a polar amino alkyl functional group is of particular interest (for other types of bonded-phase, see reference 86). Being basic, amino alkyl functional group imparts a quite different chromatographic selectivity when compared to the slightly acidic surface of silica gel. It may function as either a Bronsted acid or base, depending on the chemical structure of the solute. It also possesses strong hydrogen bonding properties, which generally results in excellent separation of polyfunctional compounds i.e., -NH2 column has been used for the separation of steroids, cardiac glycosides, carbohydrates, and peptides. For further review, see references 84-87.

C. RESULTS AND DISCUSSION

Three bonded phase columns were tried: μ-Bondapak C_{18} column, μ-Bondapak NH_2 column, and μ-Bondapak carbohydrate analysis column (Waters Associates). The sample peaks were detected at 229 nm without derivatization. Two column sizes, 3.9 mm x 30 cm and 3.9 x 60 cm, were tried either by a recycling technique or by connecting two columns (3.9 mm x 30 cm) together. Isochratic and gradient elution methods have also been applied for each column and mobile phase. Four pairs of cardiac glycosides (structures shown in Figure 26), different only in the stereochemistry at C1' (anomeric pair), were studied. The best resolutions were obtained using μ-bondapak carbohydrate analysis column (3.9 mm x 30 cm) with 50% (for glucosides) and 60% (for mannosides) of ethanol in isooctane as the mobile phase. The resolution spectra of glucosides and mannosides were shown in Figure 27 and 28. The plots of the retention capacity value and the % ethanol composition tried is given in Figure 29 and 30. Several other mobile phases were also tried, such as various compositions (5% to 95%) of methanol in water and acetonitrile in water.

As mentioned previously in this chapter, the resolution power of the conventional chromatographic methods (thin layer and liquid chromatography using silica gel or alumina) is not enough to separate the unprotected anomeric pairs of cardiac glycosides. The development of the bonded phase, with controllable physical properties, has sharply increased the resolution power of the chromatographic technique.

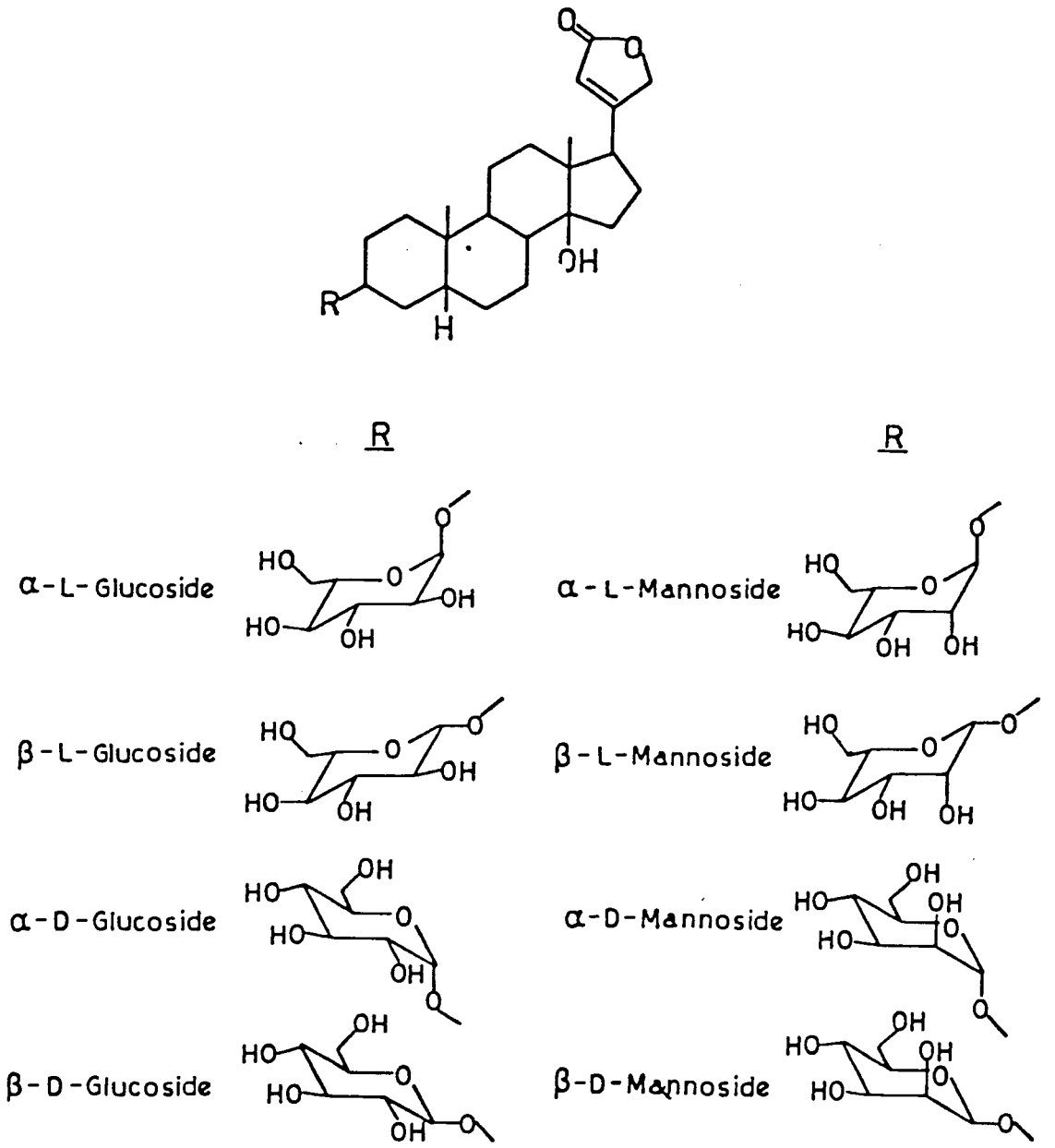


Figure 26. Structures of cardiac glycosides used in the HPLC study.

