spectrum resembled that of $B_{20}H_{17}OH^{4-}$ (isomer ii) with peaks at 7.4, 24.3, 28.8, 31.1, 46.7, and 48.5 ppm. Decoupling left peaks at 7.1, 22.5, 26.6, 29.4, 34.8, 43.3, and 47.9 ppm.

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Studies on Cyclophosphamide Metabolites and Their Related Compounds. 2.[1] Preparation of an Active Species of Cyclophosphamide and Related Compounds

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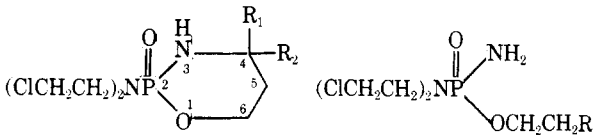
A synthetic study was made on the active metabolite of cyclophosphamide. Ozonolysis of *O*-(3-butenyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate, prepared by reaction of $POCl_3$ with 3-buten-1-ol followed by treatment with *N,N*-bis(2-chloroethyl)amine (nor mustard) and NH_3, afforded 2-[bis(2-chloroethyl)amino]-4-hydroperoxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (4-hydroperoxycyclophosphamide). Deoxygenation of 4-hydroperoxycyclophosphamide by triphenylphosphine yielded 4-hydroxycyclophosphamide in a pure crystalline state. These products exhibited high cytostatic activity in both *in vitro* and *in vivo* experiments. The results give confirmatory evidence for the hypothesis that C_4-hydroxylation on the 1,3,2-oxazaphosphorinane ring of cyclophosphamide is necessary for its activation.

Cyclophosphamide [2-bis(2-chloroethyl)aminotetrahydro-2*H*-1,3,2-oxazaphosphorine-2-oxide (1)][2] is an antitumor agent now clinically used in the treatment of various kinds of human cancer. The drug is known to be activated to a cytotoxic form during *in vivo* metabolic degradation and extensive studies have been made to elucidate the structure of the active species.[3] In 1963, Brock and Hohorst[4] found that enzymatic oxidation in liver microsomes is responsible for the activation of cyclophosphamide and recent studies by Hill, *et al.*,[5] and also by us,[1] have shown that 4-ketocyclophosphamide (**2**) and its ring-opened carboxylic acid **3** are excreted as the cyclophosphamide metabolites in animal urine indicating that the first *in vivo* metabolic reaction of cyclophosphamide involves oxidation at the C-4 position on the 1,3,2-oxazaphosphorinane ring. Hill, *et al.*,[5a] first proposed that 4-ketocyclophosphamide is either the active form of cyclophosphamide or a precursor of the active form. but both **2** and **3** were later proved to be cytostatically less active than cyclophosphamide in *in vivo* experiments.[1,5b,6,7] This suggests that the activation takes place in an earlier phase of the C-4 oxidation. Thus, 4-hydroxycyclophosphamide (**4**) and the ring-opened aldehyde **5** have been proposed as the alternative active species of cyclophosphamide.[1,6-8]

This paper is a full account of a previous communication[9] in which we have demonstrated the first unambiguous chemical synthesis of 4-hydroxycyclophosphamide and provided confirmatory evidence that cyclophosphamide is indeed activated by the C-4 hydroxylation. Prior to this work, we[10] had found that 4-ketocyclophosphamide can be reduced by lithium aluminum hydride to a potentially cytotoxic product which possibly has a cyclic structure **4**. However, the product could not be obtained in a pure state and was not unambiguously characterized because of extreme instability. Our alternative synthetic plan to obtain the product was the preparation of aldehyde **5** since the target compound **4** might possibly be in equilibrium with the ring-opened isomer. Thus, *O*-(3-hydroxypropyl)- and *O*-(2-cyanoethyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidates (**6**, **7**) were prepared and some aldehyde forming reactions from the alcohol **6**[11,12] and from the nitrile **7**[13,14] were attempted under various conditions; but these efforts, as well as acid-catalyzed hydrolysis of *O*-(3,3-diethoxypropyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (**8**), were unsuccessful (see Chart I). Ozonolysis of *O*-(3-butenyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (**9a**) also failed to give the aldehyde **5**. However, careful examination of the ozonolysis of **9a** led us to find that a very cytotoxic product,

Chart I



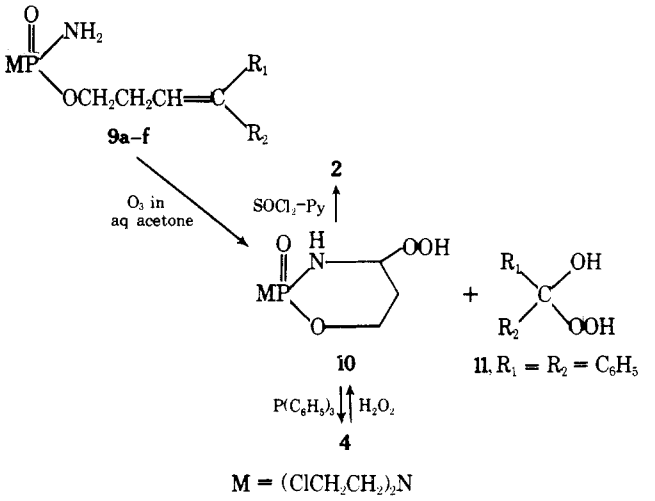
1, $R_1 = R_2 = H$
2, $R_1, R_2 = O = H$
4, $R_1 = OH$; $R_2 = H$

3, R = COOH
5, R = CHO
6, R = CH_2OH
7, R = CN
8, R = $CH(OEt)_2$
9a, R = $CH{=}CH_2$

which is readily convertible into 4-hydroxycyclophosphamide (4), the suggested active form of cyclophosphamide, can be obtained in a crystalline state. Ozonization of **9a** was carried out in aqueous acetone (acetone–H_2O, 2:1) with a slight excess of ozone at 0° and after evaporation of acetone the aqueous residue was extracted with $CHCl_3$ without treating the reaction mixture with reducing agent. From the $CHCl_3$ extract 2-[bis(2-chloroethyl)amino]-4-hydroperoxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (4-hydroperoxycyclophosphamide, **10**), mp 107–108° (with violent decomposition), was obtained in a low yield (10%) after column chromatography on silica gel–EtOAc. The yield of **10** was increased to 50–60% when an excess amount of hydrogen peroxide was added to the ozonized reaction mixture. The structure of 4-hydroperoxycyclophosphamide (**10**) was assigned on the basis of elemental analyses (C, H, N, Cl) and molecular weight determinations which agreed with the formula $C_7H_{15}N_2O_4PCl_2$ corresponding to an adduct of cyclophosphamide plus a molecule of oxygen. Compound **10** was capable of liberating iodine from an aqueous potassium iodide solution and gave a positive color reaction with $TiCl_4$–H_2SO_4 reagent,[15] indicative of the existence of a peroxide linkage. Treatment of **10** with $SOCl_2$–pyridine resulted in formation of 4-ketocyclophosphamide in good yield. These chemical properties are consistent with the assigned structure **10** which was unequivocally confirmed by nmr evidence. The nmr spectrum of **10** in DMSO-d_6 solution exhibited peaks corresponding to one proton as a pair of double doublets at δ 4.71 and 5.11 which were collapsible to a pair of triplets on H–D exchange and thus attributable to the C_4 proton [δ 4.96, J(P,C_4-H) = 24.5 Hz, J(NH,C_4-H) = 5.0 Hz, J(P,NH) = 7.0 Hz]. In addition, the spectrum showed peaks due to two H–D exchangeable protons at δ 5.81 as a double doublet and at δ 11.51 as a sharp singlet, which were obviously assignable to the protons of NH and OOH, respectively. The crystal structure of 4-hydroperoxycyclophosphamide (**10**) prepared by us has recently been determined by Camerman,[16] showing that the C_4-oxygen functional group of **10**, like that of 4-peroxycyclophosphamide,[17] is axially oriented and cis to the oxygen which is bonded to the phosphorus atom. The molecular conformation proposed by Camerman has been also found in solution studies of a number of related 4-functionalized 1,3,2-oxazaphosphorinanes including 4-hydroperoxycyclophosphamide.[18]

