(25 ml) at 0°, and the solution was allowed to stand overnight. Fractionation of the solution gave 3-piperidinopropenal, bp 115–120° (0.3 mm), yield 4.17 g (30%). This product was refluxed with piperidine perchlorate (6.12 g, 0.033 mole) in 18 ml of EtOH for 15 min. The product separated on cooling, yield 7.5 g (82%), mp 129–130°. *Anal.* ($C_{13}H_{23}ClN_2O_4$) C, H, Cl, N, O.

1-(5-Pyrrolidino-2,4-pentadienylidene)pyrrolidinium Perchlorate (2a).—Pyrrolidine (14.2 g, 0.2 mole) was added to a solution of the sodium enolate of glutaconaldehyde[6] (15.6 g, 0.1 mole) in 300 ml of MeOH. The reaction mixture was refluxed for 15 min and cooled. Acidification with $HClO_4$ yielded the product, mp 184–186°, yield 11.5 g (38%). The product was identical with that prepared by reaction of pyrrolidine with 1-(2,4-dinitrophenyl)pyridinium chloride.

(6) P. Baroniggetti, *Ber.*, **59**, 1166 (1926).

The Effects of Some Steroidal Alkylating Agents on Experimental Animal Mammary Tumor and Leukemia Systems[1]

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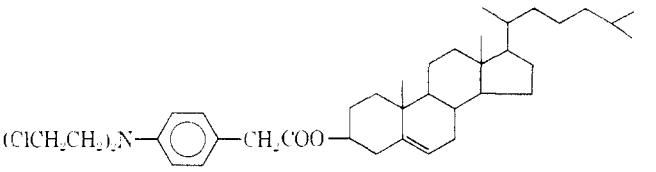
Received February 17, 1969

A series of steroid esters of *p*-[N,N-bis(2-chloroethyl)amino]phenylacetic acid (BCAPAA), steroidal sulfides of *p*-(N,N-bis-2-chloroethylamino)thiophenol, and a variety of steroidal ethyleneimine derivatives were synthesized and tested for antitumor activity in a number of experimental tumor systems. Activity was found only in those instances in which the steroid and potential oncolytic agent were connected by ester or heterocyclic ether linkages. The steroidal BCAPAA esters were of particular interest showing excellent inhibition of a DMBA-induced and transplantable mammary adenocarcinoma, and marked increase in survival when tested on a variety of rat leukemias. Factors such as route of administration, vehicle, and the nature of the steroid had definite effects and are discussed in detail in the body of the paper. The steroidal BCAPAA esters were judged to be less toxic than some of the well-known nitrogen mustards in general use.

Steroidal alkylating agents have received sporadic attention as potential antitumor agents for some 15 years. The workers in this field were hopeful that useful advantage might be made of the lipophilic nature of the steroid molecule in the transport of various nitrogen and sulfur mustards or that hormonal steroids such as androgens and estrogens could deliver the alkylating agent to a specific target tissue. In his classical treatise on biological alkylating agents, Ross, reviewing this field up to 1960,[3] concluded that the few steroidal nitrogen mustards prepared at this date had no outstanding antitumor properties. In 1961, Burstein and Ringold made an androgen nitrogen mustard which was nontoxic at doses up to 500 mg/kg.[4] Antitumor activity was not reported. In 1962, Rao and Price[5] made a variety of steroidal nitrogen mustards, a few of which were reported active in inhibition of the Gardner ascites tumor. In 1966, Schneider, Hamsher, and Beyler[6] reported that a few steroidal ethyleneimine derivatives displayed modest activity in an unspecified C_3H mouse tumor. The reports cited in most cases involved compounds in which the $(ClCH_2CH_2)_2N$ moiety or ethyleneimine grouping was linked to the steroid by an N–C bond. Moreover, all the references cited were notable for the paucity of biological data. In contrast to these unpromising results were the reports of a Russian group,[7] Degteva, Larionov, and coworkers.[8] These workers described the preparation of 3β-hydroxy-5-cholestene *p*-[N,N-bis-(chloroethyl)amino]phenylacetate which they called phenesterin (1) and have made detailed studies of the activity of phenesterin against a variety of solid tumor systems including Sarcoma 45, Walker carcinosarcoma, and alveolar liver carcinoma RS-1,[8a,b] and recently in



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brain tumors.[8c] At levels of 100–200 mg/kg in rats and mice, and subcutaneous administration in olive oil vehicle, these workers reported remarkable inhibition of the above-mentioned tumor systems; however, the compound was inactive against Ehrlich and Sarcoma 180 mice tumors. In addition phenesterin was found to be relatively nontoxic, with an LD_{50} for rats (single injection) of 2.0 g/kg.[8d] Degteva, Larionov, and coworkers claimed that the action of the cholesterol ester was different from that of the parent free acid[8b] and have stressed the importance of the ester function.

With these latter encouraging results in hand, we decided to reinvestigate steroidal nitrogen mustards and steroidal ethyleneimine derivatives with the following objectives: (a) to prepare a number of steroid analogs of phenesterin varying the steroid and the location of the BCAPAA ester on the steroid selection; (b) to prepare a number of steroidal ethyleneimines in which the active alkylating function would be located on an ester or rela-

(1) (a) These studies were supported by the Endocrine Evaluation Branch, General Laboratories and Clinics, National Cancer Institute, National Institutes of Health, Bethesda, Md., under Contract No. SA-43-ph-4351. (b) Steroids. LXXXIII. Previous paper in this series: F. I. Carroll, A. Philip, A. M. Ferguson, and M. E. Wall, *J. Heterocyclic Chem.*, **5**, 805 (1968).

(2) Endocrine Evaluation Branch, General Laboratories and Clinics, National Cancer Institute, National Institutes of Health, Bethesda, Md.

(3) W. C. J. Ross, "Biological Alkylating Agents," Butterworth and Co. Ltd., London, 1962, p 142.

(4) S. H. Burstein and H. J. Ringold, *J. Org. Chem.*, **26**, 3084 (1961); this paper also gives a good review of previous studies.

(5) G. V. Rao and C. C. Price, *ibid.*, **27**, 205 (1962).

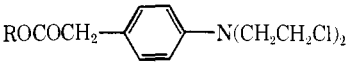
(6) F. S. Schneider, J. Hamsher, and R. E. Beyler, *Steroids*, **8**, 553 (1966).

(7) We wish to thank Dr. Ihor Masnyk for calling these studies to our attention, and providing English translation for some of the key references.

(8) (a) L. F. Larionov, G. A. Degteva, and N. A. Lesnaya, *Vopr. Onkol.*, **8**, 12 (1962); (b) S. A. Degteva, *ibid.*, **10**, 52 (1964); (c) S. A. Degteva and L. F. Larionov, *ibid.*, **12**, 51 (1966); (d) E. N. Shkodinskaya, E. M. Kurdyukova, O. S. Vasina, and A. Ya. Berlin, *J. Gen. Chem. USSR*, **32**, 945 (1962).

