The entire screening procedure was conducted for each buffered solution rather than the *N*-chloramine solution in the procedure.

SUMMARY

The lone-pair ionization energies for the N, O, and Cl heteroatoms in four derivatives of piperidine were measured from UV photoelectron spectra. The interaction between the various lone pairs in each piperidine was studied. The hydrolytic stability and antimicrobial activity of the *N*-chloro piperidines also were determined. The current results suggest that it may be possible to correlate the stability of the nitrogen lone pair, as determined from photoelectron spectroscopy, with the antibacterial activity of the corresponding *N*-chloramines.

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Cardiotonic Steroids I: Importance of 14β-Hydroxy Group in Digitoxigenin

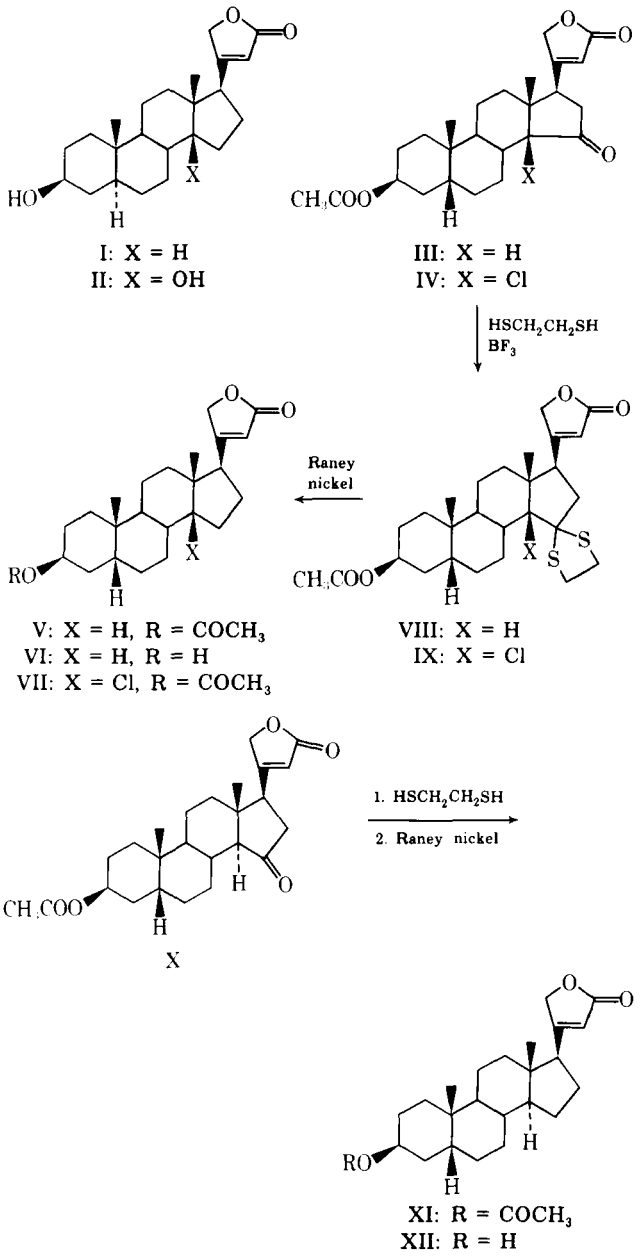
BALA K. NAIDOO‡, THOMAS R. WITTY‡, WILLIAM A. REMERS‡ˣ, and H. R. BESCH, Jr.†

Abstract □ Analogs of digitoxigenin and its 3-acetate, in which the stereochemistry and nature of the substituent at the 14-position are varied, were synthesized and assayed for inhibition of myocardial Na^+,K^+-adenosine triphosphatase. Among the 3-acetates, the 14β-H and 14α-H analogs were less active than 3-acetoxydigitoxigenin by a factor of 1000, with the 14β-chloro analog slightly more active than the others. The corresponding 14β,15β-epoxide was 0.03 as active as 3-acetoxydigitoxigenin, but the isomeric 14α, 15α-epoxide was virtually inactive. In the 3-OH series, digitoxigenin was significantly more active than any analog. The 14β-H analog was 10-fold more active than the 14α-H analog. These results suggest the desirability of *cis*-C,D-ring fusion and a highly selective function for the 14β-OH group of digitoxigenin, possibly based upon its ability to receive a hydrogen in hydrogen bonding. Syntheses of the 14β-H, 14α-H, and 14β-Cl analogs were accomplished by converting the known 15-ketones to ethylene thioketals and desulfurizing with Raney nickel. Attempts to prepare a 14β-NH_2 analog were unsuccessful.