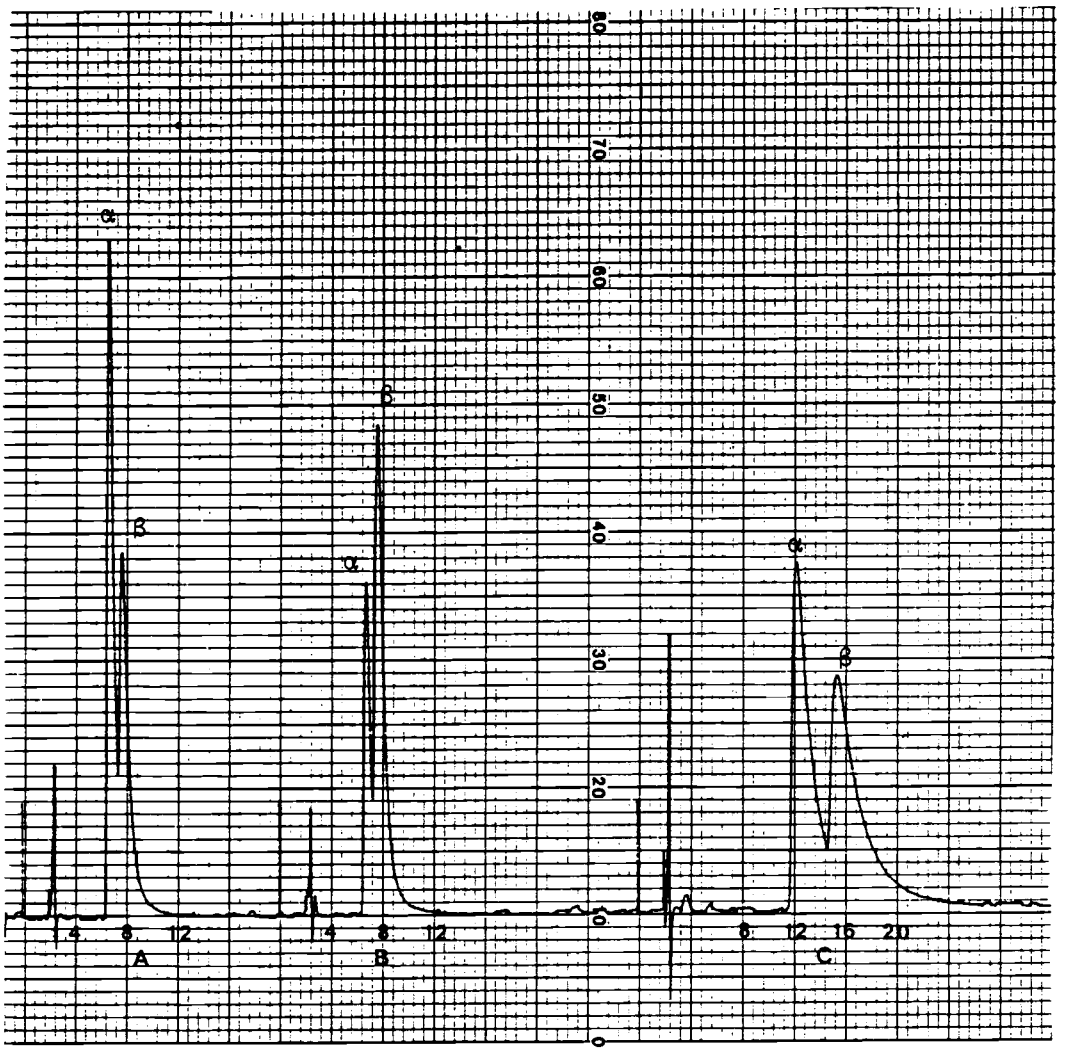


Figure 27. Separation of α- and β-L-glucosides (A), α- and β-D-glucosides (B), and α- and β-D-mannosides (C). Column: μ-Bondapak carbohydrate analysis column (30 cm x 3.9 mm). Mobile phase: 50% ethanol in isooctane. Flow rate: 1.5 ml/min. Pressure: 1,800 psi. (Times shown in the chart are in minute).

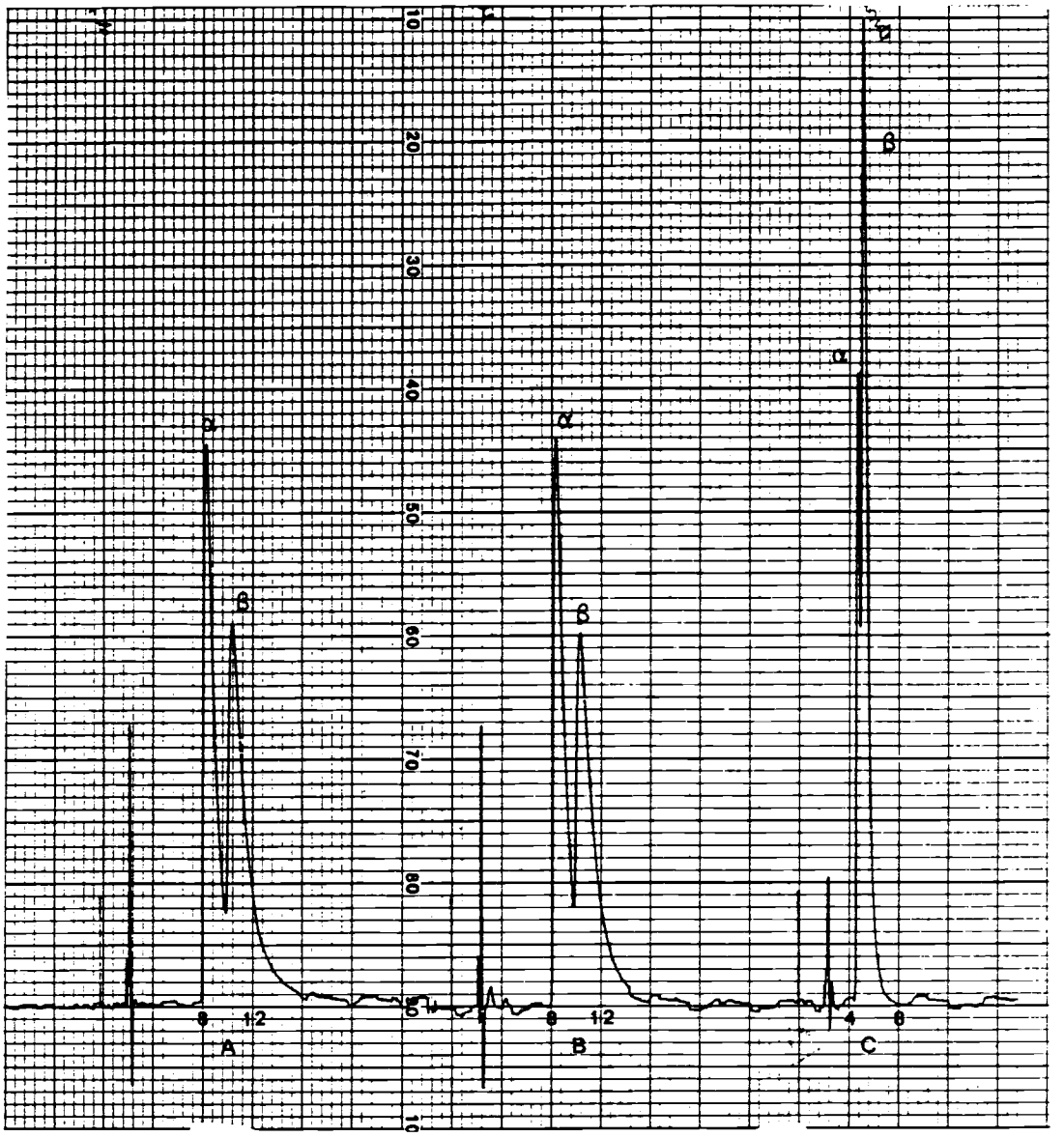


Figure 28. Separation of α- and β-L-mannosides (A), α- and β-D-mannosides (B), and α- and β-D-glucosides (C). Column: μ-Bondapak carbohydrate analysis column (30 cm x 3.9 mm). Mobile phase: 60% ethanol in isooctane. Flow rate: 1.5 ml/min. Pressure: 1,800 psi. (Times shown in chart are in minute).

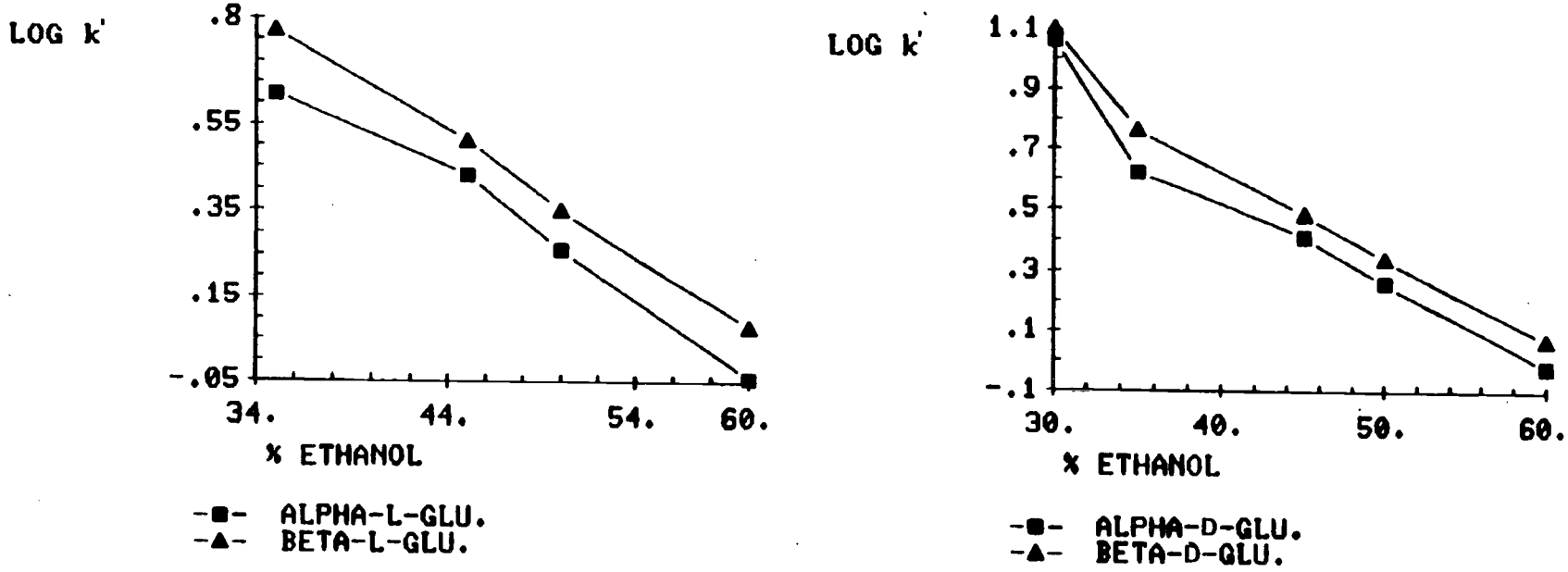


Figure 29. Plot between log k′ and % ethanol in mobile phase. Column: μ-Bondapak carbohydrate analysis column. Mobile phase: ethanol in isooctane. Flow rate: 1.5 ml/min. Pressure: 1,800 psi. The biggest k′ ratio (β vs α) was obtained with 35% ethanol for both pairs of glucosides.

