d, 1 H, $H_{10\alpha}$), 4.18 (m, 1 H, CHOH), 5.40 (br s, 1 H, vinylic), 6.0, 6.37 (br d, d, 2 H, $J$ = 16 Hz, vinylic side chain), 6.4 (br s, 2 H, aromatic); ir (neat) 3370, 1613, 1572 $cm^{-1}$. Anal. ($C_{24}H_{34}O_3$) C, H.

On treatment with $Ac_2O$ in pyridine (steam bath, 3 h) 4b was converted to its diacetate (97% yield): ir (neat) 1770, 1740 $cm^{-1}$.

**Pharmacology.** Mice were used in all tests. The MED's for ataxia and increased reactivity to sensory stimuli were determined by administering the drug intravenously (iv) as a solution in 0.06 ml of polyethylene glycol (PEG) 400 per 25-g mouse. Control mice, administered with PEG 400 only, showed a transient mild depression of behavior which disappeared in 2–3 min whereas the drug effects were longer lasting. For oral (po) preparations the drug was given as a suspension in 0.5% methylcellulose. The *mouse fighting test* (foot shock–induced fighting behavior in mice) was carried out for potential tranquilizer activity using a modified Tedeschi procedure described by us earlier.[8] The (±)-*Dopa potentiation test* consisted of determining the potentiation of motor responses to a challenge dose of (±)-Dopa following pretreatment with pargyline and the test compound. The antinociceptive activity was determined using the mouse *hot-plate test*. Both these tests have been described by us previously.[8]

**Acknowledgment.** We wish to thank Dr. N. P. Plotnikoff of Abbott Laboratories, N. Chicago, Ill., for the mouse fighting and Dopa test results.

## References and Notes

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# Cardenolide Analogues. 1. A 17β-Unsaturated Aldehyde

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A 17β-unsaturated aldehyde analogue [3β,14β-dihydroxy-5β-pregn-17β-*trans*-20-en-22-al (**7**)] of the cardenolides was synthesized and studied. In earlier studies by Rappoport, unsaturated aldehydes were found to be highly active electrophiles, more active, for example, than unsaturated nitriles or methyl esters. The synthesis followed in part a scheme previously reported by Thomas for the syntheses of the 17β-unsaturated nitrile **9** and the 17β-unsaturated methyl and ethyl esters **8** and **10**. Both **9** and **8** are more $Na^+,K^+$-ATPase inhibiting and slightly less inotropic than digitoxigenin (**1b**). However, the unsaturated aldehyde **7** was less $Na^+,K^+$-ATPase inhibiting ($I_{50}$ = 9.9 ± 0.7 × $10^{-7}$ M) and less inotropic (100% increase in contractile force at 8.5 ± 1.0 × $10^{-6}$ M) than **1b** ($I_{50}$ = 4.6 ± 1.6 × $10^{-7}$ M; 100% increase at 3.0 ± 1.0 × $10^{-7}$ M).

Structure–activity relationships of the digitalis analogues have recently been reviewed.[1-3] Irrespective of the pharmacological mechanisms operating, several models have been proposed to describe the role of the lactone ring in cardenolide–receptor binding. Two which account quite well for biological data are (1) a Michael attack by $Na^+,K^+$-ATPase on the unsaturated lactone ring;[4] and (2) ionic binding at the β carbon of the unsaturated lactone system and at the oxygen of the lactone carbonyl.[1] Since the strength of the dipole between these two atoms also determines reactivity in Michael reactions, the two mechanisms are quite similar. That is, sterically unhindered substrates possessing a reactive, polarizable π-cloud system should bind well.

