Cardiotonic Steroids

by

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ABSTRACT

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Cardiotonic steroids are of major importance in heart therapy and methods for their synthesis not only are of interest per se, but also as a means for obtaining analogs of potential pharmacological importance.

Synthesis of the A/B ring system of sarmentosigenin E, a C-5, C-19-functionalized aglycone, is described. Oxidation of 5α-chloro-syn-19-oximinocholestane-3β,6β-diol 3-acetate (1) gave 5α-chloro-3β-hydroxy-6-oxocholestane-19-nitrile 3-acetate (18) which was allowed to react with alcoholic potassium hydroxide to form 3β,5β-dihydroxy-6-oxocholestane-19-nitrile. This diol was reduced with sodium borohydride to afford 3β,5β,6β-trihydroxycholestane-19-nitrile, which on treatment with methanolic hydrogen chloride followed by hydrolysis furnished 3β,5β,6β-trihydroxycholestan-19-oic acid 6,19-lactone (23) embodying the A/B ring system of sarmentosigenin E. Reduction of 5β,6β-epoxy-19-oximinocholestan-3β-ol (3) or the corresponding nitrile (4) with lithium aluminum hydride afforded 19-norcholest-5(10)-ene-3β,6β-diol (5) via a fragmentation reaction.

In studies directed toward the elaboration of the remaining structural features of cardiac aglycones, bromination of 14-hydroxy-3-oxo-5β,14β-card-20(22)-enolide (25) with phenyltrimethylammonium tribromide (PTT) followed by dehydrobromination with benzyltrimethylammonium mesitoate (BTAM) afforded 14-hydroxy-3-oxo-14β-carda-4,20(22)-dienolide (42).

Ammonolysis of digitoxigenin (24) afforded two isomeric lactol amides, (20S,21S)-3β,21-dihydroxy-14β,21-oxidonorcholan-23-oic acid amide (43) and (20S,21R)-3β,21-dihydroxy-14β,21-oxidonorcholan-23-oic acid amide (44), both of which when treated with warm glacial acetic acid cyclized stereoselectively to the corresponding lactams, (20S,21S)-3β-hydroxy-21-amino-14β,21-oxidonorcholan-23-oic acid lactam (45) and (20S,21R)-3β-hydroxy-21-amino-14β,21-oxidonorcholan-23-oic acid lactam (46). The pharmacological evaluation of the above lactams is described and the implications of these results for the mode of action of cardiotonic steroids is discussed.

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PART I

INTRODUCTION

Cardiotonic steroids are biologically active substances found in small amounts in the seeds, leaves, stems, roots or bark of a wide variety of plants, particularly those of the other Apocynaceae. A few are also present in the venom of certain toads. In plants, cardiotonic steroids occur as the glycosides of rare desoxy sugars, whereas in the skin secretions of poison toads they occur as the free sterol or genin, or in conjugation with suberylarginine (Bufotoxins).

Pharmacologically, cardio-active steroids exert a specific and powerful action on the heart muscle when administered in very small amounts to man or animals. Generally, all of them are characterized by a high degree of cardiac toxicity and excessive doses, which by most means are quite small, can cause death in systole.

The toxic properties of plant extracts containing cardiac glycosides have been known to natives of many parts of the world. Digitalis plants and in particular the purple foxglove, Digitalis purpurea, were used for the preparation of poison for trials by ordeal and as arrow and dart poisons. Preparations from the dried leaves and seeds found some early use in medicine, mainly for external application to promote wound healing and internally for the treatment of dropsy. The use of digitalis in heart therapy was first introduced in 1785 by the Scottish physician William Withering who recognized the effectiveness of the drug in the treatment of only certain forms of dropsy, but apparently failed to associate this

finding with the cardiac actions of the drug. In spite of Withering's findings, digitalis continued to be used quite indiscriminately in all sorts of unrelated disorders. Studies carried out by many investigators in the last sixty years have firmly established the main value of digitalis as a specific in the therapy of congestive heart failure.[1]

The importance of cardiac glycosides in drug therapy cannot be overemphasized as they remain unchallenged as the most useful agents in the treatment of congestive heart failure. However, and in spite of their highly specific action on the heart, they also possess poor therapeutic indexes which makes them less than ideal. It has been stated that normal therapeutic doses represent 50 to 60 per cent of the toxic dose, so that symptoms of toxicity are not uncommon.

The nature of the mechanism of action of cardiac glycosides is not known, although several interesting hypotheses have been postulated in recent years. One of these hypotheses, proposed by K. Repke,[2] has received considerable attention and it is based on the observation that digitalis glycosides have the ability to inhibit the enzymic activity of several Na^+ - K^+-ATPase systems, including cardiac Na^+ - K^+-ATPase.

The first insight into the structural nature of the active principles of digitalis plants was furnished by the work of Windaus in 1915, and the extensive investigations of W.A. Jacobs[3] provided the basic foundations for the elucidation of structural problems. Knowledge in this field was greatly implemented by the more recent work of A. Stoll and T. Reichstein.[3]

Progress in the synthesis of cardiotonic steroids has been slow and difficult. Methods for their synthesis not only are of interest <u>per se</u>, but also as a means for obtaining analogs of potential pharmacological

importance. Syntheses of digitoxigenin[4] and periplogenin[5] have been disclosed in recent years. Neither of these aglycones has a functional group at C-19, and analysis[3,6-9] of the relationship between chemical constitution and biological activity in the cardiac glycosides indicates that concomitant oxygenation at C-19 and C-5 enhances cardiotonic action.

Based on this analysis, we were interested in synthesizing such C-5, C-19 functionalized aglycones, but in addition to these functionalities, we were particularly interested in synthesizing aglycones with halogen substituents at key positions on the steroid nucleus. Our interest in this approach stems mainly from the very large success that has been attained in the syntheses of considerably more active steroid hormones by introduction of halogen substituents at various positions on the steroid molecule.

The work described in this thesis is an effort in this direction. The synthesis of the A/B ring system of sarmentosigenin E (3β,5,6β,14-tetrahydroxy-5β-card-20(22)-enolide-19-oic acid 6,19-lactone)[10] is described, as well as syntheses of other potential intermediates for the synthesis of the aforementioned compounds. The synthesis and pharmacological evaluation of the acetate of isodigitoxigeninic acid lactam (20S, 21R)-3β-Acetoxy-21-amino-14β, 21-oxidonorcholan-23-oic acid lactam)[11]* is also described.

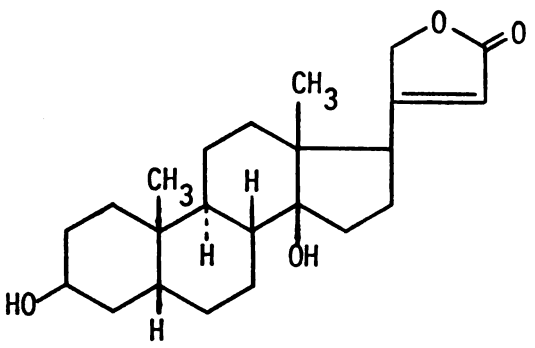
*At the time this thesis was being prepared, there appeared in the literature a claim for the preparation of this compound and other closely related compounds (See ref. No. 11), but the melting points differ widely from those reported here.

PART II

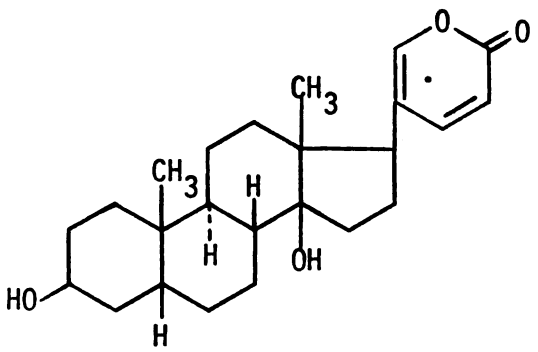
CARDIAC-ACTIVE STEROIDS

A. General considerations

Cardiac-active steroids can be devided into two groups, the cardenolides and the bufadienolides.[3] All of these compounds possess, as implied by the title name, a steroid ring system of a specific configuration. The highest cardiotonic activity in either group is shown by those members in which the rings are A/B-cis, B/C-trans and C/D-cis fused. Digitoxigenin, an aglycone obtained by mild acid hydrolysis of the glycoside digitoxin, is an example of the more abundant cardenolides. The characteristic functional features of all cardenolides are the presence of an α,β-unsaturated, five-membered lactone ring in the 17β-position and a 14β-hydroxyl group.



Cardenolide: Digitoxigenin



Bufadienolide: Bufalin

Fig. 1. Representative examples of Cardenolides and Bufadienolides.

The bufadienolides on the other hand, bear a doubly unsaturated six-membered lactone ring (α-pyrone) in the 17β-position, and as the cardenolides, they also carry a 14β-hydroxyl group.

Structural variations are common in both series and these usually involve the configuration at C-3 and C-5, the presence of double bonds

in the steroid nucleus of a few cases, and more often, the presence of additional oxyen functions at other positions of the steroid nucleus.

Table 1. Structural Variations of Cardiac Aglycones

Aglycone	C-19	Substituents
Digitoxigenin Derivatives (Cardenolides)		
Periplogenin	CH_3	5β-OH
Sarmentogenin	CH_3	11α-OH
Digoxigenin	CH_3	12β-OH
Gitoxigenin	CH_3	16β-OH
Strophanthidin	CHO	5β-OH
Sarmentosigenin A	CHO	5β-OH, 6β-OH
Ouabagenin	CH_2OH	1β-OH, 5β-OH, 11α-OH
Uzarigenin	CH_3	5α-isomer
Bufalin Derivatives (Bufadienolides)		
Telocinobufagin	CH_3	5β-OH
Gamabufotalin	CH_3	11α-OH
Bufotalin	CH_3	16β-acetoxy
Bufotalidin	CHO	5β-OH
Hellebrigenin	CHO	5β-OH
Scillarenin	CH_3	Δ^4-dehydro derivative
Scillirosidin	CH_3	6β-OAc, 8β-OH, Δ^4-dehydro

The latter type of variation is of particular interest because, as will

be shown later, the number and location of additional oxygen functions have a marked effect on the activity of these compounds. In addition, the orientation (equatorial or axial) of these functions appear to be important in determining whether the compound will be more or less active.

Table 1. includes examples of some natural modifications found in both series of cardiac aglycones.

The cardenolides are all products of plant synthesis, they occur as the glycosides of several types of aldohexose sugars, and quite often the same aglycone is found in various glycosides of different plants, so that their distribution is widespread. The sugar residues are always attached to the aglycone moeity through the hydroxyl group at C-3.

The bufadienolides are products of synthesis in both plants and animals. Thus, the first four bufadienolides listed in Table 1. are all derived from the poisonous secretions of certain toads, whereas the last three are of plant origen. Those of animal origen occur as the free genin or as conjugates of suberylarginine (Bufotoxins), while those of plant origen occur as the glycosides.

Continuing research in the chemistry of natural products constantly reveals new plant sources of cardiac glycosides.

B. Analysis of Structure-Activity Relationships

The correlation between pharmacological activity and structure of the cardiac glycosides and aglycones has been extensively reviewed.[3,6-9] For the most part, available data on the activity of these compounds is based on cat toxicity studies.[12,13] This method of assessing activity, while useful in determining the relative potency of cardiac glycosides, yields no information on their therapeutic value. Nevertheless, the method has been used extensively if not exclusively, in evaluating all known cardiac glycosides; and analysis of the results has led to a number of useful generalizations. A structural modification is considered favorable or unfavorable depending on whether the LD is decreased or increased.

The biological activity of the cardiac glycosides resides on the aglycone portion of the molecule, and the sugar(s) although inactive by themselves undoubtedly confer better solubility properties to the glycoside molecules and this may account for their better absorption and distribution to cardiac tissue as compared to the free aglycones. In the cardenolide series, the order of activity shows the following pattern: Trioside < dioside < monoside > aglycone.[13] However, in the bufadienolides the free genins or aglycones display a higher order of activity than their glycosides or than the corresponding cardenolides aglycones.

Digitoxigenin, the aglycone of digitoxin, is the simplest member in the cardenolide series and appears to encompass the minimum structural features necessary for cardiotonic activity. The characteristic ring fusion, A/B-cis, B/C-trans and C/D-cis, is a common feature to all of

the most active cardiac glycosides and aglycones. A few naturally occurring glycosides possess the A/B-*trans* ring fusion, but these are considerably less active.

Introduction of a double bond at C-4(5) has little effect on the cardiotonic activity and likewise, a double bond at C-5(6) also results in cardiac-active compounds.[7] Based on the observation that many semisynthetic cardiac aglycones with modified A and B rings are biologically active, Wolff and Ho[14] proposed that the ring A is not in contact with the cellular receptor. However, the fact that simple epimerization of the 3β-hydroxyl to the 3α-form results in considerable loss of activity[9] requires a different explanation.

The 14β-hydroxyl group has been considered essential for cardiotonic activity, although recent evidence[15] suggests that the hydroxyl group *per se* is not as important as is the nature of the fusion between rings C and D, a C/D-*cis* fusion being the indispensable structural requirement for activity.

The α,β-unsaturated lactone ring at the 17β-position appears to be essential for cardiotonic activity. Inversion to the 17β-H cardenolides results in totally inactive compounds, and saturation of the double bond to the corresponding dihydro-derivatives greatly reduces the cardiotoxicity. The double bond in the lactone ring of cardiac glycosides has been considered essential for their characteristic effects. However, Vick *et al.*[16] compared the effects of dihydro-ouabain, dihydrodigoxin and dihydrodigitoxin with their parent compounds on the heart-lung preparation of the dog and found that the dihydro-derivatives acted qualitatively similar to the parent glycosides, and that although less potent in most respects, they produce their therapeutic effects (returning the failing

heart to prefailure conditions) with a smaller portion of their lethal doses than do the parent glycosides.

Opening of the lactone ring by alkaline hydrolysis results in total loss of activity.

The unsaturated lactone moeity of the cardiac glycosides has been viewed as the most important group necessary for cardiac activity. It has been described as the "active center"[17] of the aglycone molecule. However, an unsaturated lactone is not active unless it is attached to the appropriate supporting group.[17] Portius and Repke[18] have proposed that the α,β-unsaturated carbonyl acts as a proton acceptor in hydrogen bonding. Based on this concept, Repke[2] has proposed that "transport ATPase" is the receptor for cardiac glycosides and that the α,β-unsaturated carbonyl, through hydrogen bonding provides the first one-point attachment to the receptor as shown in Fig. 2. on the following page.

In this scheme, stabilization of the initial one-point attachment is envisaged to occur through multi-point hydrophobic bonding between the fixing steroid group (Haftgruppe) and the complementary surface of enzyme.[18]

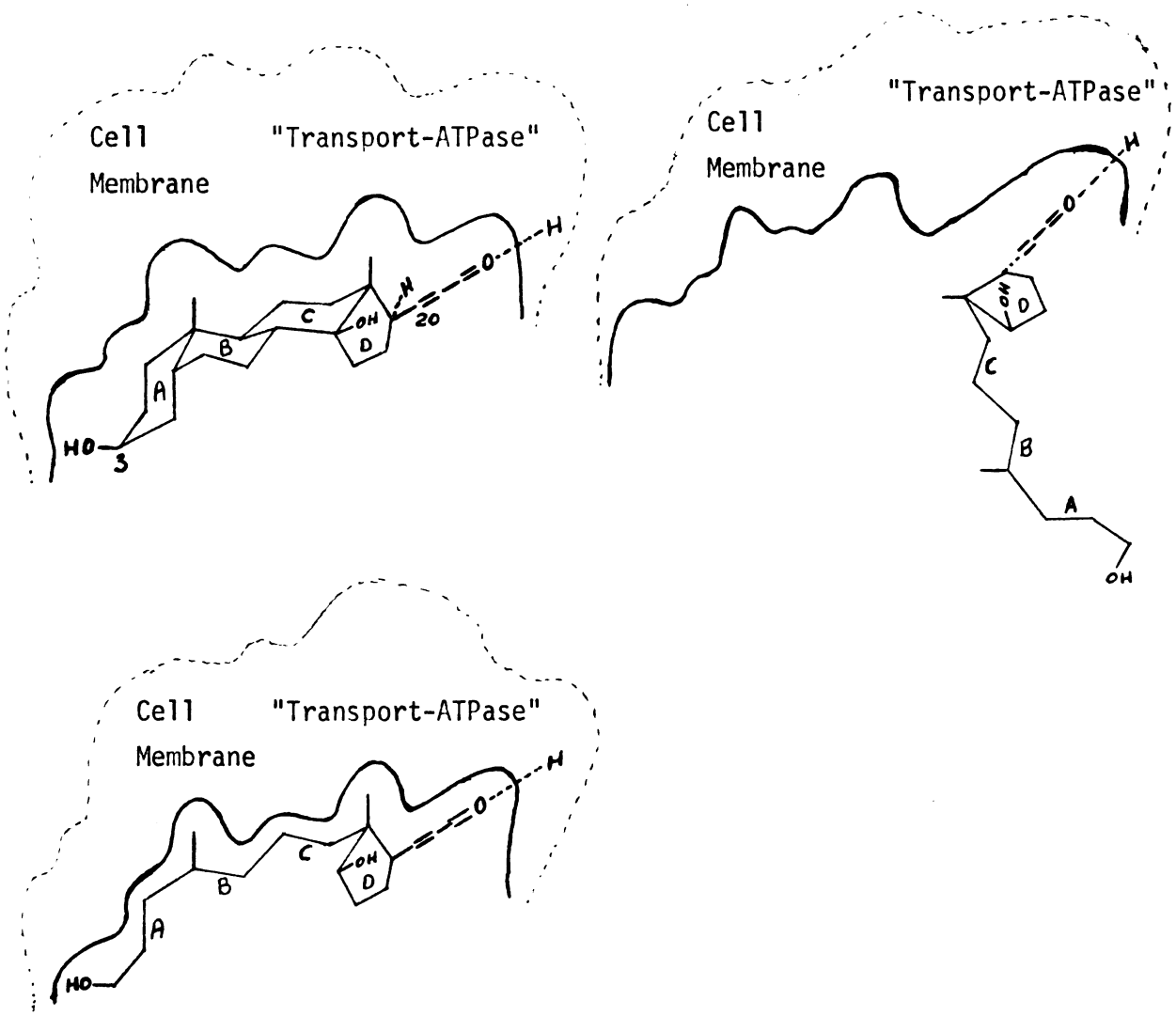
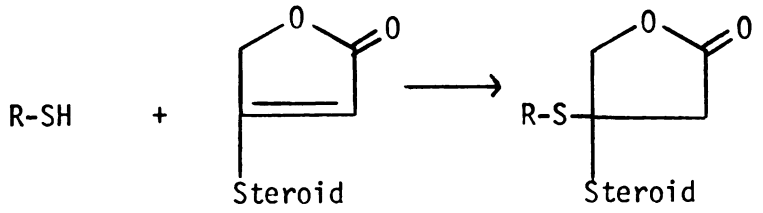


Fig. 2. Schematic representation of hypothetical binding of digitoxigenin and 17β-H-digitoxigenin to "transport-ATPase."

A second hypothesis proposed by Glynn,[17] postulates that the unsaturated lactone ring of cardiac glycosides may interact with essential sulfhydryl groups at the receptor site through covalent bond formation.



This suggestion has been investigated by Wolff and Chang[19] who prepared a series of compounds in which the steroid nuclear features of the natural cardiac aglycones are retained, but their lactone rings are replaced by halo-acetate residues which are considered to be specific inhibitors of enzyme -SH groups by virtue of their ability to alkylate such groups. The degree of cardiac toxicity (cat lethality) displayed by these modified aglycones lends support to the above line of thought.

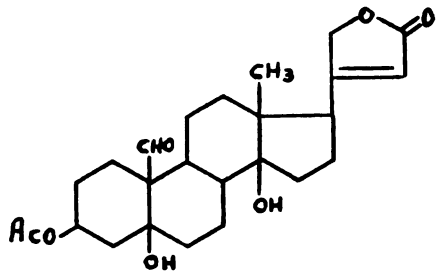
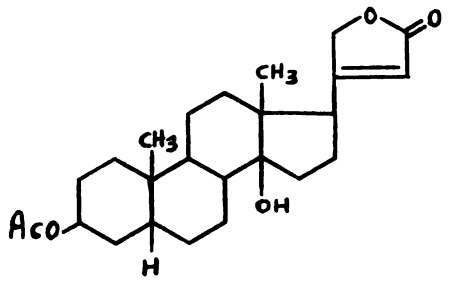
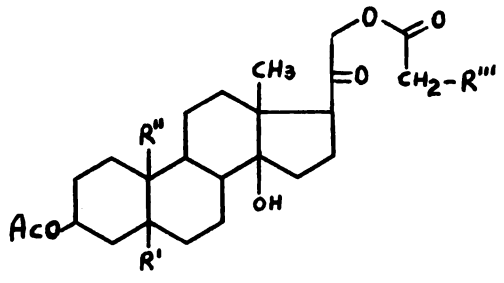
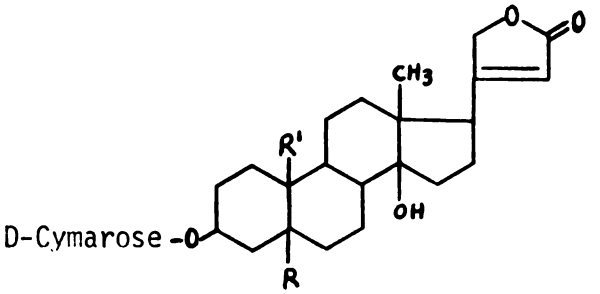
Compound	LD (Cat) (mg/Kg)
	0.08
	0.56
	
a) R' = OH; R'' = CH_2OAc; R''' = I	0.96
b) R' = OH; R'' = CH_2OAc; R''' = Cl	2.15
c) R' = H ; R'' = CH_3; R''' = I	1.71
d) R' = H ; R'' = CH_3; R''' = Cl	3.8

Table 2. Comparison of Cardiotoxic Potencies of Natural and Synthetic Aglycones.[19]

Introduction of additional hydroxyl groups into the steroid nucleus of the aglycone moeity has a marked effect on activity. The activity may be increased or decreased depending on the configuration and location of the hydroxyl groups.

Thus, the presence of either an 11α or 12β hydroxyl group, both of which have the equatorial orientation, enhance the activity; whereas the presence of a 1β, 6β, or 11β hydroxyl, all of which are axially oriented, results in a decrease in activity.[7] The change of direction in the activity in going from the equatorial to the axial hydroxyl appears to point to an steric factor as a possible cause for the effect on the activity. Introduction of a 5β-hydroxyl group enhances the activity. This group is axial with respect to ring A, but equatorial with respect to ring B.

The presence of an aldehyde function at C-19 increases the activity, and reduction of this group to the hydroxymethyl derivative results in a further increase in activity. However, oxidation of the C-19 aldehyde group to the corresponding 19-carboxylic acid results in a substantial decrease in the cardiac activity.[20]



Monoside	R	R'	LD (Cat) (mg/Kg)	Lit.Ref.
Somalin	H	CH_3	0.288	21
Periplocymarin	OH	CH_3	0.154	22
Cymarin	OH	CHO	0.110	23
Cymarol	OH	CH_2OH	0.099	24

Table 3. Effect of C-5 and C-19 Oxygenation on the Biological Activity of Cardiac Glycosides.