TABLE I

STEROID ESTERS OF *p*-[N,N-BIS(2-CHLOROETHYL)AMINO]PHENYLACETIC ACID



R[a]	Position	Yield,[b] %	Mp, °C	Formula[d]
Cholesterol	3β	86	91.5–93[c]	
Deoxycorticosterone	21	83		$C_{33}H_{43}Cl_2NO_4$
Estrone	3	89		$C_{36}H_{35}Cl_2NO_4$
Estradiol	3,17β	53		$C_{42}H_{50}Cl_4N_2O_4$
Testosterone	17β	61		$C_{31}H_{41}Cl_2NO_4$
Pregnenolone	3β	80	128–30	$C_{33}H_{45}Cl_2NO_4$
Dehydroepiandrosterone	3β	48	124–26	$C_{31}H_{41}Cl_2NO_3$

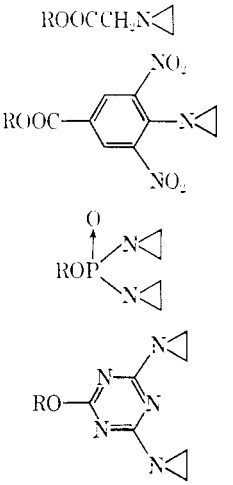
[a] A typical example is given in the Experimental Section. [b] Yield based on analytically pure material. [c] Lit.[8d] mp 89–89.5°. [d] All compounds except the first were analyzed for C, H, Cl, N.

tive heterocyclic ether linkage capable of enzymatic cleavage similar to the steroidal BCAPAA esters; (c) to prepare a number of selected steroidal alkylating agents in which the nitrogen mustard or ethyleneimine would be located in a moiety which should be difficult to remove enzymatically from the steroid; and (d) to compare groups a–c under strictly comparable conditions in a variety of solid tumor and leukemia systems. This report presents the preparation of the various compounds and initial biological information obtained to date in a continuing program.

Chemistry.—The preparation of *p*-[N,N-bis(2-chloroethyl)amino]phenylacetyl chloride hydrochloride and its reaction with various steroidal alcohols to give the desired esters is based on the original procedure of Shkodinskaya, *et al.*,[8d] modified by us as required for preparation of 10–50-g quantities. A flowsheet of the procedure is shown in Chart I. The steroidal BCAPAA esters prepared by this route are shown in Table I. The compounds described were purified by repeated chromatography on silica gel and/or Florisil until analysis by thin layer chromatography (tlc) indicated that the product was pure. Although in some cases a crystalline product was not obtained, in every instance acceptable analytical constants were obtained.[9] In addition all the compounds were characterized by ir and nmr spectroscopy. Characteristically, all the ir spectra of all the esters showed a strong band at 1725–1735 cm^{-1}. The *p*-[N,N-bis(2-chloroethyl)amino]phenylacetyl moiety present in all the compounds of this group showed characteristic nmr peaks: the benzylic protons appearing as a singlet in the region δ 3.48–3.70, the protons of the 2-chloroethyl groups as a singlet at δ 3.67, and the aromatic protons as a typical A_2B_2 multiplet centered in the region of δ 6.92–7.00. All of these bands were well downfield from the steroid methyl and methylene peaks and hence were useful for characterization. The esterification reaction proceeded readily with steroidal phenolic and aliphatic hydroxylic groups at the 3 position, and with the 21- and 17-hydroxyl functions. Although a variety of relatively simple steroids were easily converted to the BCAPAA esters, we were unable to obtain the desired product with cortisone or hydrocortisone. In these cases the esterification reaction proceeded readily at C-21 but the subsequent product was unstable and could not be obtained in pure form.

(9) Although the steroid BCAPAA esters are reasonably stable in dry, pure form, they rapidly decompose in the hot crystallizing solvents such as methanol, hexane, acetone, ethyl acetate, etc. Accordingly, if a compound was not readily crystallizable, it was not subjected to prolonged attempts, particularly since acceptable analyses were obtained.

A series of steroidal sulfides of *p*-(N,N-bis-2-chloroethylamino)thiophenol were prepared in order to determine whether a C–S bond in this form could be cleaved. The parent compound, *p*-(N,N-bis-2-chloroethylamino)-thiophenol is moderately active.[10] The steroid sulfides would be expected to be inactive but might be a good "latent" form (ref 3, pp 178–179) if the R–S bond could be cleaved. Steroid sulfides at position C-21, 7α, and 16α were prepared as shown in Table II. The C-21 sulfides were prepared by displacement of the corresponding brosylates by the anion of *p*-(N,N-bis-2-chloroethylamino)thiophenol,[10a] the others by addition of the same anion to a conjugated double bond, respectively, to the 16α position of a Δ^{16}-12,20-diketo steroid and to the 7α position of a 3-keto-$\Delta^{4,6}$-diene. Table III describes the physical properties of a variety of steroidal ethylenimine derivatives. Two basic types were prepared: (a) ethylenimines attached directly to the steroid by a C–N linkage, and (b) compounds in which the ethylenimine moiety is on a function which could be enzymatically cleaved away from the steroid and includes structures of type

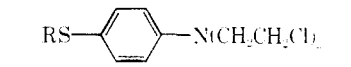


The steroidal ethylenimines with direct C–N linkages were prepared by reaction of an appropriate steroid oxide with ethylenimine or by a Michael-type addition to an appropriate conjugated mono- or diene giving a series of 7α-, 16α-, 6β-, and 17α-aziridinyl compounds. The methods and the stereochemistry of the resulting compounds in both series is well established involving

(10) (a) M. H. Benn, Ph.D. Thesis, University of London, 1957; (b) M. H. Benn, L. N. Owen, and A. M. Creighton, *J. Chem. Soc.*, 2800 (1958).

TABLE II

STEROID SULFIDES OF *p*-BIS(2-CHLOROETHYL)AMINOTHIOPHENOL

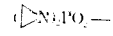
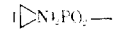
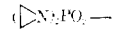
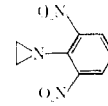
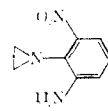
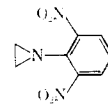
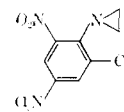
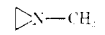
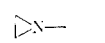
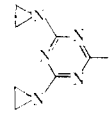
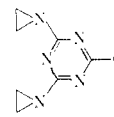
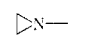
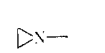
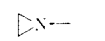
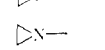
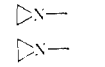


R	Position	Method[a]	Recrystn solvent	Yield, %[b]	Mp, °C	Formula	Analyses
5α-Pregnane-12,20-dione	16α	A	C_6H_6–hexane	89	91–92	$C_{33}H_{45}Cl_2NO_4S$	C, H, N
Deoxycorticosterone	21	B		77	[c]	$C_{31}H_{41}Cl_2NO_3S$	C, H, Cl, N, S
Hydrocortisone	21	C	Me_2CO–hexane	55	139–143	$C_{31}H_{41}Cl_2NO_4S$	C, H, Cl, N, S
Testosterone	7α	A	C_6H_6–hexane	61	179–182	$C_{29}H_{39}Cl_2NO_2S$	C, H, Cl, N, S

[a] An example of each procedure is given in the Experimental Section: A, $RCH{=}CHR' + p\text{-}HSC_6H_4N(CH_2CH_2Cl)_2$ in refluxing benzene containing piperidine; B, $ROBrOS + HSC_6H_4N(CH_2CH_2Cl)_2$ in refluxing C_6H_6 containing piperidine. [b] Based on analytically pure material. [c] The product was noncrystalline.