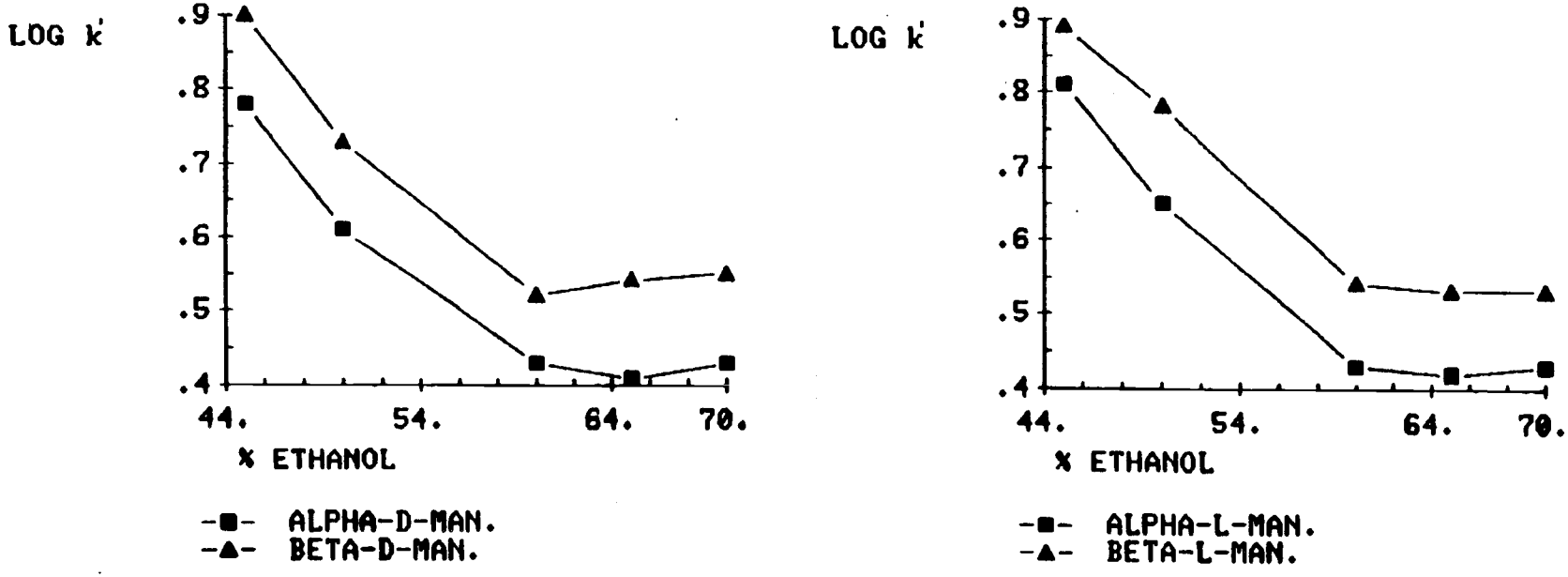


Figure 30. Plot between log k′ and % ethanol in mobile phase. Column: μ-Bondapak carbohydrate analysis column. Mobile phase: ethanol in isooctane. Flow rate: 1.5 ml/min. Pressure: 1,800 psi. The best resolutions were obtained with 60% ethanol for both pairs of mannosides. No significant change in k′ values were observed by increasing persentage of ethanol above 60% (to 70%)

The carbohydrate analysis column afforded a better resolution over C_{18} or NH_2 columns. With the C_{18} column, the retention is dependent on the steroid portion through a hydrophobic (London) force, whereas with carbohydrate column (a modified amino bonded phase), the retention is dependent on the sugar portion through hydrogen bonds. Apparently, the stereochemistry at C1' affects the hydrogen bonding capacity (possibly due to the steric effect), which may, in turn, contribute to the separation of the anomeric pairs. The effect of the stereochemistry at C1' on the steroid's hydrophobic bond capacity, however, seems not enough to result in the separation of the anomeric pairs. The best separation can be obtained with C_{18} column (3.9 mm x 30 cm) is shown in Figure 31. Broad, overlaping peaks and long elution time clearly demonstrate the inefficiency of the C_{18} column for the separation of the anomeric cardiac glycoside mixtures.

The narrow peaks obtained with the carbohydrate analysis column preclude the application of a gradent elution method. Change in the ethanol composition in the mobile phase will gradually elevate the baseline, making it difficult to measure the peaks' heights. The gradient elution method is also time inefficient since the column must be reequilibrated prior to each sample injection.

A μ-Bondapak NH_2 column was tried with 35% and 45% ethanol in isooctane and not investigated further. With those mobile phases, the NH_2 column afforded same elution time but with only 50% resolution efficiency of the carbohydrate analysis column.

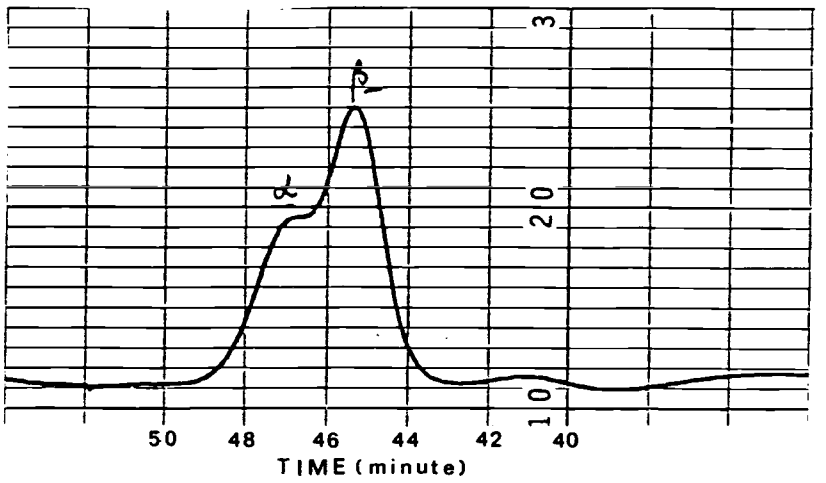


Figure 31. Separation of α- and β-D-glucosides. Column: μ-Bondapak C_{18} (30 cm x 3.9 mm). Mobile phase: 40% methanol in water. Flow rate: 30 ml/h. Pressure: 1000 psi.

The results show that the resolution achieved with the carbohydrate analysis column is sufficient for analytical purposes. Although baseline separation was not obtained, a R_S value of 1 (see Figure 32) makes it a powerful tool for a simple quantitative analysis. The narrow peaks obtained also make possible using a direct peak height measurement instead of more complicated peak area measurement method, although peak area method is less subject to minor variation in operating conditions. Our attempts to expand the use of carbohydrate analysis column to preparative scale, however, failed due to the limitation of the column capacity. The column's capacity is generally dependent on the ratio of the K' values. It was reported by Waters that a 3.9 mm x 30 cm column should be able to separate up to 4 mg of mixture which had a K' value ratio of 1.2. However, we found out that the actual column capacity for separation anomeric mixtures of cardenolide glycosides was less than 0.35 mg per injection.