From the foregoing discussion it is quite evident that the nature of the substituent at the C-19 of the aglycone moeity has a profound effect on the cardiotoxic properties of cardiac glycosides. Some synthetic mofidications of the C-19 substituent have been studied by several investigators. Chen and Anderson[24] studied the activity of methyl cymarylate and strophanthidol-3,19-diacetate and found them to be inactive. Lingner et al.[25] prepared several derivatives of C-19 aldehydes and alcohols, including such derivatives as acetals, ethers, oximes and urethanes. Cat toxicity studies of these compounds showed that the oximes are quite active, whereas most of the other derivatives while preserving the pharmacological activity were not as potent. Wolff and Ho[14] prepared a series of C-19-halocardenolides and found that these compounds also retained the pharmacological activity.

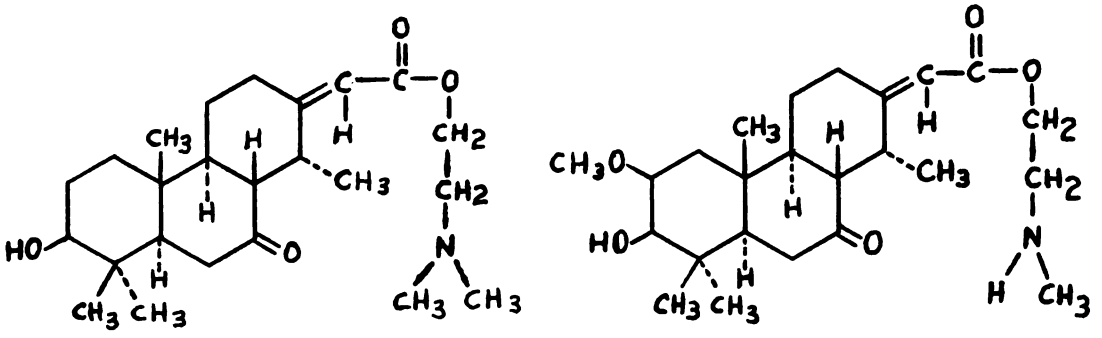
C. Inotropic and Cardiotoxic Effects of non-Digitalis Substances

The presence of the α,β-unsaturated lactone ring in the cardiac aglycones has been considered indispensable for cardiotonic activity.[20] However, the mere presence of such a group within a molecule does not necessarily indicate that the compound will have cardiotonic activity. The 17β-H cardenolides possess the same α,β-unsaturated lactone ring and yet they are totally inactive. As stated by Glynn,[17] the lactone ring is not active unless it is attached to an appropriate group, and in the cardiac glycosides such an appropriate group is represented by a steroid nucleus of specific stereochemistry. An additional requirement for the lactone to be active is that it be linked in a specific fashion to the appropriate group. Obviously, the inactive 17β-H cardenolides possess the appropriate group for attachment of the lactone ring, but do not fulfill the steric requirements.

A group of naturally occurring substances known as the erythrophleum alkaloids are of particular interest because of their digitalis-like action upon the isolated mammalian heart. One of these substances, erythrophleine, was first isolated from the bark of Erythrophleum guineense in 1875-1876 by Gallois and Hardy[26] who described its digitalis-like action on the heart of the frog. Since then, several other related alkaloids have been isolated from the bark of Erythrophleum guineense and Erythrophleum couminga. The digitalis-like cardiotonic activity of these alkaloids has been later confirmed by many investigators, and accumulating evidence appears to indicate that the erythrophleum alkaloids and the cardiac glycosides (digitalis) produce their characteristic cardiac effects by interaction with the same or similar receptor sites.

Besides their cardiac action, the erythrophleum alkaloids possess potent local anesthetic effects,[27] but the risk of cardiac toxicity has prevented their use in medicine.

The structures[28] of two members of the erythrophleum alkaloids are shown in figure 3.



Cassaine Erythrophleine

Fig. 3 Representative examples of Erythrophleum Alkaloids

Chemically, the erythrophleum alkaloids are esters of dimethyl- or methylaminoethanol with tricyclic diterpenoid acids. The presence of a double bond in conjugation with the carbonyl ester is reminiscent of the double bond in the lactone ring of the cardiac aglycones. Hydrolysis of the esters leads to complete loss of activity.[29] Presumably the methyl or dimethylaminoethanol residues serve a function similar to the sugar residues of the cardiac glycosides, i.e., increased solubility in body fluids, increased and better distribution to tissues of target organ; however, this function appears to be of greater importance in the erythrophleum alkaloids.

Chen and coworkers[30] have shown that erythrophleine sulfate when administered to cats by intravenous infusion, produced electrocardiographic changes similar to those produced by the cardiac glycosides; and that toxicity-wise (cat lethality), the order of activity is very similar to the digitalis glycosides. Further studies by the same authors[27] showed that among the erythrophleum alkaloids, coumingine is the most potent member, followed by erythrophleine, coumingaine, norcassaidine, cassaine, homophleine, and acetylcassaine.

Maling and Krayer[29] studied the effects of several erythrophleum alkaloids on the heart-lung preparation of the dog and found that all had a positive inotropic effect in doses of the same order of magnitude as the digitalis glycosides.

Further evidence that the erythrophleum alkaloids may act at similar receptor sites as the digitalis glycosides comes from the observation by Bonting et al.[31] that erythrophleine, like ouabain, produced the same inhibitory effect on "transport-ATPase".

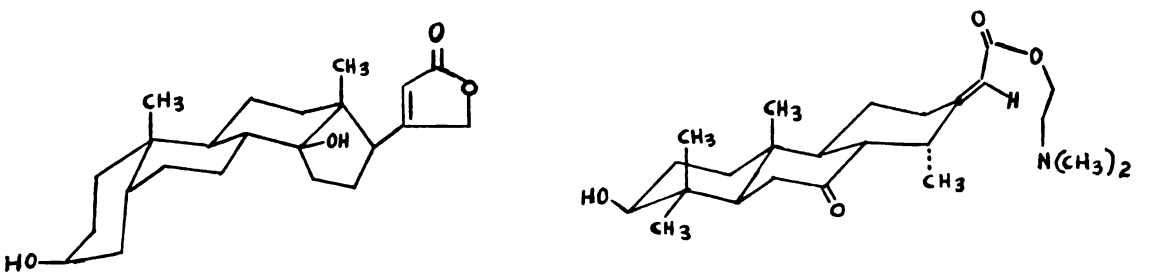
The parallel pharmacological effects shown by both the digitalis glycosides and the erythrophleum alkaloids have prompted many investigators to search for structural similarities which may account for such parallelism in activity.

The importance of the lactone ring as the "active center" of the aglycone molecule has been emphasized by most researchers in the field. That the steroid portion of the molecule is also important, is clearly shown by the profound effects on activity produced by relatively minor changes on this portion of the molecule.

The erythrophleum alkaloids possess neither a lactone ring nor a steroid nucleus. However, the presence of a carbonyl function in conjugation with a C = C double bond in the side chain of cassaine corresponds to the α,β-unsaturated carbonyl of the lactone ring in cardiac aglycones. As is well known, saturation of the double bond results in considerable loss of cardiotonic activity in both types of compounds,[16,32] and the inhibitory effect of the two dihydro-derivatives on "transport-ATPase" is also reduced several fold.

Based on the above observations, Portius and Repke[18] have postulated that the "effective grouping" (Wirkgruppierung) in the digitalis and erythrophleum compounds is embodied in the conjugated carbonyl system.

The supporting group in the erythrophleum alkaloids, a tricyclic diterpenoid nucleus, at first glance resembles the steroid group of the cardiac aglycones; however, analysis of molecular models show that they are stereochemically different.



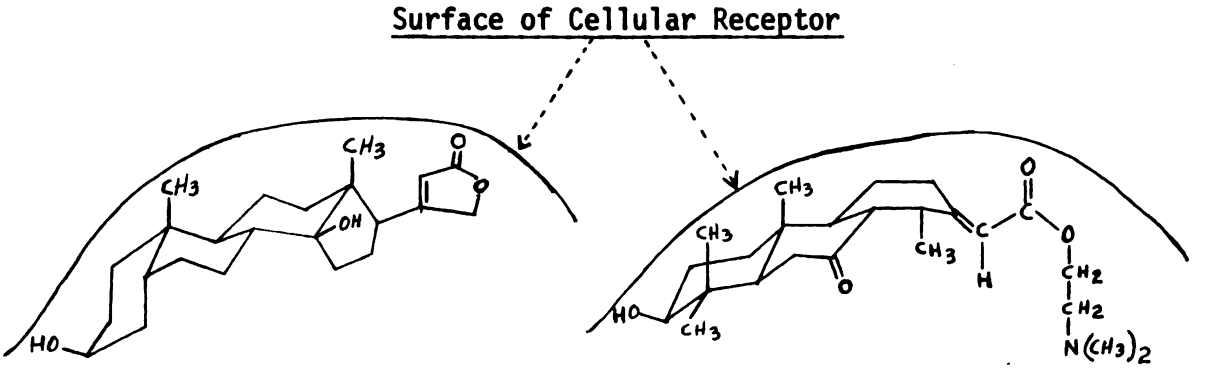
Digitoxigenin Cassaine

Fig. 4 Stereochemistry of representative members of the Cardiac Aglycones and Erythrophleum Alkaloids

Conformational analysis shows that in the cardenolide compounds the overall shape of the steroid nucleus is very much fixed and stabilized by

the type of ring fusion, and that although ring A is capable of minor conformational changes, such changes would not be sufficient to alter the shape of the critical portion of the molecule. This would lend support to the proposal by Wolff and Ho[14] that ring A is not in contact with the cellular receptor.

On the other hand, the tricyclic diterpenoid nucleus of the erythrophleum alkaloids can undergo facile conformational changes at both ends of the ring system so that at one time or another, this supporting group may assume a shape which more nearly resembles the complementary surface of the cellular receptor. The best "functional shape" assumed by the diterpenoid nucleus need not correspond exactly to the shape of the steroid nucleus, although the similarities become more striking when one examines the effects of such conformational changes as shown in Fig. 5.



Digitoxigenin Cassaine

Fig. 5 Effects of conformational changes on the overall shape of Cassaine. The ring bearing the α,β-unsaturated carbonyl function has been flipped to the boat form.

The importance for cardiac action of the conjugated carbonyl function, common to the digitalis glycosides and erythrophleum alkaloids, has prompted several groups of investigators to search for other cardiotonic substances possessing these structural features.

A Preliminary search for digitalis-like action among simple unsaturated lactones was undertaken by Chen and Anderson[30] who were unable to demonstrate any activity among the compounds tested. However, subsequent re-evaluation by Chen et al.[33] showed that β,γ-angelica lactone, the lactone of 21-hydroxy-$\Delta^{20,22}$-norcholenic acid, methyl coumalate, and ethyl coumalate caused systolic standstill on the frog's ventricle when injected in adequate doses into the abdominal lymph sac, but the typical electrocardiographic changes produced by the cardiac aglycones could not be demonstrated when these lactones were bioassayed by the cat method.

Krayer[34] demonstrated that angelica lactone improved the performance of the heart which had been made hypodynamic by perfusion with low-calcium salt solution.

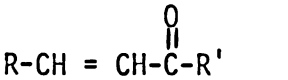
It has been reported that both l- and d-ascorbic acids, which can be considered as α,β-unsaturated lactones, had similar action upon the isolated frog heart as the angelica lactones.[35] Both ascorbic acids when administered to the isolated frog heart in concentrations of 1:10,000 led to an increase in the height of contraction which progressed to irreversible systolic standstill within one to two hours. However, the same authors[35] concluded that the effects observed were not caused by the ascorbic acids themselves nor by their products of dehydrogenation, but that instead the effects could be accounted for by formation of hydrogen peroxide during the process of dehydrogenation

of ascorbic acid solutions. The effects on the heart were absent when the ascorbic acid solutions were adequately protected against dehydrogenation.

Mendez[36] studied the effects of t-butyl hydrogen peroxide on the isolated frog's heart and noted that the effects were comparable to those shown by the angelica lactones. He extended this study to the angelica lactones based on the same approach, and concluded that the effects of such substances on the isolated frog's heart was also ascribable to the formation of peroxides in the test solutions. Potentiation of the cardiotonic activity of several lactones by hydrogen peroxide has been reported.[37]

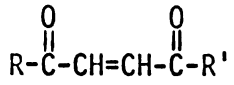
Giarman[37,38] studied the cardiotonic and cardiotoxic properties of several lactone antibiotics and synthetic analogs on the isolated frog heart. The simple unsaturated lactones displayed varying degrees of cardiotonic and cardiotoxic activities. Substitution with alkyl groups diminished the cardiotonic activity, while saturation of the double bond abolished both cardiotonic and cardiotoxic activity.

Bennett et al.[39] compared the effects of simple, open-chain conjugated carbonyl compounds on the isolated hypodynamic papillary muscle of the cat, and found them to possess positive inotropic activity. The basic chemical nucleus necessary for activity was postulated as a carbonyl group in conjugation with a double bond:



This type of structure corresponds to the α,β-unsaturated carbonyl in the lactone ring of cardiac aglycones, and more closely, to the side chain of the erythrophleum alkaloids.

Compounds with extended conjugation of the double bond system,



where R and R' are both methyl groups gave the most active compound of the series. Considerable activity was shown by compounds having the general structure $X\text{-}CH_2\text{-}CH{=}CH\text{-}\overset{O}{\overset{\|}{C}}\text{-}R$, where X = F, Br, I and R = OH or OCH_3. Activity was retained in all structures if the C=C double bond was substituted by an acetylenic bond, but saturation of the double bond had an adverse effect.

D. On The Mechanism of Action of Cardiac Glycosides

The precise mechanism by which the cardiac glycosides and related cardiotonic substances produce their characteristic effects on cardiac muscle is not known.

This aspect of drug action has been the subject of a great deal of research in the past few years, and although very suggestive evidence has led to a number of interesting hypotheses, the matter is in no way settled.

The present trend in theory was probably initiated by Schatzmann[40] who showed that the active movements of sodium and potassium in red cells were prevented by low concentrations of strophanthin. This finding led many investigators to focus their attention on the active transport of sodium and potassium across cell membranes, and since then (1953) the effects of cardiac glycosides on the movement of sodium and potassium, and to some extent calcium, have been studied in a great variety of tissues,[41] including such tissues as skeletal muscle, cardiac muscle, intestinal and uterine smooth muscle, nerve axon, mouse ascites tumor cells, lens, frog skin, kidney, gall bladder, salivary gland and the alga Nitella.

Similar and consistent observations derived from studies of the tissues cited above, has led to the conclusion that the main effects of cardiac glycosides on ion movements are on the active uptake of potassium and the active extrusion of sodium.[41]

Since the movement of potassium and sodium across red cell membranes appears to occur by two pathways, one active and one passive, it was interesting to note that the cardiac glycosides had no effect on the passive movement of ions, only the active pathway was inhibited by

very low concentrations of these agents.[41,42]

It is known that the active transport of sodium and potassium across cell membranes is intimately associated with the splitting of adenosinetriphosphate (Revs. by Glynn[41] and Skou[43]) by an enzyme system localized on or near the surface of the cell membrane, and that this enzyme system is selectively activated by sodium and potassium ions. From studies carried out in many tissue cells, it is now well established that the active movement of potassium and sodium ions indeed involves the splitting of ATP by Na^+ - K^+-activated ATPase systems, and that ion transport is effectively inhibited by the action of cardiac glycosides on such ATPase systems.

Extension of these studies to the erythrophleum alkaloids have shown that these compounds also are able to inhibit ion movements in red cells[44,45] and inhibit the activity of Na^+ - K^+-activated ATPase systems prepared from several tissue sources.[31]

The close correlation between cardiotonic activity and Na^+ - K^+-ATPase inhibition by digitalis glycosides and erythrophleum alkaloids, and the fact that ATPase inhibition by both types of compounds is partially reversed by increasing the external potassium concentration has led to the suggestion that "Transport ATPase" is the receptor for both types of compounds.[9,18] Exactly how the cardiac glycosides interact with Na^+ - K^+-ATPase and how the ensuing inhibition leads to a positive inotropic effect is not clear.

Matsui and Schwartz[46] recently studied the specific binding of digoxin to Na^+ - K^+-ATPase from cardiac tissue by the use of $[^3H]$ - digoxin, and proposed a mechanism for the inhibition of "Transport-ATPase" by cardiac glycosides. The binding required ATP and Mg^{++}

and was stimulated by Na^+ and depressed by K^+. Addition of active cardiac glycosides significantly reduced the binding of $[^3H]$-digoxin, while inactive ones had no effect. When ATP was substituted by acetyl phosphate, binding was also observed, but in this case Na^+ had no stimulatory effect, while potassium still depressed the binding. Since ATP or acetyl phosphate was required for binding, it was suggested that digoxin binds with a phosphorylated conformation of the enzyme and that digoxin inhibition of the ATPase could then be explained in terms of the stability of the resulting digoxin-phosphorylated enzyme complex. This suggestion is best summarized by the following diagram taken from the work of the authors cited above.[46]

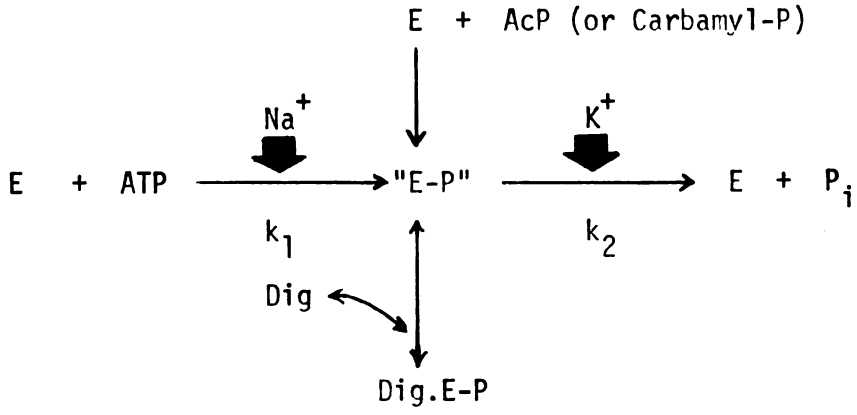


Fig. 6 Inhibition mechanism of a (Na^+ - K^+)-ATPase by cardiac glycoside. E, enzyme; E-P, phosphorylated intermediate of the enzyme; Dig., digoxin; Dig.E-P, digoxin-phosphorylated enzyme complex; k_1 and k_2 represent velocity constants of Na-accelerated phosphorylation step and K-accelerated dephosphorylation step, respectively.

Formation of E-P, either in the presence or absence of Na^+ and hydrolysis of E-P in the presence of K^+, represent the (Na^+ - K^+)-dependent ATPase system. Digoxin presumably binds to E-P to form a relatively stable complex. Matsui and Schwartz further suggested that the binding site for the glycoside appears to be neither the binding site for Na^+ nor for K^+ and furthermore, the Dig.E-P complex must be relatively stable in order to explain the glycoside-induced inhibition of the (Na^+ - K^+)-ATPase. They concluded that the cardiac glycoside probably acts as an allosteric inhibitor by virtue of stabilizing effects on some intermediary form of the enzyme.

The nature of the binding forces involved are not known, although it has been suggested that covalent bond formation between the lactone ring of the cardiac aglycones and essential sulfhydryl groups in the enzyme may be involved.

Kupchan et al.[47] demonstrated that strophanthidin-3-iodoacetate irreversibly inhibits the activity of "transport-ATPase" and concluded that this was due to alkylation of the enzyme at the site where cardiotonic steroids bind.

Of particular interest is the observation by Erjavec and Adamic[48] who compared the cardiotonic and cardiotoxic potencies and Na^+ - K^+-ATPase inhibitory activity of a series of cardiac glycosides, and suggested that the action of cardiac glycosides on the movements of Na^+ and K^+ across cell membranes might be more closely related to the toxic actions of these drugs than to their myocardial stimulant action.

A number of other theories concerning the mechanism of action of cardiac glycosides have been suggested, and one that has received

considerable attention in the last few years concerns the effects of these drugs on the calcium of heart muscle. Knowledge of the role of calcium in what is known as the "excitation-contraction coupling" has stimulated much of the work done along this line.

The effects of calcium on the force of contraction of frog heart muscle have been studied by Wilbrandt and Koller[49] who observed that the force of contraction depends on the ratio of $[Ca^{++}]/[Na^{+}]^2$ in the outside solution. This observation has been later confirmed by the work of other investigators[50] who further suggested that the effect is due to the competition of calcium and sodium ions for a common carrier at the surface. When the membrane deporarizes, the calcium carrier moves inward,carrying calcium into the fibers and thus initiating a contraction.

More recently, Niedergerke[51] has shown that strips of frog ventricle that are made to contract either by lowering the external sodium concentration or by inducing depolarization with high external potassium, take up additional amounts of calcium from the medium and that the final increase in tension roughly paralleled the increase in calcium uptake.

It has been suggested[41] that the cardiac glycosides might increase the force of contraction of heart muscle, either by increasing the liberation of "active calcium" at the inner surface of the membrane, or by delaying its inactivation. Exact correlation of the effects of cardiac glycosides and calcium on muscle contractility is made more difficult on account of the role that might be played by large amounts of unexchangeable and bound intracellular calcium.

The direct effect of cardiac glycosides on the contractile proteins of heart muscle has been investigated in an effort to explain their

cardiotonic action. Robb and Mallov[52] showed that cardiac actomyocin fibers contract more rapidly and more forcible in the presence of very low concentrations of cardiac glycosides, and concluded that the cardiotonic action of cardiac glycosides was probably due to this direct effect on the contractile proteins.

The effects of cardiac glycosides on the action potential mechanism has been looked at by some investigators, but no direct correlation has been made in this respect. The effects observed usually have been better explained in terms of net ion fluxes, mainly potassium and sodium.

From what has been said in this section, it is clear that the cardiac glycosides do have a definite effect on ion fluxes across cell membranes, but the relationship between these ion fluxes and the precise mechanism or mechanisms leading to an increase in force of contraction of cardiac muscle remains to be established beyond doubt.

PART III
CHEMISTRY

A. Introduction

Because of the unique stereochemistry of cardiotonic steroids and the type and location, on the steroid nucleus, of functional groups necessary for activity, their synthesis has met with considerable difficulty. Several methods for introducing the α,β-unsaturated lactone ring at the 17β-position of conventional steroids have been developed.[53-63] However, the drastic experimental conditions required by some of these methods, preclude their use when such procedures are applied to 14β-hydroxy steroids, which dehydrate readily to the corresponding Δ^{14}-anhydro derivatives.

Methods for the syntheses of 14β-hydroxy steroids have also been reported.[64-68] While introduction of the lactone ring alone, or synthesis of 14β-hydroxy steroids in the absence of the lactone ring offers no major problems, concomitant introduction into the steroid nucleus of both the lactone ring and the 14β-hydroxyl is not a simple matter. Attempts to introduce both of these functionalities into the same steroid molecule usually lead to isomeric mixtures with very little of the desired product being formed.

As shown in the discussion of the structure-activity relationships of the cardiac glycosides (PART II, Sec. B), the nature of the C-19 substituent has a profound effect on the activity of these compounds, and introduction of a 5β-hydroxyl group leads to a further increase in activity.

Functionalization of the 19-angular methyl group in conventional steroids is of particular importance not only from the standpoint of

cardiac glycoside activity, but also because such functionalization affords useful intermediates for preparation of biologically important steroid hormones.

Prior to development of chemical methods for functionalization of the 19-angular methyl group, the only way of attaining this was by means of enzymatic[69] and microbiological hydroxylations.[70,71] These methods, however, afford very small amounts of C-19 functionalized steroids and are further complicated by additional hydroxylations at other positions of the steroid nucleus.