Rappoport and co-workers[5] have recently reported that α,β-unsaturated systems of general structure $CH_2$=CHX have the following Michael reactivity order in vitro: PhCO > $SO_3Ph$ > CHO > $CH_3CO$ > COOPh > p-$CH_3PhSO_2$ > $COOCH_3$ > CN > $CONH_2$ > $PO(OEt)_2$ > p-$NO_2Ph$. For example, in the addition of morpholine to the olefin in absolute methanol at 30°, $k_{rel}$ (X=CHO) = 123, $k_{rel}$ ($COOCH_3$) = 1.0, $k_{rel}$ (CN) = 0.53. Based on the previously discussed models, one might anticipate that cardenolide analogues differing only in the nature of the 17β-unsaturated side chain might approximate the Rappoport order. Thomas has found that the unsaturated nitrile **9** and methyl ester **8** are slightly more $Na^+,K^+$-ATPase inhibiting than digitoxigenin (**1b**) and slightly less inotropic.[6] We therefore thought it would be interesting to employ the most reactive substrate in the Rappoport series, i.e., the α,β-unsaturated aldehyde. [While the PhCO and $SO_3Ph$ derivatives were more reactive in the Rappoport studies, the bulk of the phenyl group would be expected to negate any activity in cardenolides analogous to **9** and **8**. For example, Thomas has found that the ethyl ester **10** has essentially no $Na^+,K^+$-ATPase inhibitory nor

Table I

| Compd | $Na^+,K^+$-ATPase inhibition[a,b] | | Inotropic act.,[d] concn for 100% increase in contractile force[f] |
|---|---|---|---|
| | $I_{50}$ without preincubation | $I_{50}$ with 10 min preincubation[c] | |
| 7 | $9.9 \pm 0.7 \times 10^{-7}$ M | $1.2 \pm 0.3 \times 10^{-6}$ M | $8.5 \pm 1.0 \times 10^{-6}$ M[e] |
| 1b | $4.6 \pm 1.6 \times 10^{-7}$ M | $5.0 \pm 1.7 \times 10^{-7}$ M | $3.0 \pm 1.0 \times 10^{-7}$ M |
| Ouabain | $5.0 \pm 0.6 \times 10^{-7}$ M | $4.0 \pm 1.0 \times 10^{-8}$ M | [g] |

[a] $I_{50}$ values are for two to four runs. Appropriate $Mg^{2+}$ and $Na^+$ tubes were included to determine the basal activity of the $Na^+,K^+$-ATPase. This was then substracted from activity in the presence of $Mg^{2+}$, $Na^+$, and $K^+$. [b] The steroids were added in ethanol to the $Na^+,K^+$-ATPase tubes. In no case was more than 20 μl of ethanol added per tube. Independent studies showed that significant inhibition (over 3%) of the enzyme preparation by ethanol does not occur until over 25 μl of ethanol is added. [c] Steroid added to $Na^+,K^+$-ATPase medium lacking $K^+$ to allow the steroid and enzyme time to begin binding. After 10 min, KCl was added to begin $Na^+,K^+$-ATPase activity. [d] Average for each with five left atria. [e] No significant change was observed when 2 μg/ml of propranolol was added to the baths in two separate experiments (data not shown). [f] Uncorrected for the slight propylene glycol depression shown in Figure 2. [g] Ouabain was used only on an internal control with $Na^+,K^+$-ATPase preincubation experiments. The primary compound for comparison of biological activity with 7 is 1b, both aglycons.

inotropic activities.[6]] The compound prepared and studied is 3β,14β-dihydroxy-5β-pregn-17β-*trans*-20-en-22-al (7).

**Chemistry.** Digitoxin (1a) was hydrolyzed to digitoxigenin (1b) as previously reported.[7] Acetylation of 1b with acetic anhydride in pyridine gave 3-acetyldigitoxigenin (1c) which was then converted to the tetrahydroxy-5β-pregnane 4 as shown in Scheme I. This conversion employed a modification of a procedure previously reported by Thomas,[1,6,8] and neither we nor Thomas were able to purify intermediates 2 and 3 sufficiently for complete characterization. Sodium periodate oxidation of 4 produced 3β,14β-dihydroxy-17β-formyl-5β-androstane (5). Reaction of 5 with sodium diethyl 2-(cyclohexylamino)vinylphosphonate[9,10] gave the imine 6 which upon hydrolysis with oxalic acid in a two-phase system of water and benzene gave 3β,14β-dihydroxy-5β-17β-*trans*-20-en-22-al 7. All the spectral data are in agreement with the assigned structure of 7. In particular, the trans stereochemistry of the $C_{20}$–$C_{21}$ double bond is shown be the large coupling constant of the $C_{20}$ proton with the $C_{21}$ proton ($J$ = 16 Hz). By comparison, the analogous coupling constant for a cis olefin would be less than 8 Hz.[11]