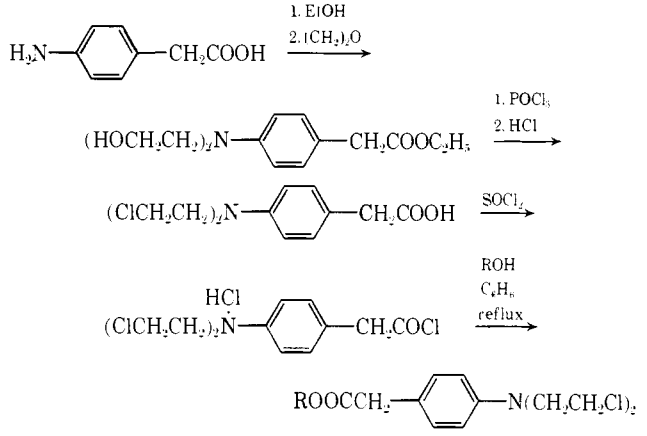
TABLE III

STEROID ETHYLENIMINE DERIVATIVES

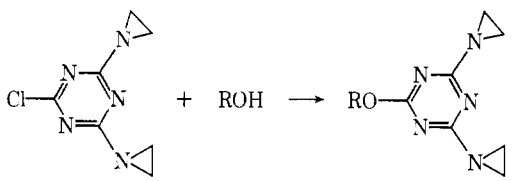
Steroid	Oncolytic agent	Position	Method[a]	Recrystn solvent	Yield,[b] %	Mp, °C	Formula[c]
Cholesterol		3β	Aa	Hexane	73	151–152[d]	$C_{30}H_{52}N_2O_2P$
2α-Methyldehydrotestosterone		17β	Ab	C_6H_6–hexane	37	150–153	$C_{24}H_{36}N_2O_2P$
Estrone		3	Ab	Me_2CO–hexane	72	128–131[e]	$C_{22}H_{29}NO_3P$
Cholesterol		3β	Ba	EtOH	84	203–205	$C_{36}H_{51}N_3O_6$
Cortisone		21	Bb	*i*-PrOH	78	242–247 dec	$C_{30}H_{35}N_3O_{10}$
Deoxycorticosterone		21	Bb	EtOH	74	217–219	$C_{30}H_{35}N_3O_8$
Cortisone		21	Bb	CH_2Cl_2–EtOH	73	203–205	$C_{30}H_{33}N_3O_{10}$[f]
3β,17β-Dihydroxy-5-androstene		17α	C[g]	EtOAc–$CHCl_3$	66	182–190	$C_{22}H_{35}NO_2$[g]
3β-Hydroxy-5α-androstan-17-one		6β	C	CH_2Cl_2–hexane	32	257–264	$C_{21}H_{33}NO_3$
Estrone		3	D	*i*-PrOH	74	170 dec	$C_{25}H_{29}N_5O_2$
Diethylstilbestrol			D	*i*-PrOH	83	190 dec	$C_{32}H_{34}O_2N_{10}\cdot H_2O$
Testosterone		7α	Ea	Me_2CO	39	209–210	$C_{21}H_{31}NO_2\cdot H_2O$
3β-Acetoxy-5α-pregnane-12,20-dione		16α	Eb	Et_2O	87	146–148	$C_{25}H_{37}NO_4$
3-Hydroxypregnane-11,20-dione acetate		16α	Eb	Et_2O–hexane	87	135.5–138	$C_{25}H_{34}NO_4$
Progesterone		7α	Ea	EtOAc	43	213–219	$C_{24}H_{33}NO_2$
Pregnenolone acetate		16α	Eb	EtOAc	87	158–159[h]	$C_{25}H_{37}NO_3$
Cholesterol		3β			75	90–97	$C_{30}H_{51}NO_2$

[a] An example of each procedure is given in the Experimental Section: A, ROH + $POCl_3$ followed by treatment with ethylenimine in (a) C_6H_6 or (b) THF; B, ROH + 3,5-$(NO_2)_2$-4-ClC_6H_2COCl or 3,5-$(NO_2)_2$-2-ClC_6H_2COCl in C_6H_6 or CH_2Cl_2 containing pyridine followed by treating the product with ethylenimine in (a) DMF containing Et_3N or (b) CH_2Cl_2 containing Et_3N; C, $RCH{-}\underset{\diagdown\,\diagup}{O}{-}CHR$ + ethylenimine; D, ROH + 2-chloro-4,6-bis(1-aziridinyl)-*s*-triazine in Me_2CO containing K_2CO_3; E, $RCH{=}CHR$ + ethylenimine (a) neat or (b) containing Et_3N. [b] Calculation based on pure product isolated. [c] K. A. Petrov, A. I. Gavriolova, and V. P. Korotova [*Zh. Obshch. Khim.*, **36**, 853 (1966)] report mp 144–146°. [d] Lit.[e] mp 130–131°. [e] C: calcd, 76.47; found, 75.33. [f] Lit.[6] mp 152–154°. [g] All compounds analyzed correctly for C, H, N, unless otherwise noted. [h] C, H analysis only.

CHART I



familiar diaxial cleavage in the case of the epoxides,[11] and diaxial, rearside attack in the case of addition of ethylenimine to the conjugated systems.[12,13] The ir spectra, which showed a typical absorption at 3060–3080 cm^{-1} due to the C–H stretching found in the aziridinyl ring, represented a good method for characterizing this group of compounds. Steroidal 3,5-dinitro-2- or -4-aziridinylbenzoyl esters were prepared from the corresponding 2- or 4-chloro derivatives by reaction with ethylenimine. The steroid bis(1-aziridinyl)phosphinate esters were prepared by treating the appropriate monohydroxy steroid with $POCl_3$ and treating the resultant $ROPOCl_2$ derivative with ethylenimine. These compounds were crystalline and characterized by analysis and ir spectra which show typical bands at 3080 (C–H stretch of aziridinyl ring) and 1275 cm^{-1} (P=O stretch). The nmr spectra show an eight-proton doublet at δ 2.17 (aziridinyl protons coupled to phosphorus). The steroidal bis(1-aziridinyl)-*s*-triazinyl ethers could be prepared by reaction of phenolic steroids with 2-chloro-4,6-bis(1-aziridinyl)-*s*-triazine. The structure



assignments were based mainly on the elemental analysis and the nmr spectra which show an eight-proton singlet at δ 2.38 (aziridine proton). The 3β-hydroxy-5-cholesteneaziridinyl acetate is an extraordinarily reactive compound. The compound can be made readily by preparing 3β-hydroxy-5-cholestene chloroacetate and treating the latter with ethylenimine.

Biological Data.—The majority of the compounds listed in Tables I–III were tested for antitumor activity through both the contract programs of the Endocrine Evaluation Branch[14] (EEB) and the Cancer Chemotherapy National Service Center (CCNSC) of the National Cancer Institute. Under the auspices of the latter, they were evaluated in the Walker carcinosarcoma 256 (intramuscular) and the L1210 lymphoid leukemia. In the EEB program the compounds were evaluated initially in either the R3149 acute monocytic leukemia of the Fischer/344 rat or the 13762 DMBA-induced and transplantable mammary adenocarcinoma (normal line) of the same imbred rat, depending upon the steroid moiety of the individual compounds. For example, based on prior experience, corticoids were more likely to show biological activity in a leukemia system than in a mammary tumor, while with androgens or estrogens the opposite could be expected. In addition to the R3149 leukemia and the 13762 mammary tumor some of the steroidal alkylating agents reported herein were tested in other mammary and leukemia systems (Tables IV and IX). A paper to be published later will report in greater detail the antitumor and other biological parameters of these and related compounds.