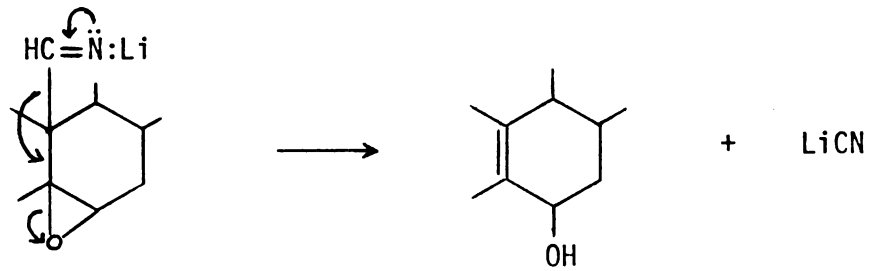
With the advent of synthetic methods in the past few years, C-19 functionalized steroids have become more readily available and introduction of exygen functions on the C-19 methyl group has become less difficult.

Current synthetic approaches utilize selective intramolecular reactions and some of the methods reported include ultraviolet irradiation of 6β-nitrites,[72] ultraviolet irradiation of 6β-hypochlorites,[73,74] lead tetraacetate oxidation of 6β-hydroxy steroids,[75] lead tetra acetate oxidation of 11β-hydroxy steroids in the presence of iodine[76] and ultraviolet irradiation of 11-oxo steroids.[77]

In our efforts to synthesize cardiac aglycones with oxygen functions at both C-5 and C-19, we utilized the 19-oximino-5α-chloro-6β-hydroxy steroid intermediates which have been prepared previously by Wolff et al.[78-80] These intermediates possess functionality at both C-5 and C-19, and are readily available from conventional steroids by ultraviolet irradiation of the 5α-chloro-6β-nitrite intermediates followed by isomerization of the resulting nitroso dimer to the corresponding 19-oximino compounds.

B. Discussion

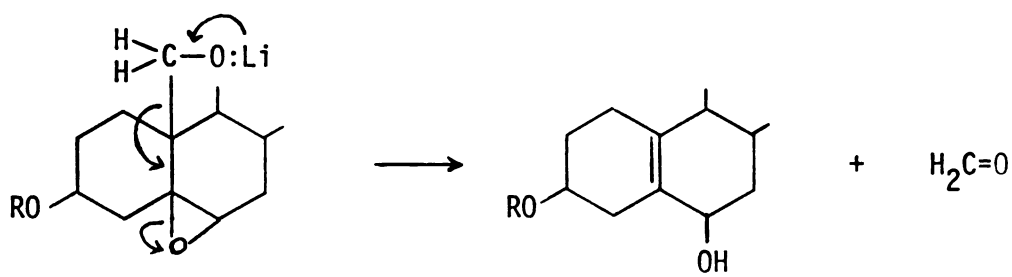
5β,6β-Epoxy steroids, which have the desired 5β-oxygen linkage, are available from 5α-chloro-6β-hydroxy steroids, and in principle it should be possible to reduce a 5β,6β-epoxide to the desired 5β-ol with lithium aluminum hydride.[81] Treatment of the 19-acetate of 1 with alkali gave the epoxide 3 which on lithium aluminum hydride reduction gave the 19-norsteroid 5. Moreover, the nitrile 4, prepared from 1 by successive treatment with hot acetic anhydride and alkali, on treatment with lithium aluminum hydride also gave 5. Presumably in both cases this fragmentation[82] is due to formation of an intermediate imine-metal complex, in which the electron deficiency resulting from ionization of the 5β-bond is neutralized by heterolysis of the 10β-bond, and by donation of the lone pair of electrons on the nitrogen.



Efforts were then made to degrade the oxime group in 1 prior to further work. Treatment of 1 with hydrochloric acid in methanol gave only the acetal 6[83] and none of the intermediate aldehyde. Attempted oxidation of 1 with N-bromoacetamide in ether, followed by saponification of the intermediate acetate 7, gave the cyclic hydroxamic acid derivative 8 instead of the expected 6-keto compound. The acetate 7 was further identified by conversion to its oxime methyl ether derivative 9.

Lee and Wolff[84] reported formation of similar cyclic hydroxamic acids by treatment of 18-oximino-20-hydroxypregnane derivatives with NBA under the same conditions.

Treatment of 1 with sodium nitrite and acetic acid[85] gave predominantly 10 together with some 11. It was not possible to hydrolyze selectively the 19-acetate function in 10 to obtain 11; treatment of 10 with methanolic ammonia at pH 9 gave only the epoxide 13, whereas attempted hydrolysis in 50% acetic acid gave acetals 6 and 16. Formation of 16 probably occurs *via* either 12 or 13; the epoxide ring is opened by the aqueous acid to form a 5α,6β-diol intermediate which then cyclizes. An attempt to reduce directly the hemiacetal acetate 10 to a 19-hydroxyl derivative with lithium aluminum hydride in ether gave mainly the triol 17, presumably *via* either 12 or 13. That the position of the hydroxyl function is 6β rather than 5β was shown by its ease of acetylation. A minor product from the reduction was the fragmentation product 5, which presumably also arose *via* 12 or 13 through loss of formaldehyde.



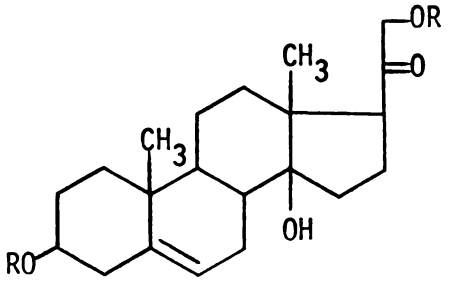
Rowland[86] has shown that 6-oxo-5α-bromo steroids are converted readily to 6-oxo-5β-hydroxy steroids because of a neighboring group effect. Attempts were made to secure a 6-ketone having an aldehyde

function at C-19. Reduction of 11 with sodium borohydride gave 14. In t-butyl alcohol, the reaction of 14 with N-bromosuccinamide, a reagent which normally oxidizes secondary alcohols selectively,[87] gave only 15. Again oxidation of 11 itself with N-bromosuccinamide in t-butyl alcohol gave only 15, whereas in ether no reaction was observed.

Compound 23, embodying the A/B ring system of sarmentosigenin E, ultimately was obtained in the following way. Oxidation of 1 with chromic acid in pyridine solution[88] proceeded with concomitant dehydration of the oxime function[89] to afford 18. Compound 18 was changed readily by alkali to a gelatinous diol, which on benzoylation gave crystalline 20. In harmony with the hindered 5β-configuration of the tertiary hydroxyl group, acetylation of the gelatinous diol even under conditions known to affect tertiary alcohols,[90,91] gave only the monoacetate 19.

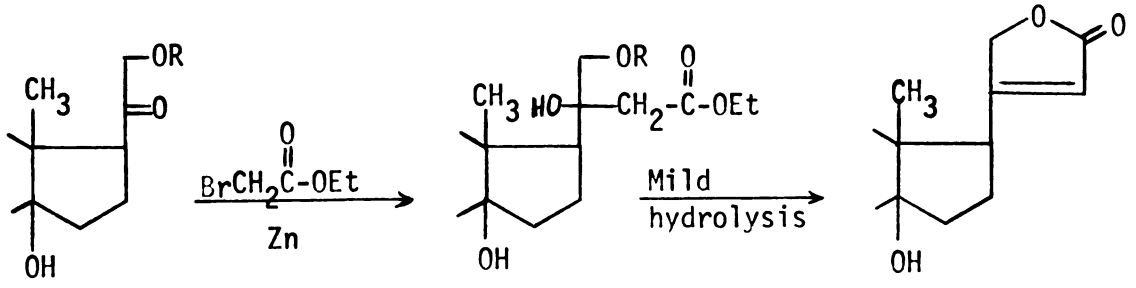
Reduction of 20 with sodium borohydride and subsequent acetylation gave 21. The 6β-configuration of the hydroxyl group in 21 was established by its conversion to 22 via the corresponding imino ether hydrochloride. Hydrolysis of the imino ether hydrochloride for 2 hr in 5% alcoholic potassium hydroxide solution at 27° gave only the diol lactone 23. The lability of the benzoate group is owing to the 1,3-diaxial relationship of the 3β,5β-diol system,[92] and is confirmatory evidence for this stereochemical assignment.

At this time it became necessary to secure a steroid intermediate with a number of desirable structural features in order to ultimately be able to synthesize a cardiac aglycone with oxygen functionalities at both C-5 and C-19. The problem consists essentially in (a) finding an adequate intermediate possessing the appropriate β-side chain at C-17 and a β-hydroxyl group at C-14, (b) concomitant oxygenation at both C-5 and C-19 by the same or a modified reaction sequence as shown for the cholestane series, and (c) construction of the lactone ring at C-17. Ideally, such an intermediate should possess the structural qualities as shown on the model structure given below.

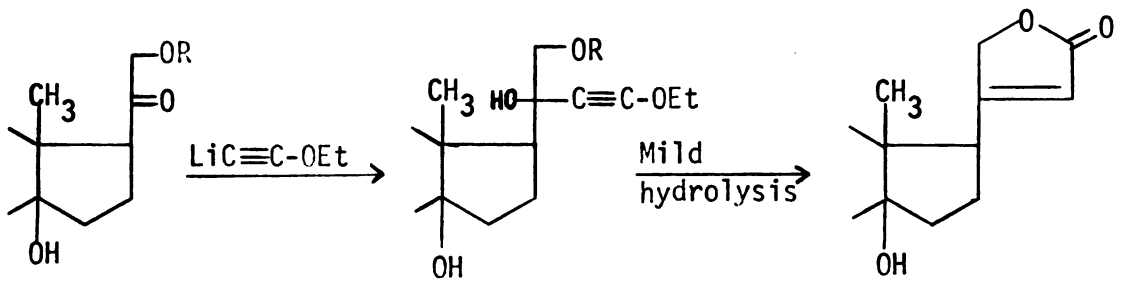


R = Ac

The Δ^5-double bond is essential for the C-5, C-19 functionalization as described for the cholesterol series, and the 17β-chain which in this case corresponds to a 21-hydroxypregnene-20-one, is specially suited for the construction of the lactone ring.



or



In order to obtain the appropriate intermediate, digitoxigenin (24) was degraded in the following way: Oxidation of digitoxigenin with 8 N chromic acid solution[93] afforded the genone 25. Compound 25 was subjected to ozonolysis in ethyl acetate-methylene chloride solution at -72°; the intermediate ozonide was decomposed with zinc dust in glacial acetic acid, and the resulting 21-oxalate ester was hydrolyzed with potassium bicarbonate in aqueous methanol. Recrystallization of the crude 3,20-dione 27 from methanol afforded the 3,3-dimethyl ketal 28 directly. Acetylation of 28 with acetic anhydride in pyridine gave the 21-acetate 29, which was then reduced to the 20-ol 30 with lithium tri-t-butoxyaluminum hydride in tetrahydrofuran. Regeneration of the 3-keto function in 30, followed by saponification of the 21-acetate and subsequent benzoylation gave the dibenzoate 33. Bromination of the ketone 33 in tetrahydrofuran solution at -12° with phenyltrimethylammonium tribromide[94] (PTT) afforded the intermediate bromoketone 34 in very good yield. Dehydrobromination of 34 with benzyltrimethylammonium mesitoate[95] (BTAM) in acetone solution gave the 3-keto-Δ^4-intermediate 35.

In an attempt to obtain a Δ^5-intermediate, 35 was converted to its

dienol acetate 36 with isopropenyl acetate in the presence of catalytic amounts of p-toluenesulfonic acid; this intermediate was then reduced with sodium borohydride in aqueous methanol, followed by benzoylation of the resulting triol (37) to give 38. During the enolization step we were not aware that the 14β-hydroxyl group had been acetylated; however, this became apparent upon the isolation of 38 the nmr spectrum of which showed a resonance peak corresponding to an acetate methyl. The hindered nature of the 14β-acetate ester may explain its resistance to hydrolysis by the alkaline aqueous sodium borohydride solutions. This is in sharp contrast to the readily hydrolyzed benzoate esters at C-20 and C-21 under the same conditions.

Additional proof for the acetylation of the 14β-hydroxyl group was obtained by formation of the 3,14-diacetate 39 obtained by treatment of digitoxigenin (24) with isopropenyl acetate in the presence of p-toluenesulfonic acid.

Prior to this discovery, it was thought possible to modify the A/B ring system of digitoxigenin (24) without disturbing the lactone ring. Bromination of digitoxigenone 25 with phenyltrimethylammonium tribromide[94] (PTT) in tetrahydrofuran afforded the bromoketone 41 without causing bromination of the double bond of the lactone ring. Dehydrobromination of 41 with benzyltrimethylammonium mesitoate[95] (BTAM) in tetrahydrofuran solution gave 42 in very good yield. Because of the poor solubility of the mesitoate salt in acetone, and the prolonged periods of refluxing required to ensure complete reaction, it was decided to use a solvent with a higher boiling point and better solvent properties. Tetrahydrofuran turned out to be a far superior solvent for this purpose, dehydro-

bromination proceeding smoothly and in a shorter time.

We were particularly interested in the 14β-acetate 39 for a number of reasons. First of all, the 14β-hydroxyl group, due to its tertiary nature and the availability of hydrogen atoms on neighboring carbons, is readily lost by dehydration under acidic conditions. Secondly, this is the first time that such group has been made to react to form a derivative other than the dehydration product; and thirdly, we sought in this reaction the possibility of protecting the lability of this hydroxyl group.

Efforts were then made to regenerate digitoxigenin from the diacetate 39 prior to any further work. For this purpose the diacetate 39 was subjected to ammonolysis in methanol solution. TLC of this reaction mixture showed two new spots very close together neither of which corresponded to the starting material, digitoxigenin. With the ammonolysis treatment one could reasonably expect removal of the two acetate residues without much difficulty, but apparently the molecule was modified further. In order to determine the ultimate course of this reaction, digitoxigenin (24) was subjected to the same ammonolysis conditions and again, TLC showed the same two identical spots. Separation of the mixture afforded lactol-amides 43 and 44.

Under the conditions of the reaction, these compounds apparently are formed by isomerization of the lactone double bond from the α,β to the β,γ-position followed by opening of the isomerized lactone to the amido-aldehyde intermediate and subsequent cyclization to the lactol-amides.

Molecular models show that the lactol rings of these compounds could assume the more stable chair conformation and that the most stable isomer

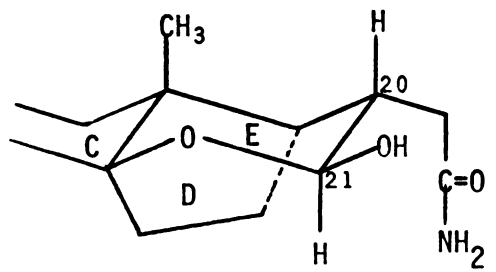
would be the one in which the amide chain at C-20 and the hydroxyl group at C-21 are trans-diequatorial, 43. A second, less stable isomer, would be 44 in which the amide chain at C-20 is still equatorial, but the hydroxyl group is axial. Since in this reaction two new centers of asymmetry are introduced (at C-20 and C-21), four isomers are theoretically possible, but severe repulsive steric interactions presumably prevent formation of the other two isomers. Lactol amide 43 is formed in the highest yield, whereas lactol amide 44 is formed in only very low yield.

That compounds 43 and 44 differ only by the orientation of the hydroxyl group at C-21 was shown by oxidation (CrO_3-pyridine) of either lactol to the same lactone 49. A second product isolated from the oxidation of 43 was the 3-ketolactam 50.

Heating of lactol amide 43 to 200° or treatment with warm glacial acetic acid caused rapid cyclization to the lactam 45. Similar treatment of lactol 44 gave the epimeric lactam 46.

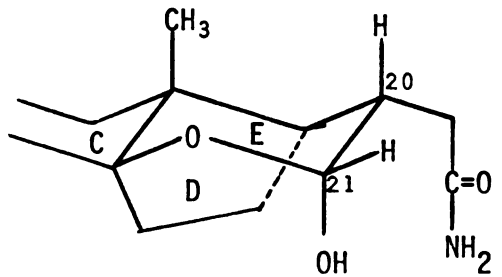
Consideration of the steric factors discussed previously, indicates that each lactol cyclizes in a stereospecific fashion to yield the corresponding lactams. Thus, lactol 43 cyclizes to give lactam 45 in which the lactam ring is trans-fused to the oxygen-containing ring E, and lactol 44 cyclizes to form product 46 in which the lactam ring is cis-fused to the oxygen-containing ring E.

Nmr spectra of the lactol amides show a hydroxyl proton resonance at very low field compatible with the resonance of a H-bonded proton, and this may explain the stereospecific cyclization to the corresponding lactams.



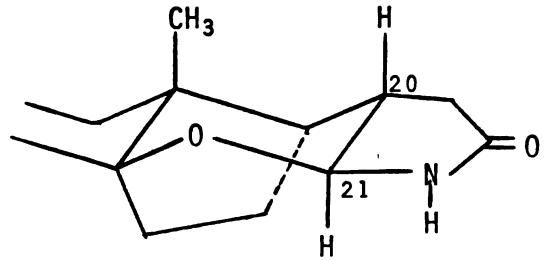
43. (20S,21S)

mp 271-273° (soften at 180-188°)



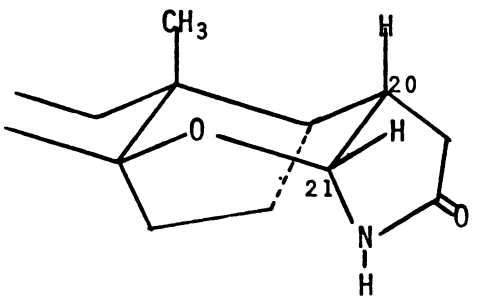
44. (20S,21R)

mp (double) 200-203° and 263-265°



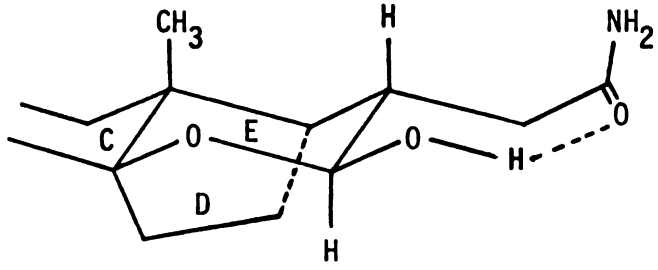
45. (20S,21S)

mp 275-277°



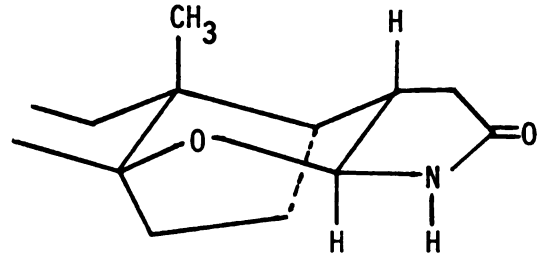
46. (20S,21R)

mp 266-268°



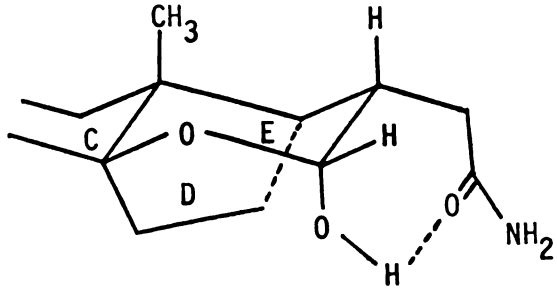
43

Nmr (Pyr.-d_5) δ 1.00 (C-19 methyl), 1.26 (C-18 methyl), 4.36 (3α-H), 4.80 ($-NH_2$), 5.43 (3β-OH), 5.76 (C-21 H), 9.08 (C-21 OH).



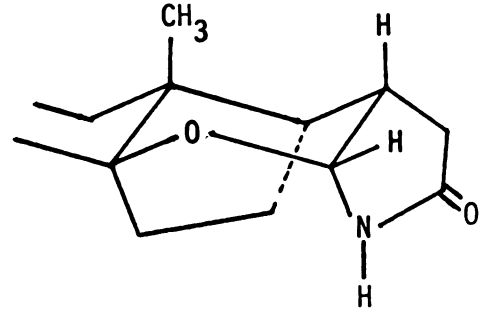
45

Nmr ($CDCl_3$) δ 1.00 (C-18 and -19 methyls), 4.16 (3α-H), 5.45 (J = 6 Hz, C-21 H), 5.96 (=NH).



44

Nmr (Pyr.-d_5) δ 1.00 (C-19 methyl), 1.18 (C-18 methyl), 4.38 (3α-H), 4.83 (C-21 H), 4.86 ($-NH_2$), 5.50 (3β-OH), 9.55 (C-21 OH).



46

Nmr ($CDCl_3$) δ 0.99 (C-19 methyl), 1.13 (C-18 methyl), 4.15 (3α-H), 5.31 (J = 5 Hz, C-21 H), 6.68 (=NH).

Acetylation of each lactam with acetic anhydride in pyridine gave the corresponding 3-acetate esters 47 and 48.

Finally, ammonolysis of digitoxin in methanol solution followed by cyclization in acetic acid gave the lactam trioside 51.