Deoxygenation of **10** with triphenylphosphine in CH_2Cl_2 at 0° yielded 2-[bis(2-chloroethyl)amino]-4-hydroxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (4-hydroxycyclophosphamide, **4**) in 40% yield in a pure crystalline state: mp 47.5–48.5°. Compound **4** was found to be somewhat unstable and gradually released acrolein in EtOH at room temperature. The structure of **4** was confirmed by elemental analyses [*Anal.* ($C_7H_{15}N_2O_3PCl_2$) C, H, N, Cl] and by the nmr spectrum (DMSO-d_6 solution) in which the C_4 proton was observed at δ 4.90 as a doublet of multiplets with J(P,C_4-H) = 21 Hz. The structure was further supported by the fact that **4** could be reconverted into **10** by the action of hydrogen peroxide. The synthetic 4-hydroxycyclophosphamide was found to exhibit positive aldehyde reactions with Fuchsin and Fehling reagent suggesting that it may be in equilibrium with the ring-opened form. It has recently been reported that 4-hydroxycyclophosphamide (**4**) is indeed in equilibrium with the ring-opened form (**5**) and that the equilibrium concentration of **4** is larger than that of **5** (**4**/**5** = 1.69) in phosphate buffer solution (pH 7) at 37°.[19] However, we could not obtain any spectral evidences supporting the equilibrium in the ir ($CHCl_3$ solution) and nmr (D_2O solution) spectrum of **4** at room temperature (Scheme I).

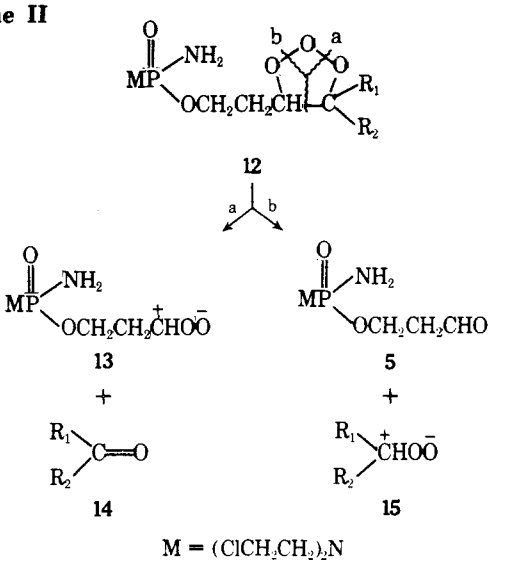
Scheme I



M = $(ClCH_2CH_2)_2N$

The formation of the cyclic hydroperoxide (**10**) from **9a** is a type of reaction analogous to the ozonolysis of olefinic alcohols giving α-hydroperoxy cyclic ethers[20] and is understandable by the Criegee's mechanism of olefin ozonolysis.[21] As shown in the Scheme II, cleavage of the primary ozonide **12** can occur in two directions, a and b, to give the fragments **13** + **14** and **5** + **15**, respectively. The cyclic hydroperoxide **10** is formed by cyclization of the zwitterion fragment **13** (cleavage a), while the aldehyde fragment **5** (cleavage b) presumably undergoes decomposition directly or after cyclization to **4**. The increase of the yield of **10** by addition of hydrogen peroxide to the ozonization mixture

Scheme II



M = $(ClCH_2CH_2)_2N$

Table I. Yield of 4-Hydroperoxycyclophosphamide (10) in the Ozonolysis of 9a–f

Compd	R_1	R_2	Yield of 10, %[a]
9a	H	H	55
9b	H	Me	33
9c	Me	Me	22
9d	H	$CH_2C_6H_5$	22
9e	H	C_6H_5	12
9f	C_6H_5	C_6H_5	16

[a] Shown by per cent of the isolated amount of 10 after addition of H_2O_2.

may be a result of the additional formation of **10** by reaction of H_2O_2 with **5** (or the cyclic isomer **4**). However, addition of *tert*-butyl hydroperoxide was also found to increase the yield of **10** (45%) but no product incorporating *tert*-butyl group was obtained. Consequently, the role of the added hydroperoxide is considered to be merely prevention of dimerization[22] or decomposition of **10.** Next we investigated the effect of a substituent at the terminal olefinic carbon upon the formation of 4-hydroperoxycyclophosphamide (**10**). *O*-(3-Alkenyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidates (**9b–f**) were prepared and ozonized in aqueous acetone at 0°, and **10** was isolated after addition of hydrogen peroxide. The results are summarized in Table I. Table I shows that the simplest olefin **9a** ($R_1 = R_2 = H$) gives the best yield, whereas a methyl or phenyl substituent on the terminal olefinic carbon decreases the formation of **10.** In the case of **9f** ($R_1 = R_2 = C_6H_5$), the zwitterion species formed by cleavage b could be isolated as a hydrate **11** in a considerable yield. These results indicate, at least qualitatively, that the formation of the cyclic hydroperoxide **10** (*i.e.*, cleavage a of the primary ozonide **12**) is unfavored when one of R_1 and R_2 (or both of which) is methyl or phenyl group. This may be attributable to the hyperconjugative and π-conjugative effects of these substituents,[23–25] by which the positive charge on the terminal carbon bearing these groups is perhaps stabilized in the transition state of the cleavage and cleavage b giving the fragments **5** + **15** is therefore more favored than cleavage a.