**Biology.** The in vitro $Na^+,K^+$-ATPase inhibitory activities of 7 and 1b (as the reference) were determined with rat brain $Na^+,K^+$-ATPase using methods previously reported from our laboratories.[12,13] Rat brain $Na^+,K^+$-ATPase is used in these studies for two reasons: (1) the ease of preparation of high-activity enzyme; and (2) the activity of heart and brain enzyme is of the same order.[12] Further, each compound was tested with preincubation, i.e., mixing steroid, enzyme, and media lacking $K^+$ for 10 min prior to addition of KCl (to begin the enzyme's activity). These conditions allow for steady-state binding of cardenolide to $Na^+,K^+$-ATPase.[2] If a significant difference is noted (e.g., one order of magnitude as with ouabain, Table I) it would indicate that binding is relatively slow and/or is affected by the simultaneous presence of the various ligands so as to reach a steady state over a prolonged period of time. It is now well known that binding of cardenolide to $Na^+,K^+$-ATPase is affected by a variety of experimental conditions,[2,13] and slow attainment of steady-state binding in the presence of $Na^+$ and $K^+$ may be due to the opposing effect of these cations on the binding of the drug to the enzyme. Nonetheless, for the purpose of comparison of various drugs, one may follow either of the procedures (i.e., without or with preincubation) provided that the experimental conditions are strictly comparable. In the present study effort was made to achieve this. However, it would seem that since preincubation of the drug with the enzyme (under optimal binding conditions) would result in steady-state binding, the values of $I_{50}$ so obtained should be a better measure for the comparison of the efficacy of various cardenolides in inhibiting $Na^+,K^+$-ATPase. Thus, useful information is gained from both types of experimental approach.

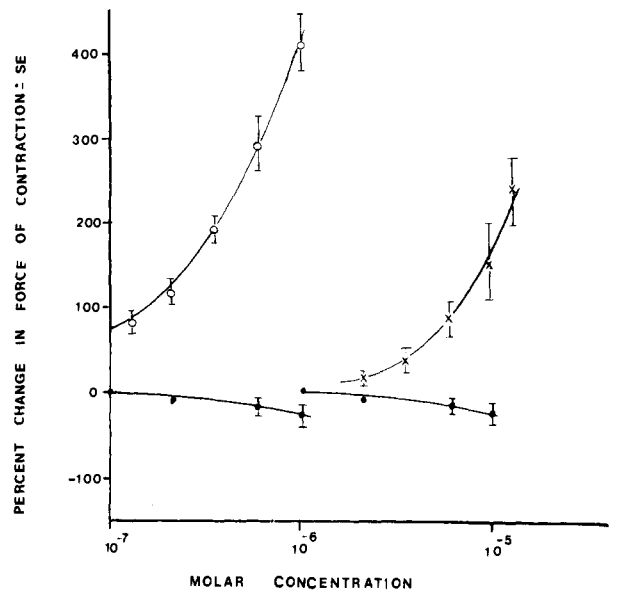
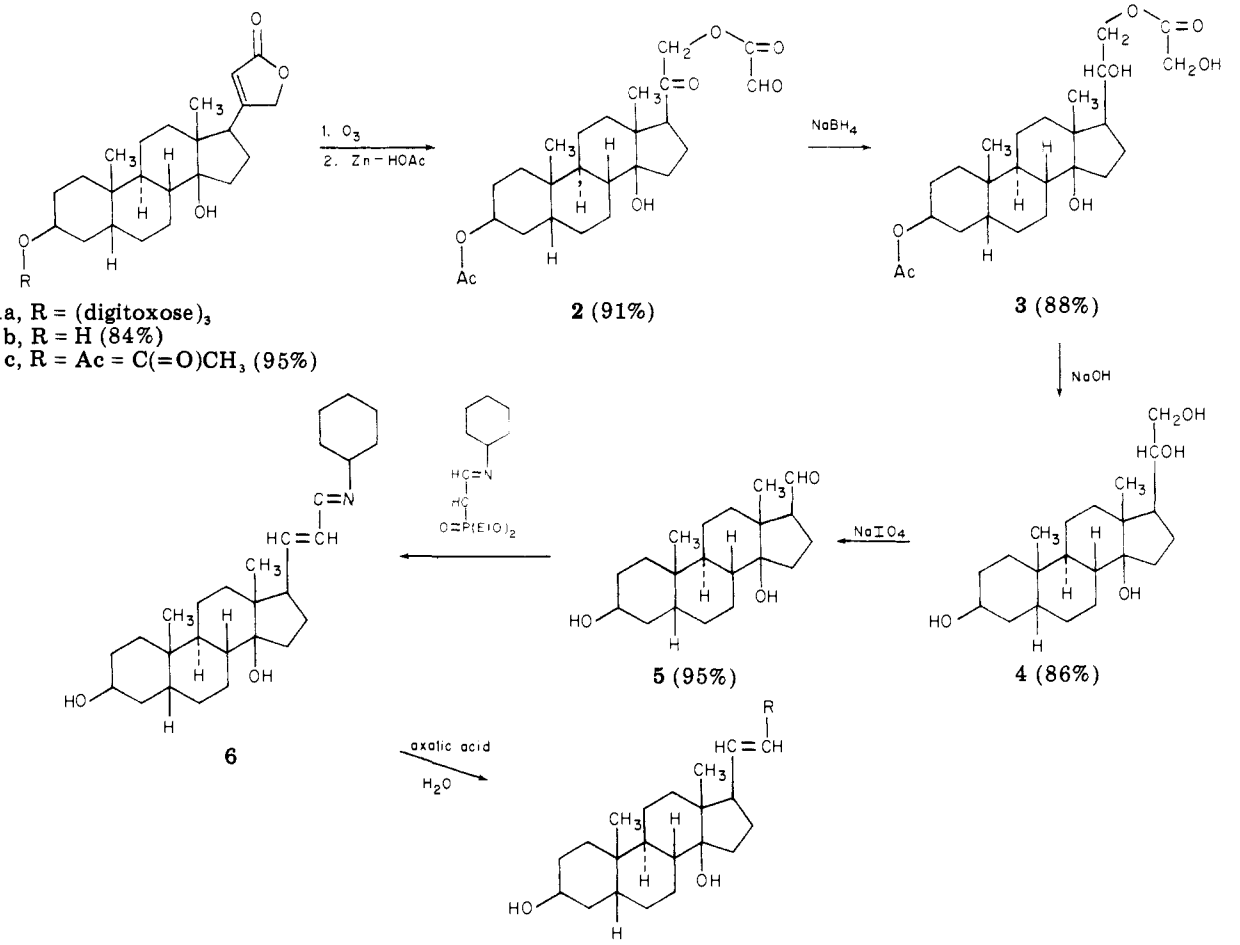