Fig. 7 Reaction Sequence in the Cholestane Series

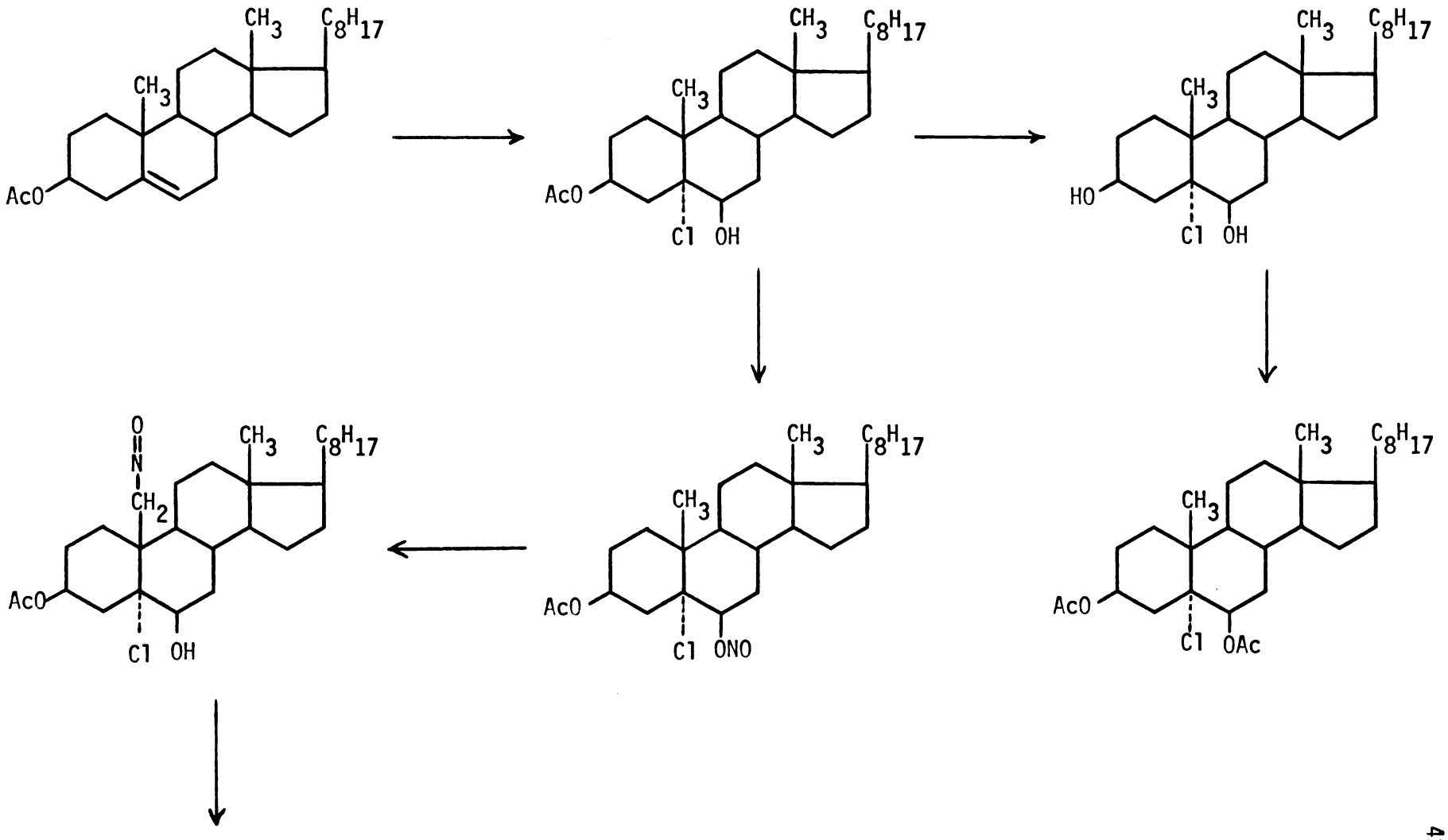
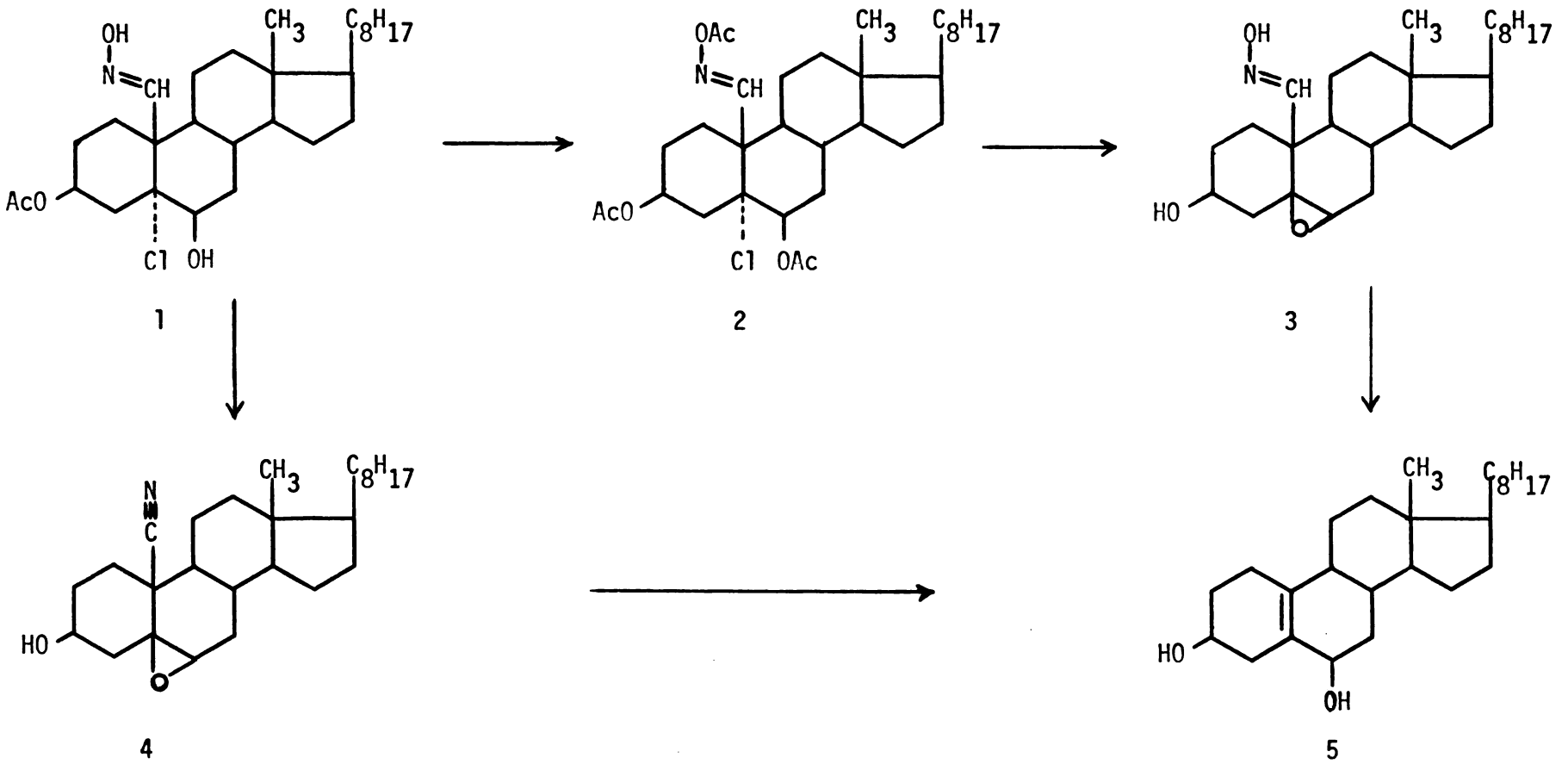


Fig. 7 (continued)



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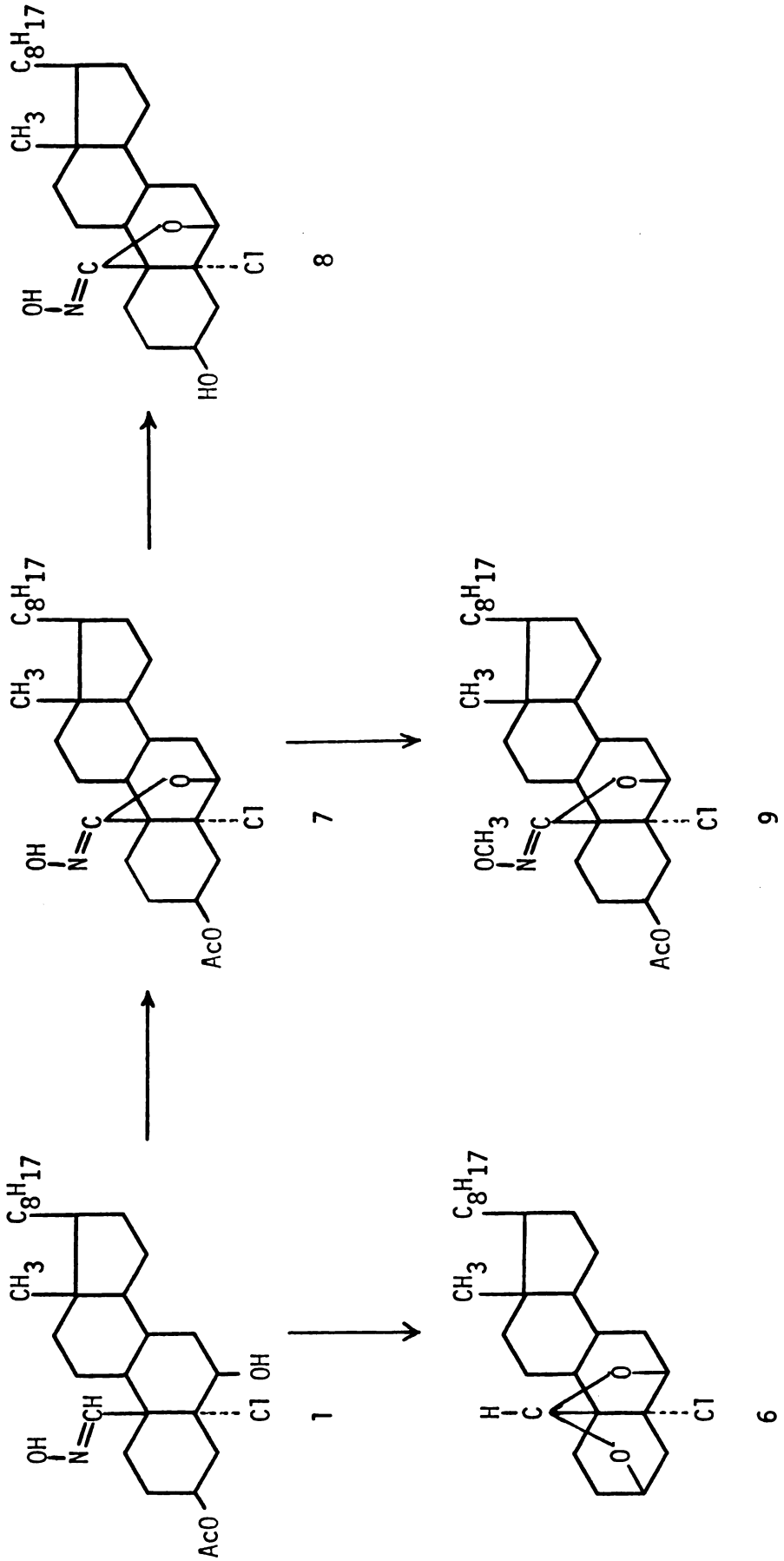

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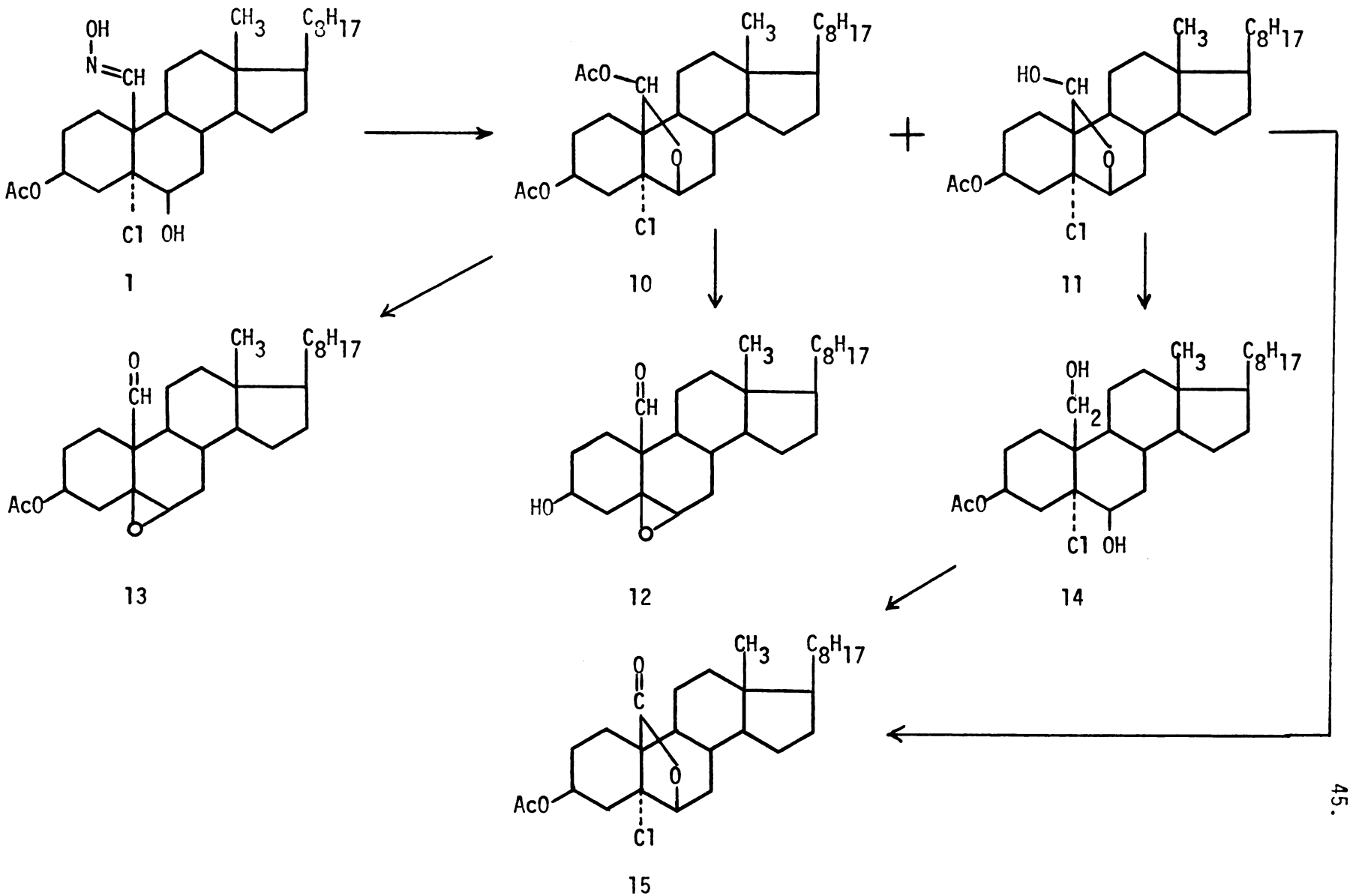

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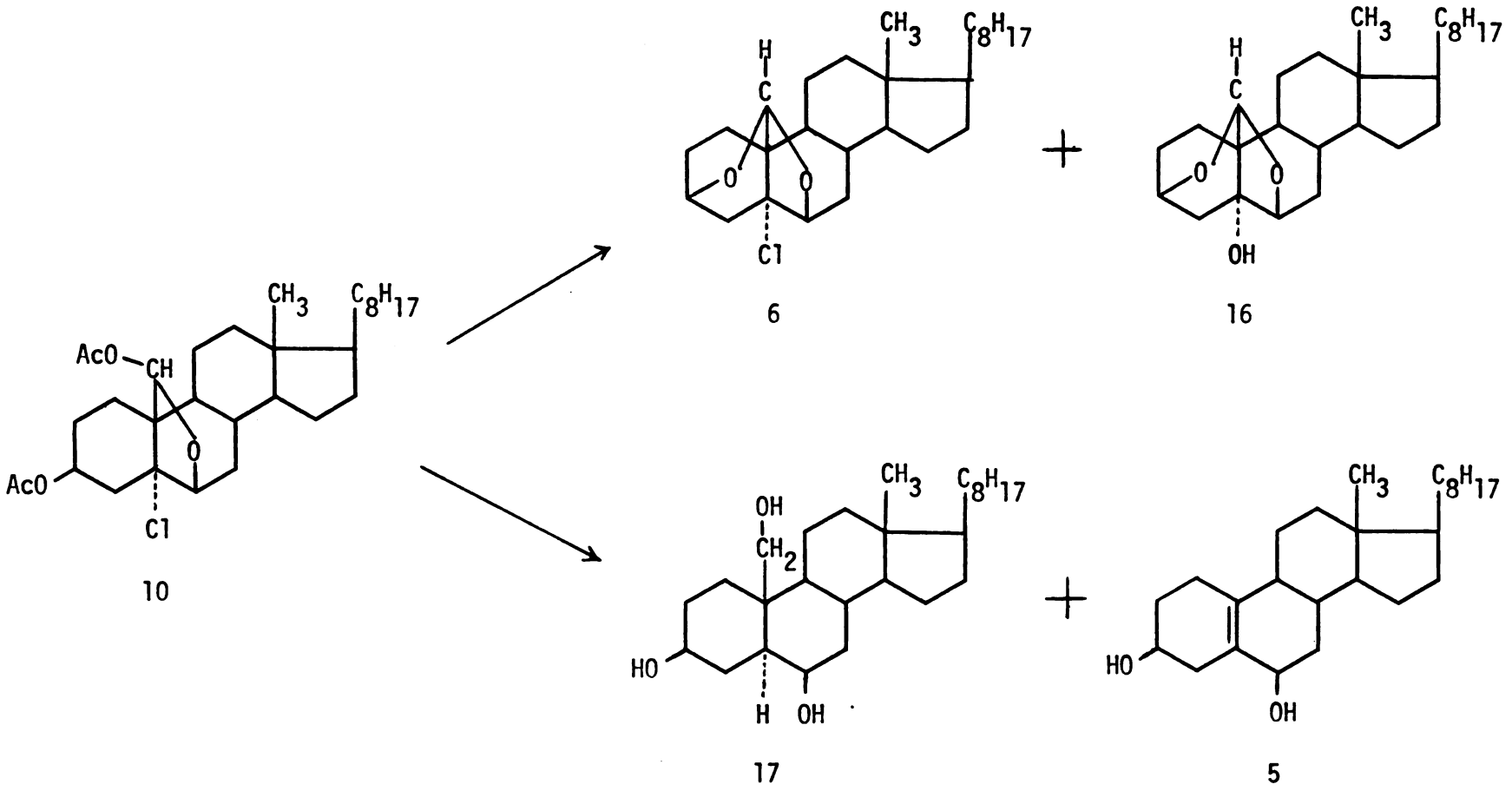

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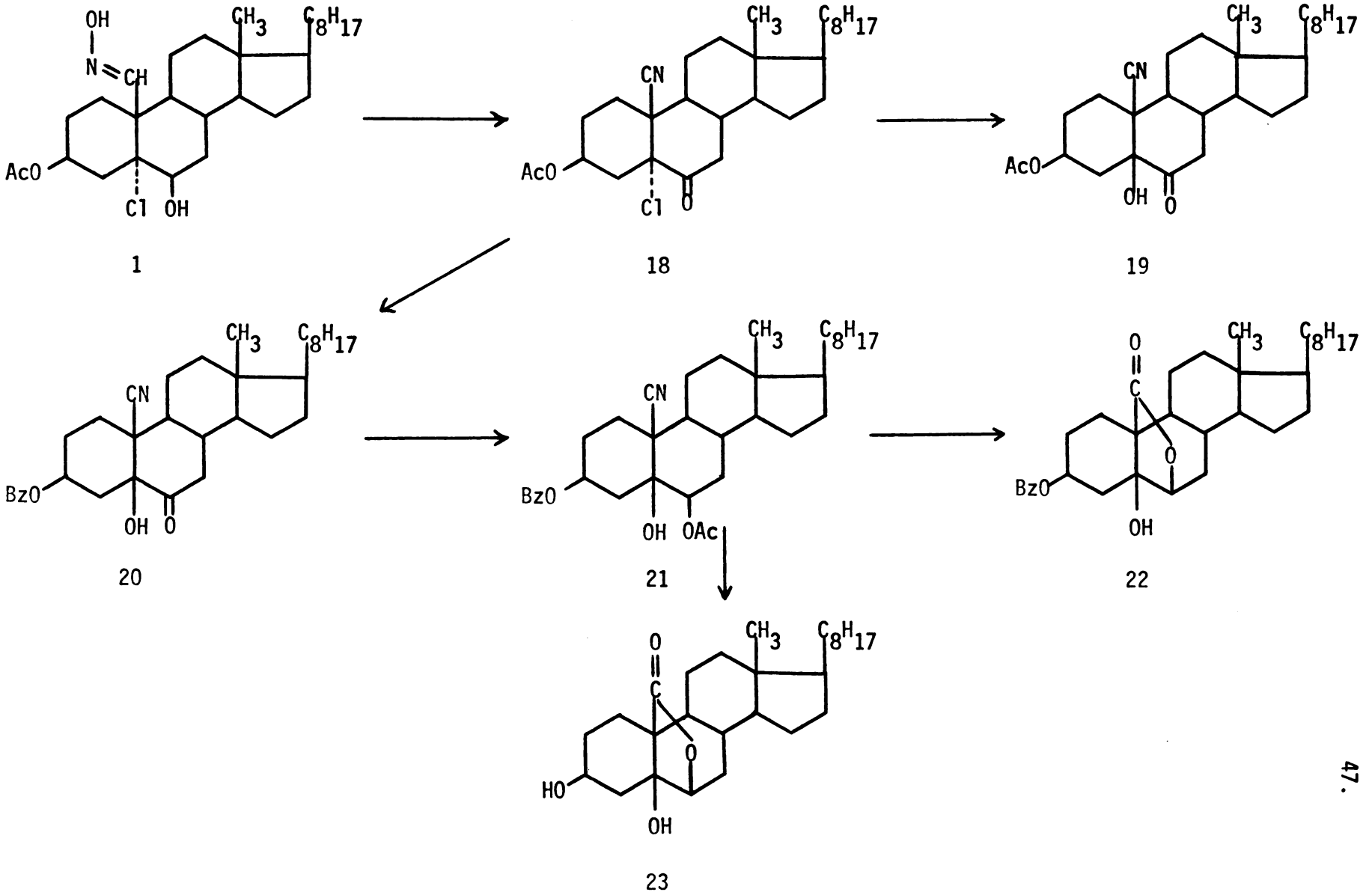
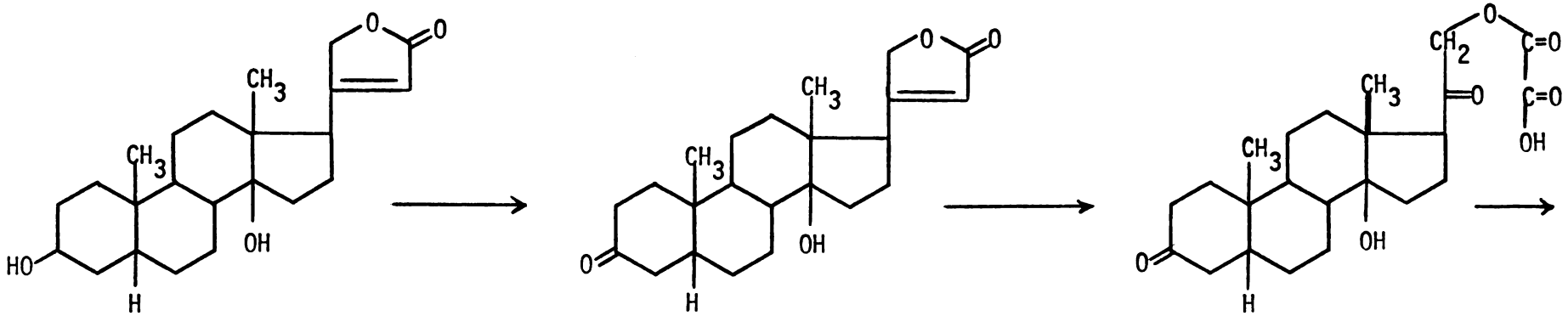
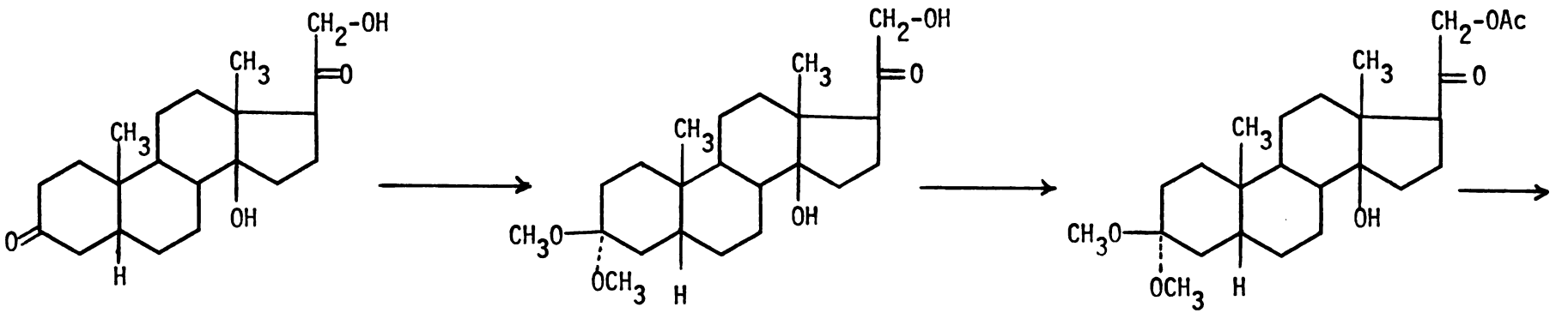