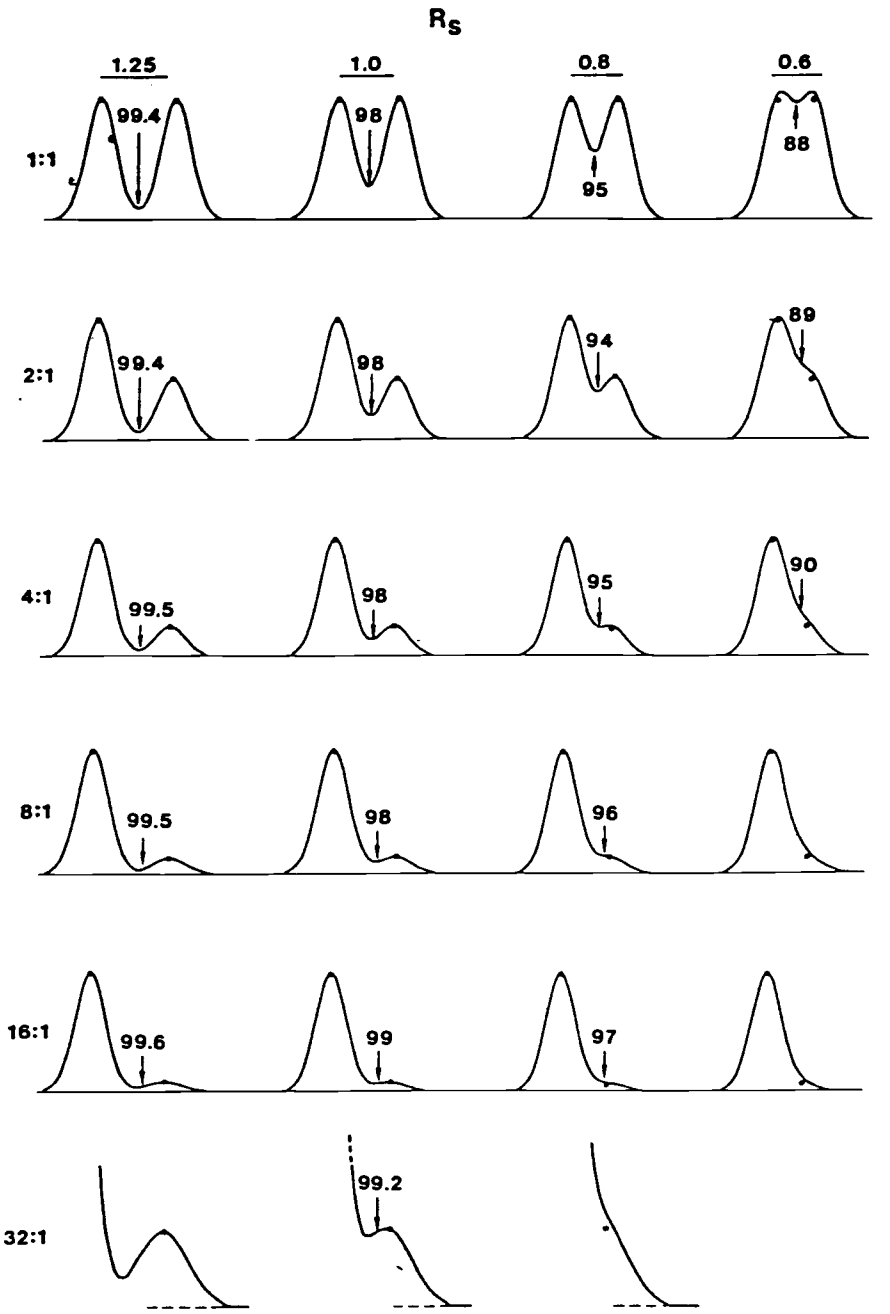


Figure 32. Standard curves for estimating resolution (R_S). For each peak height ratio, four R_S values are shown. True peak heights are indicated by black dots, and cut points for equal purity of each component by arrow. The numbers above the arrows indicate their purity (from reference 85).

EXPERIMENTAL SECTION

GENERAL PROCEDURES

NMR spectra were taken at the Oregon State University NMR spectroscopy laboratory, Department of Chemistry, using a Bucher 400 MHz spectrometer. Samples were dissolved in $CDCl_3$ or CD_3OD with 0.1% TMS as an internal standard. IR spectra were run as KBr pellets or as liquid films on NaCl discs using a Beckman model 7 spectrometer with polystyrene as reference. UV spectra were recorded in absolute methanol on a UV spectrometer (Varian CARY 219) at Oregon State University, Biochemistry Department. Mass spectra were obtained at the Department of Agricultural Chemistry, using a FINNIGAN MAT model 4023 GC-MS computer system equipped with pulsed positive-negative ion module. The optical rotations were taken in absolute methanol or chloroform using a Perkin Elmer model 141 polarimeter with a standard cell at the College of Pharmacy. Elemental analyses were performed by MHW Laboratories, Phoenix, Arizona. Melting points were measured on a Thomas Hoover apparatus and were uncorrected.

Thin layer chromatographies were conducted on .25 mm EM silica gel 60F 254 precoated glass TLC plate (EM Reagents). The following solvent combination were used:

System A	CH_2Cl_2 - EtOAc - MeOH	(20:2:1)
System B	CH_2Cl_2 - EtOAc - MeOH	(20:2:1)
System C	Benzene - EtOAc	(4:1)
System D	CH_2Cl_2 - Et_2O	(4:1)

TLC plates were visualized by either UV light at 254 nm or by heating after spraying with a solution of 2% $CaSO_4$ in 2N H_2SO_4.

The preparative TLC plates were coated with silica gel 60 PF 254 with $CaSO_4$ (EM Reagent) (20 x 20 cm, .80 mm thickness). The same solvent systems used in analytical TLC were used for developing preparative TLC. Visualization was done with UV light at 254 nm. Extraction of the samples was done with a mixture of CH_2Cl_2:EtOAc:Acetone (1:1:1).

Flash chromatography was done following the procedure reported by Still, Kahn and Mitra[80] with some modification. Nitrogen was used as the driving force instead of air and reagent grade solvent was used instead of distilled solvents. Samples were collected using an automatic fraction collector (TOYO fraction collector, Type SF 160K). Each tube was checked by TLC and the tubes containing the desired sample were collected.

The "usual work-up" refers to dilution with water, extraction with CH_2Cl_2, washing with 5% $NaHCO_3$ solution, and water, drying over $MgSO_4$ and then rotoevaporated.

SYNTHESIS OF COMPOUNDS

Tetra-O-acetyl-α-L-Rhamnose (2). To a solution of 5 g (30.5 mmol) of α-L-rhamnose in 80 ml dry pyridine was added 80 ml of acetic anhydride at 0°C. After the mixture was stirred for 2 hours at 0°C and refrigerated for 24 hours, it was added portion wise into 300 ml of ice water. The product was extracted with CH_2Cl_2 (3 x 100 ml). The combined extract was washed with 1N HCl (2 x 100 ml), neutralized with 5% $NaHCO_3$, washed with distilled water, dried over $MgSO_4$, and then evaporated to afford 9.364 g (92.5%) colorless oil of 2. ^1H NMR ($CDCl_3$) δ 6.03 (1H, C1-H), 5.30 (2H, C2-H + C3-H), 5.15 (1H, C4-H) 3.97 (1H, C5-H), 13 (3H, C6-H).

2,3,4-Tri-O-acetyl-6-deoxy-L-arabino-hexopyranosyl bromide (3). To the colorless oil of 2 (2 g, 6.02 mmol) was added 3 ml of acetic acid, 1.5 ml of acetic anhydride and 10 ml of 31% HBr in glacial acetic acid solution. The reaction was completed in 2 hours at room temperature as shown by TLC and the crude product of 3 was then used directly for the next step of reacton without isolation or further characterization.

3,4-Di-O-acetyl-1,2,6-trideoxy-L-arabino-1-hexenopyranose (4, diacetyl-L-rhamnal). To a 12 ml of cold (-10°C) buffer solution (.5 g of NaAc $3H_2O$ in 12 ml of 50% acetic acid in water) was added 4 g of zinc dust. After being stirred for 5 minutes, 1.2 ml of aqueous 20% $CuSO_4$ solution was added and the mixture was stirred further for 20 minutes. The reaction mixture of 3 was then added dropwise under constant stirring. The mixture was stirred for 3 hours at -10°C, then was filtered and worked up in the usual way to afford 1.10 g (86%) of 4

(colorless oil). ^1H NMR ($CDCl_3$) δ 6.43 (1H, d, $J_{1,2}$ = 10 Hz, C1-H), δ 5.1 (1H, dd, $J_{1,2}$ = 10 Hz, $J_{2,3}$ = 1-2 Hz, C2-H); IR 1755 (C=C), 1652 (C-C) cm^{-1}.

3β,14β-Dihydroxycard-20(22)-enolide (5, digitoxigenin). To a solution of 25 g (32.7 mmol)of digitoxin in 750 ml MeOH was added 750 ml of 0.08 N H_2SO_4. After the mixture was heated over a water bath (80°C) for 2 h, it was neutralized to pH 7 with 5% $NaHCO_3$, evaporated until about 500 ml of solution remained, and then kept refrigerated for 12 h for the crystallization to complete. The crystals were filtered, dried in vacuum and recrystallized in MeOH and EtOAc to obtain 9.59 g (25.6 mmol) of pure digitoxigenin (5, 76%).