Assays with the L1210 leukemia and the Walker 256 carcinosarcoma were performed according to the specifications[15] of the CCNSC. The R3149 leukemia and the other rat leukemias were described by Dunning, *et al.*,[16] the methods for testing chemical agents were included in the same publication. The 13762 mammary tumor was described by Segaloff.[17] Compound evaluation methods (unpublished) were essentially the same as those used for solid tumors by many investigators, *e.g.*; fragments 2–3 mm^3 were implanted subcutaneously; 24 hr later drug administration was initiated and continued once daily; in the case of the 13762 mammary system the drug was continued for 21 days; 1 day later the animals were sacrificed, the tumors were excised, and the weights were recorded. Groups of ten inoculated animals were assigned randomly as controls or to the various agents to be tested. The compounds were administered most frequently *via* the subcutaneous route; however, oral and intraperitoneal evaluations also were carried out in several instances. Steroid suspending medium (0.9% NaCl, 0.4% polysorbate 80, 0.5% carboxymethylcellulose, and 0.9% benzyl alcohol) was used chiefly as the diluent, but sesame oil, peanut oil, and saline were used in some of the experiments.

A summary of the data obtained to date with active compounds is shown in Table IV. Except for the diethylstilbestrol derivative inactive compounds are not included, although they were tested in many of the same systems and at the same or similar dosages *via* the same routes of administration. In general, no activity was found in the systems studied to date with the compounds in which the oncolytic agent and the steroid were connected by a stable bond which could not be readily cleaved by hydrolytic and/or enzymatic action. Thus all the nitrogen mustards with a stable steroid–sulfur bond (Table II) or ethylenimine compounds (Table III), in which the active agent was linked to the

(11) L. Fieser and M. Fieser, "Steroids," Reinhold Publishing Corp., New York, N. Y., 1959, pp 7–15.

(12) D. K. Fukushima and T. F. Gallagher, *J. Amer. Chem. Soc.*, **73**, 196 (1951).

(13) For the stereochemistry of the addition of anions to the 3-keto-$\Delta^{4,6}$ steroidal dienes see R. M. Dodson and R. C. Tweit, *ibid.*, **81**, 1224 (1959).

(14) Data reported herein with the exception of those on Walker 256 and L1210 were obtained in the contract laboratories of Dr. W. F. Dunning, University of Miami, Coral Gables, Fla., and Dr. Arthur Bogden, Mason Research Institute, Worcester, Mass.

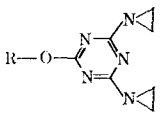
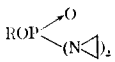
(15) Protocols for Screening Clinical Agents and Natural Products Against Animal Tumors and Other Biological Systems, *Cancer Chemotherapy Rept.*, **25**, 1 (1962).

(16) W. F. Dunning, M. R. Curtis, M. L. Stevens, and F. Dumenigo, *Cancer Res. Suppl.*, **27**, 696 (1967).

(17) A. Segaloff, *Recent Progr. Hormone Res.*, **22**, 351 (1966).

TABLE IV

SUMMARY OF ANTITUMOR EFFECTS OF STEROIDAL ONCOLYTIC AGENTS

R	Substitution	Activity,[a] dose (mg/kg/day) — Tumor Systems[b] 1	2	3	4	5	6	7	8	9	10
					$ROOCCH_2C_6H_4NX_2$[c]						
Cholesterol	3β	−, 50–400 sc		++, 5–20 sc	±, 10 *po*	−, 2.5–10 sc	+, 10 *po*	+, 10 sc	+++, 10 sc	T, 10 *po*	
Estrone	3	−, 100–400 sc	+++, 330 ip	+++, 10 *po*	++, 1.25–2.5 sc	+++, 2.5 sc	++, 2.5 sc	++, 2.5 sc	+++, 2.5 sc	T, 2.5 sc	+, 2.5 sc
Estradiol	3,17β	−, 100–400 sc	+++, 75–600 sc	++, 1–10 sc	+, 2.5 sc	++ 2.5 sc	+, 2.5 sc	+++, 2.5 sc	+++, 2.5 sc	+, 2.5 sc	± 2.5 sc
Testosterone	17β	−, 100–400 sc	++, 150–600 sc	+++, 5–10 sc, *po*							
Deoxycorticosterone	21	−, 100–400 sc	+++, 330 ip	+++, 5–10 sc	−, 1.0 sc		+++, 5.0 sc		+++, 5.0 sc	++, 5.0 sc	
											
Estrone	3	−, 25–400 sc	++, 12.5–300 sc	+, 5–10 sc			−, 1.0 sc	+, 1.0 sc	±, 1.0 sc		−, 1.0 sc
Diethylstilbestrol	2,2'	−, 100–400 ip	−, 12.5–400 sc	−, 0.1–5.0 *po*			−, 1.0 sc		−, 1.0 sc		−, 1.0 sc
											
Estrone	3	−, 100–400 sc	+, 40–200 sc	+, 5–20 sc	−, 0.1–10 sc, *po*					−, 2.2 sc	
Cholesterol	3β	−, 50–400 sc	++, 400 ip		−, 0.1–10 sc						

[a] $X_2 = (CH_2CH_2Cl)_2$. [b] Tumor systems: 1, L1210 lymphoid leukemia, BDF_1 mouse; 2, Walker 256 carcinosarcoma (intramuscular), random bred rat. The following systems were all maintained in Fischer/344 rats: 3, 13762 mammary adenocarcinoma; 4, R3149 acute monocytic leukemia; 5, IRC741 acute monocytic leukemia (Dunning); 6, R3323 acute monocytic leukemia; 7, R3330 subacute monocytic leukemia; 8, R3309 chronic leukemia; 9, R3230 mammary adenocarcinoma; 10, R2426 mammary adenocarcinoma, August/7322 rat. [c] Activity is based on increase in host survival days, *e.g.*, leukemias, and/or inhibition of tumor growth, *e.g.*, mammary tumor, and in some instances both end points: − = <35% effective; ± = >35–50%; + = >50–65%; ++ = >65–80%; +++ = >80–100% and over; T = toxic.