Fig. 8 Reaction Sequence in the Digitoxigenin Series





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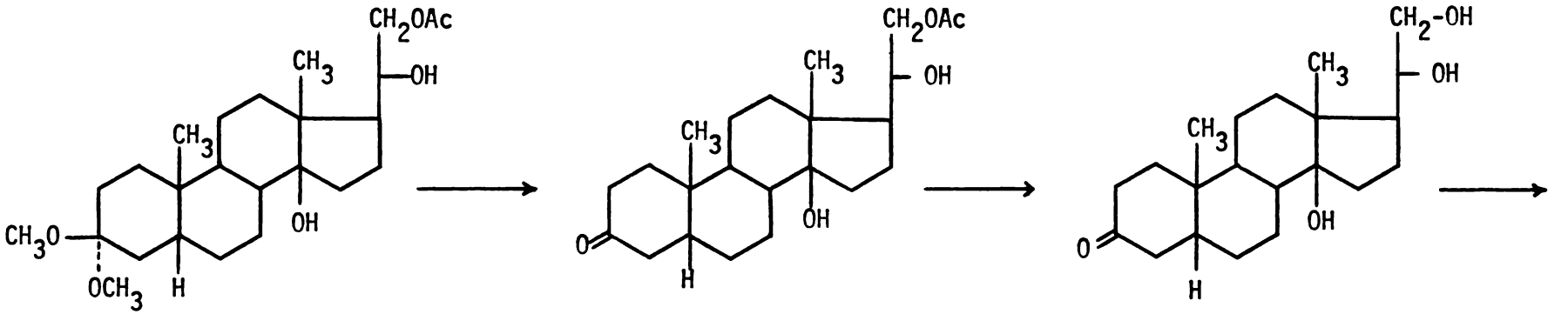

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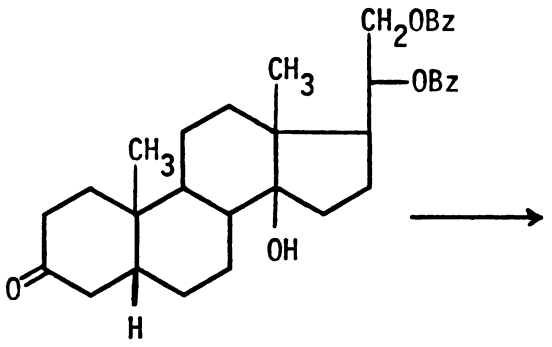

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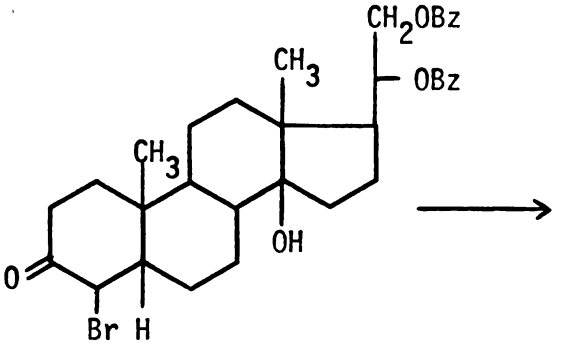

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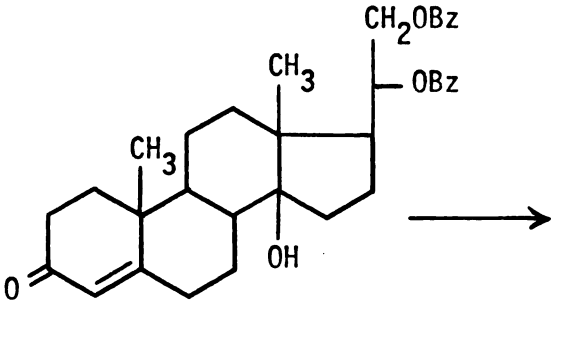

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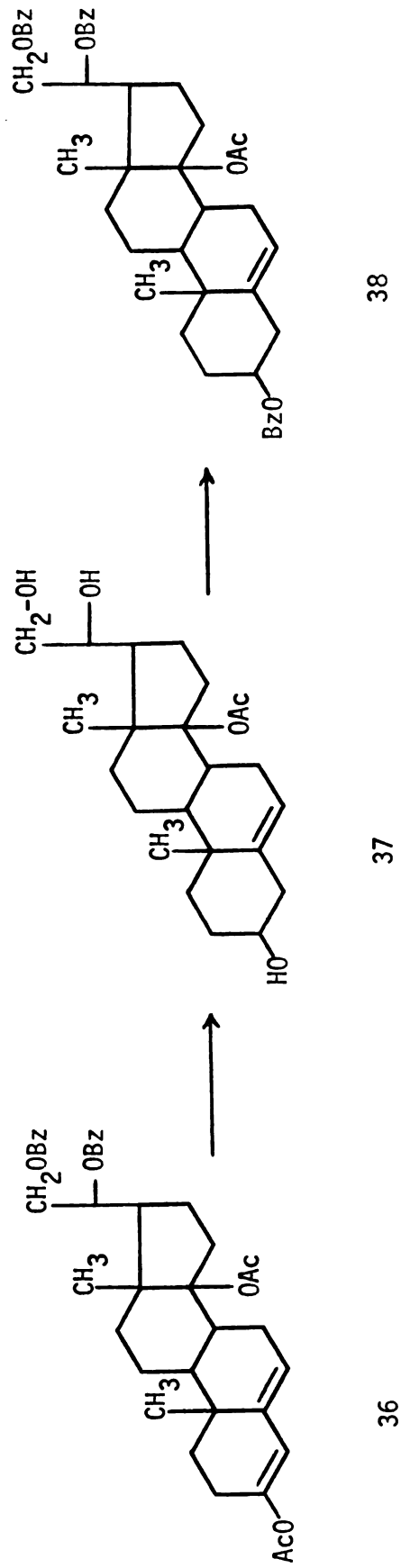

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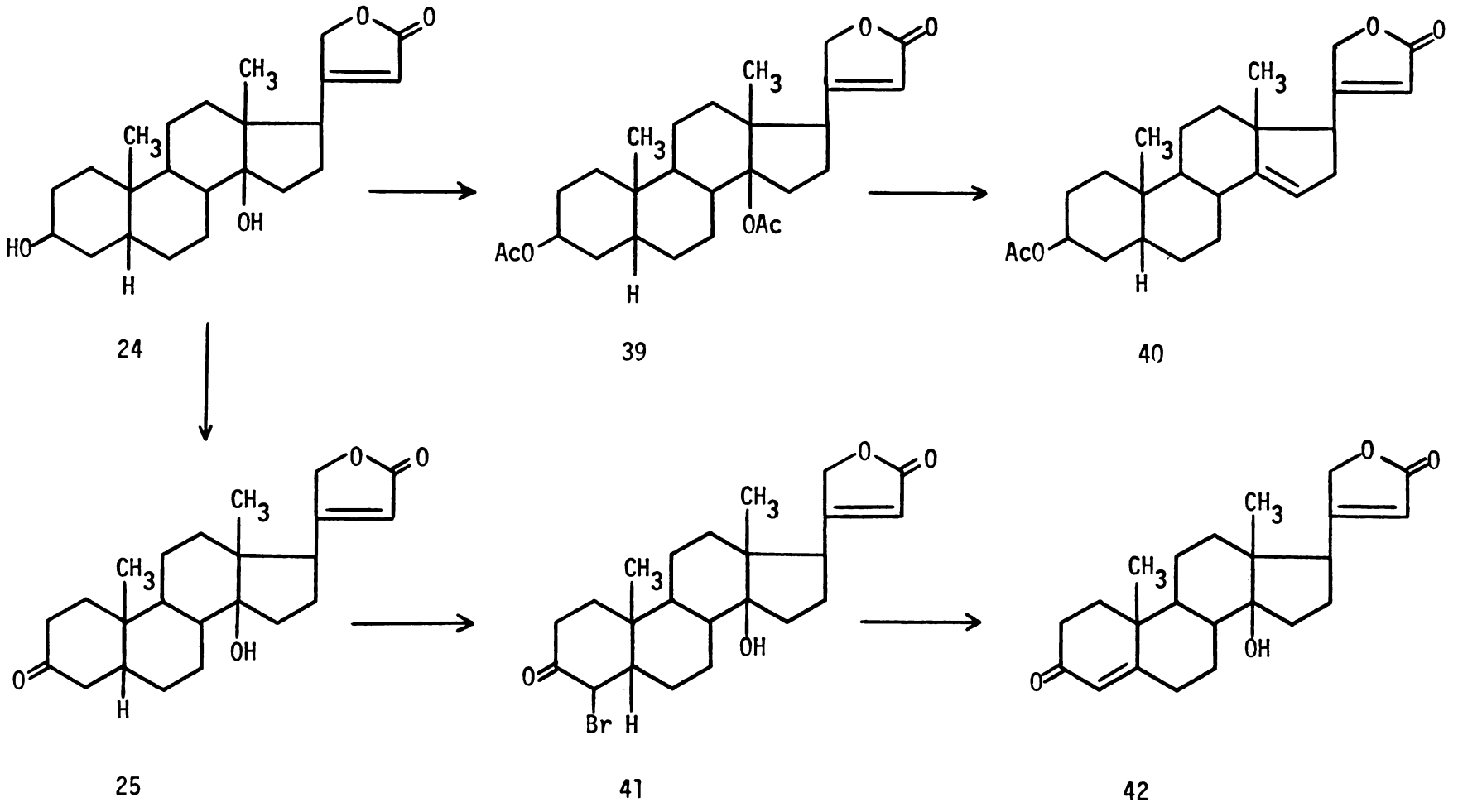

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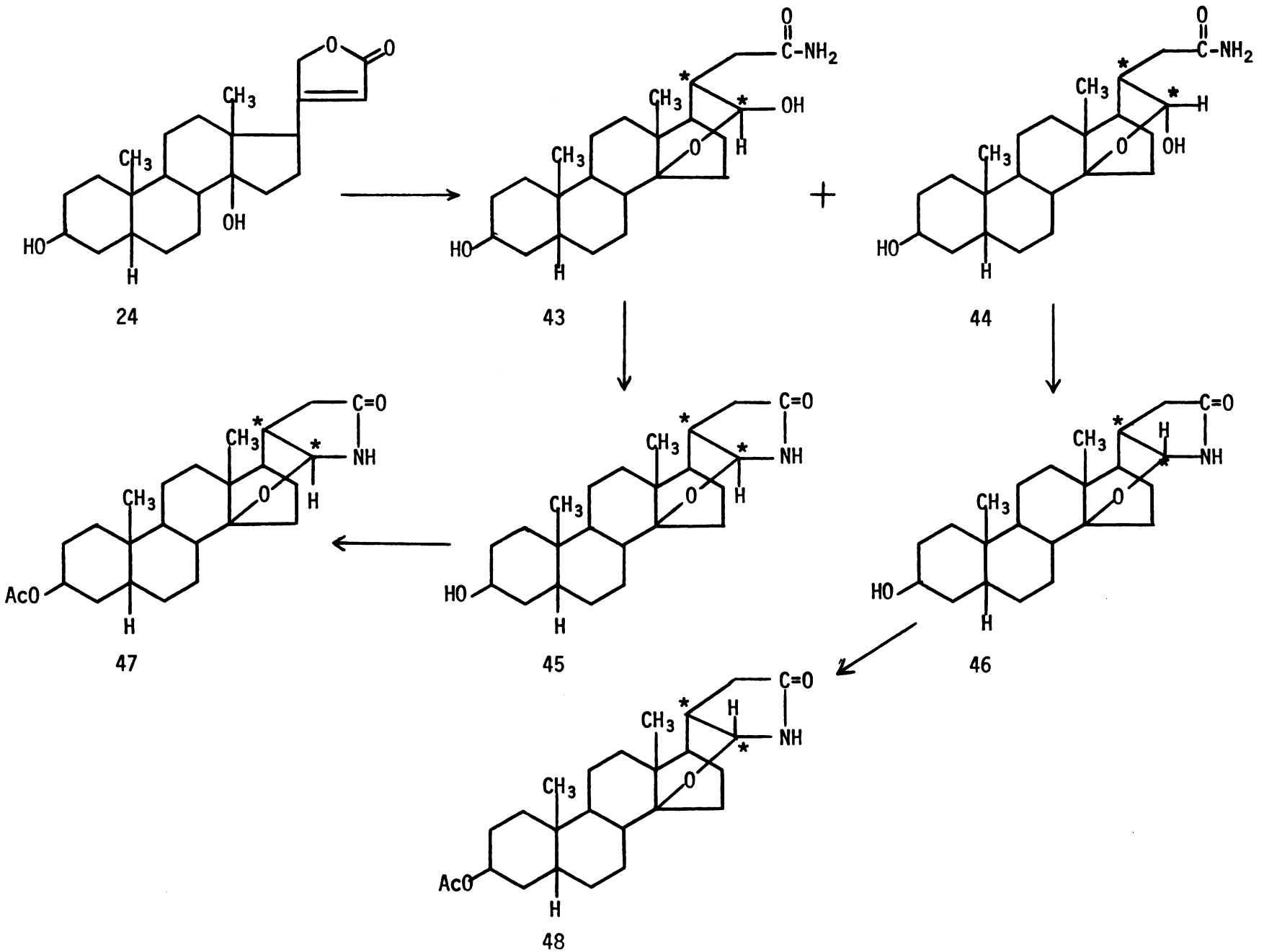

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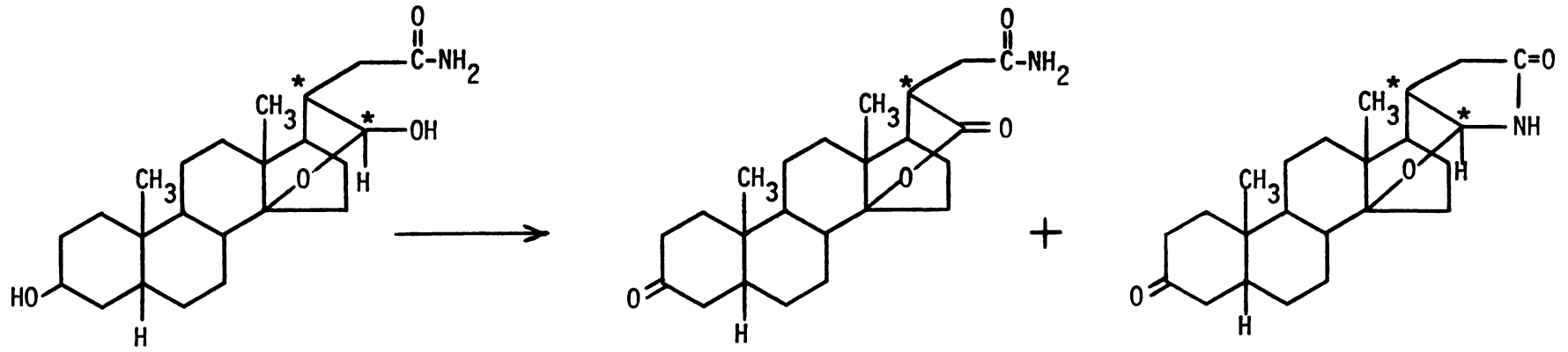

43 49 50

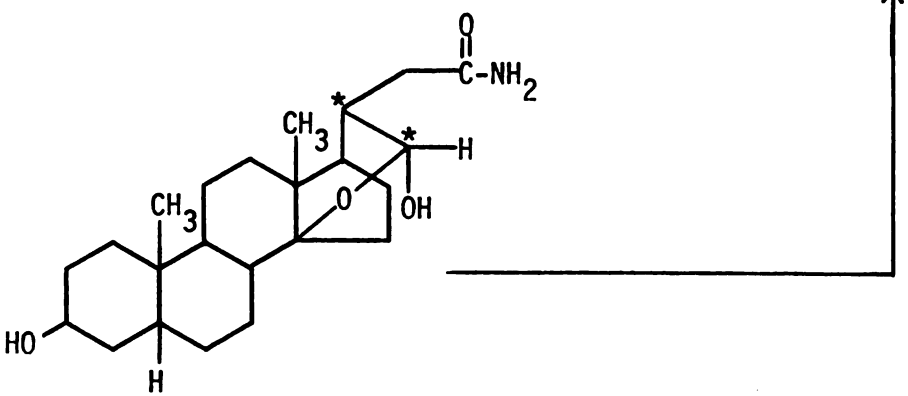

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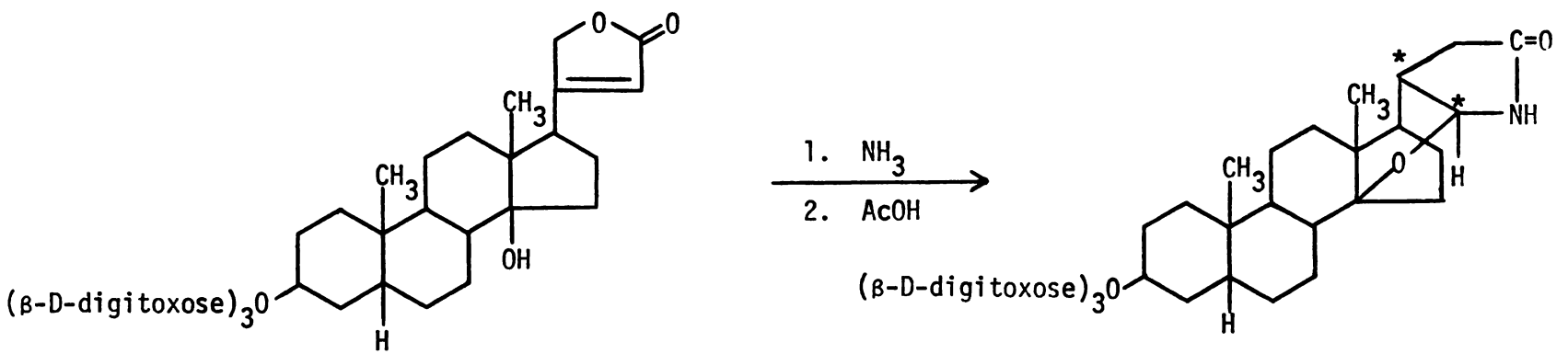
Fig. 8 (continued)



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C. Experimental

Melting points were determined with a Thomas-Hoover apparatus equipped with a corrected thermometer. Microanalysis were performed by the Microanalytical Laboratory, Chemistry Department, University of California, Berkeley, Calif. Optical rotations were obtained in a 0.5 dm tube with a Rudolph photoelectric polarimeter. Infrared spectra were obtained with a Beckman IR-5 or Perkin Elmer 337 instrument. Nmr spectra were obtained on a Varian A-60A spectrometer on samples in deuteriochloroform or deuteriopyridine solution, where indicated, using tetramethylsilane as internal standard. Resonance positions are reported in δ (ppm) values where possible; unresolved humps are described in Hz. units (60 MHz). Ozonolyses were performed on a Towers Ozone Apparatus GE 150. Mass spectrometric analysis were performed by Mr. William Garland on a MS-902 spectrometer.

5α-Chlorocholestane-3β,6β-diol 3-Acetate.[96] -- A mixture of 32.0 g of cholesteryl acetate, 450 ml of ether, 800 ml of 16% sodium hypochlorite solution and 1200 ml of water was treated with 60 ml of glacial acetic acid, added in small portions, and shaken vigorously for 30 minutes. After separation of the ether layer, the aqueous phase was extracted with 450 ml of ether. The combined ether extract was washed successively with 400 ml of 5% sodium bicarbonate solution and water and dried over sodium sulfate. Evaporation of the solvent under vacuum gave a solid residue which was collected and washed with petroleum ether. One recrystallization from methanol afforded 14.7 g (41%) of crystalline product, mp 199-201°.

5α-Chlorocholestane-3β,6β-diol. -- A solution of 5α-chlorocholestane-3β,6β-diol 3-acetate[96] in methanol containing 1% concentrated hydrochloric acid was kept at 27° for 18 hrs and concentrated. The resulting precipitate was recrystallized from methanol to give colorless needles: mp 164-166°; ν_{max}^{KBr} 3448, 1042 cm^{-1}; $[\alpha]_D^{28}$ -12° (c 0.44, $CHCL_3$).

Anal. Calcd for $C_{27}H_{47}CLO_2$: C, 73.85; H, 10.69. Found: C, 73.55; H, 10.56.

5α-Chlorocholestane-3β,6β-diol 3,6-Diacetate. -- A mixture of 10.0 g of 5α-chlorocholestane-3β,6β-diol 3-acetate,[96] 1.0 g of p-toluenesulfonic acid, 100 ml of glacial acetic acid, and 20 ml of acetic anhydride was shaken until a clear solution was obtained, kept for 5 hrs at 27°, and diluted with 60 ml of water added in small portions. The resulting precipitate was removed and recrystallized from ethanol to afford 9.0 g (83%) of colorless needles: mp 108-110°; $[\alpha]_D^{25}$ - 47° (c 1, $CHCL_3$); ν_{max}^{KBr} 1754, 1250 cm^{-1}.

Anal. Calcd for $C_{31}H_{51}ClO_4$: C, 71.16; H, 9.83; Cl, 6.78. Found: C, 71.01; H, 9.68; Cl, 7.01.

5α-Chloro-syn-19-oximinocholestane-3β,6β-diol 3-Acetate (1).[80] -- A solution of 10.0 g of 5α-chlorocholestane-3β,6β-diol 3-acetate[96] in 70 ml of pyridine was treated with nitrosyl chloride at 15-20° until a heavy precipitate of pyridine hydrochloride appeared and the solution became dark brown. The reaction mixture was poured into ice water with vigorous stirring and the resulting precipitate was collected by filtration, washed with cold water, triturated with a small volume of cold methanol to remove colored impurities, and dried under vacuum at 25°.

There was obtained 8.0 g (75%) of cream-colored nitrite ester, mp 121-123°, which was not recrystallized because of decomposition upon heating. Under an atmosphere of dry nitrogen, purified by passage through potassium pyrogallate solution, the above nitrite ester in 200 ml of toluene was irradiated for 3 hours at 0° by means of an immersed 200-w high pressure mercury arc equipped with borosilicate filter. The course of the reaction was followed by periodic testing for unreacted nitrite by means of a color-spot test[97] with diphenylamine in concentrated sulfuric acid. Upon completion of the reaction, the precipitated 19-nitroso dimer was filtered and immediately refluxed for 3 hrs in 180 ml 2-propanol. Evaporation of the solvent under reduced pressure afforded 3.95 g (49%) of pure, crystalline oxime, mp 218-219°. Recrystallization from methanol gave needle-like crystals with mp 220-221°.

19-Acetoxyimino-5α-chlorocholestane-3β,6β-diol Diacetate (2). -- A solution of 1.0 g of 1 and 0.10 g of p-toluenesulfonic acid in 30 ml of glacial acetic acid containing 5 ml of acetic anhydride was kept for 8 hrs at 27° and poured into water. The product was extracted with ether and the ether extract was washed with water and dried (Na_2SO_4) and evaporated. The product was obtained from methanol as colorless needles: mp 133-134°; ν_{max}^{KBr} 1779, 1739 cm^{-1}; $[\alpha]_D^{28}$ -27° (c 1.02, $CHCl_3$).

Anal. Calcd for $C_{33}H_{52}ClNO_6$: C, 66.70; H, 8.82; N, 2.36. Found: C, 66.66; H, 8.78; N, 2.62.

5β,6β-Epoxy-19-oximinocholestane-3β-ol (3). -- A solution of 1.6 g of 2 and 5.0 g of potassium hydroxide in 40 ml of methanol was heated under reflux for 24 hrs, cooled, poured into water, and neutralized carefully with 5% hydrochloric acid. The resulting precipitate was removed

and recrystallized from methanol to give 1.1 g of colorless needles: mp 229-231°; $[\alpha]_{\underline{D}}^{28}$ -56° (c 0.98, $CHCl_3$).

Anal. Calcd for $C_{27}H_{45}NO_3$: C, 75.13; H, 10.51; N, 3.24. Found: C, 75.24; H, 10.33; N, 3.51.