Figure 1. (○) Digitoxigenin (1b), uncorrected for propylene glycol depression, $n$ = 5. (X) 17β-Unsaturated aldehyde 7 uncorrected for propylene glycol depression, $n$ = 5. (●) Propylene glycol equal to the cumulative amount added in curves above for 1b and 7, $n$ = 3. SE calculated by the method of Snedecor.[15]

The inotropic activities of 7 and 1b were assayed using a guinea pig left atrial preparation. The compounds were dissolved in propylene glycol such that 5 μl of the resulting stock solution yielded a 50-ml bath of $1 \times 10^{-5}$ M with 7 and $1 \times 10^{-7}$ M with 1b. The concentrations for a 100% increase in contractile force are shown in Table I, along with the cumulative depressions caused by equivalent doses of propylene glycol. The dose–response curves are shown in Figure 1.

As can be seen from Table I and Figure 1, while the 17β-unsaturated aldehyde 7 is active, it is not as active as digitoxigenin 1b. On the other hand, as noted in the introduction, Thomas has reported that the unsaturated nitrile 9 and the methyl ester 8 are slightly more $Na^+,K^+$-ATPase inhibiting than 1b and only slightly less inotropic.[6] If the reactivity order reported by Rappoport would apply to digitalis analogues one would expect that 7 would be as much as 246 or 123 times more active than

Scheme I




9 or 8, respectively. The fact that it is not indicates that the activity of digitalis analogues cannot be explained solely on the basis of electrophilic reactivity, and a Michael reaction might not be occurring in vivo.

Finally, addition of 2 μg/ml of propranolol in a separate experiment to baths of 7 did not cause any significant change in contractile force, thus showing that the inotropic activity of 7 is not due to β-adrenergic stimulation.