TABLE V

RESULTS OBTAINED USING 13762 MAMMARY TUMOR OF THE FISCHER/344 RAT

Compd	Dose, mg/kg/day *po* (21 days)	No. of rats sac./initial	Mean tumor wt, g	% of controls	Mean body wt ratio[c]
Controls (sesame oil)	...[b]	10/10	1.99 ± 0.8		1.48
Cyclophosphamide	1.62	10/10	1.33 ± 0.3	69	1.70
	3.25	10/10	1.18 ± 0.5	59	1.54
	6.50	7/10	0.48 ± 0.2	24	1.41
Phenesterin (1)	5.0	10/10	0.83 ± 0.4	42	1.56
	10.0	10/10	0.16 ± 0.1	8	1.27
	20.0	9/10	0.12 ± 0.0	6	1.25

[c] Body weight ratio is the mean of the final body weight of the hosts per group divided by the mean of the initial body weight of the same group. [b] Each control animal is given a 0.5-ml dose daily, of the same vehicle used for the drug preparation.

steroid by a C–N bond, were completely inactive.[18] On the other hand, for all of the active compounds shown in Table IV, the oncolytic agent is linked to the steroid by a more easily cleaved ester or heterocyclic ether linkage

All the compounds studied in the L1210 leukemia were inactive according to the criteria of the CCNSC. On the other hand, most of the same materials were active in the Walker 256 system. In this system and the 13762 mammary tumor it was of considerable interest to find that an ether of bisaziridinyltriazine with estrone showed an antitumor effect while the ether with diethylstilbestrol, which contains two of the bisaziridinyltriazine moieties, was inactive in these and in all others studied. The most active agents of those studied to date were the steroidal esters of *p*-[*N*,*N*-bis(2-chloroethyl)amino]phenylacetic acid. As shown in Table IV, all of these compounds produced a good to excellent antitumor response in the 13762 mammary tumor. At this time it is not possible to report that one of these agents is definitely superior to another in this system. However, in some of the rat leukemias the ester of estrone, the 3,17β-diester of estradiol, and the corresponding desoxycorticosterone-21-phenylacetic acid nitrogen mustard were more effective than the cholesterol ester (phenesterin, **1**).

Antitumor results from one of the earliest experiments with the cholesteryl ester in the 13762 system indicated that route of administration and the vehicle used as a solvent might be factors important to the efficacy of the agent. The results of two experiments formulated to test the effects of each are shown in Table VI. As can be seen, oral administration was superior to either the subcutaneous or intraperitoneal routes; the sesame oil as the diluent appeared superior to the steroid suspending medium. Metabolic studies are in progress with the doubly labeled phenesterin in an attempt to determine why oral administration is more effective with this compound. As can be noted in Table IV, the corresponding estradiol, testosterone, and deoxycorticosterone esters were active by the subcutaneous route of administration, but it remains to be proven that they would have been more effective if given orally.

The results of a comparative study with the oncolytic agent, cyclophosphamide, and phenesterin (**1**) in the 13762 mammary system are shown in Tables V and VII. Table V shows the results with the routine 21-day study while Table VII shows additional groups that were maintained for a longer period of treatment (63 days). A higher dose of cyclophosphamide was not included because in two of three previous experiments with cyclophosphamide at 7.50 mg/kg/day given orally in steroid suspending medium, all animals were dead before the end of the 21-day treatment period. With the shorter test period phenesterin gave better antitumor activity than cyclophosphamide at all three dosages and only one animal died that received the highest level, 20 mg/kg/day; conversely three of the animals that were given the highest level of cyclophosphamide, 6.50 mg/kg/day, died. For the longer period of drug administration the antitumor activity of the two agents appeared equivalent at the highest levels; however, with cyclophosphamide only two rats survived beyond 23 days at which time only one animal receiving the highest dose of phenesterin had died. In this experiment and others to be reported in a later paper, the steroid BCAPAA esters appeared to be much less toxic than this oncolytic agent, as well as others commonly used. The 3.25-mg/kg dose of cyclophosphamide is roughly equivalent to the amount of BCAPAA in the 10-mg/kg dose of phenesterin.

All of the data reported above for the 13762 mammary system were obtained in animals in which drug administration was initiated 24 hr after tumor implantation. Table VIII reports results of four experiments which were obtained in rats with well-established tumors, *e.g.*, those having the tumors implanted a minimum of 22 days prior to initiation of therapy. Phenesterin and cyclophosphamide then were given daily for 21 days. Both compounds suppressed the growth of the tumor, and with tumor measurements made several times per week phenesterin was found to cause actual regression of the tumors. In the one experiment with cyclophosphamide, and using only a few animals, tumor regression was not measurable, and tumor inhibition occurred only at the higher dose of 15 mg/kg/day. The older animals appeared to be more resistant to the toxicity of cyclophosphamide than were the younger animals in the previous experiments. It is recognized that with the exception of one of the experiments the number of animals used was small; however, with phenesterin three different experiments were done and the antitumor effects were reproducible in each instance.

TABLE VI

RESULTS OBTAINED WITH PHENESTERIN (**1**) ADMINISTERED BY THREE DIFFERENT ROUTES AND IN TWO DIFFERENT VEHICLES TO FISCHER/344 RATS BEARING THE 13762 DMBA-INDUCED AND TRANSPLANTABLE MAMMARY TUMOR

No. of animals T/C[a]	Mean tumor wt T/C, g ± SD	% of controls	Mean body wt ratio[b] T/C
	10 mg/kg/day *po* for 21 days in sesame oil		
10/10	0.46 ± 43/1.44 ± 0.95	32	1.33/1.77
10/10	0.33 ± 0.30/3.39 ± 1.0	9	1.37/1.67
	10 mg/kg/day *po* for 21 days in steroid suspending medium		
10/9	0.49 ± 0.31/1.23 ± 0.58	40	1.64/1.69
9/10	1.02 ± 1.3/2.73 ± 0.60	37	1.38/1.68
	10 mg/kg/day sc for 21 days in sesame oil		
10/10	0.98 ± 0.81/1.68 ± 0.80	58	1.56/1.82
10/10	2.66 ± 1.2/2.79 ± 0.7	95	1.68/1.67
	10 mg/kg/day sc for 21 days in steroid suspending medium		
10/10	1.52 ± 0.83/1.72 ± 0.86	88	1.77/1.73
10/10	3.21 ± 1.1/3.21 ± 1.6	100	1.53/1.63
	10 mg/kg/day ip for 21 days in sesame oil		
10/9	1.02 ± 0.74/0.99 ± 0.41	103	1.71/1.64
10/10	1.44 ± 0.60/2.93 ± 1.1	49	1.37/1.46
	10 mg/kg/day ip for 21 days in steroid suspending medium		
10/8	1.36 ± 1.2/0.64 ± 0.56	213	1.70/1.45
10/10	3.39 ± 1.0/3.79 ± 1.9	89	1.53/1.46

[a] Treated/controls. [b] Body weight ratio is the mean of the final body weight of the hosts per group divided by the mean of the initial body weight of the same group.

(18) In this connection, N. F. Bukva and G. H. Gass, *Cancer Chemotherapy Rept.*, **51**, 431 (1967), have reported some activity of steroidal 16-aziridinyl compounds similar to the pregnendone acetate and 3-hydroxypregnane-11,20-dione derivatives (Table III) in inhibition of CH/AN mammary carcinoma (milk factor) (intraperitoneal administration). Inspection of their data indicates that very high doses 25–50 mg were required to produce a modest response, *i.e.*, T/C 30–45%. The similar compounds in our studies were investigated at much lower dose levels and largely by subcutaneous administration.

TABLE VII

RESULTS OBTAINED WITH THE 13762 MAMMARY TUMOR OF THE FISCHER 344 RAT

Compd	Dose, mg/kg/day *po* (63 days)[e]	No. of rats surv./initial	Mean tumor wt, g	% of controls	Mean body wt ratio[a]
Controls (sesame oil)	...[b]	2/10	31.7 ± 17.4	...	1.37
Cyclophosphamide	1.62	7/10	28.6 ± 12.8	90	1.71
	3.25	10/10	31.0 ± 12.6	98	2.05
	6.50	0/10[c]	0.38 ± 0.1	1	0.97
Phenesterin (1)	5.0	7/10	10.8 ± 8.2	34	1.50
	10.0	4/10	2.61 ± 2.6	8	1.10
	20.0	0/10[d]	0.68 ± 0.5	2	0.96

[a] Body weight ratio is the mean of the final body weight of the hosts per group divided by the mean of the initial body weight of the same group. [b] Each control animal is given a 0.5-ml dose daily, of the same vehicle used for the drug preparation. [c] Only two rats lived beyond 23 days. [d] Only one rat died before day 30. [e] Survivors sacrified on day 65.