3-β-(4'O-Acetyl-2',3',6'-trideoxy-α-L-arabino-2'-hexenopyranosyl) oxy-14-hydroxy-5β,14β-card-20(22)-enolide (6) and 3β-(4'-O-Acetyl-2',3',6'-trideoxy-β-L-arabino-2-hexenopyranosyl)oxy-14-hydroxy-5β-14β-card-20(22)- enolide (9). To a mixture of 20 ml of dry tetrahydrofuran and 10 ml of dry CH_2Cl_2 was added under constant stirring 2 g (5.32 mmol) of 5, 4.173 g (18.63 mmol) of 4, and 15 minutes later 0.065 ml (0.7 mmol) of $POCl_3$. The reaction mixture was stirred at room temperature for 20 h. The usual work up afforded 5.98 g of oil which after separation with flash chromatography (Ch_2Cl_2:Et_2O = 8:1) afforded 1.511 g (52.5%) of 6 and 0.81 g (27.8%) of 9.

The NMR spectra are as follows:

6: ^1H ($CDCl_3$) δ 5.85 (1H, s, C22-H), 5.75-5.90 (2H, m, C2'-H + C3'-H). ^{13}C ($CDCl_3$) δ (174.81 (C23), 174.62 (C20), 170.57 (4' acetyl carbonyl), 129.12 (C2'), 123.71 (C3'), 117.58 (C22), 93.39 (C1'), 85.50 (C14),

71.06 (C4'), 64.80 (C5').

9: ^{1}H NMR ($CDCl_3$) δ 5.85 (1H, s, C22-H), 5.75-5.90 (2H, m, C2'-H + C3'-H), 5.05 (1H, d, $J_{1',2'}$ = 6 Hz, C2'-H). ^{13}C ($CDCl_3$) δ 174.71 (C23), 174.44 (C_{20}), 170.41 (4' acetyl carbonyl), 131.43 (C2'), 127.30 (C3'), 117.61 (C22), 94.07 (C1').

3-β-(4'-O-Acetyl-2',3',6'-trideoxy-α-L-arabino-hexopyranosyl)oxy-14-hydroxy-5β-14β-card-20(22)-enolide (7). A solution of 100 mg of 6 in 20 ml of ethanol was hydrogenated over 5 mg of preactivated 5% $Pd/CaCO_3$ at room temperature and atmospheric pressure for 8 h. The catalyst was removed by filtration. The filtrate was rotoevaporated to afford 82 mg (82%) of 7, which was directly used for the next step of reaction. ^{1}H NMR ($CDCl_3$) showed no peaks at δ 5.75 - 5.90. UV λ max (MeOH) 218 nm, Log ε = 4.30.

3β-(2',3',6'-trideoxy-α-L-arabino-hexopyranosyl)oxy-14-hydroxy-5β,14β-card-20(22)-enolide (8). To a solution of 70 mg of 7 in 10 ml of dry MeOH was added 0.1 ml of 25% $Ba(OCH_3)_2$ suspension in MeOH. After stirring at room temperature for 12 h, 1 ml of IRA-410 (HCO_3^-) and 1 ml of IR-120 (H^+) resin were added for 1 h. The resins were removed by filtration and the filtrate was evaporated. The crude product was purified by preparative T.L.C. (solvent A) and recrystallizaton in CH_2Cl_2 and hexane to afford 32 mg (51%) of pure 8. ^{1}H NMR ($CDCl_3$) δ 5.89 (1H, s, C22-H), 5.0 (1H, dd, $J_{21A,21B}$ = 18 Hz, $J_{21A,22}$ = 1.2 Hz, C21-H_A), 4.82 (1H, dd, $J_{21A,21B}$ = 18 Hz, $J_{21B,22}$ = 1.6 Hz, C21-H_B), 4.81 (1H, broad singlet, C1'-H); ^{13}C NMR (see Table 3); $[M]_D$ = -23.9; Mp 208°-209°C. Anal ($C_{29}H_{44}O_6$) C.H.; UV λmax (MeOH) 218 nm, Log ε =

4.29.

3-β-(2',3',6'-Trideoxy-β-L-arabino-hexopyranosyl)oxy-14-hydroxy-5β,14β-card-20(22)-enolide (11). The conversion of 9 to 11 was done in the same manner as the conversion of 6 to 8. The only difference was that the recrystallization of 11 was done in EtOAc. ^{1}H NMR ($CDCl_3$) δ 5.86 (1H, s, C22-H), 5.0 (1H, dd, $J_{21A,21B}$ = 18 Hz, $J_{21A,22}$ = 1.2 Hz, (C21-H_A), 4.81 (1H, dd, $J_{21A,21B}$ = 18 Hz, $J_{21B,22}$ = 1.6 Hz, C21-H_B), 4.48 (1H, dd, $J_{1',2'a}$ = 2 Hz, $J_{1',2'e}$ = 9.2 Hz, C1'-H); ^{13}C NMR (see Table 3). $[M]_D$ = +70.14; UV λmax (MeOH) 218 nm. Log ε = 4.32; Mp. 171-173°C; Anal ($C_{29}H_{44}O_6$) C.H.

Methyl 4,6-O-benzylidine-α-D-glucopyranoside (13). To 300 ml of benzaldehyde was added 100 g of 12 and 80 g of $ZnCl_2$. After stirring at room temperature for 96 h, the reaction mixture was poured into 3 l of ice water and kept refrigerated for 12 h. The precipitate was filtered, washed with a large quantity of petroleum ether to give 117.4 g (80.8%) white crystal of 13. ^{1}H NMR ($CDCl_3$) δ 7.45 (5H, m, phenyl protons), 5.55 (1H, s, acetal proton), 4.78 (1H, d, $J_{1,2}$ = 3 Hz, C1-H), 3.15 (3H, s, 1-OCH_3); Mp 164-165°C.

Methyl 2,3-di-(p-toluenesulfonyl)-4,6-O-benzylidene-α-D-gluco-pyranoside (14). To a solution of 55 g (0.2 mol) Of 13 in 350 ml of dry pyridine was added 120 g (0.6 mol) of p-toluenesulfonyl chloride, stirred at room temperature for 72 h, and then poured into 2 l of ice water. The usual work up (the organic portion was washed with 1N HCl solution prior to washing with 5% $NaHCO_3$) afforded 110.98 g (83.9%) of 14 which was used for next step of reaction without further purification.

Methyl 2,3-anhydro-4,6-O-benzylidene-α-D-allopyranoside (15). To a solution of 110 g (0.166 mol) of 14 in 1.5 l of dry CH_2Cl_2 was added 500 ml of 2 M $NaOCH_3$ in MeOH. After being kept refrigerated for 44 h, then at room temperature for 12 h, the reaction mixture was washed with ice water (2 x 500 ml), dried over $MgSO_4$ and rotoevaporated to dryness. Recrystallization of the residue with CH_2Cl_2 and Et_2O afforded 45.66 g (92.7%) white crystals of 15. ^1H NMR ($CDCl_3$) δ 5.53 (1H, s, acetal proton), 4.85 (1H, d, $J_{1,2}$ = 2 Hz, C1-H), 7.40 (5H, m, phenyl proton), 3.45 (2H, d, $J_{5,6}$ = 2 Hz, C6-H); Mp 200-201°C.

Methyl 2,3-anhydro-4-O-banzoyl-6-bromo-α-D-allopyranoside (16). To a solution of 5 g (18.9 mmol) of 15 in 500 ml of dry CCl_4 was added 5 g (28 mmol) of N-bromo-succinimide and 25 g (0.12 mol) of $BaCO_3$. The mixture was refluxed for 2 h, and evaporated to dryness. The residue was suspended in 150 ml of Et_2O, filtered, and the Et_2O layer was then washed twice with 50 ml portion of water, dried over $MgSO_4$, and rotoevaporated to give 6.3 g (92.6%) of 16 (oil). ^1H NMR ($CDCl_3$) δ 5.3 (1H, broad singlet, C4-H), 4.9 (1H, d, $J_{1,2}$ = 2 Hz, C1-H), 3.25 (3H, m, C6-H + C5-H), 3.15 (3H, s, 1-OCH_3 proton).

Methyl 2,3-anhydro-4-O-benzoyl-6-deoxy-α-D-allopyranoside (17). A solution of 4 g (11.8 mmol) of 16 in 80 ml of MeOH was hydrogenated over 0.5 g of freshly prepared 20% Pd/C and 4 ml of diethyl amine at room temperature and atmospheric pressure for 5 h. The catalyst was removed by filtration. The filtrate was evaporated, and the pure 17 was obtained by flash chromatography eluted with 5% of EtOAc in benzene to yield 2.36 g (76%) of 17 (colorless oil). ^1H NMR ($CDCl_3$) δ 4.9 (1H, d,

$J_{1,2}$ = 2.8 Hz, C1-H), 5.07 (1H, dd, $J_{3,4}$ = 9.6 Hz, $J_{4,5}$ = 1.3 Hz, C4-H), 3.48 (3H, s, 1-OCH_3), 1.22 (3H, d, $J_{5,6}$ = 6.4 Hz, C6-H).