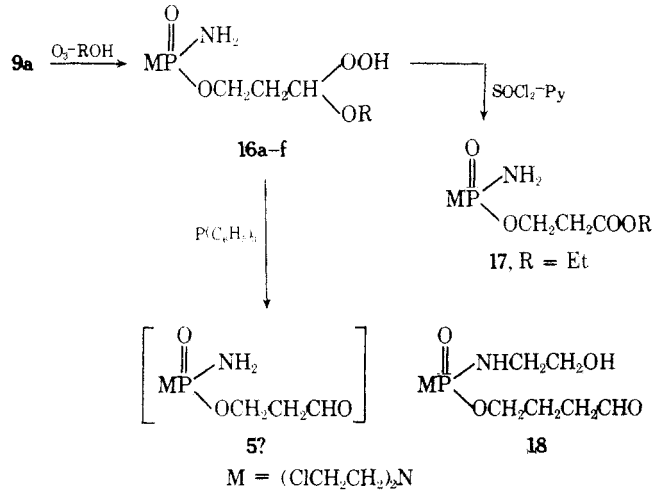
As generally known, the zwitterion fragment produced by cleavage of a primary ozonide can be captured by adduct formation with alcohol.[21] Thus, ozonolysis of **9a** in ethanol yielded *O*-(3-ethoxy-3-hydroperoxy)propyl-*N,N*-bis(2-chloroethyl)phosphorodiamidate (**16a**) in 50% yield. **16a** was obtained as an unstable oil which could be purified by column chromatography on silica gel with acetone elution at room temperature. The structure of **16a** was confirmed by elemental analyses [*Anal.* ($C_9H_{21}N_2O_5PCl_2$) C, H, N; Cl: calcd, 20.90; found, 21.80] and by the fact that dehydration of **16a** with $SOCl_2$–pyridine yielded *O*-(2-ethoxycarbonylethyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (**17**) in good yield. Ozonolysis of **9a** in the presence of other alcohols also yielded corresponding open-chain hemiacetal hydroperoxides **16b–f** (Table II). Deoxygenation of **16a** with triphenylphosphine in CH_2Cl_2 at −20° yielded a colorless mixture which gradually turned out to an yellow, turbid solution on standing for 3 hr at room temperature. By column chromatography with silica gel–acetone at room temperature, the deoxygenation product was found to be completely decomposed. However, the freshly prepared solution of the equimolar mixture of **16a** and triphenylphosphine in $CDCl_3$ exhibited an weak ir band at 1717 cm^{-1} and an nmr signal at δ 9.80 ppm as a triplet with J = 1.2 Hz at room temperature, possibly indicating the formation of an aldehyde species **5,** although an attempt to isolate **5** as its 2,4-dinitrophenylhydrazone resulted in the isolation of acrolein 2,4-dinitrophenylhydrazone. The intensities of the ir peak at 1717 cm^{-1} and nmr signal at δ 9.80 ppm progressively decreased on standing at room temperature, but they were found to be essentially unchanged within 15 min. It is noted that Struck, *et al.*,[26] have reported the synthesis of aldehyde **5,** which was identified as an enzymatically produced, active metabolite of cyclophosphamide and named aldophosphamide.[8,27] The ir and nmr data of the deoxygenation mixture of **16a,** however, are somewhat different from those of aldophosphamide[26] (ir 1705 cm^{-1}; nmr δ 7.6 ppm) but are in agreement with those of a structurally related compound **18** (*N*-3-hydroxypropylhomoaldophosphamide) which has been synthesized as a stable oil and has an ir band at 1722 cm^{-1} and an nmr signal at δ 9.97 ppm as a triplet with J = 1.2 Hz in $CDCl_3$ solution.[28] We have repeatedly examined the methods reported by Struck, *et al.*,[26,29] to obtain aldophosphamide for the purpose of comparison with our product but have not been able to isolate the product corresponding to aldophosphamide and the structure of the deoxygenation product of **16a** could not be confirmed convincingly (Scheme III).

Table II. Open-Chain Hemiacetal Hydroperoxides 16a–g

Compd[a]	R	Formula	Analyses	Yield,[b] %
16a	Et	$C_9H_{21}N_2O_5PCl_2$	C, H, N; Cl[c]	50
16b	Me[d]	$C_8H_{19}N_2O_5PCl_2$		49
16c	*i*-Pr	$C_{10}H_{23}N_2O_5PCl_2$	H, Cl; C,[e] N[f]	24
16d		$C_{13}H_{21}N_2O_5PCl_2$	C, H, N	25
16e		$C_{12}H_{25}N_2O_5PCl_2$	H, N, Cl; C[g]	33
16f		$C_{11}H_{20}N_2O_5PCl_2$	C, H; N[h]	13

[a] All compounds **16a–f** were obtained as oils. [b] Isolated yield of product after column chromatography on silica gel–acetone. [c] Cl: calcd, 20.90; found, 21.80. [d] Could not be purified to the analytical grade. [e] C: calcd, 34.01; found, 33.50. [f] N: calcd, 7.93; found, 7.43. [g] C: calcd, 39.70; found, 40.86. [h] N: calcd, 6.88; found, 5.98.

Scheme III



The fact that the C-4 peroxylated 1,3,2-oxazaphosphorinane ring system can be produced by the ozonolysis of *O*-alkenylphosphorodiamidate is of synthetic interest, since no phosphorus-containing heterocyclic peroxide has previously been synthesized, although a number of heterocyclic α-peroxyamines are known.[30] After the preliminary

Table III. Cyclic Hydroperoxides

Compd	Formula	Analyses	Mp,[a] °C	Yield, %[b]	Chemical shift, δ[c] C_4-H	NH	OOH	Coupling constant, Hz
10	$C_7H_{15}N_2O_4PCl_2$	C, H, N, Cl	107–108 dec	55	4.96 d of dd	5.81 dd	11.51 s	$J(P, C_4\text{-H}) = 24.5$, $J(NH, C_4\text{-H}) = 5.0$, $J(P, NH) = 7.0$
20a	$C_8H_{17}N_2O_4PCl_2$	C, H, N, Cl	99–100 dec	25	4.82 d of t		11.58 s	$J(P, C_4\text{-H}) = 20.6$
20b	$C_{10}H_{21}N_2O_4PCl_2$	C, H, N, Cl	112–114 dec	11	4.92 d of t		11.55 s	$J(P, C_4\text{-H}) = 21.4$
22a	$C_6H_{13}N_2O_4PCl_2$	C, H, N, Cl	135–135.5 dec	33	5.27 d of dd	6.44 dd	11.67 s	$J(P, C_4\text{-H}) = 22.0$, $J(NH, C_4\text{-H}) = 2.9$, $J(P, NH) = 21.0$
22b	$C_7H_{15}N_2O_4PCl_2$	C, H, N, Cl	124–126 dec	28	4.90 d of t	6.31 dd	11.74 s	$J(P, C_4\text{-H}) = 23.2$, $J(NH, C_4\text{-H}) = 3.0$, $J(P, NH) = 19.9$
22c	$C_7H_{15}N_2O_4PCl_2$	C, H; N,[d] Cl[e]	Oil	3		6.35 d	11.40 s	$J(P, NH) = 18.0$

[a]Determined in open capillary and uncorrected. [b]Isolated yield after H_2O_2 addition. [c]Determined in DMSO-d_6 solution using TMS as an internal standard. [d]N: calcd, 9.56; found, 8.99. [e]Cl: calcd, 24.20; found, 23.68.