Keyphrases □ Digitoxigenin, 14β-H, 14α-H, 14β-Cl, and acetate analogs—synthesis and inhibition of Na^+,K^+-adenosine triphosphatase □ Cardiotonic steroids—synthesis of digitoxigenin analogs (14β-H, 14α-H, 14β-Cl, and acetates), inhibition of Na^+,K^+-adenosine triphosphatase □ Structure–activity relationships—digitoxigenin analogs (14 substituents) and inhibition of Na^+,K^+-adenosine triphosphatase

It has been accepted generally that the 14β-OH group of cardiotonic steroids and their glycosides is essential to their inotropic activity (1). Derivative structures such as the corresponding 14(15)-enes and epoxides are considered much less active in assays based upon lethality in cats (1), and 14-epidigitoxigenin showed no activity in a Na^+,K^+-adenosine triphosphatase assay (2). These observations tend to confirm the need for a *cis*-C,D-ring fusion as well as a hydroxy group at the 14-position. However, recent reports (3, 4) on uzarigenin derivatives showed that the 14β-H analog (I) was one-third as active as uzarigenin itself (II) in the isolated frog heart, indicating that the 14β-hydroxy group is not indispensible for positive inotropic activity. This report prompted a more extensive investigation of the requirements in stereochemistry and the nature of substituents at the 14-position of cardenolides for inotropic activity.

The digitoxigenin nucleus was chosen for investigation since it is more active than the uzariginen nucleus due to its *cis*-A,B-ring fusion. This factor appeared important for structure–activity relation-



Scheme I

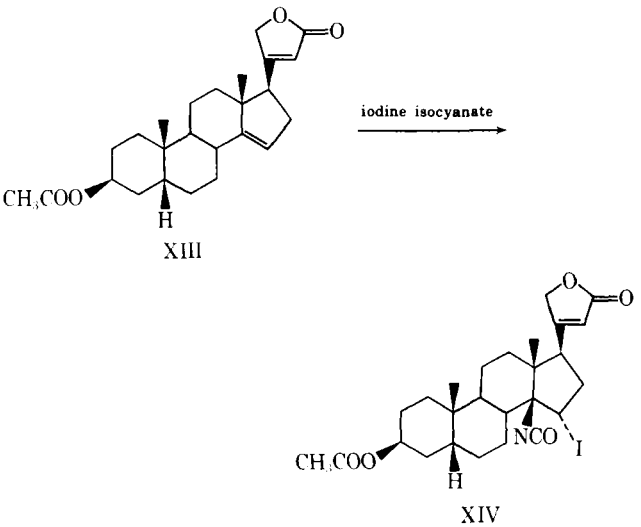
ships, and it suggested potential clinical utility if the digitoxigenin analogs retained good activity. The 14β-H analog (VI) of digitoxigenin was chosen as a target compound by analogy to 14β-H,14-dehydroxyuzarigenin (I), and the known (5) 14α-H epimer (XI) was chosen to check the C,D-ring fusion requirement. The 14β-chloro group (of analog VII) seemed important since it would have the same size and stereochemistry as the 14β-hydroxy group of digitoxigenin but not its ability to hydrogen bond to the receptor. The 14β-amino group would be able to participate in hydrogen bonding. Finally, the known 14β,15β-epoxide (XXI) is interesting as an analog because it has oxygen in nearly the same position as that of the 14β-OH group (although the steroid nucleus is distorted). It could receive a hydrogen in hydrogen bonding but it could not furnish one, in contrast to the hydroxy group. Since the 3-acetates of these target analogs were intermediates in their syntheses, it was reasonable to assay both the 3-acetates and the 3-hydroxy target compounds, using digitoxigenin and its 3-acetate as standards.

