4 h. Solvent and excess anhydride were removed at reduced pressure. The residue was chromatographed on silica gel (150-mL dry volume), eluting first with $CHCl_3$–CH_3OH (4:1) to remove less polar impurities and then with $CHCl_3$–CH_3OH–H_2O (140:60:9) to elute 3.6 g (95%) of **17** as a colorless oil: NMR ($CDCl_3$–CD_3OD) δ 5.12 (m, 1 H, OCH), 4.23 (m, 2 H, $POCH_2$), 4.00 (m, 2 H, CH_2OP), 3.75–3.34 (m's, 16 H, CH_2O's, CH_2N), 3.39 (s, 3 H, OCH_3), 3.22 (s, 9 H, $N(CH_3)_3$), 2.08 (s, 3 H, $COCH_3$), 1.56 (m, 4 H, OCH_2CH_2's), 1.29 (m, 8 H, $(CH_2)_4$); IR (neat) 1735 (C=O) cm^{-1}. Anal. ($C_{23}H_{48}O_{10}PN{\cdot}H_2O$) C, H, P, N.

7-(Acetyloxy)-4-hydroxy-*N*,*N*,*N*-trimethyl-3,5,9,12,15-pentaoxa-4-phosphapentacosan-1-aminium, 4-Oxide, Hydroxide, Inner Salt (18). This compound was prepared by using the method described above from **16** (6.7 g, 13.8 mmol), acetic anhydride (35.2 g, 340 mmol), and triethylamine (14 g, 140 mmol) to give 6.5 g (89%) of **18** as a colorless gel: NMR ($CDCl_3$–CD_3OD) δ 5.14 (m, 1 H, HCO), 4.25 (m, 2 H, $POCH_2$), 4.00 (m, 2 H, CH_2OP), 3.70–3.30 (m's, 14 H, CH_2O's, CH_2N), 3.22 (s, 9 H, $N(CH_3)_3$), 2.08 (s, 3 H, $COCH_3$), 1.58 (m, 2 H, CH_2CH_2O), 1.26 (m, 14 H, $(CH_2)_7$), 0.87 (m, 3 H, terminal CH_3); IR (neat) 1727 (C=O) cm^{-1}; MS (FAB), *m/z* 528 (M + H). Anal. ($C_{24}H_{30}O_9P$-$N{\cdot}0.5H_2O$) C, H, P. N.

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Isolation, Synthesis, and Antitumor Evaluation of Spirohydantoin Aziridine, a Mutagenic Metabolite of Spirohydantoin Mustard

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Spirohydantoin mustard (SHM), a central nervous system directed nitrogen mustard with anticancer activity, was metabolized in the presence of mouse liver postmitochondrial supernatant (9000*g* fraction) to a nonpolar alkylating metabolite. The metabolite was isolated by thin-layer chromatography of chloroform or ethyl acetate extracts of incubation mixtures, and its structure was established by mass spectral analysis, synthesis, and cochromatography. The metabolite, spirohydantoin arizidine, was mutagenic for *Salmonella typhimurium* TA1535 in the Ames assay but inactive as an antitumor agent against P388 leukemia in vivo.

Spirohydantoin mustard (SHM, see Scheme I) was designed and synthesized as a central nervous system directed alkylating agent.[1] SHM is active against several experimental murine leukemias and solid tumors, including the ependymoblastoma mouse brain tumor. In preparation for possible clinical studies, preclinical toxicology studies have been completed, and phase I trials have been initiated. In previous studies SHM was observed not to be a direct-acting mutagen in the *Salmonella*/mammalian microsome mutagenicity assay (Ames assay) but is converted by mouse liver postmitochondrial supernatant (9000*g*, S9) to a mutagenic metabolite in the presence of NADPH.[2] The structural identity of this metabolite has not yet been established. Continuing investigations have demonstrated the production of an additional, direct-acting, mutagenic metabolite by mouse liver S9 fraction in the presence of NADPH. Isolation, identification, synthesis, and antitumor and mutagenic activities of this metabolite are reported here.