5β,6β-Epoxy-3β-hydroxycholestane-19-nitrile (4). -- A solution of 2.0 g of 1 in 15 ml of acetic anhydride was heated under reflux for 3 hrs., cooled, and diluted with water. The product was extracted with ether, and the ether solution was washed with water, dried (Na_2SO_4), and evaporated to give a gum which resisted crystallization. A solution of the gum in 25 ml of 5% potassium hydroxide in methanol solution was kept for 18 hrs at 27°, poured into water, and extracted with ether. Evaporation of the washed and dried (Na_2SO_4) ether extract gave a residue which was recrystallized from methanol to furnish 0.75 g of colorless needles: mp 195-196°; $[\alpha]_{\underline{D}}^{28}$ -14° (c 1.24, $CHCl_3$). Analysis showed this material to be a methanolate.

Anal. Calcd for $C_{27}H_{43}NO_2 \cdot CH_3OH$: C, 75.46; H, 10.63. Found: C, 75.80; H, 10.44.

The solvent-free compound, mp 194-196°, was obtained by drying the methanolate at 185° at 0.01 mm.

Anal. Calcd for $C_{27}H_{43}NO_2$: C, 78.40; H, 10.38; N, 3.39. Found: C, 78.30; H, 10.10; N, 3.62.

19-Norcholest-5(10)-ene-3β,6β-diol (5). A. From 3. -- A solution of 0.10 g of 3 and 0.10 g of lithium aluminum hydride in 20 ml of tetrahydrofuran was heated under reflux for 18 hrs, cooled, and treated with excess ethyl acetate. The mixture was poured into 100 ml of 5% acetic acid and the product was extracted with ether. The ether layer was washed with 5% sodium bicarbonate solution and water, dried (Na_2SO_4),

and evaporated. The residue was recrystallized from methanol to give 0.04 g of colorless crystals: mp 175-176°; $[\alpha]_D^{28}$ +103° (c 1.32, $CHCl_3$); nmr 0.71 (C-18 methly), 0.81, 0.91 (C-21, C-26, C-27 methyls), 3.82 (doublet) (6α-H), 4.08 (multiplet)(3α-H) ppm; lit.[98] mp 165-168°, $[\alpha]_D$ +98.

Anal. Calcd for $C_{26}H_{44}O_2$: C, 80.35; H, 11.41. Found: C, 79.94; H,11.12; (N, 0.0).

B. From 4. -- A solution of 0.50 g of 4 and 0.50 g of lithium aluminum hydride in 15 ml of tetrahydrofuran was heated under reflux for 18 hrs, cooled, decomposed with ethyl acetate, acidified with 5% acetic acid, and extracted with ether. The washed and dried ether extract was evaporated to give 0.06 g of colorless crystals of 5, identical with the previous preparation.

C. From 10. -- The benzene filtrate from the preparation of 17 was evaporated and the residue was recrystallized from acetonitrile to give 0.04 g of product.

5α-Chloro-3β,19-6β,19-diepoxycholestane (6). -- A solution of 0.50 g of 1 in 25 ml of methanol was acidified to pH 1 with 2N hydrochloric acid and heated under reflux for 18 hrs. The precipitate which was obtained on cooling was filtered and recrystallized from methanol to afford 0.05 g of colorless plates: mp 137-138°; $[\alpha]_D^{28}$ + 39° (c 1.80, $CHCl_3$); nmr 0.72 (C-18 methyl), 0.82, 0.95 (C-21, C-26, C-27 methyls), 3.97 (3α-H), (6α-H), 5.20 (C-19 H) ppm.

Anal. Calcd for $C_{27}H_{43}ClO_2$: C, 74.53; H, 9.96. Found: C, 74.35; H, 9.91.

The reaction mixture filtrate was made alkaline with 20% sodium hydroxide and the product was extracted into ether. The washed and dried ether extract was evaporated, and the residue was recrystallized from methanol to give 0.10 g of 4 mp 194-195°.

5α-Chloro-6β,19-epoxy-19-oximinocholestan-3β-ol 3-Acetate (7). -- To a solution of 1.0 g of 1 in 100 ml of ether there was added a suspension of 0.50 g of N-bromoacetamide in 20 ml of 70% methanol. After 5 minutes, the mixture turned yellow. After 15 minutes, the solution was washed with water and 5% sodium bicarbonate solution and dried (Na_2SO_4). The filtered solution was evaporated and the residue solidified on treatment with methanol. Recrystallization from acetonitrile gave 0.80 g (80%) of colorless plates: mp 161-163°; $[\alpha]_D^{25}$ -15° (c 1, $CHCl_3$); ν_{max}^{KBr} 3333, 1724, 1695, 1266, 1235 cm^{-1}; nmr 0.64 (C-18 methly), 0.81, 0.90 (C-21, C-26, C-27 methyls), 2.00 (Acetate), 4.38 (6α-H), 4.71-5.26 (low hump)(3α-H) 7.53-7.86 (low hump)(N-OH proton) ppm.

Anal. Calcd for $C_{29}H_{46}ClNO_4$: C, 68.54; H, 9.12. Found: C, 68.43; H, 9.12.

5α-Chloro-6β,19-epoxy-19-oximinocholestan-3β-ol (8). -- A solution of 0.50 g of 7 in 25 ml of absolute ethanol containing 5% potassium hydroxide was kept at 27° for 5 hrs and diluted with 150 ml of ether. The resulting solution was washed with brine and dried over sodium sulfate. The residue obtained from evaporation of the ether was washed with petroleum ether and recrystallized from methanol to afford 0.45 g (98%) of colorless needles: mp 236-237°; $[\alpha]_D^{25}$ -17° (c 1, $CHCl_3$); ν_{max}^{KBr} 3448-3226, 1695 cm^{-1}.

Anal. Calcd for $C_{27}H_{44}ClNO_3$: C, 69.57; H, 9.54. Found: C, 69.43; H, 9.73.

5α-Chloro-6β,19-epoxy-19-O-methyloximinocholestan-3β-ol Acetate (9). --

A. Using Diazomethane. -- To an ice-cold solution of 3.0 g of 7 in 100 ml of anhydrous ether containing 1.0 ml of boron trifluoride etherate, there was added excess ethereal diazomethane, and the mixture was kept in an ice bath for 3 hrs. A small amount of precipitate was removed by filtration and the clear filtrate was allowed to evaporate. The residue was recrystallized from methanol to furnish 1.60 g of colorless crystals: mp 121-123°; $[\alpha]_D^{25}$ +7° (c 1, $CHCl_3$); ν_{max}^{KBr} 1739, 1672, 1242, 1047 cm^{-1}; nmr 0.65 (C-18 methyl), 0.81, 0.90 (C-21, C-26, C-27 methyls), 2.0 (acetate methyl), 3.83 (19-oxime-O-methyl), 4.35 (6α-H), 4.73-5.31 (low hump)(3α-H) ppm.

Anal. Calcd for $C_{30}H_{48}ClNO_4$: C, 69.00; H, 9.27. Found: C, 68.98; H, 9.05.

B. Using Methyl Iodide. -- A stirred solution of 1.00 g of 7 in 15 ml of purified dimethylformamide was kept at 27° and treated in five equal portions with 5.0 ml of methyl iodide and 5.0 g of silver oxide during 30 minutes. The mixture was stirred for 24 hrs, diluted with 150 ml of ether, filtered and evaporated in vacuo. The residue was recrystallized from methanol to afford 0.65 g of the product, mp 121-123°.

5α-Chloro-6β,19-epoxycholestan-3β,19-diol Diacetate (10). -- To a warm solution of 10.0 g of 1 in 700 ml of glacial acetic acid there was added a solution of 15 g of sodium nitrite in 40 ml of water. The resulting solution was kept for 30 minutes at 50° and poured into ice water. The product was extracted with ether and the ether extract was washed with 5% sodium bicarbonate solution and water and dried (Na_2SO_4). Evaporation of the filtered solution gave a residue which was recrystallized from aqueous

2-propanol and methanol to afford 5.2 g of colorless needles: mp 155-157°; $[\alpha]_D^{27}$ +31° (c 1, $CHCl_3$); ν_{max}^{KBr} 1733, 1242-1227 cm^{-1}; nmr 0.71 (C-18 methyl), 0.81, 0.91 (C-21, C-26, C-27 methyls), 2.05 (C-3 acetate methyl), 2.8 (19-Ac) 4.30 (doublet)(6α-H), 5.00-5.60 (low hump)(3α-H), 6.30 (C-19 H) ppm.

Anal. Calcd for $C_{31}H_{49}ClO_5$: C, 69.31; H, 9.19. Found: C, 69.10; H, 9.23.

5α-Chloro-6β,19-epoxycholestane-3β,19-diol 3-Acetate (11). -- The 2-propanol mother liquor from the recrystallization of 10 was diluted with water and extracted with ether. The ether extract was washed with water, dried, and evaporated and the residue was crystallized from alcohol and then methanol to give 1.2 g of colorless crystals, mp 143-146°. The analytical sample, obtained from methanol, had mp 150-152°; ν_{max}^{KBr} 3333, 1739, 1242 cm^{-1}; nmr 0.67 (C-18 methyl), 0.81, 0.91 (C-21, C-26, C-27 methyls), 2.20 (acetate methyl), 3.30 (6α-H), 4.18 (3α-H) ppm; $[\alpha]_D^{28}$ +8° (c 1.17, $CHCl_3$).

Anal. Calcd for $C_{29}H_{47}ClO_4$: C, 70.34; H, 9.57. Found: C, 69.68: H, 9.25.

5β,6β-Epoxy-19-oxocholestan-3 -ol (12). -- A solution of 0.50 g of 10 in 40 ml of 5% methanolic KOH was kept at 27° for 4 hrs and diluted with ether and brine. The ether layer was separated, washed again with brine, dried (Na_2SO_4), and evaporated. The residue was recrystallized from methanol to give 0.35 g of colorless needles: mp 135-137°; ν_{max}^{KBr} 3333; 1718 cm^{-1}; $[\alpha]_D^{25}$ -31° (c 1, $CHCl_3$); lit.[98] mp 138-140°, $[\alpha]_D$ -10°.

Anal. Calcd for $C_{27}H_{44}O_3$: C, 77.84; H, 10.64. Found: C, 77.56; H, 10.68.

5β,6β-Epoxy-19-oxocholestan-3β-ol Acetate (13). -- A solution of 1.00 g of 10 in 100 ml of methanol at 40° was treated with gaseous ammonia to pH 9, kept at 27° for one hr, neutralized with glacial acetic acid, and poured into water. The resulting precipitate was filtered and recrystallized from aqueous methanol to give 0.50 g of colorless needles: mp 139-141°; ν_{max}^{KBr} 2703, 1739, 1250, 813 cm^{-1}; $[\alpha]_D^{25}$ -48° (c 1, $CHCl_3$); lit.[98] mp 138-140°; $[\alpha]_D$ +34°.

Anal. Calcd for $C_{29}H_{46}O_4$: C, 75.94; H, 10.11. Found: C, 75.79; H, 9.87.

5α-Chlorocholestane-3β,6β,19-triol 3-Acetate (14). -- A solution of 0.20 g of 11 in 50 ml of methanol was treated with a solution of 0.10 g of sodium borohydride in 0.5 ml of water and kept for one hr at 27°. It was poured into ice-water, kept for one hr, and extracted with ether. Evaporation of the washed and dried ether extract gave colorless crystals: mp 125-127°; ν_{max}^{KBr} 3279 cm^{-1}; $[\alpha]_D^{28}$ +11° (c 0.86, $CHCl_3$).

Anal. Calcd for $C_{29}H_{49}ClO_4$: C, 70.06; H, 10.17. Found C, 69.87; H, 9.67.

5α-Chloro-3β,6β-dihydroxycholestan-19-oic Acid 6,19-Lactone 3-Acetate (15). -- A solution of 0.10 g of 11 in 20 ml of acetone was treated dropwise with 8 N chromic acid solution[93] until a brown color persisted. The excess oxidant was decomposed by addition of 2-propanol and the reaction mixture was filtered through a cotton pledget and evaporated. The residue was recrystallized from acetonitrile to afford 0.07 g of colorless plates: mp 178-180°; ν_{max}^{KBr} 1786, 1739, 1274 cm^{-1}; $[\alpha]_D^{25}$ +3° (c 1, $CHCl_3$).

Anal. Calcd for $C_{29}H_{45}ClO_4$: C, 70.63; H, 9.20. Found: C,70.86; H, 8.95.

The same product was obtained on oxidation of 0.10 g of 11 with 0.10 g of N-bromosuccinimide in 5 ml of t-butyl alcohol at 95° for 1 hr. When 0.10 g of 11 in 50 ml of ether was treated with 0.10 g of N-bromosuccinimide in 10 ml of aqueous methanol and kept at 27° for 1 hr, only the starting material was recovered.

3β,19-6β,19-Diepoxycholestan-5α-ol (16). -- A solution of 0.10 g of 10 in 30 ml of 50% aqueous acetic acid was heated under reflux for 18 hrs and cooled. The crystalline precipitate was filtered to give 0.06 g of 6, mp 137-138° after recrystallization from acetonitrile. The filtrate was diluted with water and extracted with ether, the ether extract was washed with 5% sodium bicarbonate solution and water. The dried (Na_2SO_4), filtered solution was evaporated and the residue recrystallized from acetonitrile to give 0.02 g of halogen-free colorless needles: mp 241-243°; ν_{max}^{KBr} 3333 cm^{-1}; nmr 0.72 (C-18 methyl), 0.85, 0.95 (C-21, C-26, C-27 methyls), 3.85 (doublet)(6α-H), 4.10 (3α-H), 5.32 (C-19 H) ppm.

Anal. Calcd for $C_{27}H_{44}O_3$: C, 77.84; H, 10.64. Found: 77.93; H, 10.50.

5α-Cholestane-3β,6β,19-triol (17). -- A solution of 0.90 g of 10 and 3.0 g of lithium aluminum hydride in 100 ml of anhydrous ether was stirred at 27° for 4 hrs and decomposed with ethyl acetate. It was washed with 5% hydrochloric acid solution and water, dried (Na_2SO_4), and evaporated. The residue consisted of two main fractions, as shown by thin layer chromatography. It was slurried with benzene, and the benzene-insoluble material was recrystallized from methanol to give 0.40 g of halogen-free colorless

needles: mp 229-231°; ν_{max}^{KBr} 3333, 3175 cm^{-1}; $[\alpha]_D^{28}$ +23° (c 0.052, tetrahydrofuran).

Anal. Calcd for $C_{27}H_{48}O_3$: C, 77.09; H, 11.50. Found: C, 76.92; H, 11.24.

The benzene-soluble fraction afforded 5 (see above). Acetylation of the triol with acetic anhydride in pyridine solution at 27° during 18 hrs gave the corresponding triacetate, identified by its nmr spectrum: 0.67 (C-18 methyl), 0.82, 0.92 (C-21, C-26, C-27 methyls), 2.08, 2.12, 2.14 (acetate methyls) ppm.

5α-Chloro-3β-hydroxy-6-oxocholestane-19-nitrile Acetate (18). -- A solution of 10.0 g of 1 in 100 ml of pyridine was added to a stirred suspension prepared[88] by adding 10.0 g of chromic acid to 100 ml of pyridine. After 48 hrs the reaction mixture was poured onto a mixture of crushed ice and 200 ml of concentrated hydrochloric acid. The resulting suspension was shaken with ether and the resulting mixture was filtered through glass wool. The layers were separated and the ether phase was washed with water, dried over sodium sulfate, filtered and evaporated. The residue was first washed with methanol and then disolved in hot acetonitrile. The acetonitrile solution was filtered through alumina and concentrated to give 4.4 g (46%) of colorless crystals: mp 210-211°; $[\alpha]_D^{25}$ -86° (c 1, $CHCl_3$); ν_{max}^{KBr} 2222, 1724, 1266, 1235 cm^{-1}.

Anal. Calcd for $C_{29}H_{44}ClNO_3$: C, 71.06; H, 9.05. Found: C, 71.47; H, 9.45.

3β,5β-Dihydroxy-6-oxocholestane-19-nitrile 3-Acetate (19). -- A suspension of 1.0 g of 18 in 12.5 ml of 5% potassium hydroxide in absolute

ethanol was stirred for 1 hr, diluted with 7 ml of absolute ethanol, and stirred for an additional 4 hrs. The resulting solution was diluted with 100 ml of ether, washed three times with brine, dried over sodium sulfate, and evaporated under reduced pressure. The residue was dissolved in 5 ml of pyridine and allowed to react with 1.0 ml of acetic anhydride for 18 hrs at 27°. The solution was poured onto crushed ice containing 10 ml of concentrated hydrochloric acid and extracted with ether. The ether extract was washed with brine and water, dried (Na_2SO_4), and evaporated under reduced pressure. The residue was chromatographed on neutral alumina to afford 0.12 g (12%) of pure product, mp 174-176°. Recrystallization from acetone-petroleum ether furnished the analytical sample as colorless fine needles: mp 175-176°; $[\alpha]_{\underline{\underline{D}}}^{25}$ +12° (c 0.55, $CHCl_3$); ν_{max}^{KBr} 3448, 2222, 1754, 1724, 1250 cm^{-1}.

Better yields were obtained when the acetylation was carried out with acetic anhydride in acetic acid in the presence of p-toluenesulfonic acid.[90,91]

Anal. Calcd for $C_{29}H_{45}NO_4$: C, 73.85; H, 9.62. Found: C, 73.68; H, 9.40.

3β,5β-Dihydroxy-6-oxocholestane-19-nitrile 3-Benzoate (20). -- This compound was obtained from 3.0 g of 18 by the same method described for 19 except that benzoyl chloride was substituted for the acetic anhydride. The solution from the benzoylation step, after 3 hrs at 70°, was poured into 60 ml of 5% sodium bicarbonate solution, and the resulting precipitate was filtered after 0.5 hr and washed with 5% sodium bicarbonate solution and water. Recrystallization from acetonitrile gave 1.3 g (40%) of shiny plates: mp 230-232°; $[\alpha]_{\underline{\underline{D}}}^{25}$ +18° (c 1, $CHCl_3$); ν_{max}^{KBr} 3333, 1754, 1724 cm^{-1}.

Anal. Calcd for $C_{34}H_{47}NO_4$: C, 76.51; H, 8.88; N, 2.62. Found: C, 76.42; H, 9.01; N, 2.41.

3β,5β,6β-Trihydroxycholestane-19-nitrile 6-Acetate 3-Benzoate (21). -- To a solution of 0.90 g of 20 in a mixture of 35 ml of dioxane and 25 ml of methanol there was added, dropwise, a solution of 0.30 g of sodium borohydride in 1 ml of water. The mixture was kept at 27° for 1 hr, cooled in ice, and diluted with 200 ml of water and extracted with ether. The combined ether extracts were washed with water, dried (Na_2SO_4), filtered, and evaporated under reduced pressure to afford 0.90 g of crude reduction product, mp 219-221°. The triol momobenzoate was kept in pyridine and acetic anhydride for 18 hrs, and the solution was diluted with water. The product was filtered and recrystallized from methanol to furnish colorless needles: mp 220-222°; $[\alpha]_D^{25}$ -43° (c 1, $CHCl_3$); ν_{max}^{KBr} 3226, 1754, 1724 cm^{-1}.

Anal. Calcd for $C_{36}H_{51}NO_5$: C, 74.83; H, 8.90. Found: C, 74.77; H, 8.70.

3β,5β,6β-Trihydroxycholestan-19-oic Acid 6,19-Lactone 3-Benzoate (22). -- A solution of 0.20 g of 21 in 30 ml of methanol was treated with a stream of hydrogen chloride for 5 min., kept for 1 hr at 27°, and evaporated. The crystalline residue (imino ether hydrochloride) was dissolved in 20 ml of methanol, treated with 0.10 g of sodium borohydride in 1 ml of water, and kept for 1 hr at 27°. The mixture was diluted with water, and the resulting precipitate was filtered and recrystallized from methanol to give a nearly quantitative yield of colorless crystals: mp 248-250°; $[\alpha]_D^{28}$ +53° (c 1, $CHCl_3$); ν_{max}^{KBr} 3333, 1754, 1724 cm^{-1}.

Anal. Calcd for $C_{34}H_{48}O_5$: C, 76.08; H, 9.01. Found: C, 75.98; H, 9.02.

3β,5β,6β-Trihydroxycholestan-19-oic Acid 6,19-Lactone (23). -- A solution of 0.10 g of the crude imino ether hydrochloride described in the preparation of 22 was prepared in 5% methanolic potassium hydroxide solution and kept for 2 hrs at 27°. It was diluted with water and the resulting precipitate was collected and recrystallized from ethyl acetate to give colorless plates: mp 263-264°; ν_{max}^{KBr} 3333, 3226-3125, 1754 cm^{-1}; $[\alpha]_D^{28}$ +23° (c 0.30, $CHCl_3$).

Anal. Calcd for $C_{27}H_{44}O_4$: C, 74.96; H, 10.25. Found: C, 75.12; H, 10.22.

Digitoxigen (24).

3β,14-Dihydroxy-5β,14β-card-20(22)-enolide. -- A solution of 52 g of digitoxin in a mixture of 3.1 l. of acetone, 620 ml of water and 31 ml of concentrated hydrochloric acid was kept at 27° for 10 days. The solution was neutralized with solid sodium bicarbonate, and the acetone removed under reduced pressure. The crystalline product which separated was filtered and washed with cold water. Recrystallization from ethyl acetate afforded 20.7 g (81%) of colorless needles: mp 252°; lit.[99] mp 250°.

Digitoxigenone (25).

14-Hydroxy-3-oxo-5β,14β-card-20(22)-enolide. -- A cold solution of 20.7 g of digitoxigenin in 1.2 l. of acetone was treated dropwise with 8 N chromic acid solution[93] until a brown color persisted. The excess oxidant was decomposed by addition of 2-propanol and the reaction mixture was filtered through a cotton pledget and evaporated. The crystalline residue was collected and washed with cold water to remove green

color, and recrystallized from ethyl acetate to give 17.3 g (84%) of colorless crystals, mp 200-202°; lit. mp 203-205°,[100] mp 203-204°[101].

14,21-Dihydroxy-3,3-dimethoxy-5β,14β-pregan-20-one (28). -- A solution of 2.0 g of digitoxigenone in 10 ml of methylene chloride and 90 ml of ethyl acetate was cooled to -72° and treated with a stream of ozone for 18 minutes. Oxygen was then passed into the deep blue solution until it became colorless (about 30 min.). Evaporation of the solvent under reduced pressure gave a gummy residue, which was dissolved in 15 ml of glacial acetic acid, treated with 1.0 g of zinc dust, and stirred magnetically for 1 hr at 27°. The solution was filtered and evaporated in vacuo to give a gum. The gum was treated with a small volume of ice-cold water followed by the portionwise addition of solid sodium bicarbonate until the resulting mixture became slightly alkaline. At this point most of the solid had dissolved. The mixture was made acidic by portionwise addition of cold, dilute hydrochloric acid until no further precipitation occurred. The filtered and slightly damp precipitate was dissolved in 120 ml of methanol, stirred magnetically for 0.5 hr, and then treated with 1.64 g of potassium bicarbonate in 43 ml of water. After stirring for 1.5 hr at 27° thin layer chromatography showed only one spot. Most of the solvent was evaporated under reduced pressure and the crystalline residue was filtered and dried under vacuum. Recrystallization from methanol afforded 1.80 g (85%) of shiny plates: mp 186-188°; $[\alpha]_D^{18}$ +46° (c 1, $CHCl_3$); nmr 0.91 (C-18 methyl), 0.94 (C-19 methyl), 3.15, 3.21 (C-3 methoxy methyls), 4.30 (C-21 methylene) ppm.