Table IV. Comparative *in Vitro* Cytotoxicity of Cyclophosphamide (1), Nor Nitrogen Mustard, and Synthetic Active Species of Cyclophosphamide (4, 10) against HeLa Cells

Compd	ED_{50}, μg/ml
Cyclophosphamide (1)	>100
Nor nitrogen mustard	5.8
4-Hydroxycyclophosphamide (4)	1.0
4-Hydroperoxycyclophosphamide (10)	0.6

Table V. Comparative Antitumor Activity of Cyclophosphamide (1) and Synthetic Active Species of Cyclophosphamide (4, 10) against Yoshida Sarcoma in Rats

Compd	Dose,[a] mg/kg iv	No. of rats	Tumor wt,[b] mg	Body wt change, g	Inhibition,[c] %
1	3	5	240 ± 25	+19	86
	12	5	0	+22	100
4	0	5	1260 ± 112	+17	
	1.25	5	860 ± 58	+21	32
	5.0	5	50 ± 22	+20	96
10	0	5	1720 ± 172	+21	
	2.5	5	140 ± 60	+24	92
	10.0	5	0	+23	100

[a]Compound was administered once a day after inoculation of 10^7 cells. [b]The tumor was excised 1 week after administration. [c]Inhibition per cent of tumor weight over control.

communication of this work had been published, synthesis of the C-4 peroxylated cyclophosphamide derivatives by direct oxidation of cyclophosphamide was reported by two groups.[31,32] However, because of the poorer yield of product their method has less synthetic value than ours, which has been regarded as a promising route leading to 4-functionalized 1,3,2-oxazaphosphorinane and a number of related heterocyclic compounds have been synthesized.[33] For example, N-substituted 4-hydroperoxycyclophosphamides (**20a,b**) could be obtained by the ozonolysis of the corresponding olefins **19a,b** (Scheme IV). Five-membered analogs **22a–c** could also be obtained by the ozonolysis of *O*-(2-propenyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidates (21a–c, Table III).

Among the C-4 functionalized cyclophosphamide derivatives and some related compounds obtained so far, both 4-hydroxy- and 4-hydroperoxycyclophosphamide (**4** and **10**) exhibit high antitumor activity. Especially, it is striking that both compounds show much stronger cytotoxicity against HeLa cells *in vitro* than cyclophosphamide (Table IV). Compounds **4** and **10** also show high antitumor activity *in vivo* against Yoshida sarcoma in rats and L1210 leukemia in BDF_1 mice as shown in Tables V and VI, although their acute toxicity was found to be somewhat enhanced (Table VII). These results indicate that cyclophosphamide is indeed activated by C-4 hydroxylation and that the synthetic 4-hydroxycyclophosphamide is replaceable by 4-hydroperoxycyclophosphamide with equal biological activity both *in vivo* and *in vitro*. The biological equivalence of **4** and **10** was further revealed by their metabolic behavior. Thus, administration of **4** and **10** to rabbits resulted in the urinary excretion of 4-ketocyclophosphamide (**2**) and carboxylic acid (**3**), isolated in the essentially same ratio (**2:3** = 1:5) in both cases, as produced from cyclophosphamide.[1] It is notable that the open-chain hemiacetal hydroperoxides (**16a–f**) also exhibit considerable antileukemic activity *in vivo* (Table VI); however, there is a considerable difference in potency, especially at higher dosage, between **10** and the open-chain hemiacetal hydroperoxides. A difference was also observed in the metabolic behavior between the cyclic hydroperoxide **10** and an open chain hydroperoxide **16a** which was shown to be metabolized into carboxylic acid **3**,

Scheme IV

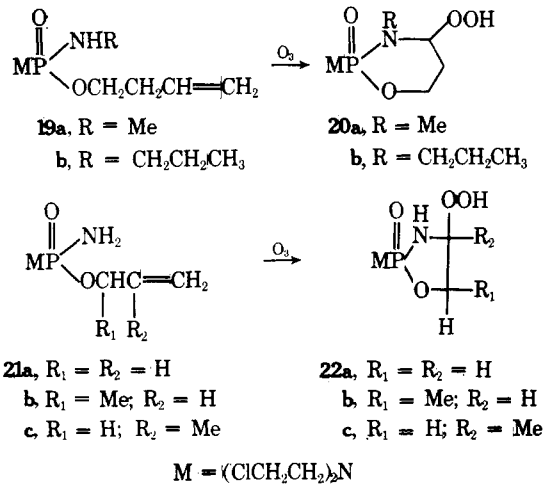


Table VI. Comparative Life-Span Activity of Cyclophosphamide (**1**), Synthetic Active Species of Cyclophosphamide (**4, 10**), and Open-Chain Hemiacetal Hydroperoxides (**16a–f**) against L1210 Leukemia in BDF_1 Mice

Compd	Dose,[a] mg/kg (route)	No. of mice	Survivors Survival days	Survivors over 30 days	ILS, %[b]
1	0 (iv)	10	9.6 ± 0.53	0	
	40 (iv)	9	12.4 ± 0.44	0	29
	80 (iv)	9	>27.9 ± 1.46	7	>191
	160 (iv)	9	>30	9	>213
4	0 (iv)	10	8.2 ± 0.32	0	
	12.5 (iv)	8	10.0 ± 0.88	0	20
	25 (iv)	8	10.4 ± 1.71	0	24
	50 (iv)	8	>16.9 ± 2.92	2	>106
	100(iv)	7	>27.9 ± 2.14	6	>240
10	0 (iv)	10	9.6 ± 0.53	0	
	18 (iv)	9	10.1 ± 0.20	0	5
	36 (iv)	9	16.0 ± 1.55	0	67
	72 (iv)	9	>30	9	>213
10	0 (ip)	10	8.9 ± 0.31	0	
	20 (ip)	9	>20.9 ± 2.89	4	135
	40 (ip)	9	>27.9 ± 1.46	7	>213
	60 (ip)	9	>27.6 ± 2.44	8	>210
16a	0 (ip)	10	7.1 ± 0.10	0	
	20 (ip)	10	11.3 ± 0.73	0	59
	60 (ip)	10	16.2 ± 2.63	2	128
16b	0 (ip)	10	8.3 ± 0.69	0	
	20 (ip)	10	13.4 ± 1.46	0	61
	60 (ip)	10	18.9 ± 2.90	3	128
16c	0 (ip)	10	8.1 ± 0.35	0	
	20 (ip)	10	12.0 ± 0.26	0	48
16d	0 (ip)	10	8.3 ± 0.69	0	
	20 (ip)	10	10.4 ± 0.35	0	25
	60 (ip)	10	10.9 ± 0.53	0	31
16e	0 (ip)	10	8.3 ± 0.69	0	
	20 (ip)	10	10.9 ± 0.82	0	31
	60 (ip)	10	8.1 ± 0.90	0	−2
16f	0 (ip)	10	8.3 ± 0.69	0	
	20 (ip)	10	9.4 ± 0.75	0	13
	60 (ip)	10	10.2 ± 0.55	0	23

[a]Compound was administered once a day after inoculation of 5×10^5 cells. [b]Per cent increase of life span over control.

possibly *via* the aldehyde species, without formation of 4-ketocyclophosphamide. The observed differences in antileukemic activity and in metabolic behavior between the two classes of hydroperoxides thus suggest that they are not regarded as the biologically equivalent species. It has recently been reported that cyclophosphamide can be oxidized both chemically and enzymatically to give two cytotoxic fragments acrolein and *N,N*-bis(2-chloroethyl)phosphorodiamidic acid (phosphoramide mustard), both of which have been suggested to be responsible for the activity exerted by cyclophosphamide *in vivo*.[34–36] Thus, there is a possibility that the antileukemic activity of both **10** and **16a–f** is also exerted by these fragments since they perhaps undergo ready decomposition after biological reduction, as exemplified chemically. Although, at present state, it is difficult to evaluate the actual role of these fragments in the *in vivo* action of **10** and **16a–f**, the apparent differences in activity between them suggest the possibilities that they exert cytotoxicity prior to decomposition or that there is a difference in life time during transpor to cancer cells or in cell membrane permeability between **10** and **16a–f** (or perhaps between **4** and **5**) if the intracellularly released acrolein and phosphoramide mustard act to produce antitumor effect of these compounds.

