Inhibition of Sodium- and Potassium-Dependent Adenosine Triphosphatase by Cardenolide Alkylating Agents[1]

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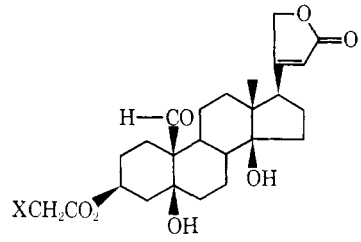
Four bromoacetylated cardenolides, namely, digitoxigenin 3-bromoacetate (**2a**), 15α-bromoacetoxydigitoxigenin 3-acetate (**2b**), digoxigenin 3,12-dibromoacetate (**2c**), and Δ^{14}-anhydrodigitoxigenin 3-bromoacetate (**3a**), were prepared and tested for their ability to inhibit Na- and K-ATPase from guinea pig brain. Three of these compounds, **2a**, **2b**, and **2c**, inhibited the enzyme to approximately the same extent. Compound **3a** had significant irreversible activity in spite of the fact that the corresponding 3-acetate (**3b**) did not appreciably inhibit the enzyme in a reversible fashion.

The naturally occurring cardiotonic steroids have long been accepted in medicine for their potent effects on the human heart. Investigations[2] into the mode of action of these compounds have demonstrated that they act as inhibitors of active ion transport. More specifically, it has been shown[3] that this interference with ion transport is a result of inhibition of Na^+- and K^+-dependent ATPase (also called transport ATPase) which, by catalyzing the cleavage of ATP to ADP, provides the energy required for active ion transport.

The dephosphorylation of a postulated[4] acyl phosphate ester is K^+ dependent and this stage is inhibited by the cardiotonic steroid, ouabain.[5] This inhibition is believed to be a result of displacement of K^+ from its attachment to the enzyme by the steroid in a competitive-type reaction.[5,6] Portius and Repke[7] have suggested that the CO group of the unsaturated lactone, characteristic of the cardiac steroids, forms an H bond with an OH of the phosphoric acid residue in the postulated phosphorylated enzyme intermediate. This H bonding prevents access of K^+ to the phosphorylated enzyme and also permits free rotation of the steroid molecule so that its correct face comes into close relationship with the complementary enzyme surface. Alternatively, Repke[8] has proposed that the H bond involves a free OH, NH_2, or SH group in the enzyme. Likewise, Glynn[9] has postulated that the unsaturated lactone ring may interact with SH or other nucleophilic groups at the K^+ transport site, although Hoffman[10] has adduced evidence that these steroids may actually inhibit in an allosteric manner rather than by direct competition with K^+.

In an effort to further elucidate the molecular mechanism of action of the cardiotonic steroids Kupchan, *et al.*,[11] used the concept of active-site-directed inhibition.[12] They found that strophanthidin 3-iodoacetate and bromoacetate (**1**, X = I and Br, respectively) are both active-site-directed inhibitors of Na- and K-



ATPase obtained from guinea pig brain and also exhibit high cardiotonic activity in the guinea pig atrial perparation.

As part of a study to further define the nature of the inter-action between the cardiotonic steroids and Na- and K-ATPase we have prepared 4 bromoacetylated cardenolide derivatives—*viz.*, digitoxigenin 3-bromoacetate (**2a**), 15α-bromoacetoxydigitoxigenin 3-acetate (**2b**), digoxigenin 3,12-dibromoacetate (**2c**), and Δ^{14}-anhydrodigitoxigenin 3-bromoacetate (**3a**)—in order to test the ability of each to irreversibly inhibit Na- and K-ATPase.

Synthesis.—Digitoxigenin acetate (**2d**) served as the starting material in the synthesis of 15α-bromoacetoxydigitoxigenin 3-acetate (**2b**). Dehydration of **2d** to give Δ^{14}-anhydrodigitoxigenin 3-acetate (**3b**) was accomplished using the procedure of Bach, *et al.*[13] Treatment of **3b** with *m*-chloroperbenzoic acid gave the 14,15α-epoxide **4** which was hydrolyzed with $HClO_4$[14] to yield 15α-hydroxydigitoxigenin 3-acetate (**2e**). Bromoacetylation of **2e** to give **2b** was achieved by heating with $BrCH_2COBr$ in dioxane–pyridine at 70° for 16 hr.