RESULTS AND DISCUSSION

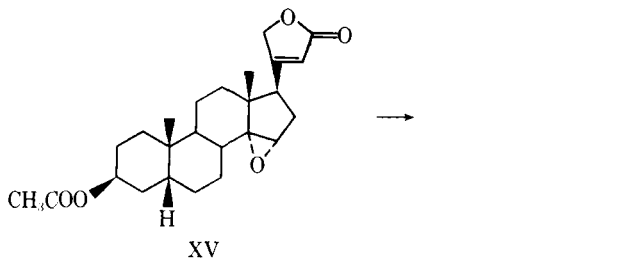
Synthesis—The 3-acetates (V and XI, respectively) of 14β-H- and 14α-H-dehydroxydigitoxigenins were prepared from the known 15-oxo derivatives (III and X, respectively) (6–8) by conversion to thioketals and desulfurization with deactivated Raney nickel (Scheme I). They were then hydrolyzed to the 3-hydroxy analogs VI and XII. Compound XII was also prepared by catalytic hydrogenation of 14(15)-anhydrodigitoxigenin according to the known (5) procedure. The 14β-chloro analog was prepared as its 3-acetate (VII) by a parallel route from the known (8) 14β-chloro-15-oxo compound (IV). However, it was not possible to hydrolyze VII to the corresponding 3-hydroxy compound due to an insufficient amount of VII. An alternative route to this 3-hydroxy compound involving hydrolysis of IV (9) prior to thioketal formation was attempted but was unsuccessful. Subsequent attempts to repeat the preparation of VII were only partially successful since the pure product was not obtained.

Two routes to the 14β-amino analog were investigated. In one route (Scheme II), the 14(15)-ene (XIII) was converted into the corresponding 15α-iodo-14β-isocyanato derivative XIV by treatment with iodine isocyanate. The stereochemistry of XIV is assigned on the basis of the mode of addition of iodine isocyanate to double bonds. Thus, an iodonium ion should form on the less hindered α-side and be opened by the attack at carbon-14 of isocyanate ion from the β-side of the molecule. This mechanism is analogous to the mechanisms that are presumably involved in the addition of hypobromous acid to XIII and and the acidic ring opening of 14α,15α-epoxide (XV); in each of these cases, the 14β,15α-product is formed (7, 8). The facile elimination of the elements of iodine isocyanate from XIV supports this structural assignment. Unfortunately, this elimination occurred during all attempts to convert XIV into the desired 14β-NH_2 analog and this route was abandoned. The second route (Scheme III) was based upon hydrazoic acid ring opening of 14α,15α-epoxide (XV). This reaction gave, in low yield, a product that appeared to be the azidohydrin XVI, according to its IR, NMR, and mass spectra, but it could not be purified because of its instability.

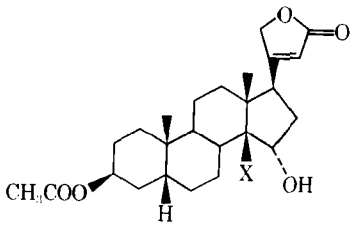
Biological—Several cardenolides and certain intermediates leading to them were tested for their ability to inhibit a Na^+,K^+-stimulated, Mg^{+2}-dependent adenosine triphosphatase isolated from calf or canine myocardium (10). The ability of a large number of cardenolides to inhibit specifically this adenosine triphosphatase has been shown to correlate well with their positive inotropic activity (11, 12). As recorded in Table I, digitoxigenin acetate has a K_I of 2×10^{-8}. The next most active compound was the 14β,15β-epoxide (XXI), which had about 0.03 of this activity. The 14β-H, 14α-H, 14β-Cl, and 14(15)-ene analogs were about 0.001 as active,



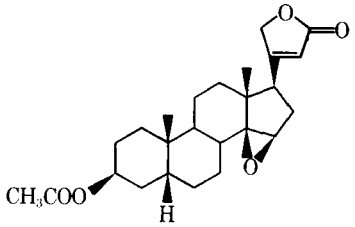
Scheme II



XV



XVI: R = $COCH_3$, X = N_3
XVII: R = $COCH_3$, X = OH
XVIII: R = $COCH_3$, X = Cl
XIX: R = H, X = OH
XX: R = H, X = Cl



XXI

Scheme III

with the 14β-Cl analog (VII) the most active of this group. Little difference was found between the acetates (V and XI) of the 14β-H and 14α-H compounds, due possibly to the low solubility of the former. However, the corresponding 3-hydroxy analogs (VI and XII) and the 14β-H compound were 10 times as active, which indicates the superiority of the *cis*-C,D-ring fusion. The 14α,15α-epoxide (XV) was so inactive that its K_I could not be determined conveniently.