Table VII. Comparative Acute Toxicity of Cyclophosphamide (**1**) and Synthetic Active Species of Cyclophosphamide (**4, 10**)

Compd	Animal	LD_{50}, mg/kg (route)
1	Rat	160 (iv), 150 (ip)
	Mouse	380 (iv), 400 (ip)
4	Rat	139 (iv)
10	Rat	115 (iv), 131 (ip)
	Mouse	235 (iv), 181 (ip)

Table VIII. *O*-Alkenyl-*N,N*-bis(2-chloroethyl)-phosphorodiamidates

Compd	Formula	Analyses	Yield, %[a]
9a	$C_8H_{17}N_2O_2PCl_2$	C, H, N, Cl	73
9b	$C_9H_{19}N_2O_2PCl_2$	C, N: H[c]	32
9c	$C_{10}H_{21}N_2O_2PCl_2$	C, H, N, Cl	68
9d[b]	$C_{15}H_{23}N_2O_2PCl_2$		39
9e[b]	$C_{14}H_{21}N_2O_2PCl_2$		29
9f	$C_{20}H_{25}N_2O_2PCl_2$	C, H, N, Cl	61
19a[b]	$C_9H_{19}N_2O_2PCl_2$		40
19b	$C_{11}H_{23}N_2O_2PCl_2$	H, N; C,[d] Cl[e]	55
21a	$C_7H_{15}N_2O_2PCl_2$	C, H, N, Cl	21
21b	$C_8H_{17}N_2O_2PCl_2$	C, H, N, Cl	18
21c	$C_8H_{17}N_2O_2PCl_2$	C, H, N, Cl	23

[a]Yield of the isolated product after column chromatography. [b]Could not be purified to analytical grade; used for the ozonolysis without further purification. [c]H: calcd, 6.61; found, 7.09. [d]C: calcd, 41.65; found, 40.97. [e]Cl: calcd, 22.35; found, 21.89.

Experimental Section

Melting points were determined in open glass capillary tubes using a Yamato MP-I apparatus and were uncorrected. Ir data were determined with a JASCO IRA-1 spectrometer in Nujol mull or in film. Nmr data were determined with a Varian Model A-60 spectrometer with tetramethylsilane as an internal standard. Column chromatography was carried out using silica gel (Davision Chemical Co., grade 950). Where analyses are indicated only by symbols of the elements, analytical results obtained for these elements were within ±0.4% of the theoretical values. *O*-(4-Substituted 3-butenyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidates (**9b–f**), *O*-(3-butenyl)-*N,N*-bis(2-chloroethyl)-*N'*-substituted phosphorodiamidates (**19a,b**), and *O*-[2-propenyl- and 1- (or 2-) substituted 2-propenyl]-*N,N*-bis(2-chloroethyl)phosphorodiamidates (**21a–c**), which gave satisfactory ir and nmr data, were prepared in a manner similar to that used for the preparation of **9a** in the yields shown in Table VIII. Olefinic alcohols used for the preparation of these intermediates were obtained by $LiAlH_4$ reduction of the corresponding olefinic acids according to the usual procedure.

***O*-(3-Hydroxypropyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (6).** To a stirred solution of $POCl_3$ (69.5 g, 450 mmol) in CH_2Cl_2 (50 ml) was added dropwise a solution of monobenzyltrimethylene glycol (14 g, 85 mmol) in CH_2Cl_2 (50 ml) at −12 to −10°. After stirring at −10 to −8° for 4 hr, the reaction mixture was concentrated under reduced pressure below 30° to remove CH_2Cl_2 and remaining $POCl_3$. The residual oil was dissolved again in CH_2Cl_2 (100 ml) and bis(2-chloroethyl)amine hydrochloride (8.0 g, 45 mmol) was added to the solution; the Et_3N (20 g, 200 mmol) was added dropwise with stirring at −10 to −8°. After stirring for 2 hr at the same temperature, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure to give a colorless oil which was submitted to column chromatography, eluting with Et_2O. The Et_2O eluate was concentrated and the residue was dissolved in CH_2Cl_2 (100 ml); then the solution was saturated with NH_3 with stirring in an ice–water bath. After stirring for 1 hr at

room temperature, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure and the residue was chromatographed with EtOAc to afford *O*-(3-benzyloxypropyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate as a colorless oil [*Anal.* ($C_{14}H_{23}N_2O_3PCl_2$) C, H, N, Cl; nmr ($CDCl_3$) δ 4.50 ($OCH_2C_6H_5$)] which was then submitted to catalytic reduction with 10% Pd/C (1.5 g) in MeOH (50 ml) at atmospheric pressure and room temperature. The reaction mixture was filtered and concentrated to give an oily residue which was purified by column chromatography, eluting with Me_2CO to give **6** as a colorless oil (3.5 g, 15% from $POCl_3$), which crystallized on standing at 0°: mp 40–41°. *Anal.* ($C_7H_{17}N_2O_4PCl_2$) C, H; N: calcd, 10.04; found, 9.52.

***O*-(2-Cyanoethyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (7).** $POCl_3$ (120 g, 785 mmol), ethylene cyanohydrin (10 g, 140 mmol), bis(2-chloroethyl)amine hydrochloride (15.1 g, 85 mmol), and excess amount of NH_3 were allowed to react according to the same procedure cited above. The final reaction mixture saturated with NH_3 was filtered and the filtrate was washed with water, dried over anhydrous Na_2SO_4; and concentrated to give a crude crystalline product which was recrystallized from *n*-hexane–Et_2O to afford **7** (3.5 g, 8%) as colorless prisms: mp 99–100°; ir ν_{max} (Nujol) 2300 cm^{-1} (CN). *Anal.* ($C_7H_{14}N_3O_2PCl_2$) C, H, N, Cl.