Methyl 4-O-benzoyl-2,6-dideoxy-2-iodo-α-D-allopyranoside (18). To a solution of 4.36 g (32.5 mmol) of LiI in 175 ml of dry Et_2O was added 1.72 g (6.5 mmol) of 17. A clear yellow solution was obtained after stirring at room temperature for 4 h. The reaction was neutralized with acetic acid and evaporated to dryness. The residue was then worked up in the usual manner to afford 2.50 g (97%) of 18 (oil). ^1H NMR ($CDCl_3$) δ 5.45 (1H, broad singlet, C4-H), 5.0 (1H, d, $J_{1,2}$ = 2 Hz, C1-H), 3.75-4.0 (3H, C2-H + C3-H + C5-H), 1.30 (3H, d, $J_{5,6}$ = 6 Hz, C6-H); IR 3550 (OH) cm^{-1}.

1,2,6-Trideoxy-D-allo-1-hexenopyranose (19). To a solution of 2.4 g (6.15 mmol) of 18 in 150 ml of dry Et_2O was added slowly 20 ml of 1.5 M(30 mmol) of CH_3Li solution in Et_2O. After being refluxed for 30 minutes, the reaction mixture was neutralized with acetic acid and evaporated to dryness. This crude product was used directly for next step of reaction without further isolation or characterization.

3,4-Di-O-acetyl-1,2,6-trideoxy-D-allo-1-hexenopyranose (20). The crude product of 19 was dissolved in 30 ml of dry pyridine, cooled to 0°C and added portionwise 30 ml of acetic anhydride. After being stirred at 0°C for 2 h and kept refrigerated for 12 h, the reaction mixture was worked up in the usual manner. The pure 20 was obtained by flash chromatography eluted with 10% of EtOAc in benzene. Yields of 20 (oil) is 0.6 g (45.6% calculated from 18). ^1H NMR ($CDCl_3$) δ 6.45 (1H, d, $J_{1,2}$ = 8 Hz, C1-H), 5.40 (1H, dd, $J_{1,2}$ = 8 Hz, $J_{2,3}$ = 5 Hz,

C2-H), 1.21 (3H, d, $J_{5,6}$ = 6 Hz, C6-H); IR 1740 (C=O), 1638 (C=C) cm^{-1}.

3β-(4'-O-Acetyl-2',3',6'-trideoxy-β-D-allo-2'-hexenopyranosyl)oxy-14-hydroxy-5β,14β-card-20(22)-enolide (21). Compound 21 was synthesized from 20 (0.6 g, 2.8 mmol), digitoxigenin (5, 0.525 g, 1.4 mmol), and 0.2 mmol of $POCl_3$ in the same manner as the L-analogue (6). Yield of 21 (amorphous powder) 0.451 g (61%), which was used for the next step of reaction without further purification. ^1H NMR ($CDCl_3$) δ 5.85 (1H, s, C22-H), 5,75-5.90 (2H, m, C2'-H + C3'-H), 4.8 (1H, d, $J_{1',2'}$ = 2 Hz, C1'-H), 3.95 (1H, bs, C3-H); ^{13}C NMR ($CDCl_3$) δ 129.16 (C2'), 123.72 (C3'), 93.05 (C1'); Anal ($C_{31}H_{44}O_7$) C. H.

In addition to 21, 0.081 g (11%) of α anomer (24) was also obtained.

3β-(2',3',6'-trideoxy-β-D-allo-hexopyranosyl)oxy-14-hydroxy-5β,14β-card-20(22)-enolide (23). Compound 23 was obtained by subsequent reduction and hydrolysis of compound 22 in the same manner as the corresponding L-analogue (6→8). Yields of 23 48.80% as calculated from 21. ^1H NMR ($CDCl_3$) δ 5.89 (1H, s, C22-H), 5.0 (1H, dd, $J_{21a,21b}$ = 18 Hz, $J_{21a,22}$ = 1.2 Hz, C21-H_a), 4.82 (1H, dd, $J_{21a,21b}$ = 18 Hz, $J_{21b,22}$ = 1.6 Hz, C21-H_b), 4.80 (1H, bs, C1'-H); ^{13}C NMR see Table 3; UV λmax (MeOH) 218 nm, Log ε = 4.33; $[M]_D^{22}$ = +70.14; Mp 205-206°C; Anal. ($C_{29}H_{44}O_{20}$) C. H.

3β-14β-Dihydroxy-17β-formyl-5β-androstane (29). The ozonolysis was carried out by flowing ozone through a solution of 15 g of 25 in 100 ml of dry CH_2Cl_2 for 5 h. The ozonide was then reduced with 6 g of zinc dust and 150 ml of acetic acid for 3 h. The usual workup afforded

14.052 g (87%) of **26**, which was used for next step of reaction without further purification. To a solution of 14 g (31 mmol) of **26** in methanol was added portion wise 2.35 g (62 mmol) of $NaBH_4$, stirred for 30 minutes, neutralized with acetic acid to pH 7, and evaporated. The usual workup on the residue afforded 13.97 g (98.5%) of **27**, which was subjected to hydrolysis without further purification. To a solution of 13.5 g of **27** in 100 ml methanol was added 300 ml of 5% KOH in methanol solution, refluxed for 20 minutes, concentrated the reaction mixture, then poured into 600 ml of water. After being neutralized with 25% HCl to pH 7, the mixture was kept refrigerated for 12 h, and then the crystals were filtered with suction. Washed the crystal with water, and then dried. Yields of **28** 10.95 g (crude). To a solution 10.75 g (29 mmol) of crude **28** in 200 ml of methanol was added 9.2 g (30 mmol) of $NaIO_4$ solution in 30 ml of water. At the end of 30 minutes, the reaction mixture was filtered through celite, and the filtral was evaporated. The usual workup on the residue afforded 7.92 g (81.3%) of pure **29** after recrystallization in EtOAc. ^1H NMR ($CDCl_3$) δ 9.71 (1H, d, $J_{17,20}$ = 4 Hz, C20-H), 4.1 (1H, bs, C3-H); IR (KBr) 3450 (OH), 2785 (CHO), 1705 (C=O) cm^{-1}; Mp 77-80°C.

3β,14β-Dihydroxy-5β-pregn-17β-trans-20(22)-ene-22-methyl formate (30). To a suspension of 0.97 g (40 mmol) of NaH in 50 ml of dry tetrahydrofuran was slowly added a solution of 6.4 ml (40 mmol) of trimethyl phosphonoacetate in 85 ml of dry tetrahydrofuran. After being stirred for 20 minutes, a solution of 5 g (15.6 mmol) of **29** in 75 ml of dry tetrahydrofuran was added dropwise into the mixture, and the

stirring was continued for another 40 minutes. The reaction mixture was poured into 2 l of acetic buffer solution (450 g of NaAc and 115 ml of acetic acid in 2 liter of water, pH 8-9), refrigerated for 12 h, and the crystals were filtered with suction, washed with water, and dried in vacuum. Recrystallization in EtOAc afforded 5.61 g (95.5%) of pure 30. ^{1}H NMR ($CDCl_3$) δ 7.16 (1H, dd, $J_{20,22}$ = 15.5 Hz, $J_{17,20}$ = 10.5 Hz, C20-H), 5.6 (1H, d, $J_{20,22}$ = 15.5 Hz, C22-H), 4.12 (1H, bs, C3-H), 3.7 (3H, s, C21-H); Mp 166-168°C; IR (KBr) 3450 (OH), 1735 (C=O), 1652 (C=C); Anal. ($C_{23}H_{36}O_4$) C. H.