The relatively high potency of the 14β,15β-epoxide (XXI) is surprising in view of Chen's (13) observation that the 3-hydroxy analog of this epoxide was not toxic to cats. However, that observation might be due to pharmacokinetic considerations. At the receptor enzyme, XXI appears to be more like digitoxigenin acetate than any of the other analogs tested in the present study, despite certain conformational distortions in XXI. Thus, its epoxide ring deforms the C-ring from a perfect chair formation, which causes small changes throughout the molecule in the positions of atoms relative to those in digitoxigenin acetate. Molecular models do show that the 14-oxygen in XXI is in almost the same position as the corresponding oxygen in digitoxigenin acetate.

Since the 14β-Cl analog (VII), which has correct C,D-ring fusion and substituent size at the 14-position, is poorly active whereas the 14β,15β-epoxide is relatively potent, it appears that the 14-oxygen fulfills a specific role. This role possibly is one of receiving a hydrogen in hydrogen bonding with some functional group on the adenosine triphosphatase. The 14β-OH group of digitoxigenin could furnish the hydrogen as well as receive it. The requirement of a 14β-oxygen group is supported by both the very poor activity of the 14α,15α-epoxide (XV) and the previously reported inactivity of 14-epidigitoxigenin relative to digitoxigenin (2).

In a previous report, Shigei *et al.* (14) were surprised to find that 15α-hydroxydigitoxigenin (XIX) was inactive in the frog heart whereas the corresponding 14β-Cl analog (XX) was 0.1–0.3 as active as digitoxigenin. Since the intermediate 3-acetates (XVII and XVIII) corresponding to these compounds had been prepared, they were tested for inhibition of Na^+,K^+-adenosine triphosphatase. As shown in Table I, the 14β-Cl,15α-OH derivative (XVIII) was approximately three times more potent than the corresponding compound without the 15α-OH substituent (VII) and about four times more active than 15α-hydroxydigitoxigenin 3-acetate (XVII).

Table I—K_I Values for the Inhibition of Na^+,K^+-Adenosine Triphosphatase

	3-OH,5β-Card-20(22)-enolide		K_I
	14β-OH	3-acetate	2×10^{-8}
V	14β-H	3-acetate	3.5×10^{-5a}
XI	14α-H	3-acetate	3.5×10^{-5}
VII	14β-Cl	3-acetate	1.1×10^{-5}
XIII	14(15)-ene	3-acetate	7.8×10^{-5}
XV	14α,15α-epoxide	3-acetate	$>3 \times 10^{-5}$
XXI	14β,15β-epoxide	3-acetate	6.2×10^{-7}
XVII	14β-OH,15α-OH	3-acetate	1.7×10^{-5}
XVIII	14β-Cl,15α-OH	3-acetate	4.2×10^{-6}
	14β-OH (digitoxigenin)		6×10^{-8}
VI	14β-H		1.6×10^{-6}
XII	14α-H		1.5×10^{-5}
	14(15)-ene		2.0×10^{-5}

[a] Not completely soluble.

During this investigation, it was reported that an impure sample of 14β-H analog (VI) was about 0.1 as active as digitoxigenin in a frog heart preparation (14).

EXPERIMENTAL[1]

3β-Acetoxy-15-oxo-5β,14β-card-20(22)-enolide, Cyclic Ethylene Mercaptole (VIII)—A mixture of 0.2 ml of 1,2-ethanedithiol, 0.26 ml of boron trifluoride etherate, and 100 mg of 3β-acetoxy-15-oxo-5β,14β-card-20(22)-enolide (III) (6–8) was kept at room temperature for 24 hr, treated with 0.5 g of solid sodium carbonate, and extracted with two portions (200 ml) of chloroform. The combined extracts were washed successively with 5% aqueous sodium hydroxide and water, dried (sodium sulfate), and concentrated under reduced pressure. The white solid residue (92 mg) was purified by chromatography on silica gel with chloroform–acetone (5:1) as solvent. This procedure gave 76 mg (65%) of VIII as white flakes, mp 262–263°; IR ($CHCl_3$): 5, 6, and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 490.