***O*-(3,3-Diethoxypropyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (8).** To a solution of the freshly prepared 3-hydroxypropanal[37] (14.8 g, 200 mmol) in absolute EtOH (45 ml) was added ethyl orthoformate (29.6 g, 200 mmol) and a small amount of ammonium nitrate (300 mg); then the mixture was refluxed for 40 min at 80–90°. After evaporation of EtOH under reduced pressure, the resulting residue was distilled to give 3,3-diethoxypropan-1-ol [bp 44–44.5° (2 mm)] as a colorless oil (14 g, 50%). A solution of 3,3-diethoxypropan-1-ol (740 mg, 5 mmol) in CH_2Cl_2 (10 ml) was added dropwise to a stirred mixture of bis(2-chloroethyl)phosphoramidic dichloride[38] (1.15 g, 4.5 mmol) and Et_3N (500 mg, 5 mmol) in CH_2Cl_2 (20 ml) at room temperature, and the mixture was refluxed in an oil bath (50–60°) for 10 hr. After standing overnight at room temperature the reaction mixture was washed with water, dried over anhydrous Na_2SO_4, and concentrated to give an oily residue which was dissolved in Et_2O (50 ml). The Et_2O solution was then saturated with NH_3 while cooling in an ice–water bath and allowed to stand overnight at room temperature. The mixture was filtered and concentrated to give a colorless, oily residue which was submitted to column chromatography, eluting with Me_2CO to afford **8** as a colorless oil (265 mg, 15.1%): nmr ($CDCl_3$) δ 1.22 (t, 6 H, $2CH_2CH_3$), 1.97 (q, 2 H, $-CH_2CH-$), 2.87 (br, 2 H, NH_2), 3.3–3.9 (m, 12 H, $6CH_2$), 4.08 (q, 2 H, $POCH_2$), 4.65 (t, 1 H, $-CH-$); ir ν_{max} (film) 3280 (NH_2), 1225 (PO), 1050 cm^{-1} (POC). *Anal.* ($C_{11}H_{25}N_2O_4PCl_2$) C, H, N, Cl.

***O*-(3-Butenyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (9a).** To a stirred solution of $POCl_3$ (15.3 g, 100 mmol) in CH_2Cl_2 (50 ml) was added dropwise a solution of 3-buten-1-ol (7.2 g, 100 mmol) in CH_2Cl_2 (20 ml) at $-10 \pm 2°$, and the mixture was stirred for 4 hr at the same temperature. Bis(2-chloroethyl)amine hydrochloride (17.8 g, 100 mmol) and CH_2Cl_2 (700 ml) were added to the mixture, then Et_3N (31 g, 300 mmol) was added dropwise with stirring at −5 to −10°, and after stirring for 3 hr the mixture was filtered. Excess ammonia was passed into the filtrate while cooling in an ice–water bath, and the mixture was stirred at room temperature for 2 hr. After standing overnight at room temperature the reaction mixture was filtered and the filtrate was concentrated to give an oily residue which was subjected to column chromatography. The column was first eluted with Et_2O to remove unreacted 3-buten-1-ol and other unidentified impurities; then it was eluted with Me_2CO to give pure **9a** as a colorless oil (20 g, 73%) which crystallized on standing at 0° (mp 20°). *Anal.* ($C_8H_{17}N_2O_2PCl_2$) C, H, N, Cl.

2-[Bis(2-chloroethyl)amino]-4-hydroperoxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (4-Hydroperoxycyclophosphamide, 10). Method a. To a solution of **9a** (2.73 g, 10 mmol) in aqueous Me_2CO (Me_2CO–H_2O, 2:1) (30 ml) O_3 was passed at a rate of *ca.* 48 mg/min for 15 min (total amount of O_3, 720 mg; 15 mmol) with stirring in an ice–water bath. After standing overnight at 0°, Me_2CO was removed from the ozonized solution by evaporation under reduced pressure and the resulting pale yellow aqueous residue was extracted with $CHCl_3$. The $CHCl_3$ extract was washed with water, dried over anhydrous Na_2SO_4, and concentrated to give a pale yellow oil which was subjected to column chromatography, eluting with EtOAc. The eluted fractions were monitored by thin-layer chromatography and the pure fractions were collected and concentrated under reduced pressure to give an oily residue which was crystallized by adding an Et_2O–Me_2CO mixture. The crystals were collected by suction and recrystallized from Me_2CO to give pure **10** (290 mg, 10%) as colorless prisms: mp 107–108° dec; mol wt calcd 293.09, found 287 (vapor pressure osmometry in $CHCl_3$ solution); ir ν_{max} (Nujol) 3310, 3080, 1237, 1210, 1035, 926, 840 cm^{-1}; nmr (DMSO-d_6) δ 1.92 (m, 2 H, C_5–H_2), 3.01–4.02 (m, 8 H, $4CH_2$), 4.21 (m, 2 H, C_6–H_2), 4.96 (d of dd, 1 H, C_4–H), 5.81 (dd, 1 H, NH), 11.51 (s, OOH). *Anal.* ($C_7H_{15}N_2O_4PCl_2$) C, H, N, Cl.

Method b. To a solution of **9a** (2.73 g, 10 mmol) in aqueous Me_2CO (Me_2CO–H_2O, 2:1) (30 ml) O_3 (720 mg, 15 mmol, 48 mg/min for 15 min) was passed with stirring in an ice–water bath; then 30% H_2O_2 (3 ml) was added to the ozonized solution and the mixture was allowed to stand overnight at room temperature. In this case the coloring of the reaction mixture was not observed. After evaporation of Me_2CO under reduced pressure the colorless aqueous residue was extracted with $CHCl_3$ from which an essentially pure colorless oil was obtained after washing with water, drying over anhydrous Na_2SO_4, and evaporation under reduced pressure. The oil crystallized by addition of small amount of Me_2CO without purification by column chromatography and the crystals were collected by suction to give **10** (1.6 g, 55%).

Method c. 9a (2.73 g, 10 mmol) was azonized by O_3 (720 mg, 15 mmol, 48 mg/min for 15 min) in Me_2CO (Me_2CO–H_2O, 2:1) (30 ml), and after addition of *tert*-butyl hydroperoxide (3 ml) to the ozonized solution the reaction mixture was allowed to stand overnight at room temperature. The reaction mixture was treated according to the same procedure described above to afford crystalline **10** (1.31 g, 45%) without column chromatography.

Ozonolyses of *O*-(4-Substituted 3-butenyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidates (9b–f), *O*-(3-Butenyl)-*N,N*-bis(2-chloroethyl)-N′-substituted Phosphorodiamidates (19a,b), and *O*-[2-Propenyl- and 1- (or 2-) substituted 2-propenyl]-*N,N*-bis(2-chloroethyl)phosphorodiamidates (21a–c). General Procedure. An *O*-alkenylphosphorodiamidate (10 mmol) was ozonized with 15 mmol (720 mg) of O_3 at a rate of *ca.* 50 mg/min in 30 ml of aqueous Me_2CO (Me_2CO–H_2O, 2:1), and after addition of 30% H_2O_2 (3 ml) the reaction mixture was allowed to stand overnight at room temperature. Then the product (cyclic hydroperoxide) was isolated according to the same procedure described above (method a). Occasionally some cyclic hydroperoxides could be obtained in a crystalline state without column chromatography.