TLC of chloroform or ethyl acetate extracts of incubates of [^{14}C]-SHM with mouse liver S9 fraction indicated by radioscanning the presence of an alkylating metabolite of SHM of the following R_f values in the following solvents: chloroform–methanol (95:5) 0.26; chloroform–acetone (1:1), 0.17; chloroform–acetone (1:2), 0.15; and toluene–acetone (1:1), 0.27. The alkylating property of the metabolite was determined by its reaction with 4-(*p*-nitrobenzyl)pyridine on the TLC plates. The metabolite, which accounted for 5% of the extractable activity while SHM accounted for 40%, was purified further by TLC in one or more of these solvent systems. The isolated metabolite fraction was analyzed by fast atom bombardment (FAB) MS and yielded a molecular ion peak [$(M + 1)^+$] of *m/z* 238. Electron-impact (EI) MS yielded the following data:

m/z (rel abund)	possible struct
237 (50)	M^+
209 (100)	$[M - (CH_2)_2]^+$ or $[M - CO]^+$
182 (70)	$[M - (CH_2N(CH_2)_2 + H]^+$

The data are consistent with a structure in which the mustard group of SHM has been monodechloroethylated and the resulting (2-chloroethyl)amino group has cyclized to an aziridine, yielding spirohydantoin aziridine (SHAZ, see Scheme I). There is precedent for this proposed oxidative metabolism of the nitrogen mustard moiety leading to a monodechloroethylated product; cyclophosphamide, a leading anticancer and immunosuppressive drug, has been shown to be metabolized along an identical pathway by a purified cytochrome P-450 fraction from rat liver microsomes, leading to the corresponding structure with the nitrogen mustard moiety dechloroethylated to a (chloroethylamino) group.[3] Structural proof was obtained

Table I. Mutagenic Activity of SHAZ against *Salmonella typhimurium* TA1535

sample	amt, μg or μg SHM equiv/plate	induced His$^+$ revertants[a] expt 1	induced His$^+$ revertants[a] expt 2
metabolite	2	41	
SHAZ	1	15	21
SHAZ	10	227	238
SHAZ	100	1841	2000

[a] These values represent the difference between the observed number of revertants and the average number of spontaneous revertants, which was 13 ± 2 in these experiments, on four solvent control plates.

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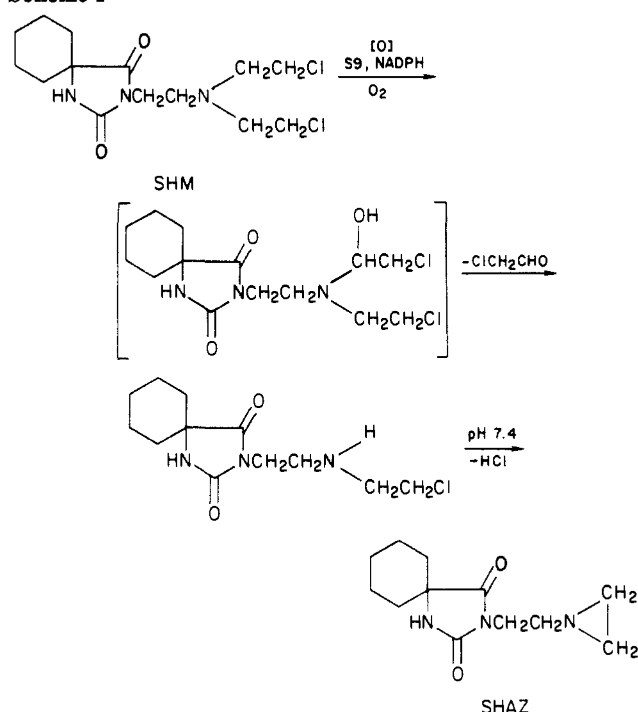
Table II. Activity of SHAZ and SHM against P388 Leukemia in Female CDF1 Mice

	treatment ip		% drug deaths		therapeutic response		
agent[a]	mg/kg	schedule	leukemic mice	normal mice[b]	% ILS[c,d]	approx no. tumor cells alive after Rx	approx log cge in tumor burden after Rx[e]
SHAZ	60	day 1 only	50	0	−30	toxic	
	45		33			toxic	
	30		16		0	4.8×10^6	0
	30		0	0	0	4.8×10^6	0
	20		0	0	0	4.8×10^6	0
	10		0		0	4.8×10^6	
SHAZ	20	qd 1–9 days	83	0	−25	toxic	
	10	83	0		−40	toxic	
	5		83		−5	toxic	
SHM	15	day 1 only	40	50	+35	toxic	
	10		0	33	+40	8.9×10^3	−2.7
	10		83	33	−45	toxic	
	5		10		+30	4.3×10^4	−2.1
SHM	20	qd 1–9 days	100		−60	toxic	
	10		100		−50	toxic	
	5		100		−45	toxic	