Anal.—Calc. for $C_{27}H_{38}O_4S_2$: C, 66.10; H, 7.75; S, 13.08. Found: C, 65.87; H, 7.79; S, 12.94.

3β-Acetoxy-15-oxo-5β,14α-card-20(22)-enolide, Cyclic Ethylene Mercaptole—This compound was prepared by the procedure described for VIII. From 80 mg of 3β-acetoxy-15-oxo-5β,14α-card-20(22)-enolide (X) (6–8) was obtained 63 mg (73%) of the thioketal as white powder, mp 269–272°; IR ($CHCl_3$): 5.59, 5.71, and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 490.

Anal.—Calc. for $C_{27}H_{38}O_4S_2$: C, 66.10; H, 7.75; S, 13.08. Found: C, 65.91; H, 7.88; S, 12.86.

3β-Acetoxy-5β,14β-card-20(22)-enolide (V)—A mixture of 68 mg of VIII, Raney nickel prepared from 3 g of alloy and deactivated by heating with acetone (15), and 40 ml of ethanol was heated at reflux temperature for 150 min, filtered, and concentrated. The residual solid (51 mg) was purified by chromatography on silica gel with chloroform–acetone (5:1) as the solvent. Recrystallization from ether gave a low yield of V as white plates, mp 180–181°; IR ($CHCl_3$): 5, 6, and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 400.

Anal.—Calc. for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06. Found: C, 75.36; H, 8.55.

3β-Acetoxy-5β,14α-card-20(22)-enolide (XI)—This compound was prepared by the procedure described for V. From 50 mg

[1] Melting points were determined on a Mel-Temp apparatus and are uncorrected. IR absorption spectra were recorded using $CHCl_3$ solutions or KBr pellets and a Perkin-Elmer 237B spectrophotometer. NMR spectra were determined in $CDCl_3$ using tetramethylsilane as the reference on a Jeol 60-MHz spectrometer. Mass spectra were determined on a Consolidated Electronics Corp. 21-110B mass spectrometer. Microanalyses were carried out by Midwest Microlab Ltd., Indianapolis, Ind.

of the thioketal (prepared from X) was obtained a low yield of XI, mp 171–173°; IR ($CHCl_3$): 5.61 and 5.77 (butenolide, acetate) μm; the mass spectrum showed M^+ at *m/e* 400.

Anal.—Calc. for $C_{25}H_{36}O_4$: C, 74.96; H, 9.06. Found: C, 75.21; H, 8.85.

3β-Hydroxy-5β,14β-card-20(22)-enolide (VI)—A solution of 20 mg of V in 3 ml of 70% aqueous ethanol containing 5% HCl was heated at reflux temperature for 3 hr, cooled in an ice bath, neutralized with 5% $NaHCO_3$ solution, and concentrated under reduced pressure. The residue was extracted with chloroform and this extract was washed with water, dried (magnesium sulfate), and concentrated. The crude product was purified by chromatography on silica gel with chloroform–acetone (5:1) as the solvent, followed by recrystallization from ether–hexane. This procedure gave 11 mg (60%) of VI as white plates, mp 168–170°; IR: 2.85 (OH), 5.6, and 5.71 (butenolide) μm; the mass spectrum showed M^+ at *m/e* 358.

Anal.—Calc. for $C_{23}H_{34}O_3$: C, 77.05; H, 9.56. Found: C, 77.41; H, 9.35.

3β-Hydroxy-5β,14α-card-20(22)-enolide (XII)—This compound was prepared by the procedure described for VI. From 18 mg of XI was obtained 8 mg (50%) of XII as white needles, mp 223–224° [lit. (5) mp 224–225°]; IR: 2.86 (OH), 5.6, and 5.71 (butenolide) μm; the mass spectrum showed M^+ at *m/e* 358.