TABLE VIII

RESULTS OBTAINED WITH PHENESTERIN AND CYCLOPHOSPHAMIDE IN FISCHER 344 RATS BEARING WELL-ESTABLISHED 13762 MAMMARY TUMORS

Compd, dose (mg/kg/day)	No. of animals T/C[a]	Mean tumor wt T/C, g ± SD	% of controls	Mean body wt ratio[b] T/C
Phenestrin, 10	20/20[c]	7.32 ± 3.4/63.2 ± 25.7	12	1.39/2.17
	4/4[d]	16.4 ± 9.3/49.8 ± 32.5	33	1.04/1.34
	5/5[e]	22.5 ± 9.0/84.6 ± 16.6	27	0.78/1.24
Cyclophosphamide, 7.5	3/2[e]	44.9 ± 25.7/59.5 ± 18.2	75	1.14/1.17
Cyclophosphamide, 15	3/2[e]	13.3 ± 15.2/59.5 ± 18.2	22	0.93/1.17

[a] T/C = treated group/control group. [b] Body weight ratio is the mean of the final body weight of the hosts per group divided by the mean of the initial body weight of the same group. [c] Tumors implated 22 days prior to initiation of therapy, 21 days of therapy. [d] Tumors implanted 30 days prior to initiation of therapy, 7 days of therapy. [e] Tumors implanted 28 days prior to initiation of therapy, 3 weeks of therapy.

TABLE IX

RESULTS OBTAINED WITH THREE STEROID ESTERS OF *p*-[N,N-BIS(2-CHLOROETHYL)AMINO]PHENYLACETIC ACID IN VARIOUS LEUKEMIAS OF THE INBRED FISCHER 344 RAT

$ROOCCH_2C_6H_4N(CH_2CH_2Cl)_2$

R	Substitution	Leukemic systems[b]	Dose, mg/kg/day (no. of doses)	No. of rats T/C[a]	Mean tumor diameter, cm T/C	%	Mead survival, days T/C	%	Wt change, g T/C
Cholesterol	3β	R3149	10 sc (10)	10/10	2.1/2.6	81	12.6/11.7	108	−5/0
			10 *po* (9)	10/10	1.1/2.2	50	20.4/13.8	148	−7/3
		R3323	10 *po* (10)	9/9	[c]		16.0/10.1	158	−12/−1
		R3330	10 sc (18)	10/10	[c]		28.6/18.8	152	−19/−7
		R3399	10 sc (13)	10/10	0.0/1.9	0	26.2/14.3	183	−7/−5
		IRC741	2.5 sc (16)	10/10	1.1/1.2	92	20.5/22.1	93	−24/−8
			10 sc (16)	10/10	2.0/2.4	83	21.6/20.1	107	−36/−7
Estrone	3	R3149	2.5 sc (13)	10/10	0.50/1.5	33	16.0/12.6	127	−27/−12
		R3149	2.5 sc[d] (10)	10/10	0.70/3.0	23	24.8/14.4	172	0/5
		R3323	2.5 sc (9)	10/9			11.0/10.0	110	−5/10
		R3323	2.5 sc (10)	10/9			14.2/10.0	141	−1/6
		R3323	2.5 sc[d] (10)	10/9			21.8/10.6	206	−20/−8
		R3330	2.5 sc (18)	8[e]/9			35.4/20.3	174	−34/−9
		R3399	2.5 sc (16)	10/10	1.0/2.6	38	33.2/16.4	202	−27/−3
		R3432	2.5 sc (20)	10/10	0.0/2.9	0	71.0/41.0	173	−33/3
		IRC741	2.5 sc (14)	10/10	0.0/2.5	0	35.5/17.8	199	−35/0
Estradiol	3,17β	R3149	2.5 sc (10)	10/9	2.9/2.9	100	17.8/10.8	165	−8/−9
			2.5 sc[d] (10)	10/10	0.9/3.0	30	20.5/14.4	142	2/5
		R3323	2.5 sc (10)	10/9			15.5/9.9	157	−22/−6
		R3323	2.5 sc[d] (10)	10/9			13.7/9.8	140	−16/−10
		R3330	2.5 sc (18)	10[f]/10			51.0/18.9	270	−32/6
		R3399	2.5 sc (17)	9/10	0.0/3.0	0	30.3/15.7	193	−31/−7
		R3432	2.5 sc[d] (20)	5/10	Toxic				
		R3432	2.5 sc (20)	10[g]/10	0.0/3.1	0	62.7/39.8	158	−20/8
		IRC741	2.5 sc (16)	10/10	1.2/1.2	100	31.1/22.1	141	−29/−8
		IRC741	2.5 sc[d] (14)	0/10	1.5/2.4	63	33.3/18.2	183	−18/9

[a] T/C = treated/controls. [b] Leukemias: R3149 acute monocytic, R3323 acute monocytic (no solid tumor), R3330 subacute monocytic (no solid tumor), R3399 chronic, R3432 chronic lymphocytic, IRC741 acute monocytic. [c] No solid tumor formed at the site of inoculation. [d] sc = in peanut oil. [e] Two animals classed as cures since they survived over 90 days before reinoculation. [f] Eight of ten animals classed as cures since they survived over 90 days before reinoculation. [g] Seven of ten animals classed as cures, survival over 90 days before reinoculation.

Table IX reports in detail the antileukemic results obtained with cholesterol, estrone, and estradiol esters of BCAPAA in at least five leukemias of the Fischer/344 rat. Comparable data on the deoxycorticosterone ester beyond those in Table IV are not presented in Table IX because of incomplete testing. The importance of the vehicle of administration to the efficacy of phenesterin was referred to earlier; it is again indicated by the results with the estrone ester given in peanut oil or the steroid suspending vehicle to animals with R3149 and R3323 leukemias. All three esters were highly effective in the R3399 chronic leukemia, and, in fact, it was the only leukemia in which the cholesteryl ester had an outstanding activity. Both the estrone and estradiol esters were highly effective in several of the rat leukemias. The results were of particular interest in the IRC741 leukemia, known to be responsive to alkylating agents[19] but never effectively treated with steroids.[16] R3323 and R3330 were reported by Dunning, *et al.*,[16] to be responsive to several compounds of estrogenic structure; however, neither estrone nor estradiol alone was significantly effective in increasing host survival time as were these BCAPAA esters. Of particular interest also with these two agents were the animals that were "cured" of their leukemia, *e.g.*, in the R3330 and R3432 systems.

Additional antitumor and toxicological experiments must be completed before the five most effective compounds to date can be ranked as to superiority.