2-[Bis(2-chloroethyl)amino]-4-hydroxytetrahydro-2*H*-1,3,2-oxazaphosphorine 2-Oxide (4-Hydroxycyclophosphamide, 4). To a suspension of 4-hydroperoxycyclophosphamide (**10**, 1.2 g, 4 mmol) in CH_2Cl_2 (12 ml), a solution of triphenylphosphine (1.4 g, 5.3 mmol) in CH_2Cl_2 (6 ml) was added dropwise for 5 min with stirring at −50°; then the cooling bath was set aside and the reaction mixture was stirred for 30 min. The mixture was extracted three times with 10 ml of aqueous 1% NaCl solution and the combined aqueous extract was filtered to remove the insoluble unidentified materials. The aqueous filtrate was re-extracted two times with 100 ml of CH_2Cl_2, and the combined CH_2Cl_2 extract was dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to leave a residue in which a small volume (*ca.* 0.5 ml) of CH_2Cl_2 still remained. Et_2O (2–3 ml) was added to the residue, and after standing at 0° for 1 hr the precipitated crystals were collected by suction to give **4** (0.45 g, 40%). Precipitation of the product occasionally failed when the CH_2Cl_2 extract was completely concentrated. In such a case, a small portion of *n*-hexane was added to the Et_2O solution of the product and the mixture was allowed to stand overnight at −20°. Recrystallization of **4** from the CH_2Cl_2–Et_2O mixture afforded the analytically pure colorless needles: mp 47.5–48.5°; ν_{max} (Nujol) 3240, 3180, 1240, 1215, 1195, 1053, 980 cm^{-1}; nmr (DMSO-d_6) δ 1.80 (m, 2 H C_5–H_2), 3.00–3.87 (m, 8 H, $4CH_2$), 4.20 (m, 2 H, C_6–H_2), 4.90 (d of m, 1 H, C_4–H), 5.19 (m, 2 H, NH, OH). *Anal.* ($C_7H_{15}N_2O_3PCl_2$) C, H, N, Cl.

Dehydration of 4-Hydroperoxycyclophosphamide (10) with $SOCl_2$–Pyridine. To a stirred solution of **10** (291 mg, 1 mmol) in a mixture of CH_2Cl_2 (20 ml) and pyridine (2 ml) was added dropwise a solution of $SOCl_2$ (0.5 ml) in CH_2Cl_2 (5 ml) at −50 to −30°, and after stirring at −30 to −20° for 3 hr the reaction mixture was concentrated under reduced pressure to give a brown residue which was dissolved in CH_2Cl_2 (50 ml) and washed with water. The CH_2Cl_2 layer was dried over anhydrous Na_2SO_4 and concentrated to give a crystalline residue which was washed with Me_2CO–Et_2O to afford 4-ketocyclophosphamide[1] (**2**, 232 mg, 85%).

Formation of 4-Hydroperoxycyclophosphamide (10) from 4-Hydroxycyclophosphamide (4) by the Action of H_2O_2. To an aqueous solution (1 ml) of 4-hydroxycyclophosphamide, which was

generated by reacting 4-hydroperoxycyclophosphamide (**10**, 100 mg, 0.34 mmol) with triphenylphosphine (107 mg, 0.4 mmol) according to the procedure cited above, 30% H_2O_2 (0.5 ml) was added and the mixture was allowed to stand overnight at room temperature. The reaction mixture was extracted with $CHCl_3$ and the extract was dried over anhydrous Na_2SO_4 and concentrated to give a crystalline residue which was washed with Me_2CO to afford **10** (43 mg, 40%).

Open-Chain Hemiacetal Hydroperoxides (16a–f) by the Ozonolyses of 9a. General Procedure. 9a (10 mmol) was dissolved in 20 ml of an alcohol (ROH) and the solution was ozonized by 15 mmol (720 mg) of O_3 at a rate of *ca.* 50 mg/min. After the calculated amount of O_3 has been introduced, the alcohol (ROH) was removed by evaporation under reduced pressure below 30°, and the resulting oily residue was submitted to column chromatography. The column was first eluted with Et_2O to remove unidentified impurities; then it was eluted with Me_2CO. The fractions eluted with Me_2CO were monitored by thin-layer chromatography and pure fractions were collected and concentrated under reduced pressure below 30° to give the hemiacetal hydroperoxides in the yields shown in Table II. All products (**16a–f**) were obtained as colorless oils which gave satisfactory nmr data.

Dehydration of *O*-(3-Ethoxy-3-hydroperoxypropyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (16a) with $SOCl_2$–Pyridine. To a stirred solution of **16a** (337 mg, 1 mmol) in a mixture of CH_2Cl_2 (20 ml) and pyridine (1 ml) was added dropwise a solution of $SOCl_2$ (0.3 ml) in CH_2Cl_2 (5 ml) at −40 to −30°, and the mixture was stirred at the same temperature for 3 hr. After additional stirring for 30 min at room temperature, the reaction mixture was washed four times with 5 ml of water and the CH_2Cl_2 layer was submitted to column chromatography, eluting with Me_2CO. The pure fractions monitored by thin-layer chromatography were combined and concentrated under reduced pressure yielding *O*-(2-ethoxycarbonylethyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate as an essentially pure colorless oil (175 mg, 55%) which was identical with an authentic specimen[1] by ir comparisons.

Deoxygenation of *O*-(3-Ethoxy-3-hydroperoxypropyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (16a) with Triphenylphosphine. To a stirred solution of **16a** (337 mg, 1 mmol) in CH_2Cl_2 (20 ml) was added dropwise a solution of triphenylphosphine (262 mg, 1 mmol) in CH_2Cl_2 (5 ml) at −20 to −10°, and after stirring for 30 min at the same temperature the reaction mixture was concentrated under reduced pressure below 25° to give a pale yellow oily residue which contained a small amount of crystalline triphenylphosphine oxide. The ir ($CDCl_3$ solution) spectrum of the freshly prepared crude oily product had a broad band at 1717 cm^{-1} at room temperature, and it had a nmr ($CDCl_3$) signal at δ 9.8 (t, J = 1.2 Hz) corresponding to –CHO group at room temperature. A solution of the crude product in CH_2Cl_2 (20 ml) gave an yellow, turbid solution on standing for 3 hr at room temperature. Brown resinous material, water-soluble but unidentified, gradually precipitated. The precipitate was removed by decantation; then the CH_2Cl_2 mixture was filtered to give a pale yellow, clear solution. To the solution was added 5 ml of 1% 2,4-dinitrophenylhydrazine solution in 99% EtOH and the mixture was allowed to stand at room temperature for 3 hr. After concentration of the reaction mixture under reduced pressure the crystalline residue was washed with cold MeOH and recrystallized from MeOH to give *ca.* 20 mg of yellow prisms of the 2,4-dinitrophenylhydrazone of acrolein (mp 165°) which was identified by a mixture melting point determination with an authentic specimen.