Methyl 4,6-O-benzylidene-2-deoxy-allo-hexopyranoside (31). To a solution of 25 g (95 mmol) of 15 in 500 ml of dry tetrahydrofuran was added portion wise 7 g (190 mmol) of $LiAlH_4$, refluxed for 2 h, cooled to 0°C, subsequently added 200 ml of EtOAc and 100 ml of water, filtered and rotoevaporated until all organic solvent had evaporated. The usual workup on the residue gave 23.5 g (90%) of 31. ^{1}H NMR ($CDCl_3$) δ 7.3-7.6 (5H, m, aromatic protons), 5.65 (1H, s, acetal proton), 4.8 (1H, bs, C4-H), 4.35 (1H, dd, $J_{1,2}$ = 1-2 Hz, C1-H), 4.2 (1H, m, C6-H), 3.8 (1H, t, $J_{5,6}$ = 10 Hz, C5-H), 3.6 (1H, dd, $J_{2e,3}$ = 10 Hz, $J_{2a,3}$ = 2 Hz, C3-H), 3.4 (3H, s, 1-O-CH_3), 2.18 (1H, dd, $J_{2e,2a}$ = 18 Hz, $J_{1,2a}$ = 2 Hz, C2-Ha), 1.95 (1H, td, $J_{2a,2e}$ = 18 Hz, $J_{1,2e}$ = $J_{2e,3}$ = 2 Hz, C2-He); IR (KBr) 3500 (OH) cm^{-1}; Mp 123-126°C.

2-Deoxy-D-ribo-hexopyranose (32). A suspension of 9.5 g of 31 in 200 ml of 0.1 N H_2SO_4 was refluxed under N_2 gas for 4 h, cooled to room temperature, added 10 g of resin IR 400 (OH^-), and stirred for 15 minutes. The resin was filtered, and the filtrate was evaporated to

dryness. Recrystallization of the residue in methanol and methylene dichloride afforded 4.13 g (70%) of 32. ^{1}H NMR (CD_3OD) δ 5.09 (1H, dd, $J_{1,2a}$ = 2.08 Hz, $J_{1,2e}$ = 9.6 Hz, C1-H), 4.03 (1H, q, J = 3.08 Hz, C3-H); Mp 136-139°C; Anal ($C_6H_{12}O_5$) C,H.

1,3,4,6-Tetra-O-acetyl-2-deoxy-β-D-ribo-hexopyranose (33). To a solution of 2.4 g (14.6 mol) of 32 in 40 ml of dry pyridine was added 40 ml of acetic anhydride at 0°C. After being kept refrigerated for 24 h, the usual work up on the reaction mixture afforded 4.5 g (91.8%) of 33 (colorless oil). MS M^+: 329; IR 1740 (broad, C=O) cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 6.07 (1H, dd, $J_{1,2a}$ = 2.6 Hz, $J_{1,2e}$ = 9.3 Hz, C1-H), 3.54 (1H, q, $J_{3,2a} = J_{3,2e} = J_{3,4}$ = 3.2 Hz, C3-H), 4.93 (1H, dd, $J_{3,4}$ = 3.2 Hz, $J_{5,4}$ = 9.4 Hz, C4-H), 4.30 (1H, m, C6-H_A), 4.20 (1H, m, C5-H), 4.15 (1H, m, C6-H_B).

3β-(3',4',6'-tris-O-acetyl-2'-deoxy-β-and α-D-ribo-hexopyranosyl) oxy-14β-hydroxy-5β-pregn-17β-trans-20(22)-ene-22-methyl formate [34(β) and 36(α)]. To a solution of 2 g (5.3 mmol) of 30 in 80 ml of dry benzene and 20 ml of dry methylene dichloride (refluxed for 10 minutes, and then cooled to room temperature) was subsequently added 2.5 g (7.6 mmol) of 33, 0.94 g (53 mmol) of anhydrous para toluene sulfonic acid, and the reaction mixture was stirred at room temperature for 3 h (molecular sieves 4Å was used to insure a strict water free condition). The reaction mixture was poured into 300 ml of ice water, and was worked up in the usual manner. The crude product (4.083 g) was subjected to flash chromatography eluted with CH_2Cl_2:EtOAc=8:1. The first fraction afforded 1.097 g (30.86%) of 34 as amorphous powder. ^{1}H

NMR ($CDCl_3$ δ 7.18 (1H, dd, $J_{20,22}$ = 15.5 Hz, $J_{17,20}$ = 10.5 Hz, C20-H), 5.60 (1H, d, $J_{20,22}$ = 15.5 Hz, C22-H), 4.1-4.9 (6H, m, C1'-H + C3'-H + C4'-H + C5'-H + C6'-H), 4.92 (1H, bs, C3-H), 0.93 (3H, s, C19-H), 0.81 (3H, s, C18-H); ^{13}C NMR ($CDCl_3$) δ 169.91 (C23), 155.16 (C20), 119.28 (C22), 96.44 (C1'), 85.89 (C14), 73.78 (C21), 75.48 (C3). 34 was subjected for hydrolysis without further purification.

The second fraction afforded 0.978 g (27.5%) of 36 as an amorphous powder, which was also hydrolized without further purification. ^1H NMR ($CDCl_3$) δ 7.18 (1H, dd, $J_{20,22}$ = 15.5 Hz, $J_{17,20}$ = 10.5 Hz, C20-H), 5.60 (1H, d, $J_{20,22}$ = 15.5 Hz, C22-H), 4.1-5.3 (6H, m, C1'-H + C3'-H + C4'-H + C5'-H + C6'-H), 3.88 (1H, bs, C3-H), 0.93 (3H, s, C19-H), 0.85 (3H, s, C10-H); ^{13}C NMR ($CDCl_3$) δ 169.72 (C23), 155.18 (C20), 119.31 (C22), 95.20 (C1'), 85.88 (C14), 73.99 (C21), 70.87 (C3).

3β-(2'-deoxy-β-D-ribo-hexopyranosyl)oxy-14β-hydroxy-5β-pregn-17β-trans-20(22)-ene-22-methyl formate (35). To a solution of 552 mg of 34 in 30 ml of dry methanol was added 0.5 ml of 25% $Ba(OCH_3)_2$ suspension in methanol, and the reaction was carried out at room temperature for 12 h. Five g of IR410 (HCO_3^-) resin and 5 g of IR120 (H^+) resin were then subsequently added, and the stirring was continued for another 30 minutes. The resins were removed by filtration, and the filtrate was rotoevaporated to dryness. The residue was recrystallized in EtOAc to afford 30.1 mg (67.6%) of pure 35. ^1H NMR (CD_3OD) δ 7.15 (1H, dd, $J_{20,22}$ = 15.5 Hz, $J_{17,20}$ = 10.5 Hz, C20-H), 5.55 (1H, d, $J_{20,22}$ = 15.5 Hz, C22-H), 4.91 (1H, dd, $J_{1',2'a}$ = 2 Hz, $J_{1',2'e}$ = 8 Hz, C1'-H), 4.04 (1H, bs, C3-H), 3.65 (3H, s, C21-H), 0.92 (3H, s, C19-H), 0.82 (3H, s,

C18-H); ^{13}C NMR (CD_3OD) δ 169.10 (C23), 157.51 (C20), 119.72 (C22), 96.90 (C1'), 86.92 (C14), 75.51 (C3), 74.18 (C21); $[M]_D^{22}$ + 8.91°; IR (KBr) 3450 (OH), 1705 (C=O), 1645 (C=C) cm^{-1}; Mp 183-185°C; Anal. ($C_{29}H_{46}O_9$) C. H.

3β-(2'-deoxy-α-D-ribo-hexopyranosyl)oxy-14β-hydroxy-5β-pregn-17β-trans-20(22)-ene-22-methyl formate (37). The hydrolysis of 36 was carried out in the same manner at 34. 300 mg of 36 afforded 191 mg (72%) of pure 37. ^1H NMR (CD_3OD) δ 7.16 (1H, dd, $J_{20,22}$ = 15.5 Hz, $J_{17,20}$ = 10.5 Hz, C20-H), 5.62 (1H, d, $J_{20,22}$ = 15.5 Hz, C22-H), 5.0 (1H, bs, C1'-H), 4.05 (1H, bs, C3-H), 3.65 (3H, s, C21-H), 0.95 (3H, s, C19-H), 0.87 (3H, s, C10-H); ^{13}C NMR (CD_3OD) δ 169.05 (C23), 157.46 (C20), 119.71 (C22), 96.67 (C1'), 86.84 (C14), 73.97 (C21), 70.53 (C3); IR (KBr) 3440 (OH), 1743 (C=O), 1650 (C=C) cm^{-1}; $[M]_D^{22}$ + 99.37°; Mp 173-177°C; Anal. ($C_{29}H_{46}O_8$) C. H.