The placement of the bromoacetyl group at position 3 of digitoxigenin (**5**) was carried out at room temp using $BrCH_2COBr$ in dioxane–pyridine. It was found

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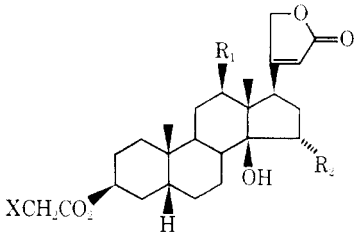
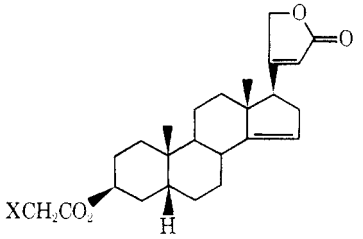


2a, X = Br; $R_1 = R_2 = H$
b, X = R_1 = H; $R_2 = O_2CCH_2Br$
c, X = Br; $R_1 = O_2CCH_2Br$; R_2 = H
d, X = $R_1 = R_2$ = H
e, X = R_1 = H; R_2 = OH

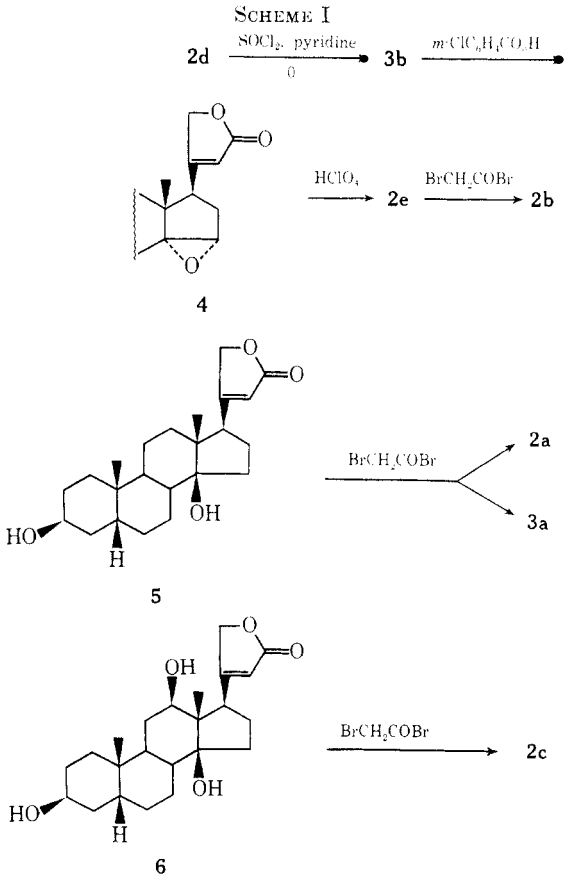


3a, X = Br
b, X = H

that when the reaction mixture was allowed to stand for 1.5 hr, **2a** was the major product, but when the reaction time was extended to 3 hr, **3a** was the predominant component of the reaction mixture.

When digoxigenin (**6**) was heated in dioxane–pyridine with $BrCH_2COBr$ at 75°, **2c** was obtained in 56% yield after purification on a silica gel tlc plate.

SCHEME I



Biochemical Studies.—The inhibition studies were carried out using a Na- and K-ATPase preparation obtained from guinea pig brain. The procedure of Kupchan, *et al.*,[11b] for obtaining the preparation was followed. The quantity of protein in the preparation was estimated by the method of Lowry, *et al.*,[15] and the activity of the ATPase in both reversible and irreversible inhibition studies was determined by the quantity of inorganic phosphate liberated from the ATP substrate following the procedure used by Kupchan, *et al.*,[11b] for the evaluation of strophanthidin 3-haloacetates.

Table I shows the effect of the cardenolide derivatives on brain Na- and K-ATPase. Na- and K-ATPase activity in the presence of cardenolide is expressed as a per cent of the activity of the control sample incubated without cardenolide. Irreversible inhibition of the Na- and K-ATPase was demonstrated by failure to regain the control activity after washing. The washing procedure brought the enzyme activity back to that of the control when the inhibition was of a reversible nature. The gain in activity after washing is the per cent of reversible inhibition. The activity not regained after washing is the per cent of irreversible inhibition.