Anal. Calcd for $C_{23}H_{38}O_5$: C, 70.02; H, 9.71. Found: C, 70.23; H, 9.78.

14,21-Dihydroxy-3,3-dimethoxy-5β,14β-pregnan-20-one 21-Acetate (29). -- A solution of 1.2 g of 28 in 20 ml of pyridine was treated with 1.5 ml of acetic anhydride and kept for 18 hr at 27°. The solution was poured into ice-water and the resulting precipitate was filtered and recrystallized twice from hexane to give 0.90 g (68%) of colorless needles: mp 107-109°; $[\alpha]_D^{18}$ +52° (c 1, $CHCl_3$);; nmr 0.95 (C-18, C-19 methyls), 2.16 (21-acetate methyl), 3.15, 3.21 (C-3 methoxy methyls), 4.01 14β-OH proton), 4.71 (C-21 methylene) ppm.

Anal. Calcd for $C_{25}H_{40}O_6$: C, 68.78; H, 9.23. Found: C, 68.83; H, 9.40.

14,20,21-Trihydroxy-5β,14β-pregnan-3-one 20,21-Dibenzoate (33). -- A cold solution of 2.0 g of 29 in 50 ml of freshly distilled, anhydrous tetrahydrofuran was treated with 4.0 g of lithium tri-t-butoxyaluminum hydride dissolved in 100 ml of cold tetrahydrofuran and the mixture was kept for 3 hrs at 0°. The solution was partially evaporated under vacuum, a small volume of ice water was added, and the remaining tetrahydrofuran evaporated under reduced pressure. The gelatinous residue was acidified with 3 ml of glacial acetic acid, diluted with water, and extracted with ether. The ether extract was washed with 5% sodium bicarbonate solution and water, dried (Na_2SO_4), and evaporated to a gum under reduced pressure. To a solution of the gum in 50 ml of tetrahydrofuran there was added 10 ml of 2.5% aqueous trifluoroacetic acid solution and the mixture stirred for 2 hrs at 27°. The solution was neutralized with 0.5 g of solid potassium bicarbonate and evaporated in vacuo at room temperature. A solution of the residue in 180 ml of methanol was then treated with 2.5 g of potassium bicarbonate in 70 ml of water and stirred

for 18 hrs at 27°. Most of the solvent was evaporated under reduced pressure and the remaining aqueous phase was saturated with sodium chloride and extracted with ethyl acetate. The ethyl acetate extract was washed with water, dried (Na_2SO_4), and evaporated to a gummy residue which gave one spot on thin layer chromatography. It was dissolved in 15 ml of pyridine and allowed to react with 1.5 ml of benzoyl chloride for 1 hr at 27°. The solution was poured into ice-water and extracted with ether. The ether extract was washed successively with cold 5% sodium bicarbonate solution, cold 5% hydrochloric acid solution and water, dried (Na_2SO_4), and evaporated. The crystalline residue was recrystallized from acetone-hexane to afford 1.64 g (64%) of colorless needles: mp 164-166°; $[\alpha]_D^{18}$ -5° (c 1, $CHCl_3$); nmr 1.00 (C-18 methyl), 1.06 (C-19 methyl) ppm, 254-297 (multiplet) (C-21 methylene), 322-349 (broad peak)(C-20 H), 436-455, 470-490 (multiplets)-(aromatic protons) Hz.

Anal. Calcd for $C_{35}H_{42}O_6$: C, 75.24; H, 7.58. Found: C, 74.97; H, 7.80.

3-Oxo-14,20,21-trihydroxy-14β-pregn-4-ene 20,21-Dibenzoate (35). -- A stirred solution of 0.5 g of 33 in 15 ml of tetrahydrofuran was kept at -12° and treated in small portions with 0.485 g of phenyltrimethylammonium tribromide[94] (PTT) over a 3 hr period. After an additional 0.5 hr of stirring, thin layer chromatography showed only one spot corresponding to intermediate product 34. The solution was then treated with 3 ml of acetone and 1 ml of water and evaporated under reduced pressure at 20°. Addition of ice to the gummy residue resulted in the formation of a white precipitate which was allowed to stant at 0° for 0.5 hr. The precipitate was filtered, washed with ice-cold water, and dried under vacuum. There was obtained

0.55 g (96%) of crude bromoketone. A solution of the crude bromoketone in 175 ml of acetone was then treated with 1.1 g of benzyltrimethylammonium mesitoate[95] (BTAM) and refluxed for 24 hrs. The filtered solution was evaporated under reduced pressure and the resulting residue was taken up in ether. The ether solution was washed successively with water, 5% sodium bicarbonate solution, and again with water, dried (Na_2SO_4), and evaporated. The residue was crystallized from acetonitrile to afford 0.30 g (60% overall) of colorless needles, mp 211-214°. The analytical sample, obtained by an additional recrystallization from acetonitrile, had mp 215-216°; $[\alpha]_D^{18}$ +55° (c 1, $CHCl_3$); nmr 1.08 (C-18 methyl), 1.15 (C-19 methyl) ppm, 254-298 (multiplet)(C-21 methylene), 327-? (broad peak, partially hidden by C-4 proton)(C-20 H), 5.76 (ppm)(C-4 H), 440-460, 472-493 (multiplets)(aromatic protons) Hz.

Anal. Calcd for $C_{35}H_{40}O_6$: C, 75.51; H, 7.24. Found: C, 75.39; H, 7.13.

The same compound was obtained in very low yield by treatment of the intermediate bromoketone with ethoxycarbonyl hydrazine[102] followed by acid hydrolysis of the resulting ethoxycarbonyl hydrazone intermediate.[103]

3β,14,20,21-Tetrahydroxy-14β-pregn-5-ene 3,20,21-Tribenzoate 14-Acetate (38). -- A solution of 0.20 g of 35 and 0.04 g of p-toluenesulfonic acid monohydrate in 15 ml of isopropenyl acetate was stirred during 36 hrs at 30°. The solution was neutralized with solid sodium bicarbonate and evaporated under reduced pressure at 27°. The residue was taken up with ether and the ether solution was washed with water, dried over sodium sulfate, and evaporated to give a gum 36. Crystallization was unsuccessful even after purification by preparative thin layer chromatography. A solution of the gum in 5 ml of methanol was treated with 0.15 g of sodium borohydride dissolved in 1 ml of water and kept at 27° for 24 hrs. The slightly cloudy solution that resulted upon dilution with ice-water was saturated with sodium chloride and extracted with ethyl acetate. The washed and dried ethyl acetate extract was evaporated in vacuo to give a gum 37. The gum was dissolved in 5 ml of pyridine and allowed to react with 0.5 ml of benzoyl chloride for 2 hrs at 27°. The reaction solution was poured into ice-water and extracted with ether, and the ether was washed with 5% sodium bicarbonate solution and water. The dried (Na_2SO_4) solution was evaporated under reduced pressure to give a gummy residue, which resisted crystallization. Purification by preparative thin layer chromatography and crystallization from methanol afforded 0.06 g of small, colorless needles, mp 144-148°. The analytical sample, obtained by a second recrystallization From methanol, had mp 148-150°; nmr 1.10 (C-18, C-19 methyls), 2.16 (acetate methyl) ppm, 246-294 (multiplet)(C-21 methylene), 317-340 (multiplet)-(3α-H, C-6 H, C-20 H), 437-462, 472-491 (multiplet)(aromatic protons) Hz.

Anal. Calcd for $C_{44}H_{48}O_8$: C, 74.97; H, 6.86. Found: C, 75.05; H, 6.87.

3β,14-Dihydroxy-5β,14β-card-20(22)-enolide 3,14-Diacetate (39). -- A solution of 0.15 g of p-toluenesulfonic acid in 30 ml of isopropenyl acetate was refluxed for 0.5 hr and then cooled to 35°. To the above solution there was added 1.0 g of digitoxigenin (24) and the resulting mixture was stirred for 78 hrs at 35-40°. The solution was neutralized with 0.30 g of sodium bicarbonate in 4 ml of water, and evaporated under reduced pressure to give a crystalline residue. The product was collected, washed with cold water, dried, and again washed with a 50-50 mixture of hexane and anhydrous ether. Recrystallization from methanol afforded 0.96 g (79%) of colorless prisms: mp 191-193°; $[\alpha]_D^{18}$ + 14° (c 1, $CHCl_3$); nmr 1.00 (C-18 methyl), 1.01 (C-19 methyl), 2.00, 2.05 (acetate methyls), 4.75 (C-21 methylene), 5.08 (3α-H), 5.86 (C-22 vinyl proton) ppm.

Anal. Calcd for $C_{27}H_{38}O_6$: C, 70.72; H, 8.35. Found: C, 70.80; H, 8.36.

3β-Acetoxy-5β-carda-14,20(22)-dienolide (40). -- This compound was obtained in good yield by heating a solution of 39 in acetonitrile containing trace amount of p-toluenesulfonic acid. The product was recrystallized from methanol to afford colorless needles: mp 180-182°; $[\alpha]_D^{18}$ -16° (c 1, $CHCl_3$); nmr 0.81 (C-18 methyl), 0.98 (C-19 methyl), 2.03 (acetate methyl), 4.75 (doublet)(C-21 methylene), 5.07 (3α-H), 5.25 (doublet)(C-15 H), 5.89 (doublet)(C-22 vinyl proton) ppm; lit.[104] mp 180-182, $[\alpha]_D$ -19°, lit.[105] mp 182-184°, $[\alpha]_D$ -12°.

14-Hydroxy-3-oxo-14β-carda-4,20(22)-dienolide (42). -- A stirred solution of 3.0 g of 25 in 120 ml of anhydrous tetrahydrofuran was kept at -12° and

treated with 4.0 g of phenyltimethylammonium tribromide[94] (PTT) added all at once. The reaction was complete within 30 min. as shown by thin layer chromatography. The reaction solution was treated with 5 ml of acetone and enough water to dissolve the white precipitate that formed, and evaporated under reduced pressure at 20°. Ice-water was added and the resulting precipitate was filtered, washed thoroughly with cold water, and dried under vacuum at 27°. The cream-colored granular material was digested in 10 ml of ethanol for few minutes, cooled to 0°, filtered and washed with a few ml of cold ethanol. Crystallization from acetone-ethanol afforded 2.15 g (59%) of crystalline bromoketone intermediate (41), which softened at 171° and melted at 195-196°, with decomposition. This compound appears to be rather unstable and was identified by its nmr spectrum in deuteriodimethyl sulfoxide solution: 0.81 (C-18 methyl), 1.00 (C-19 methyl), 4.10 (14 β-OH proton), 4.95 (C-21 methylene), 5.35 (doublet)(4α-H), 5.92 (C-22 vinyl proton) ppm. A solution of 1.0 g of the bromoketone (41) in 80 ml of anhydrous tetrahydrofuran was treated with 1.40 g of benzyltrimethylammonium mesitoate[95] (BTAM) and refluxed for 8 hrs. Evaporation of the solvent under reduced pressure and addition of ice-water resulted in the formation of a gummy residue, which was extracted with ethyl acetate. The extract was washed with 5% sodium bicarbonate solution and water, dried (Na_2SO_4), and evaporated *in vacuo* to afford a crystalline residue. Recrystallization from acetone-ethyl acetate gave 0.60 g (73%) of white crystals: mp 247-250° $[\alpha]_D^{18}$ +104° (*c* 1, $CHCl_3$); nmr 0.95 (C-18 methyl), 1.20 (C-19 methyl), 4.93 (multiplet)(C-21 methylene), 5.75 (C-4 H), 5.90 (C-22 vinyl proton) ppm; lit.[106] mp 234-241°, $[\alpha]_D$ +95°, lit.[107] mp 238-242°, $[\alpha]_D$ +87°, lit.[108] mp 250-264°, $[\alpha]_D$ +112°.

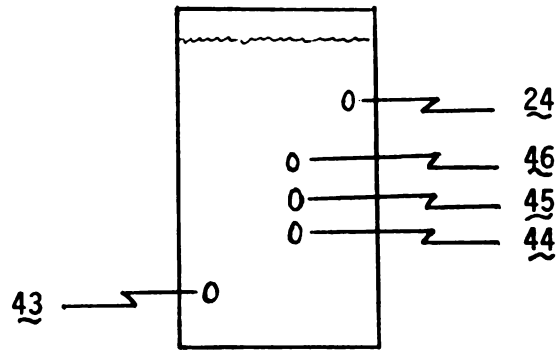
Anal. Calcd for $C_{23}H_{30}O_4$: C, 74.56; H, 8.16. Found: C, 74.42; H, 8.16.

(20S,21S)-3β,21-Dihydroxy-14β,21-oxidonorcholan-23-oic Acid Amide (43).-- A solution of 6.0 g of digitoxigenin (24) in 650 ml of methanol was treated with a stream of ammonia for 1 hr at 0°, and stirred for 10 days at room temperature. The excess ammonia was driven off by passing nitrogen through the solution. Evaporation of the solvent under reduced pressure gave a crystalline residue, which was recrystallized from methanol to afford 2.20 g of shiny plates: mp 271-273°; $[\alpha]_D^{20}$ -5° (c 1, pyridine); ν_{max}^{KBr} 3540, 3420 cm^{-1} (amide NH_2), 1670 cm^{-1} (amide C=O); nmr (Pyr.-d_5) 1.00 (C-19 methyl), 1.26 (C-18 methyl), 3.56 (methanol of solvation), 4.36 (3α-H), 4.80 ($-NH_2$), 5.43 (3β-OH), 5.76 (C-21 H), 9.08 (C-21 OH, H-bonded to carbonyl of amide) ppm.

Anal. Calcd for $C_{23}H_{37}NO_4.2CH_3OH$*: C, 65.90; H, 9.95. Found: C, 66.05; H, 9.77.

*Complete removal of methanol of solvation by heating in vacuo was impossible without causing partial cyclization.

(20S,21R)-3β,21-Dihydroxy-14β,21-oxidonorcholan-23-oic Acid Amide (44).--The mother liquor from 43 was evaporated to dryness under reduced pressure and the resulting residue was dissolved in a mixture of 25 ml of methanol and 5 ml of glacial acetic acid. The mixture was warmed on the steam cone for a few minutes and then kept for 8 hrs at 27°. Thin layer chromatography showed that the mixture contained three main fractions (A, B & C), none of which corresponded to either starting material (24) or lactolamide (43).



With the aid of a fraction collector, the mixture was separated by column chromatography on 140 g of silica gel (Merck 0.2-0.5 mm) and using as continuos eluant a mixture of 10% benzene, 20% acetone, and 70% anhydrous ether. Excellent separation afforded 0.20 g of compound 44 (fraction C) in crystalline form. Recrystallization from methanol afforded colorless needles: mp (double) 200-203°and 263-265°; $[\alpha]_D^{20}$ +44° (c 1, pyridine); ν_{max}^{KBr} 3400, 3250 cm^{-1} (amide NH_2) and 1700 cm^{-1} (amide C=O); nmr (Pyr.-d_5) 1.00 (C-19 methyl), 1.18 (C-18 methyl), 3.41 (methanol of solvation), 4.38 (3α-H), 4.83 (C-21 H), 4.86 ($-NH_2$), 5.50 (3β-OH), 9.55 (C-21 OH, H-bonded to carbonyl of amide) ppm.

Anal. Calcd for $C_{23}H_{37}NO_4.CH_3OH$*: C, 68.05; H, 9.76. Found: C, 68.50; H, 10.04.

(20S,21S)-3β-Hydroxy-21-amino-14β,21-oxidonorcholan-23-oic Acid Lactam (45)

Fraction B from the previous experiment (see experiment 44) afforded 0.35 g of this compound. Recrystallization from aqueous methanol gave shiny plates: mp 275-277°; $[\alpha]_D^{20}$ -19° (c 1, pyridine); ν_{max}^{KBr} 3521 cm^{-1} (-OH), 3448 cm^{-1} (lactam =NH), 1681 cm^{-1} (lactam C=O); nmr 1.00 (C-18 and -19 methyls), 4.16 (3α-H), 5.45 (J=6 Hz, C-21 H), 5.96 (=NH) ppm.

Anal. Calcd for $C_{23}H_{35}NO_3.CH_3OH$: C, 71.07; H, 9.69; N, 3.45. Found: C, 70.94; H, 9.41; N. 3.57.

Calculated molecular weight is 373.26167; mass spectrometric analysis gave 373.26147.

The same product was obtained by heating 43 to 200° or by treatment with warm glacial acetic acid.

(20S,21R)-3β-Hydroxy-21-amino-14β,21-oxidonorcholan-23-oic Acid Lactam (46)

Fraction A from experimental 44 afforded 0.75 g of this compound in pure form. Recrystallization from methanol-ethyl acetate gave white crystals: mp 266-268°; $[\alpha]_D^{20}$ -62° (c 1, pyridine); ν_{max}^{KBr} 3472 cm^{-1} (-OH), 3367, 3279 cm^{-1} (lactam =NH), and 1678 cm^{-1} (lactam C=O); nmr 0.99 (C-19 methyl), 1.13 (C-18 methyl), 4.15 (3α-H), 5.31 (C-21 H, doublet nature clearly seen when spectrum taken in Pyridine-d_5, J=5 Hz), 6.68 (=NH) ppm.

Anal. Calcd for $C_{23}H_{35}NO_3$: C, 73.96; H, 9.44; N, 3.75. Found: C, 73.74; H, 9.61; N, 3.81.

The calculated molecular weight is 373.261679; mass spectrometric analysis gave 373.261862.

This compound was also obtained by heating compound 44 to 200° or by treatment with warm glacial acetic acid.

(20S,21S)-3β-Acetoxy-21-amino-14β,21-oxidonorcholan-23-oic Acid Lactam (47).-- A solution of 0.32 g of 45 in 5 ml of pyridine was treated with 0.5 ml of acetic anhydride and kept for 18 hrs at 27°. The solution was poured into ice-water and the resulting precipitate was filtered, washed with water, air-dried, and crystallized from methanol. There was obtained a nearly quantitative yield of long, white prisms, mp 280-283°. The analytical sample, obtained from acetone-hexane, had mp 273-275°; $[\alpha]_D^{20}$ -39° (c 1, $CHCl_3$); ν_{max}^{KBr} 3367 cm^{-1} (lactam =NH), 1724, 1681 cm^{-1} (C=O of ester and lactam), and 1258, 1235 cm^{-1} (ester); nmr 1.00 (C-18 and -19 methyls), 2.03 (3β-acetate), 5.08 (3α-H), 5.41 (J=6.5 Hz, C-21 H), 6.41 (=NH) ppm.

Anal. Calcd for $C_{25}H_{37}NO_4$: C, 72.26; H, 8.97; N, 3.37
Found: C, 72.75; H, 8.89; N, 3.58.

The calculated molecular weight of this compound is 415.27224; mass spectrometric analysis gave a value of 415.27284.

(20S,21R)-3β-Acetoxy-21-amino-14β,21-oxidonorcholan-23-oic Acid Lactam (48). -- A solution of 0.23 g of 46 in 5 ml of pyridine was treated with 0.5 ml of acetic anhydride and kept for 18 hrs at 27°. The solution was poured into ice-water and extracted with ether. Evaporation of the washed and dried ether extract gave a crystalline residue which was recrystallized from methanol to afford 0.20 g of colorless crystals: mp 241-243°; $[\alpha]_D^{20}$ -25° (c 1, $CHCl_3$); nmr 0.99 (C-19 methyl), 1.11 (C-18 methyl), 2.03 (3β-acetate), 2.50 (multiplet, C-22 methylene), 5.08 (3α-H), 5.28 (C-21 H, doublet nature better seen when spectrum taken in benze-d_6, J=5 Hz), 6.80 (=NH) ppm.

Anal. Calcd for $C_{25}H_{37}NO_4$: C, 72.26; H, 8.97; N, 3.37.
Found: C, 72.28; H, 9.04; N, 3.41.

Calculated molecular weight is 415.27224; mass spectrometric analysis gave 415.27201.

(20S)-3-Oxo-14β-hydroxynorcholan-21,23-dioic Acid 14,21-Lactone 23-Amide (49). -- A cold solution of 0.20 g of 43 in 2 ml of pyridine was added to a stirred suspension prepared[88] by adding 0.2 g of chromic acid to 2 ml of pyridine. After 24 hrs at 27°, thin layer chromatography showed that the mixture consisted of two fractions neither of which corresponded to starting material. The reaction mixture was poured into ice-water and extracted with two 150 ml portions of ethyl acetate. the combined extracts were washed with water, dried (Na_2SO_4), and evaporated under reduced pressure. The gummy residue was chromatographed on a preparative thin layer plate using as the mobile phase a mixture of 10% benzene, 20% acetone, and 70% anhydrous ether. The upper band in the plate was cut out and extracted first with ethyl acetate and then with chloroform. The combined extracts were evaporated in vacuo and the residue was crystallized from methanol to give 0.035 g of 49 as granular crystals: mp 284-286°; $[\alpha]_D^{20}$ -3° (c 1, pyridine); nmr (Pyr.-d_5) 0.86 (C-19 methyl), 1.25 (C-18 methyl), 3.20 (C-22 methylene) ppm.

Anal. Calcd for $C_{23}H_{33}NO_4$: C, 71.29; H, 8.58; N, 3.61. Found: C, 70.94; H, 8.69; N, 3.82.

The calculated molecular weight is 387.24094; mass spectrometric analysis gave 387. 24019.

The same product was obtained on oxidation of 44 under the same conditions.

(20S,21S)-3-Oxo-21-amino-14β,21-oxidonorcholan -23-oic Acid Lactam (50).-- The lower band in the preparative thin layer plate from experiment 49 was cut out and extracted several times with ethyl acetate. The combined ethyl acetate extracts were evaporated in vacuo and the crystalline residue was

recrystallized from a small volume of ethyl acetate to yield 0.10 g of 50 as colorless needles: mp 275-276°; $[\alpha]_D^{20}$ 0.0° (c 1, ethanol); nmr (Pyr.-d_5) 0.90 (C-19 methyl), 0.98 (C-18 methyl), 5.50 (J=6.5 Hz, C-21 H) ppm.

Anal. Calcd for $C_{23}H_{33}NO_3$: C, 74.36; H, 8.95; N, 3.77. Found: C, 74.51; H, 8.83; N, 3.77.

Calculated molecular weight: 371.24603; mass spectrometric analysis gave 371.24575.

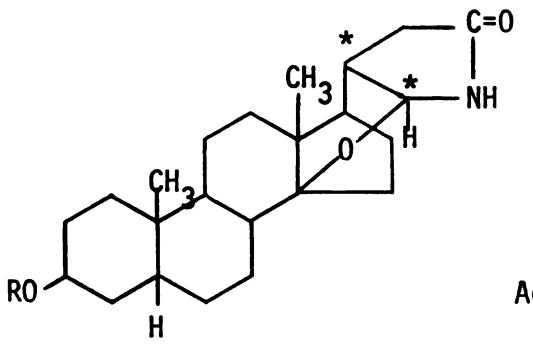
(20S,21S)-3β-Hydroxy-21-amino-14β,21-oxidonorcholan-23-oic Acid Lactam 3-Tridigitoxoside (51).-- An ice-cold solution of 2.0 g of digitoxin in 150 ml of methanol was treated with a stream of ammonia for 0.5 hr and stirred for 8 days at 27°. The solution was evaporated under reduced pressure at 25° to give a gelatinous residue which resisted crystallization. A solution of the gelatinous residue in 30 ml of methanol was treated with 5 ml of glacial acetic acid, warmed for a few minutes in the steam cone, and kept for 5 hrs at 27°. Evaporation of the solvent under reduced pressure afforded 1.15 g of amorphous, crude product. Several recrystallizations from aqueous acetone gave very small, colorless needles: mp 269-272°; $[\alpha]_D^{20}$ -11° (c 1, pyridine); nmr ($CDCl_3$+CD_3OD) 0.98 and 1.01 (C-18 and -19 methyls), 5.47 (J=7 Hz, C-21 H) ppm.