Initial studies of the hormonal properties of the compounds reported in this paper indicate that the steroid portion of the molecule, be it androgen, estrogen, or corticoid, does express its respective biological activity on the endocrine organs of the host; however, no such activity has been found in the case of phenesterin with its cholesterol moiety. The antitumor activity of these steroidal alkylating agents cannot be explained solely on the basis of their hormonal properties because neither cholesterol, estrone, estradiol, testosterone, nor deoxycorticosterone has demonstrated such effective inhibition of tumor growth in most of the systems used (unpublished data and Dunning, *et al.*[16]).

Experimental Section[20]

Ethyl *p*-[N,N-Bis(2-hydroxyethyl)amino]phenylacetate.—Ethylene oxide (90 g) was dissolved in 100 ml of cold 12% AcOH. The resulting solution was frozen in Dry Ice–Me_2CO and 90 g of ethyl *p*-aminophenylacetate was added. The mixture was sealed in a Parr pressure bomb (2-l. capacity) and allowed to stand at room temperature for 17 hr. The apparatus was vented and the two-phase solution was placed on a rotary evaporator for 30 min at room temperature. Water (200 ml) and $CHCl_3$ (300 ml) were added and the organic layer was drawn off. The aqueous layer was extracted with $CHCl_3$ (four 50-ml portions) and the extracts were combined with the original $CHCl_3$ phase. This solution was washed with saturated Na_2CO_3 solution until neutral and dried (Na_2SO_4). Evaporation of the solvent afforded 130.1 g of a crystalline solid, mp 67–70°, lit.[21] mp 70.5–71.5°.

Ethyl *p*-[N,N-Bis(2-chloroethyl)amino]phenylacetate.—Ethyl-*p*-[N,N-bis(2-hydroxyethyl)amino]phenylacetate (130 g) in 350 ml of dry C_6H_6 containing 130 ml of freshly distilled $POCl_3$ was heated under reflux with stirring for 1 hr. The solution was cooled to room temperature and poured on to 1500 ml of ice. The ice was allowed to melt and the C_6H_6 layer was drawn off. The aqueous phase was extracted with C_6H_6 (two 100-ml portions). The combined C_6H_6 solution was washed (H_2O, dilute $NaHCO_3$) and dried (Na_2SO_4). Evaporation of the solvent *in vacuo* afforded 132.2 g of a brown oil which crystallized on standing, mp 49–52°, lit.[21] mp 52–53°.

***p*-[N,N-Bis(2-chloroethyl)amino]phenylacetic Acid.**—Ethyl *p*-[N,N-bis(2-chloroethyl)amino]phenylacetate (132.2 g) was heated in 560 ml of concentrated HCl at 100° for 4 hr. The solution was cooled to room temperature and poured into 5 l. of ice and H_2O with stirring. The tan, crystalline precipitate was filtered off and dried giving 96.2 g, mp 103–105°. The aqueous filtrate was saturated with NaCl and extracted with $CHCl_3$ (four 100-ml portions). The combined extracts were neutralized with $NaHCO_3$ solution. After drying, evaporation of the solvent afforded 19 g of a solid. Crystallization from C_6H_6–C_6H_{14} gave 17.5 g, mp 104–105°, lit.[21] mp 105°.

***p*-[N,N-Bis(2-chloroethyl)amino]phenylacetyl Chloride Hydrochloride.**—*p*-[N,N-Bis(2-chloroethyl)amino]phenylacetic acid (12 g) in 95 ml of dry C_6H_6 was added dropwise with stirring to 34 ml of freshly distilled $SOCl_2$ at 0.5°. After stirring at 0° for 1 hr, a white precipitate formed. The mixture was allowed to stand at −5° overnight and the solid was filtered off under dry N_2. After washing with cold, dry C_6H_6, the solid was dried *in vacuo* over P_2O_5 giving 14.1 g of crystals.

3β-Hydroxy-5-cholestene[N,N-bis(2-chloroethyl)amino]phenyl Acetate (Table I).—A mixture of 14 g of cholesterol and 13.37 g of *p*-[N,N-bis(2-chloroethyl)amino]phenylacetyl chloride hydrochloride in 220 ml of dry C_6H_6 was refluxed for 4 hr. The solution was cooled to room temperature and concentrated to approximately 100 ml on a rotary evaporator. This solution was percolated through 125 g of Florisil and the column was washed with C_6H_6. Evaporation of the solvent yielded 21.7 g of a colorless glass. Crystallization from Me_2CO–MeOH afforded 20.1 g (86%) of crystals, mp 91.5–93°, lit.[8d] mp 89–89.5°. The ir spectrum (CH_2Cl_2) showed bands at 1725, 1615, 1515, 1357, 1005, and 805 cm^{-1}.

16α-[*p*-(N,N-Bis-2-chloroethylamino)phenylthio]-3β-acetoxy-5α-pregnane-12,20-dione (Method A, Table II).—A mixture of 0.36 g (1.0 mmole) of 3β-acetoxy-5α,16-pregnane-12,20-dione,[22] 0.250 g (1 mmole) of *p*-[N,N-bis(2-chloroethylamino)]thiophenol,[10b] and 0.5 ml of piperidine in 5 ml of C_6H_6 was stirred at reflux for 24 hr under N_2. The reaction mixture was concentrated under reduced pressure on a rotary evaporator. The remaining residue was dried at high vacuum overnight and chromatographed on silica gel. Elution with C_6H_6 gave di[*p*-(N,N-2-chloroethylamino)phenyl] disulfide. Elution with C_6H_6–AcOEt (80:20) yielded a fraction which gave 0.55 g (89%) of the desired product.

21-[*p*-(N,N-Bis-2-chloroethylamino)phenylthio]-11β,17α-dihydroxy-4-pregnene-3,20-dione (Method B, Table II).—A mixture of 17.5 g (30 mmoles) of hydrocortisone brosylate,[1b] 7.51 g (30 mmoles) of *p*-[N,N-bis(2-chloroethylamino)]thiophenol,[10b] and 9 ml of piperidine in 150 ml of C_6H_6 was refluxed for 25 hr under N_2. The cooled reaction mixture was filtered to remove piperidinium brosylate. The filtrate was concentrated at reduced pressure on a rotary evaporator. The remaining residue was dried under high vacuum and chromatographed on silica gel. Elution with C_6H_6 gave *p*-[N,N-bis(2-chloroethylamino)phenyl] disulfide. Elution with C_6H_6–AcOEt (60:40) yielded a fraction which gave 9.75 g (55%) of the desired product.

17β-Hydroxy-2α-methyl-5α-androstan-3-one Bis(1-aziridinyl)-phosphinate Ester (Method A, Table III).—To a solution of 15 g (49 mmoles) of 17β-hydroxy-2α-methyl-5α-androstan-3-one in 450 ml of THF containing 10 ml of Et_3N at 0° was added 6 ml (66 moles) of $POCl_3$ and the resulting solution was allowed to stand at room temperature for 24 hr. Et_3N (4 ml) and 1 ml of $POCl_3$ were added and the mixture was allowed to stand for 20 hr at room temperature. The solution was cooled in an ice bath and a solution of 7.7 ml (148 mmoles) of ethylenimine in 30 ml of

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(20) Melting points were determined on a Kofler hot stage microscope using a calibrated thermometer. Uv spectra were measured on a Cary Model 14 spectrophotometer. Nmr spectra were recorded on a Varian Model A-60 (TMS). Ir spectra were measured with a Perkin-Elmer 221 spectrophotometer. Mass spectra were determined on an AEI MS-902 spectrometer. Microanalyses were carried out by Micro-Tech Laboratories, Skokie Ill. Where analyses are indicated only by symbols of the elements or functions, analytical results obtained for these elements or functions were within ±0.4% of the theoretical values.