TABLE I
Na- AND K-ATPase INHIBITION STUDIES

	—% of control activity—		—% of enzyme inhibition—	
No.[a]	Unwashed	Washed	Reversible	Irreversible
2a	7.1	18.9	11.8	81.1
2b	13.3	19.6	6.3	80.4
2c	5.8	15.5	9.7	84.5
2d	67.5	100	32.5	0.0
2e	98.0	104	6.0	−4.0
3a	20.0	29.7	9.7	70.3
3b	96.6	96.7	0.1	3.3
4	93.7	108	14.3	−8.0
5	53.5	105	51.5	−5.0
6	32.3	85.8	53.5	14.2

[a] All incubations were carried out with a final cardenolide concn of 10^{-4} *M*.

Discussion

Digitoxigenin (**5**) and digoxigenin (**6**) inhibited the ATPase only before washing and this inhibition was reversible. This agrees well with the results of Repke and Portius[16] who showed that digitoxigenin inhibited Na- and K-ATPase by 50% at 1.2×10^{-4} *M*. Digitoxigenin 3-acetate (**2d**) also showed reversible inhibition in our study but less than that seen with digitoxigenin.

Both **3b** and **4** did not markedly inhibit the enzyme; the latter result is consistent with the observation of Henderson and Chen[17] that both 14,15-α- and 14,15-β-epoxy derivatives of digitoxigenin are inactive in cardiotoxicity studies. The weak reversible activity seen in **2e** corresponds well with the report of Shigei, *et al.*,[18] that 15α-hydroxydigitoxigenin does not appreciably alter heart contractility. They have suggested that the 15α-OH group may interfere with the stereochemical arrangement in the vicinity of the C and D rings.

The bromoacetates **2a**, **2b**, and **2c** all irreversibly inhibited the transport ATPase preparation to approxi-

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mately the same extent. Compound **3a** was also found to be a potent irreversible inhibitor of transport ATPase in spite of the fact that **3b** (which has very weak cardiotonic activity[19]) did not appreciably inhibit the enzyme. This is in contrast to the finding of Hokin, *et al.*,[11a] that $\Delta^{5,14}$-dianhydrostrophanthidin 3-iodoacetate does not irreversibly inhibit transport ATPase whereas strophanthidin 3-iodoacetate does.

Experimental Section[20]

3β-Hydroxy-14,15α-epoxy-5β,14α-card-20(22)-enolide 3-Acetate (4).—To 418 mg (1.1 mmoles) of **3b**[13] in 8.6 ml of $CHCl_3$ was added a soln of 535 mg of *m*-$ClC_6H_4CO_3H$ (purity 68%) in 13 ml of C_6H_6. The reaction mixt was allowed to stand for 27 hr at room temp. $CHCl_3$ (42 ml) was added followed by 16 ml of 10% Na_2SO_3 soln. The org layer was sepd and washed with 5% Na_2CO_3 soln and H_2O and after drying (Na_2SO_4) yielded 385 mg of residue. Recrystn of the residue from Me_2CO–Et_2O gave 376 mg (84% yield) of **4** as needles: mp 186–188° (lit.[14] mp 220–226° from Me_2CO–petr ether; lit.[21] mp 187–198° from MeOH–Et_2O); nmr δ 0.83 (3 H, s, 18-CH_3; calcd[22] δ 0.82), 1.02 (3 H, s, 19-CH_3; calcd[22] δ 1.01), 2.11 (3 H, s, 3-CH_3CO_2), 3.60 (1 H, m, 15-CH), 4.77 (2 H, q, J = 1 cps, 21-CH_2), 5.17 (1 H, m, 3-CH), 5.90 (1 H, q, J = 1 cps, 22-CH); mass spectrum, parent ion at *m/e* 414, a (P − H_2O) peak at *m/e* 396, and a (P − HOAc) peak at *m/e* 354.

15α-Hydroxydigitoxigenin 3-Acetate (2e).—This compd was prepd from **4** by the procedure described by Okada and Hasumuma.[14] The product was obtained in 65% yield and after recrystn from MeOH–Et_2O had mp 260–262° (lit.[14] mp 245–253° from Me_2CO–Et_2O; lit.[21] mp 247–250° from MeOH–Et_2O).