Anal. Calcd for $C_{41}H_{65}NO_{12}$: C, 64.45; H, 8.57; N, 1.83. Found: C, 64.20; H, 8.81; N, 2.04.

PART IV

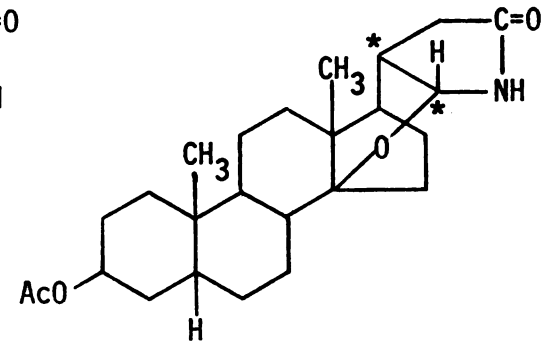
PHARMACOLOGICAL EVALUATION

Of the compounds synthesized and described in this thesis, we were particularly interested in evaluating the lactam derivatives 47, 48 and 51.



47. R = Ac

51. R = (Digitoxose)$_3$



48

One method of assaying compounds for digitalis-like activity is based on toxicity experiments carried out in cats, guinea pigs, frogs and pigeons. This method of bioassay, while useful in determining the relative potency of digitalis glycosides or digitalis-like compounds, yields no information on their therapeutic usefulness or cardiotonic activity. Unfortunately, the extensive use of this method of bioassay as a screening test for cardiotonic activity of completely different substances, may have resulted in the rejection of potentially useful and less toxic digitalis substitutes simply because they were not as toxic as digitalis. Better methods for assessing cardiotonic activity are obviously needed, and further developments in this area may eventually lead to the discovery of less toxic cardiotonic drugs.

There are several ways of testing the direct effects of drugs on the contractility of cardiac muscle. One of these methods, the one we used in the present biological evaluation, involves the use of isolated rabbit or guinea pig atria. The excised left atrium is mounted into a muscle chamber as illustrated in Fig. 9. The atrium is secured to the muscle holder by passing punctate electrodes through the lower corner. A light thread is then tied at the opposite corner of the muscle, and the free end of the thread is tied to an isometric transducer connected to a recorder. The muscle holder is made of glass tubing having a micropore at the submerged end for bubbling oxygen and carbon dioxide into the physiological solution bathing the muscle. Wire leads from the electrodes are connected to a muscle stimulator.

The chamber bath is maintained at constant temperature by pumping water through the jacket with a constant temperature circulator. A solution of the test compound is added directly to the solution bathing the muscle and the contractility events are observed directly on the recorder.

A satisfactory preparation of this type is good for 8 to 10 hours when using rabbit atria and a slightly shorter time when using guinea pig atria, so that the same muscle can be used for several test runs. The muscles are stimulated at a rate of one beat/sec. and the stimuli, which have a duration of 5 msec., are kept 20-40% above threshold.

Using the method described above, the isometric contractilities of isolated guinea pig and rabbit left atria were studied at $35.5^{o}C$ in modified Krebs-Henseleit bicarbonate solution containing 2.0 mM of Ca^{++}. The rate of spontaneous decline of twitch tension and the effects of the

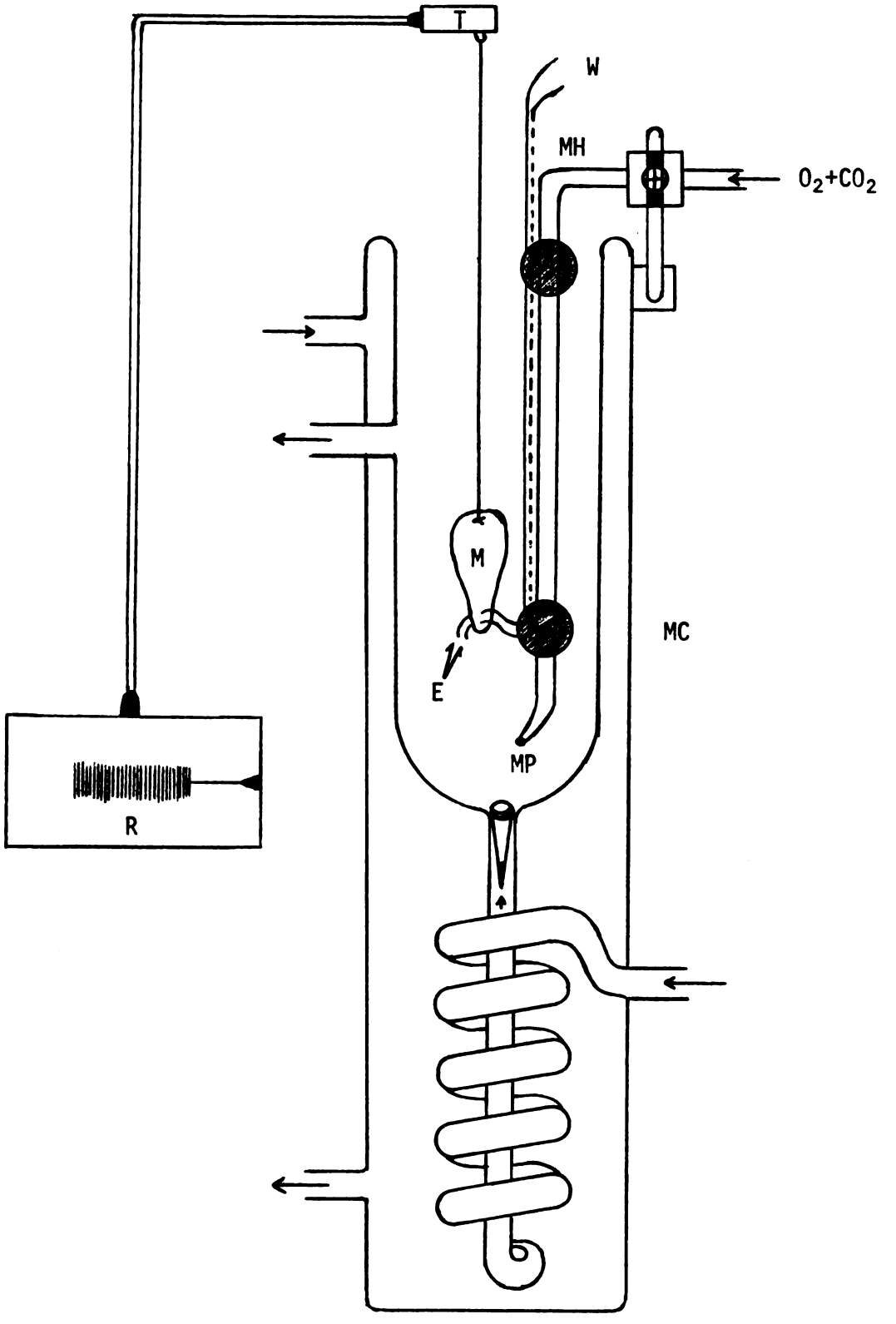


Fig. 9 Vertical chamber and muscle holder for in vitro atrial preparation. MC, jacketed muscle chamber; MH, muscle holder made of glass tubing with distal micropore(MP) for dispersing O_2-CO_2 bubbles; M, myocardial tissue; E, punctate electrodes; T, transducer; W, wires to stimulator; R, recorder.

solvent, dimethylsulfoxide, were consistent and not significantly different from each other. However, because of the quantitative variability of response from one preparation to another, each muscle was used as its own control.

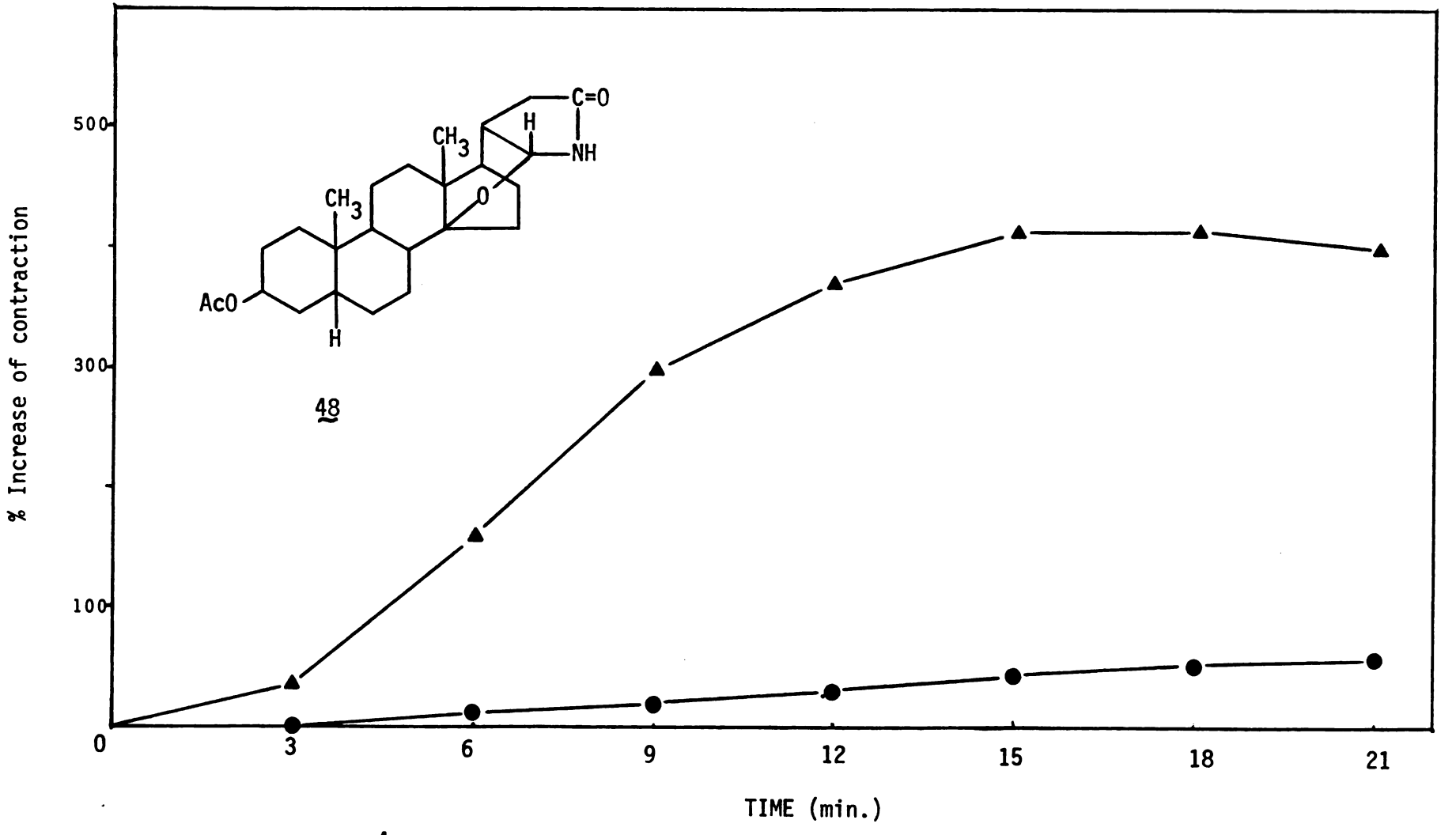
Preliminary testing of lactam 48 showed that this compound had no significant effect on the isometric contractility of isolated rabbit or guinea pig atria. However, it was observed that the inotropic effect produced by a standard dose of digitoxigenin acetate was substantially reduced if the muscle was pretreated with lactam 48. The lower curve in Fig. 10 shows the effects of 2.4×10^{-5} M dose of digitoxigenin acetate on the isometric contractility of rabbit atrium pretreated with 1.2×10^{-4} M of the lactam acetate. After 12 minutes, the force of contraction had barely increased by 25%, and after 21 minutes this had increased by about 50%. By contrast, the upper curve shows the effect of the same dose of digitoxigenin acetate on the same muscle in the absence of lactam. After 12 minutes the force of contraction had increased by about 370% and is still increasing.

Other experiments showed that if the dose of digitoxigenin acetate is increased, or conversely, if the dose of lactam acetate is decreased, the degree of inhibition or antagonism by the lactam is reduced. This alone may suggest a competitive type of inhibition.

Similar experiments were carried out with guinea pig atria in a balanced cross-over design with each of the six possible orders of administration of A:lactone* alone, B:lactam followed by lactone,

*Lactone = Digitoxigenin Acetate

Fig. 10 Effects of Digitoxigenin Acetate and Lactam Acetate (48) on the Isometric Contractility of Rabbit Left Atrium.



—●-●— 1.2×10^{-4} M of Lactam Acetate (48) followed by 2.4×10^{-5} M of Digitoxigenin Acetate.

—▲-▲— 2.4×10^{-5} M of Digitoxigenin Acetate alone.

C:lactam alone, represented once. Because the duration of inhibition is short, a standard dose of digitoxigenin acetate was chosen so as to produce a consistently large positive inotropic response in the first 10 minutes. Those muscles (about 10%) which showed toxic effects during the first 20 minutes of exposure were excluded. The results are shown in Table 4.

Table 4. Isometric contractile force of guinea pig left atria. Dimethylsulfoxide (DMSO) 0.42 ml or lactam (1×10^{-4} M final concentration) in 0.42 ml DMSO was added to the 50 ml bath at time zero. Digitoxigenin acetate (3×10^{-6} M final concentration) in 12.5 µl DMSO was added at 10 minutes. Data are expressed as percent of contractility at zero time ± standard error of the mean.

	Time (min)				
	10	13	15	20	30
Conditions	(%)	(%)	(%)	(%)	(%)
A. Lactone	98 ± 4	129 ± 7	147± 8	185 ± 14	243 ± 25
B. Lactam + Lactone	94 ± 5	103 ± 5	115 ± 5	151 ± 8	214 ± 17
C. Lactam	108 ± 4	-	-	96 ± 5	83 ± 5
D. No Treatment	94 ± 2	-	-	89 ± 4	84 ± 4
E. DMSO	98 ± 3	-	-	-	-

Quantification of the degree of inhibition for each muscle at each time interval according to the relationship given below, yielded Table 5. Letters refer to conditions as listed in Table 4.

Effect of lactone alone = A - D

Effect of lactone after lactam = B - C

$$\% \text{ Inhibition} = \frac{A - D - (B - C) \times 100}{A - D}$$

Table 5. Inhibition of guinea pig left atrial inotropic response to lactone (3×10^{-6} M) caused by prior administration of lactam (1×10^{-4}M). Lactam added at zero time, lactone at 10 minutes. Mean percentage inhibition calculated as described in the text. SEM = standard error of the mean. Probability (P) of no significant inhibition (null hypothesis) calculated by student t test (n = 6).

	Statistic		
Time (min)	Mean Inhibition (%)	SEM (%)	P
13	87.2	7.4	<.001
15	80.3	6.6	<.001
20	35.7	11.1	<.02
25	19.2	13.6	>.1
30	11.2	.4.0	>.1

In contrast to these results, compounds 47 and 51, the C-21 stereoisomers of 48 were devoid of inhibitory effects.

Exposure of guinea pig atria to 1 x 10^{-4} M lactam had no effect on the peak positive inotropic response to increasing the Ca^{++} concentration of the bath from 2 to 4 mM. However, in a few muscles, the same concentration of lactam inhibited the positive inotropic response to 0.1-0.2 µg/ml epinephrine by 21.3%±3.4 S.E.M. In one cat, I.V. administration of the lactam by 0.15 mg increments to a final dose of 7.7 mg/kg produced no EKG irregularities. The administration of digitoxigenin acetate by increments to 0.25 mg/Kg immediately following the lactam induced ventricular tachycardia and 0.6 mg/Kg was lethal, suggesting no inhibition of the toxic effects of the lactone.

The observed inhibition of the positive inotropic effect of conventional cardenolides by the lactam suggested that this compound might be useful in localizing the fundamental mechanism of the inotropic action of digitalis. One characteristic action common to all digitalis glycosides and aglycones is their ability to inhibit the enzymic activity of several Na^+ - K^+ -ATPase systems. This biochemical effect has received considerable attention as a possible mechanism for the inotropic action of digitalis,[2,41] and therefore we were interested in studying the interaction of the lactam with digitoxigenin acetate in preparations of Na^+ - K^+ -ATPase.

Lubrol extracts containing Na^+ - K^+ -ATPase activity were prepared from guinea pig cardiac microsomal fractions[109]. Total Na^+ - K^+ - Mg^{++} -ATPase activity was measured by orthophosphate release in 0.5 ml of medium containing ATP (tris salt) 5 mM, $MgSO_4$ 5 mM, KCl 10 mM, NaCl 110 mM, imidazole buffer (pH 6.8) 10 mM and enzyme preparation (25-100µg protein), following incubation at 37^{o} for 10 min. Mg^{++} -ATPase activity

determined in a similar system containing choline chloride instead of NaCl and KCl, represented 10-15% of the total ATPase activity and by subtraction gave values for Na^+ - K^+ -ATPase activity. Half-maximal inhibition of Na^+ - K^+ -ATPase activity of guinea pig cardiac preparations by ouabain and digitoxigenin acetate occurred at concentrations of 2.6×10^{-5} M and 5×10^{-6} M respectively. The lactam at concentrations between 10^{-6} and 10^{-4} M had no sognificant effect on Na^+ - K^+ -ATPase activity and did not influence the inhibitory action of the lactone when both compounds were added to the reaction media at the same time. These results are summarized in Table 6.

Table 6. Sensitivity of Na^+ - K^+ -ATPase Preparations of Guinea Pig Heart to Lactone and Lactam. Composition of the assay medium described in the text.

Conditions	Na^+ - K^+ -ATPase Activity (μMoles P_i/mg Protein/hr)	Inhibition (%)
Control	20.3 ± 1.6	-
Lactone (10^{-5}M)	7.3 ± 0.5	64.4
Lactam (10^{-4} M)	19.8 ± 1.0	3.1
Lactone (10^{-5} M) + Lactam (10^{-4} M)	6.8 ± 0.1	64.9

A number of these experiments were designed to parallel certain of the physiological studies. In these experiments, the enzyme preparations were preincubated for 10 minutes with 10^{-4} M of lactam before addition of the lactone and the reaction was then allowed to proceed for 30 minutes.

In these experiments, the degree of lactone inhibition (60%) of the Na^+ - K^+ -ATPase activity was the same whether or not the lactam was present. In similar experiments using lubrol extracts of rat brain microsomal[109] fractions and conventional rat cardiac microsomal preparations[110] the lactam had no inhibitory effect on Na^+ - K^+ -ATPase activities and no positive or negative influence on the sensitivity of such preparations to either digitoxigenin acetate or ouabain.

The following conclusions can be drawn from these experiments.

1. Compound 48, 3-acetylisodigitoxigeninic acid lactam, has a spectrum of activity not previously reported. This compound has no significant direct effects on the contractility of myocardium in vitro, and shows no significant effect on the activity of Na^+ - K^+ -ATPase preparations. These properties alone would not distinguish the lactam from many other "inactive" natural and semisynthetic cardenolides which have been reported. These direct action experiments merely indicate that in the digitoxigenin series, an intact lactone ring is required for inotropic and ATPase inhibitory effects.[111-113]
2. The physiological experiments designed to study the interaction of the lactam with a standard cardenolide were a necessary requirement in order to reveal its inhibitory effects. These experiments indicate that the lactam appears to be somewhat specific in its inhibition of cardenolide inotropism. Other agents which have been reported to inhibit cardenolide inotropism included aldosterone[114] and manganese ion.[115] However, some authors have been unable to demonstrate the inhibitory effect of aldosterone.[116] Manganese ion is a less specific inhibitory agent than the lactam since manganese inhibits the positive inotropic effect of increased extracellular Ca^{++} as well as that of ouabain. The evidence for inhibition of epinephrine inotropism by the lactam, while not conclusive, suggests

that (a) epinephrine may share an inotropic mechanism with the cardiac glycosides for a small portion of its total inotropic effect or that (b) the lactam may have weak beta adrenergic blocking effects. In the case of digitalis antagonism, the high antagonist:-agonist ratio required and the brief duration of the inhibition suggests that the affinity of the lactam for the digitalis receptor is quite low or that digitalis has more than one mechanism of action. Further experiments will be required to fully define the nature of the inhibition caused by the lactam. In a recent report,[117] it was shown that tetrodotoxin, a specific inhibitor of the Na-spike generating mechanism, caused a small but significant depression of the ouabain induced positive inotropic effect similar to that produced by the lactam.

3. The biochemical data show that in the presence of the lactam, the inhibitory effect of digitoxigenin acetate on several different Na^+ - K^+ -ATPase systems is unimpaired. These data together with the preliminary observation that the lactam did not produce EKG toxicity or change the toxic EKG manifestations of subsequent doses of the lactone is in harmony with the hypothesis that the toxic effects of cardenolides may be related to the inhibition of transport ATPase.[48] However, these results should not be interpreted to indicate that there is no relationship between ATPase inhibition and the inotropic effects of cardenolides in the absence of the lactam.

4. Finally, these results suggest that the lactam 48 because of its rigid structure might more closely resemble the actual overall shape in which the cardenolide interacts with the receptor and that it may be possible to synthesize a selective cardenolide with a significantly increased therapeutic index, or conversely, a selective inhibitor of the toxic effects of conventional cardenolides.

SUMMARY

Cardiotonic steroids are of major importance in heart therapy and methods for their synthesis not only are of interest per se, but also as a means for obtaining analogs of potential pharmacological importance.

Synthesis of the A/B ring system of sarmentosigenin E, a C-5, C-19-functionalized aglycone, is described. Oxidation of 5α-chloro-syn-19-oximinocholestane-3β,6β-diol 3-acetate (1) gave 5α-chloro-3β-hydroxy-6-oxocholestane-19-nitrile 3-acetate (18) which was allowed to react with alcoholic potassium hydroxide to form 3β,5β-dihydroxy-6-oxocholestane-19-nitrile. This diol was reduced with sodium borohydride to afford 3β,5β,6β-trihydroxycholestane-19-nitrile, which on treatment with methanolic hydrogen chloride followed by hydrolysis furnished 3β,5β,6β-trihydroxycholestan-19-oic acid 6,19-lactone (23) embodying the A/B ring system of sarmentosigenin E. Reduction of 5β,6β-epoxy-19-oximinocholestan-3β-ol (3) or the corresponding nitrile (4) with lithium aluminum hydride afforded 19-norcholest-5(10)-ene-3β,6β-diol (5) via a fragmentation reaction.

In studies directed toward the elaboration of the remaining structural features of cardiac aglycones, bromination of 14-hydroxy-3-oxo-5β,14β-card-20(22) enolide (25) with phenyltrimethylammonium tribromide (PTT) followed by dehydrobromination with benzyltrimethylammonium mesitoate (BTAM) afforded 14-hydroxy-3-oxo-14β-carda-4,20(22) dienolide (42).

Ammonolysis of digitoxigenin (24) afforded two isomeric lactol amides, (20S,21S)-3β,21-dihydroxy-14β,21-oxidonorcholan-23-oic acid amide (43) and (20S,21R)-3β,21-dihydroxy-14β,21-oxidonorcholan-23-oic acid amide (44), both of which when treated with warm glacial acetic acid cyclized stereoselectively to the corresponding lactams, (20S, 21S)-

3β-hydroxy-21-amino-14β,21-oxidonorcholan-23-oic acid lactam (45) and (20S,21R)-3β-hydroxy-21-amino-14β,21-oxidonorcholan-23-oic acid lactam (46). The pharmacological evaluation of the above lactams is described and the implications of these results for the mode of action of cardiotonic steroids is discussed.

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