## Experimental Section

Elemental analyses were performed by MHW Laboratories, Garden City, Mich. Where analyses are indicated only by symbols, they are ±0.4% of the theoretical values. Infrared spectra were obtained in chloroform or potassium bromide with a Perkin-Elmer 237B. NMR spectra were obtained in $CDCl_3$ with a Varian A-60D or XL-100 at ambient temperature as approximately 10% solutions using $Me_4Si$ as reference. Optical rotations were determined in chloroform with a Perkin-Elmer 141 polarimeter. Mass spectra were obtained at the University of Minnesota Mass Spectroscopy Lab. Melting points were determined with a Thomas-Hoover melting point apparatus and are corrected.

Thin-layer chromatographies used reusable silica gel–glass bonded[14] "replates" (Shionogi Company, Osaka, Japan), using 5% ethanol in chloroform. Preparative thin-layer chromatographies employed 1.25-mm silica gel HF-254 (Brinkman), generally using 5–10% methanol in chloroform.

Organic solvents were removed at 35° in vacuo using a Büchi rotory evaporator.

**3β,14β-Dihydroxy-5β-pregn-17β-*trans*-20-en-22-al (7).** Digitoxin (1a) was hydrolyzed to digitoxigenin (1b) as previously reported by Pettit.[7] The conversion of 1b to 3β,14β-dihydroxy-17β-formyl-5β-androstane (5) as shown in Scheme I essentially followed a procedure previously reported by Thomas.[8] Using a modified procedure of Nagata,[9] 803 mg (0.003 mol) of diethyl 2-(cyclohexylamino)vinylphosphonate[10] in 25 ml of dry tetrahydrofuran was added to 205 mg of sodium hydride (used as 57% dispersion in oil, so equivalent to 115 mg of sodium hydride, 0.003 mol) in 30 ml of dry tetrahydrofuran and allowed to stir at room temperature for 30 min. A solution of 5 (510 mg, 0.001 mol) in 15 ml of dry tetrahydrofuran was then added slowly over a period of 0.5 h and the reaction stirred for an additional 2 h. The mixture was poured into 100 ml of water and extracted with three 50-ml portions of chloroform, and the organic layers were pooled, dried over sodium sulfate, and evaporated under reduced pressure to yield an orange residue assumed to correspond to 6: ir ($CHCl_3$) 3420, 1650, 1025, 950 $cm^{-1}$.

This residue was dissolved in 50 ml of benzene and stirred with 100 ml of 5% aqueous oxalic acid for 15 h at room temperature. The organic layer was separated, washed with water, dried over sodium sulfate, and evaporated under reduced pressure to yield the crude product 7. Purification by preparative thin-layer chromatography (using silica gel HF-254 and 5% ethanol in chloroform) yielded the analytically pure 3β,14β-dihydroxy-5β-pregn-17β-*trans*-20-en-22-al (7): 410 mg (80%); mp 98–99°; ir (KBr) 3450, 1680, 1625, 1450, 1375, 1025, 950 $cm^{-1}$; NMR ($CDCl_3$) δ 9.6 (d, $J$ = 8.0 Hz, 1, $C_{22}$-H), 6.0 (1, d, $J$ = 16.0, 8.0 Hz, $C_{21}$-H), 7.1 (1, d, $J$ = 16.0, 5.0 Hz, $C_{22}$-H), 4.1 (br s, 1, $C_3$-H), 0.98 (s, 3, $C_{19}$-H), 0.89 (s, 3, $C_{18}$-H); MS $m/e$ at 346 (small), 328 (large); uv $\lambda_{max}$ EtOH 232 nm (ε 21000). Anal. ($C_{22}H_{36}O_4$) C, H.

**Biological Testing Methods.** The in vitro $Na^+$,$K^+$-ATPase inhibitory activities of the compounds were determined with rat brain $Na^+$,$K^+$-ATPase using methods previously reported in our laboratories.[12,13]