3β-Acetoxy-14-chloro-5β,14β-card-20(22)-enolide (VII)—A mixture of 48 mg of 3β-acetoxy-14-chloro-15-oxo-5β,14β-card-20(22)-enolide (IV) (8), 0.15 ml of 1,2-ethanedithiol, and 0.12 ml of boron trifluoride etherate was warmed until a solution was obtained and kept 2 days at room temperature. Solid sodium carbonate was added and the mixture was extracted with chloroform. Purification as described for the preparation of VIII gave 35 mg of the thioketal (IX) as a gum which could not be crystallized. It was treated directly with deactivated Raney nickel in refluxing ethanol as described in the preparation of V. After the silica gel chromatography, 8 mg (27% overall) of VII was obtained as white powder, mp 157–159°; IR ($CHCl_3$): 5.61, 5.72, and 5.8 (butenolide, acetate) μm; the mass spectrum showed $M^+ - HCl$ at *m/e* 398.

Anal.—Calc. for $C_{25}H_{35}ClO_4$: Cl, 8.07. Found: Cl, 7.73.

3β-Acetoxy-15α-iodo-14-isocyanato-5β,14β-card-20(22)-enolide (XIV)—A slurry of 0.25 g of silver cyanate (16) and 0.25 g of iodine in 5 ml of tetrahydrofuran was stirred 1 hr at −20° and then treated dropwise with 0.4 g of 3-acetoxy-5β-card-14(15),20(22)-dienolide (XIII) (17) in 5 ml of tetrahydrofuran. The resulting mixture was stirred 1 hr at −20°, stirred 4 hr at room temperature, filtered, and concentrated under reduced pressure. The residue was dissolved in methylene chloride, washed with 0.1 *N* sodium thiosulfate and water, dried (magnesium sulfate), and concentrated. Recrystallization of the residue (350 mg) from chloroform–ether gave a white solid, mp 167–168°; IR (KBr): 4.42 (isocyanate), 5.61, 5.73, and 5.78 (butenolide, acetate) μm; NMR ($CDCl_3$): 5.90 (m, 1, vinyl of butenolide), 5.06 (m, 1, C-3 methine), 4.72 (m, 2, methylene of butenolide), 2.02 (s, 3, acetate), and 0.97 and 0.90 (each s, each 3, angular methyls) ppm.

Anal.—Calc. for $C_{26}H_{34}INO_5$: C, 55.03; H, 6.04; N, 2.47. Found: C, 55.16; H, 6.28; N, 2.25.

Biological Assay—Na^+,K^+-adenosine triphosphatase activity was determined by continuously monitoring[2], at 340 nm, the oxidation of NADH linked *via* a series of enzymatic reactions to the hydrolysis of ATP (18). In this assay, the ADP produced by the adenosine triphosphatase is rephosphorylated to ATP by the action of pyruvate kinase, which converts phosphoenolpyruvate to pyruvate. This reaction is coupled to the oxidation of NADH *via* lactic dehydrogenase, which reduces the pyruvate to lactate. For total adenosine triphosphatase activity, each cell contained 0.1 *M* NaCl, 0.01 *M* KCl, 0.05 *M* $MgCl_2$, 0.025 *M* tromethamine–ATP, 0.025 *M* tromethamine hydrochloride (pH 7.4), 0.05 *M* NADH, 0.025 *M* phosphoenolpyruvate, about 15 units of pyruvate kinase/ml, and about 15 units of lactic dehydrogenase/ml. After temperature equilibration at 37°, the reactions were started by the addition of the Na^+,K^+-adenosine triphosphatase preparation (usually about 100 μg). The Na^+,K^+-stimulated portion of the total adenosine triphosphatase was taken as the difference in activity of the adenosine triphosphatase in the absence and the presence of 0.001 *M* ouabain. Inhibition of Na^+,K^+-adenosine triphosphatase activity by the cardenolides was measured over a 10^{-9}–10^{-3} *M* range of concentrations. With some cardenolides, the degree of inhibition did not reach its maximum until after about 15 min of incubation. The K_I values reported were extrapolated from curves describing this steady level of inhibition at each concentration of cardenolide.

[2] A Gilford recording spectrophotometer equipped with a Haake constant-temperature bath was used.

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