Isolation of Urinary Metabolites. Metabolites of 4-Hydroperoxycyclophosphamide (10). To 13 rabbits (male, mean body weight 1.4 kg) was administered subcutaneously 4-hydroperoxycyclophosphamide (**10**) with doses of *ca.* 70 mg/kg (total amount of **10**, 1.0 g), and their urine was collected after 24 hr. The collected urine (total volume of *ca.* 1000 ml) was treated according to the same procedure used for the isolation of cyclophosphamide metabolites[1] to give 4-ketocyclophosphamide (30 mg) as crude crystals which were shown to be identical with an authentic specimen[1] by ir comparison. The final carboxylic acid portion fractionated by the procedure could not be obtained in crystalline state and was found to contain a considerable amount of unidentified materials which gave a positive NBP [4-(*p*-nitrobenzyl)pyridine] test after thin-layer chromatography [R_f <0.1, acetone, silica gel (60 F_{254} precoated plate, 0.2 mm, Merck)]. The oily mixture was dissolved in EtOH (5 ml), and it was added dropwise to a stirred solution of $SOCl_2$ (1 ml) in EtOH (10 ml) at −50°. After stirring for 1 hr at the same temperature, the cooling bath was removed and the reaction mixture was stirred further for 1 hr. The reaction mixture was concentrated under reduced pressure to give an oily residue which was dissolved in 20 ml of $CHCl_3$ and washed with 5% $NaHCO_3$ aqueous solution (20 ml). The $CHCl_3$ layer, after drying over Na_2SO_4, was concentrated under reduced pressure to give an oily residue (250 mg) which was subjected to column chromatography, eluting with EtOAc. The eluted fractions were monitored by thin-layer chromatography and the pure fractions were collected and concentrated under reduced pressure to give *O*-(2-ethoxycarbonylethyl)-*N,N*-bis(2-chloroethyl)phosphorodiamidate (**17**, 150 mg) which was shown to be identical with an authentic specimen[1] by ir comparison.

Metabolites of 4-Hydroxycyclophosphamide (4). To seven rabbits (male, mean body weight 1.5 kg) was administered subcutaneously 4-hydroxycyclophosphamide (**4**) with doses of *ca.* 60 mg/kg (total amount of **4**, 600 mg), and their urine was collected after 24 hr. The collected urine (total volume of *ca.* 700 ml), after the same treatment carried out on **10**, afforded 4-ketocyclophosphamide (10 mg) and the carboxylic acid **3** which was also identified by esterification to **17** (48 mg).

Metabolite of *O*-(3-Ethoxy-3-hydroperoxypropyl)-*N,N*-bis-(2-chloroethyl)phosphorodiamidate (16a). To two rabbits (male, mean body weight 1.5 kg) was administered subcutaneously **16a** with doses of *ca.* 70 mg/kg (total amount of **16a**, 200 mg), and their urine was collected (total volume of *ca.* 200 ml). Thin-layer chromatography (silica gel, Me_2CO) of the native urine showed no spot corresponding to 4-ketocyclophosphamide (R_f 0.55) and it was revealed that the major metabolite was the carboxylic acid **3** (R_f <0.1). After the same purification procedure carried out for **10**, it was found in fact that 4-ketocyclophosphamide could not be isolated from the corresponding fraction, and the carboxylic acid **3** was obtained as the ester **17** (50 mg) by esterification with $SOCl_2$–EtOH.

Procedure for the Bioassay of *in Vitro* Cytotoxicity against HeLa Cells. HeLa cells were obtained from Dr. N. Ishida, Tohoku University (Japan). The cell cultures were maintained in Eagle's minimum essential medium (MEM) containing 10% bovine serum. For subcultivation, the suspension of cells was prepared by trypsinization of the stock cultures. Then the cells were resuspended in a fresh medium, diluted to the concentration of 1×10^5 cells/ml, and transferred to small test tubes. Three cultures thus prepared were employed as a set for each experiment.

Samples were dissolved in absolute methanol at a concentration of 4 mg/ml and subjected to serial twofold dilutions with the culture medium.

The sample was added to the cell cultures on the second day of cultivation. After 2 days of incubation with the sample, the cell population was measured by means of an electronic device, the TOA micro-cell counter (TOA Electronics, Kobe, Japan). The growth rate (%) was calculated from the following formula

$$\text{growth } \% = \frac{T - C_0}{C - C_0} \times 100$$

where C = final cell number in controls, T = final cell number in treated tube, and C_0 = cell number in the tube at the time of sample addition.

The effective dose for 50% growth inhibition (ED_{50}) was determined by plotting the logarithmic curve of the drug concentration against the growth rate.

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Anticonvulsants. 5. Derivatives of 5-Ethyl-5-phenylhydantoin and 5,5-Diphenylhydantoin

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Alkoxymethyl, acyloxymethyl, and mixed alkylalkoxymethyl or alkylacyloxymethyl derivatives of 5-ethyl-5-phenylhydantoin exhibit anticonvulsant activity. Also effective are bis(alkoxymethyl) and mixed alkylalkoxymethyl derivatives of 5,5-diphenylhydantoin. Of particular interest are 1,3-bis(methoxymethyl)-5,5-diphenylhydantoin and 3-acetoxymethyl-5-ethyl-5-phenylhydantoin, which show good activity against maximal electroshock seizures, and 3-methoxymethyl-5-ethyl-5-phenylhydantoin, which is effective against both maximal electroshock and pentylenetetrazole. None of the above compounds show greater activity against maximal electroshock seizures than the parent compounds, however.

We reported earlier[1] that both 3-acetoxymethyl- and 1,3-bis(acetoxymethyl)-5,5-diphenylhydantoin showed good activity against maximal electroshock seizures. We also reported[2] that 3-alkoxymethyl derivatives of diphenylhydantoin possess activity against maximal electroshock seizures but, unlike diphenylhydantoin,[3] were effective against chemoshock as well. We were therefore interested in investigating the activities of the corresponding derivatives of 5-ethyl-5-phenylhydantoin, itself an effective compound against both electroshock and electroshock seizures.[3] It was also of interest to determine the effects induced by introduction of an additional alkoxymethyl group at the remaining nitrogen atom of the diphenylhydantoin ring and by mixed alkylation–alkoxymethylation of the ring nitrogen atoms of both 5,5-diphenyl- and 5-ethyl-5-phenylhydantoin.

The synthetic methods used for the preparation of compounds are outlined in Scheme I.

Chemistry. The synthesis of 1,3-bis(alkoxymethyl)-5,5-diphenylhydantoins (**2** and **3**) was accomplished from 5,5-diphenylhydantoin (**1**) with the appropriate chloromethyl alkyl ether in the presence of two equivalents of base. Mixed alkylalkoxymethyl derivatives (**5** and **6**) of 5,5-diphenylhydantoin were obtained by alkylation of 3-benzyl-5,5-diphenylhydantoin[4] (**4**) with chloromethyl alkyl ethers in the presence of base. From the base-catalyzed reaction of 5-ethyl-5-phenylhydantoin (**7**) with alkyl chloromethyl ethers, 3-alkoxymethyl-5-ethyl-5-phenylhydantoins (**8–10**) were obtained. Treatment of compound **7** with excess CH_2O in the presence of base, followed by Ac_2O and pyridine, provided 1,3-bis(acetoxymethyl)-5-ethyl-5-phenylhydantoin (**12**). 3-Methoxymethyl-5-ethyl-5-phenylhy-