The inotropic activity of the test compounds was assayed utilizing a guinea pig atrial preparation. Isolated guinea pig left atria (obtained from 200-g animals) were placed in a 50-ml muscle bath and isometric tension was measured with a strain gauge transducer (Statham $\mu$C3) and recorded with an oscillographic recording system (Sanborn 150). The bath temperature was maintained at 37 °C and the atria were paced at 60 beats/min at a voltage 10% above threshold utilizing platinum field electrodes. The resting tension was set at 300 mg. The bathing solution had the following composition—NaCl, 125 mM; $NaHCO_3$, 24 mM; KCl, 4 mM; $NaH_2PO_4$, 1.2 mM; $MgSO_4$, 0.6 mM; $CaCl_2$, 1.2 mM; glucose, 23 mM—and was continuously aerated with a mixture of 95% $O_2$–5% $CO_2$. The compounds were dissolved in propylene glycol such that 5 $\mu$l of the resulting digitoxigenin (**1b**) stock solution yielded a bath of $1 \times 10^{-7}$ M, and, in the case of **7**, $1 \times 10^{-5}$ M bath solutions. After prior stabilization for 30 min of the atria in the baths without test compounds, appropriate aliquots of the stock solutions were added with a micropipet at 30-min intervals so as to obtain cumulative dose responses. The force of contraction was continuously recorded throughout the experiment and the maximal response following each dose was measured. The percent change in contractile tension at each dose was calculated and standard errors were determined. Five experiments were performed for 1b and five for **7**. In separate experiments, depression due to cumulative equivalent doses of propylene glycol was also determined and these results are quite similar to those reported by Thomas.[6] Digitoxigenin (**1b**) was studied over a range of $1 \times 10^{-7}$–$1 \times 10^{-6}$ M, and the 17$\beta$-unsaturated aldehyde **7** was studied from $1 \times 10^{-6}$ to $2 \times 10^{-5}$ M.

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# Some Novel Potential Alkylating Agents Derived from Diethylstilbestrol

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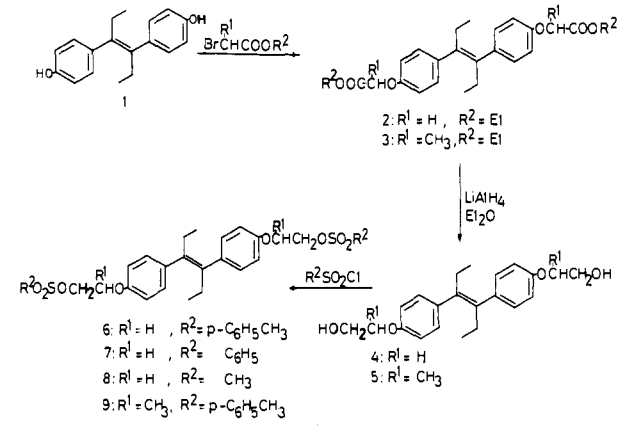
Several alkylating agents containing sulfonic esters, nitrogen mustards, and aziridine moieties attached to diethylstilbestrol through ethyl and isopropyl chains have been synthesized. The tests of some of the products for antileukemic activity in L1210 lymphoid leukemia indicated no significant activity over diethylstilbestrol.

In the course of studies aimed at developing alkylating agents that can be directed to specific target tissues, two series of steroidal alkylating agents were synthesized and pharmacologically tested for their anticancer activities. The compounds of the first series[1-4] included several steroidal nitrogen mustards derived from various steroids by replacement of their hydroxyl or ketonic functions with the nitrogen mustard unit $-N(CH_2CH_2Cl)_2$. The second[5-7] incorporated some steroidal esters of *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylacetic acid and the corresponding phenylbutyric acid, steroidal sulfides of *p*-[*N*,*N*-bis(2-chloroethyl)amino]thiophenol, and steroidal ethylenimine derivatives.

The detailed studies of the antitumor activities of these compounds[5-7] indicated that some of the esters, particularly 3$\beta$-hydroxy-5-cholestene-*p*-[*N*,*N*-bis(chloroethyl)amino]phenylacetate (phenesterin),[5] were potent in inhibition of solid tumors including Sarcoma 45, Walker carcinosarcoma, and alveolar liver carcinoma RS-1.

As a correlative study in the same field, we were interested in investigating the anticancer activities of a new series of alkylating agents in which the alkylating functions are attached to steroids or diethylstilbestrol through ether linkages. This part reports on the synthesis and antileukemic activities of some sulfonic esters, nitrogen mustards, and aziridine derivatives of diethylstilbestrol.

Scheme I




**Chemistry.** Diethylstilbestrol (1) was etherified with ethyl bromoacetate and ethyl $\alpha$-bromopropionate as reported[8] and the products **2** and **3** were reduced with