[a] Drug prepared in reagent-grade Me_2SO according to average body weight, with dose contained in 0.1 mL. [b] Concurrently treated drug toxicity controls. [c] Based on median day of death. Historical doubling time of 0.44 day was used in calculating tumor cell kill.[5] [d] Percent increase in life span of treated mice as compared to the untreated leukemia controls; P388 leukemia cell inoculum 1×10^6 cells implanted intraperitoneally. [e] log change = net change in viable tumor stem cell population at the end of treatment as compared to the start of treatment. E.g., a −3 log change means that there was a 99.9% reduction in tumor burden after treatment.

Scheme I



by synthesis from spirohydantoin and bis(2-chloroethyl)amine. The metabolite and synthetic SHAZ cochromatographed in the solvent systems listed above.

The mutagenicity of a sample of TLC-isolated metabolite and synthetic SHAZ was determined using *Salmonella typhimurium* strain TA1535 and a plate incorporation assay in the absence of S9.[4] The results are shown in Table I.

Synthetic SHAZ was evaluated against P388 leukemia in female CDF1 mice for comparison of possible antitumor activity with the activity of SHM. The results are shown in Table II. There was no concern about the stability of SHAZ in the saline injection solutions because the aziridine was observed to be stable in aqueous solution at physiological pH and temperature for at least 40 min.

The results demonstrate microsomal generation of a metabolite of SHM that is mutagenic but inactive as an antileukemic agent against this tumor. It is likely that the mutagenic metabolite arises via the metabolic pathway shown in Scheme I.

Experimental Section

Melting points were determined with a Kofler Heizbank and are corrected. NMR spectra were determined with a Varian XL-100-15 spectrometer. Mass spectra were determined with a Varian-MAT 311A spectrometer equipped for electron-impact, field desorption, field ionization, and fast atom bombardment capabilities. TLC was performed on Analtech silica gel G plates. Analytical results indicated by element symbols were within 0.3% of the theoretical values. Elemental analyses and spectral determinations were performed in the Molecular Spectroscopy Section of Southern Research Institute under the direction of Dr. William C. Coburn, Jr., and antitumor evaluation was conducted in the Cancer Screening Division under the direction of Dr. W. Russell Laster, Jr.

Chemicals. [^{14}C]-SHM (13.5 mCi/mmol), uniformly labeled in the bis(2-chloroethyl)amino group, was obtained from Dr. J. A. R. Mead, Division of Cancer Treatment, NCI. Radiochemical purity was 96–98% as determined by TLC. Spirohydantoin was a gift from Dr. John Driscoll, Division of Cancer Treatment, NCI. Other chemicals were obtained from commercial sources.

Metabolism of SHM in Vitro. [^{14}C]-SHM was incubated with mouse liver S9 fraction and extracted with ethyl acetate or chloroform as described previously.[2]

Isolation of Metabolites. Chloroform or ethyl acetate extracts of incubates of [^{14}C]-SHM with mouse liver S9 fraction were fractionated by TLC in chloroform–methanol (95:5), chloroform–acetone (1:1, 1:2), or toluene–acetone (1:1). Radioactive bands were detected by radioscanning, collected, and eluted with acetone. Further purification was accomplished by TLC in one or more of the solvent systems listed above.

Spirohydantoin Aziridine. Spirohydantoin (4.2 g, 2.5 mmol) in a solution of 13.5 g of potassium hydroxide in 100 mL of absolute ethanol was treated with 4.44 g of bis(2-chloroethyl)amine hydrochloride, and the solution was refluxed 16 h with stirring. After evaporation to dryness, the resulting residue was treated with 50 mL of ethyl acetate and 50 mL of water with vigorous stirring. The ethyl acetate layer was collected, and the aqueous

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layer was extracted with ethyl acetate (2 × 50 mL). The combined ethyl acetate extracts were washed with 25 mL of 10% potassium bicarbonate, dried over sodium sulfate, filtered, and concentrated to ca. 2 mL. The concentrate was stored at −20 °C. A supernate was decanted from a crystalline solid, which was triturated with ether and collected by filtration. Further purification to TLC homogeneity was accomplished by recrystallization from acetone: yield 250 mg; mp 166–167 °C ^{1}H NMR (Me_2SO-d_6) δ 1.0–1.1 (t, 2, CH_2 in aziridine, 1.2–1.8 (complex m, 12, cyclohexyl $(CH_2)_5$ and CH_2 in aziridine), 2.2–2.4 (t, 2, CH_2N), 3.4–3.6 (t, 2, $CH_2N(C{=}O)_2$) 9.6 (s, 1, HN); FAB MS and EI MS identical with FAB MS and EI MS, respectively, of the metabolite; high-resolution MS (obsd, theory) 237.147, 237.148. Anal. ($C_{12}H_{19}N_3O_2$) C, H; N: calcd, 17.70; found, 16.89.