1,3,4,6-Tetra-O-(para-nitro benzoyl)-2-deoxy-D-ribo-hexopyranoside (38). To a solution of 1.73 g (10.5 mmol) of 32 in 50 ml of dry pyridine was added portion wise 9.9 g (53 mmol) of para nitro-benzoyl chloride, and the reaction mixture was stirred at room temperature for 96 h. The reaction was worked up in the usual manner, and the crude product was purified by flash chromatography eluted with methylene dichloride to afford 5.53 g (69%) of 38. ^1H NMR ($CDCl_3$) δ 8.05-8.4 (16H, m, aromatic proton), 6.57 (1H, dd, $J_{1,2a}$ = 2.5 Hz, $J_{1,2e}$ = 8.6 Hz, C1-H), 6.10 (1H, q, $J_{2a,3} = J_{2e,3} = J_{3,4}$ = 3-3.5 Hz, C3-H), 5.54 (1H, dd, $J_{3,4}$ = 3 Hz, $J_{4,5}$ = 9.4 Hz, C4-H), 4.73-4.76 (2H, m, C6-H), 4.56-4.61 (1H, m, C5-H), 2.49-2.63 (2H, m, C2-H); IR (KBr) 1733 (C=O) cm^{-1}; Mp 161-165°C.

3,4,6-Tris-O-(para-nitro benzoyl)-2-deoxy-α- and β-D-ribo-1-

hexopyranosyl bromide (39). To a 40 ml saturated solution of HBr in dry methylene dichloride was added 3 g of **38**. After being stirred at room temperature for 1 h, the reaction mixture was filtered, and the filtrate was evaporated to dryness to afford 2.55 g (93.9%) of **39**. Which was used directly for glycosidation reaction without further purification. Compound **39** was a mixture of α and β anomer with a ratio of approximately 2α to 1 β (estimated from proton NMR integration). ^{1}H NMR ($CDCl_3$) δ 5.8-6.0 (1H, m, C3-H), 5.4-5.55 (1H, m, C4-H), 4.9-5.05 (1H, m, C1-H) 4.65-4.8 (1H, m, C5-H), 4.45-4.65 (2H, m, C6-h), 2.1-2.4 (2H, m, C2-H); IR (KBr) 1730 (C=O) cm^{-1}; Mp 116-119°C.

(3β-5β)-3-[3',4',6'-Tris-O-(para-nitro benzoyl)-2'-deoxy-α- and β-D-ribo-hexopyranosyl)oxy]-14β-hydroxycard-20(22)-enolide. [40(β) and 41(α)]. A mixture of digitoxigenin (**5**, 475 mg, 1.27 mmol), silver carbonate (1g, 3.63 mmol), and dry ethylene dichloride (100 ml) was heated with stirring and shielded from light. After 50 ml of the solvent was distilled, a solution of **39** (1.75 g, 2.54 mmol) in 60 ml of dry ethylene dichloride and 60 ml of dry carbon tetrachloride was added dropwise while keeping the volume of the reaction constant (3 h). At the end of 3 h, 100 ml of dry ethylene dichloride was added dropwise, again the volume of the reaction was kept constant (3 h). The reaction mixture was cooled to room temperature, and filtered. The residue was washed with ethylene dichloride. The filtrate was rotoevaporated to dryness to afford 2.24 g of amorphous powder, which was purified by flash chromatography eluted with CH_2Cl_2:Et_2O = 8.1. The first fraction afforded 440 mg (35.3%) of **41**. ^{1}H NMR ($CDCl_3$) δ 8.07-8.31 (12H, m, aro-

matic proton), 5.88 (1H, s, C22-H), 4.5-5.8 (8H, m, C21-H + C1'-H + C3'-H + C4'-H + C5'-H + C6'-H), 4.05 (1H, bs, C3-H); ^{13}C NMR ($CDCl_3$) δ 174.53 (C23 and C20), 117.73 (C22), 93.85 (C1'), 85.40 (C14), 73.47 (C21), 71.92 (C3); IR (KBr) 3440 (OH), 1725 (C=O) cm^{-1}.

The second fraction afforded 380 mg (30.5%) of 40. ^1H NMR ($CDCl_3$) δ 8.05-8.35 (12H, m, aromatic proton), 4.5-6.0 (9H, m, C22-H + C21-H + C1'-H + C3'-H + C4'-H + C5'-H + C6'-H), 4.1 (1H, bs, C3-H). ^{13}C NMR ($CDCl_3$) δ 174.54 (C23 and C20), 117.71 (C22), 96.89 (C1'), 85.54 (C14), 73.48 (C21), 71.48 (C3); IR (KBr) 3420 (OH), 1725 (C=O) cm^{-1}.

(3β-5β)-3-(2'-deoxy-β-D-ribo-hexopyranosyl)oxy-14β-hydroxycard-20(22)-enolide (42). A mixture of 225 mg of 40 in 20 ml of dry methanol and 0.2 ml of 25% $Ba(OCH_3)_2$ suspension in methanol was stirred at room temperature for 4 h. The reaction mixture was worked up in the same manner as 35, and the crude product was then recrystallized in methanol-methylene dichloride to afford 82 mg (67.74%) of pure 42. ^1H NMR (CD_3OD) δ 5.89 (1H, s, C22-H), 5.03 (1H, dd, $J_{21a,22}$ = 1.4 Hz, $J_{21a,21b}$ = 18.2 Hz, C21-Ha), 4.95 (1H, dd, $J_{1',2'a}$ = 1.9 Hz, $J_{1',2'e}$ = 11.5 Hz, C1'-H), 4.93 (1H, $J_{21a,21b}$ = 18.2 Hz, $J_{21b,22}$ = 1.8 Hz, C21-Hb), 4.09 (1H, bs, C3-H), 3.4-4.05 (5H, m, C3'-H + C4'-H + C5'-H + C6'-H), 2.83 (1H, t, $J_{16,17}$ = 6 Hz, C17-H); ^{13}C NMR (CD_3OD) δ 178.45 (C23) 177.25 (C20), 117.80 (C21), 96.89 (C1'), 86.44 (C14), 75.51 (C21), 74.15 (C3); IR (KBr) 3400 (OH), 1725 (C=O) cm^{-1}; $[M]_D^{22}$ = 4.3°; Mp 222°-224°C.

(3β-5β)-3-(2'-deoxy-β-D-ribo-hexopyranosyl)oxy]-14β-hydroxy-20R-and S-cardanolide (43). A mixture of 60 mg of 42 in 10 ml of dry methanol and 20 mg of 5% $Pd/CaCO_3$ was hydrogenated at room temperature and atmospheric pressure for 7 days. The catalyst was removed by filtra-

tion, and the filtrate was evaporated to dryness. The residue was recrystallized in CH_3OH:CH_2Cl_2:hexane to afford 52 mg (86.6%) of pure **43**. Anal. ($C_{29}H_{46}O_8$) C. H.

Compound Number	Chemical Formula	Theory %		Found%	
		C	H	C	H
8	$C_{29}H_{44}O_6$	71.28	9.08	71.46	9.05
11	$C_{29}H_{44}O_6$	71.28	9.08	71.39	9.03
21	$C_{31}H_{44}O_7$	70.43	8.39	70.26	8.17
23	$C_{29}H_{44}O_6 \cdot H_2O$	68.75	9.14	68.97	8.97
24	$C_{31}H_{44}O_7$	70.43	8.39	70.55	8.12
30	$C_{23}H_{36}O_4$	73.37	9.64	73.33	9.68
32	$C_6H_{12}O_5$	43.90	7.37	43.81	7.41
34	$C_{35}H_{52}O_{11}$	64.80	8.08	64.99	8.03
35	$C_{29}H_{46}O_8$	66.64	8.87	66.43	8.77
36	$C_{35}H_{52}O_{11}$	64.80	8.08	65.11	7.96
37	$C_{29}H_{46}O_8$	66.64	8.87	66.43	8.85
43	$C_{29}H_{46}O_8$	66.64	8.87	66.49	8.75

Table 8: Elemental Analysis Result

Experimental

High Performance Liquid Chromatography. HPLC was performed on Waters 200 series chromatographic system incorporating a Model 6000 A pump operating at a flow rate of 1.5 ml/min., a Model 660 solvent programmer, a Model U6K injector, and Waters μ-bondapak columns. An analytical or HPLC grade solvents were used. The mobile phase was prefiltered through a 0.5 μm Millipore filter (Millipore, Bedford, MA, USA), and degassed by ultrasonic stirring in vacuum for 10 minutes. Nitrogen gas was bubbled through the mobile phase throughout the experiments. The samples were synthesized in our laboratories (see reference 65). The pure glycosides were dissolved in absolute methanol at a concentration of 1 mg/ml. Four μl of sample solution was used per injection.

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