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(22) M. E. Wall and S. Serota, *Tetrahedron*, **10**, 238 (1960).

Et_3N was added dropwise with stirring. After 2 hr the solution was washed (dilute $NaHCO_3$, H_2O, saturated NaCl solution). After drying (Na_2SO_4), the solution was evaporated to give 19.5 g of a brown oil. This material was dissolved in C_6H_6 and chromatographed on 386 g of silica gel. The column was eluted with a gradient system: 5% EtOAc/C_6H_6–30% EtOAc/C_6H_6–EtOAc. This procedure gave 10.7 g of chromatographically pure material. Crystallization from C_6H_{14} afforded 8.8 g of a solid, mp 150–153°.

3β-Hydroxy-5-cholestene 4-(1-Aziridinyl)-3,5-dinitrobenzoate Ester (Method B, Table III).—To a solution of 8.21 g (31 mmoles) of 4-chloro-3,5-dinitrobenzoyl chloride in 100 ml of C_6H_6 was added 10 g (26 mmoles) of cholesterol and 10 ml of C_5H_5N. After stirring at room temperature for 2 hr, the solid material was removed by filtration and the filtrate was washed (dilute $NaHCO_3$, H_2O saturated NaCl). After drying the solution (Na_2SO_4), the liquid was evaporated to give 15.1 g of a crystalline solid. Crystallization from MeOH gave 13.8 g of a pale yellow solid, mp 170–173°. To a solution of 11.5 g of this material in 300 ml of DMF was added 10 ml of Et_3N and 2 ml of ethylenimine. This solution was stirred at room temperature for 20 min and diluted with 600 ml of EtOH. After cooling the solution to 0°, the crystalline precipitate was filtered off and dried to give 11.2 g of a yellow solid, mp 202–205°.

Estrone 4,6-Bis(1-aziridinyl)-*s*-triazin-2-yl Ether (Method D, Table III).—A mixture of 10 g (36 mmoles) of estrone, 7.31 g (37 mmoles) of 2-chloro-4,6-bis(1-aziridinyl)-*s*-triazine,[23] and 5.11 g (37 mmoles) of K_2CO_3 in 400 ml of dry Me_2CO was refluxed with stirring for 17 hr. After cooling, the reaction mixture was poured into 3 l. of H_2O with stirring. The resulting precipitate was filtered off and dried to give 16.2 g of a colorless powder. Crystallization from *i*-PrOH afforded 11.8 g of crystals, mp 156–160° dec. This material was recrystallized from *i*-PrOH to give 11.1 g, mp 170° dec.

3β-Acetoxy-16α-(1-aziridinyl)-5α-pregnane-12,20-dione (Method E, Table III).—3β-Acetoxy-5α-pregn-16-en-12,20-dione (10 g) was dissolved in 100 ml of ethylenimine and 2 ml of Et_3N was added. After standing at room temperature for 2.5 hr, the liquid was removed *in vacuo* and the residue was crystallized from petroleum ether (bp 30–60°) to give 9.9 g of crystals, mp 141–144°. Recrystallization from the same solvent gave 9.7 g, mp 145–147°.

17α-(1-Aziridinylmethyl)-3β,17β-dihydroxy-5-androstene (Method C, Table III).—A solution of 19.86 g of 3β-hydroxyspiro-17β-oxiranylandrost-5-ene[24] in 400 ml of ethylenimine containing a catalytic amount of MeONa was maintained at 100 ± 5° in a sealed pressure bomb for 10 hr. The solution was cooled to room temperature and the liquid was evaporated. The residue was dissolved in $CHCl_3$ and this solution was washed (H_2O) and dried (Na_2SO_4). Evaporation of the liquid afforded 21.9 g of a solid. Crystallization of this material gave 14.46 g of chromatographically pure material.

3β-Hydroxy-5-cholestene Aziridinylacetate.—Cholesterol chloroacetate (10 g, 22 mmoles) was combined with 50 ml of dry C_6H_6 and 100 ml of Et_3N. To this solution was added 10 ml (238 mmoles) of ethylenimine. The reaction mixture was stirred at room temperature for 24 hr, whereupon 5 ml more ethylenimine was introduced. Stirring was continued for 48 hr. The reaction mixture was then diluted with 500 ml of 1% Et_3N in C_6H_{14} and filtered through Supercel. Evaporation of the solvent afforded 10 g of crude product. This residue was combined with 8 g of crude product from two previous reactions. The total crude product was subjected to reversed-phase partition chromatography using a system composed of Et_3N–C_7H_{16}–MeCN (1:25:100). The less polar phase (2250 ml) was supported on a 10 × 95 cm column of 2500 g of silylated Supercel, and the column was eluted with 30 l. of polar phase. Fractions containing pure product were evaporated at room temperature and 0.5 mm to give 11.3 g (57%) of analytical quality cholesterol β-aziridinylacetate. The compound had no true melting point but rather softened to a gel in an evacuated capillary at 90–97°.

Acknowledgment.—We should like to thank Mrs. Lee Burger, Dr. David Rosenthal, Joan Gratz, and Mrs. Anna Ferguson for supplying some of the compounds discussed in this paper. We acknowledge with appreciation the interest and advice of Drs. Ihor Masnyk and Erwin Vollmer, Endocrine Evaluation Branch, General Laboratories and Clinics, National Cancer Institute.

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Fluorescent Alkylating Agents. 1-(β-Chloroethyl)bisbenzimidazoles[1]

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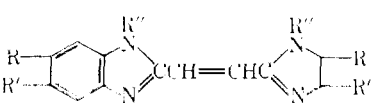
Received March 24, 1969

cis- and *trans*-1-(β-chloroethyl)bisbenzimidazoles have been synthesized as fluorescent alkylating agents. Preliminary *in vivo* study with HeLa cells shows that such compounds can be useful to demonstrate the intranuclear alkylation in dividing cells.

In spite of the numerous studies that suggested the reaction of alkylating agents with DNA or RNA or other nuclear material of a tumor cell, no direct *in vivo* evidence has ever been offered to demonstrate the uptake of an alkylating agent inside a cancer cell.

Fluorescent alkylating agents should be especially useful for such a purpose because fluorescence could be detected visually after reaction and measured fluorometrically. A histochemical fluorescent alkylating agent should possess a high degree of fluorescence so that the alkylating site would be suitably sensitive. Preferably, in *in vivo* experiments, the fluorescence should not be masked by the autofluorescence of the surrounding normal cells. The alkylating agents should not only have a good degree of substantivity but, for *in vivo* work, they should also not be easily metabolized. Since the incorporation of a benzimidazole ring into a nitrogen mustard had previously resulted in a clinically palliative alkylating agent,[2] the *cis*- and *trans*-1-(β-chloroethyl)bisbenzimidazoles (**1**) were there-



1, R = H or CH_3; R′ = H or CH_3; R″ = CH_2CH_2Cl

(1) This work was supported by U. S. Public Health Grant CA-07339 from the National Cancer Institute, National Institutes of Health, Bethesda, Md. Presented in part at the 1st Mid-Atlantic Regional Meeting of the American Chemical Society, Philadelphia, Pa., Feb 1966.

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