Acknowledgment. This investigation was supported by NIH Grant RO1 CA 30579.

Registry No. SHM, 56605-16-4; SHAZ, 102234-07-1; NH$(CH_2CH_2Cl)_2$·HCl, 821-48-7; spirohydantoin, 702-62-5.

Basically Substituted Ellipticine Analogues as Potential Antitumor Agents

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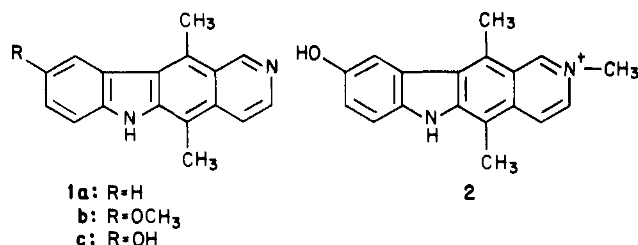
Installation of a basic side chain on the ring nitrogen of ellipticine did, as expected, improve the DNA binding properties of these molecules as measured by an ethidium displacement assay. In vivo antitumor activity was not, however, improved.

Ellipticine (**1a**) and its 9-methoxy analogue (**1b**) are constituents of plants of the Apocynacae family. In 1967, antitumor properties of ellipticine were revealed,[1] and in



1970 a study of **1b** against acute myeloblastic leukemia in man was reported.[2] More recent work has led to new derivatives such as **1c**[3] and the charged species **2**,[4] which has been said to have shown interesting results in phase II trials.[5]

The ellipticines are generally thought to exert their antitumor activity via their interaction with DNA. It was of interest, therefore, to prepare some examples of an ellipticine nucleus to which a basically substituted aliphatic side chain was appended, since such functionality has not infrequently been associated with improved DNA binding of planar molecules.

A basic side chain could be installed on the indole ring nitrogen of **1a** by alkylation of the anion formed with NaH with a corresponding alkyl halide in much the same way that the *N*-methyl analogue had been prepared.[1] Alternatively the basic side chain could be introduced on the pyridine nitrogen similar to the methodology used for the predecessor of **2**, the deshydroxy analogue.[6]

In this manner compounds **1–8** were prepared, and data on their properties are summarized in Table I.

Biology

The compounds were evaluated against the L1210 leukemia and a human colon tumor line in tissue culture. The data are shown in Table II. In vitro these analogues display cytotoxicity of the same order of magnitude as the parent, ellipticine. Against the murine P388 leukemia IPIP on a Q04DX02 schedule, however, only the quaternary analogues **7** and **8** showed a low order of activity (T/C of 145 at 25 mg/kg and 137 at 5 mg/kg, respectively), while all others were inactive at the highest toxic doses tested. In the same test system, ellipticine resulted in a T/C of 200 at 10 mg/kg. A recent patent[7] contains basically substituted quaternary analogues of **2** although the biological data presented reveals little advantage over the methyl quaternary **2**.

To ascertain whether the lack of in vivo activity was due to a reduction of the DNA binding capabilities of the analogues, they were subjected to an ethidium displacement assay. Data shown in Table III indicates the concentration at which the fluorescence of an ethidium–DNA complex was reduced by 50%.

All compounds tested were superior to ellipticine itself in DNA binding capability.

Conclusions

Addition of a basic side chain to the ellipticine structure has been demonstrated to improve DNA binding properties of the system. The poor in vivo antitumor response of the compounds despite the improved DNA binding capability must involve unfavorable pharmacokinetic, pharmacodynamic, or metabolic properties and must receive further attention.

Experimental Section

Melting points were obtained with a Thomas-Hoover melting point apparatus and are uncorrected. The ^{1}H NMR 90-MHz spectra were obtained with a Varian FM 390 or Brüker WH90

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