15α-Hydroxydigitoxigenin 3-Acetate 15-Bromoacetate (2b).—A soln of 90 mg (0.2 mmole) of **2e** in 3.6 ml of dry dioxane[23] and 2 drops of pyridine was treated with 2 drops of $BrCH_2COBr$, whereupon a granular ppt formed. The suspension was stirred at 70° for 16 hr. The reaction mixt was dild with 30 ml of H_2O, and 5% Na_2CO_3 soln was added to pH 7. The mixt was then concd to dryness, and the residue was purified by preparative tlc on silica gel (plates developed in $CHCl_3$–MeOH 10:1, major product had R_f 0.4) to give, after 1 recrystn from MeOH, 54 mg (47% yield) of **2b**. An anal. sample had mp 230–232°; nmr δ 0.90 (3 H, s, 18-CH_3), 0.93 (3 H, s, 19-CH_3), 2.01 (3 H, s, 3-CH_3CO_2), and 3.80 (2 H, s, 15-$BrCH_2CO_2$). *Anal.* ($C_{27}H_{37}BrO_7$) C, H, Br.

Digitoxigenin 3-Bromoacetate (2a).—A soln of 50 mg of digitoxigenin (**5**) in 2.5 ml of dry dioxane was treated with 2 drops of pyridine followed by 2 drops of $BrCH_2COBr$. The white suspension was stirred at room temp for 1.5 hr. The reaction mixt was dild with 10 ml of H_2O, and the ppt was filtered off, washed with H_2O, and dried *in vacuo*. Prep tlc of this solid on silica gel (plates developed in $CHCl_3$–MeOH, 96:4) gave 2 major bands. The lower band (R_f 0.2) yielded 10 mg of starting material. The higher band (R_f 0.5) gave, after recrystn from MeOH, 38 mg (57% yield) of **2a**, mp 211–212°. *Anal.* ($C_{25}H_{33}BrO_5$) C, H.

Δ[14]-Anhydrodigitoxigenin 3-Bromoacetate (3a).—The reaction leading to this compd was carried out as described in the synthesis of **2a** except that the reaction time was 3 hr. Prep tlc of the crude product on silica gel (plates developed in $CHCl_3$–MeOH, 96:4) gave a major band at R_f 0.8 which, after recrystn from MeOH, gave 41 mg (65% yield) of **3a**, mp 193–195°; nmr δ 0.82 (3 H, s, 18-CH_3), 1.01 (3 H, s, 19-CH_3), 3.85 (2 H, s, 3-$BrCH_2CO_2$), and 5.27 (1 H, m, 15-CH). *Anal.* ($C_{25}H_{33}BrO_4$), C, H, Br.

Digoxigenin 3,12-Dibromoacetate (2c).—A soln of 78 mg of digoxigenin (**6**) in 3.6 ml of dry dioxane was treated with 2 drops of pyridine and 2 drops of $BrCH_2COBr$. The white suspension was stirred for 4 hr at 75°, dild with H_2O, and adjusted to pH 7 with 5% Na_2CO_3 soln. The solvents were removed under reduced pressure, and the residue was purified by prep tlc on silica gel (plates developed in $CHCl_3$–MeOH, 10:1). The major band at R_f 0.7 gave, after recrystn from MeOH–H_2O, 72 mg (56% yield) of **2c**, mp 215–218°. *Anal.* ($C_{27}H_{36}O_7Br_2$) C, H, Br.

Acknowledgments.—The authors are indebted to Dr. John Oliver's group for the nmr spectra and to Drs. Don DeJongh and David Brent for the mass spectral data. This work was made possible by a grant from the Michigan Heart Association.

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β-Adrenergic Blocking Agents. 10. (3-Amino-2-hydroxypropoxy)anilides

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Several (3-amino-2-hydroxypropoxy)acylanilides have been synthesized. In experimental animals, they have potent β-adrenergic blocking actions on the myocardium but not at some other sites, for example, the peripheral blood vessels. Of the compounds tested 4-(2-hydroxy-3-isopropylaminopropoxy)acetanilide (practolol) was selected for clinical trial on the basis of optimal potency and selectivity.

In extension of our work on 1-amino-3-aryloxy-2-propanols related to propranolol[1] we have prepared several analogs in which the aryl residue contains an acylamino substituent. In these preliminary studies, only relatively minor variations of the substituent (R_1) on the propanolamine side chain have been made. The acylamino substituents (R_2CONH) examined have included examples of alkanoyl, aroyl, and aralkanoyl groups. In general the compounds are potent β-adrenergic blocking agents. They differ, however, from previously known active compounds in that the inhibition of β-adrenergic responses is restricted to certain sites. Thus, the most studied compound of the new

(1) Inderal.