Chemical and Biological Evaluation of Hydrolysis Products of Cyclophosphamide

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^{31}P NMR spectroscopy was used to study the products of the decomposition of cyclophosphamide (**1**) in buffered solutions at pH's ranging between 1.2 and 8.6 at 20 °C and at pH 7.4 at 37 °C. At pH 1.2, **1** undergoes a rapid breakdown ($t_{1/2}$ = 1.4 days) of the two P–N bonds, giving compounds **2** [$HN(CH_2CH_2Cl)_2$] and **3** [$H_2N(CH_2)_3OP(O)(OH)_2$] as hydrochlorides. No intermediates were detected. At pH's between 5.4 and 8.6, hydrolysis of **1** during 17 days leads to the sole and previously unknown nine-membered ring compound **13**. **13** results from the intramolecular alkylation of **1** giving the bicyclic compound **7** followed by the exothermal hydrolytic breakdown of the P–N bond of its six-membered ring. At pH 2.2 and 3.4, the two hydrolytic pathways coexist since, beside compounds **2** and **3**, the hydrochloride of compound **9** [$Cl(CH_2)_2NH(CH_2)_2NH(CH_2)_3OP(O)(OH)_2$] is formed, resulting from the acid-catalyzed breakdown of the P–N bond in the nine-membered ring compound **13**. At pH 2.2, the presence of chloride ion affected neither the stability of **1** nor the contribution of the two competing hydrolytic pathways. At pH's ranging from 3.4 to 8.6, there is little degradation of **1** since more than 95% of initial **1** was still present after 7 days at 20 °C. Under physiological conditions (pH 7.4, 37 °C) after 6 days, 45% of **1** is hydrolyzed ($t_{1/2}$ = 6.6 days), leading essentially (30% of initial **1**) to the nine-membered ring compound **13**. The rate of hydrolysis of **13** and the nature of its hydrolysis products were found to depend on pH over the range 0–8.6. After a single ip injection to mice, compounds **3**, **9**, and **13** were less toxic than **1**. They did not exhibit any direct cytotoxic efficacy on the colony-forming capacity of L1210 cells *in vitro*, and they had no antitumor activity *in vivo* against P388 leukemia.

Introduction

Cyclophosphamide (**1**) was introduced in tumor therapy in 1958 and is until now the most important clinically used alkylating agent.[1] **1** is not active itself. Therefore, **1** is named a "transport form" which requires an enzymatic activation in the liver to 4-hydroxycyclophosphamide. This first metabolite releases via aldophosphamide the "active form", phosphoramide mustard, which is the ultimate alkylating metabolite causing DNA destruction in tumor cells.

The hydrolytic cleavage of **1** was first studied by Arnold and Klose.[2,3] In a buffered system at pH 8.6 and 70 °C, the P–N bonds of **1** were cleaved producing **2** and **3** (Scheme **1**, A).[2] Under milder conditions (several days at 37.5 °C, pH 5.0 or 6.85), **1** was shown to undergo a slow breakdown with cleavage of the exocyclic P–N bond leading to **4** and **5**, which further degraded into **3** (Scheme 1, B).[3] It was then demonstrated that treatment of an aqueous solution of **1** with ethereal HCl led to compound **6** resulting from the cleavage of the endocyclic P–N bond of **1** (Scheme **1**, C).[4] More recently, various hydrolytic products were isolated and purified after reflux of **1** in unbuffered aqueous solutions for 0.5–72 h. The hydrolytic pathway referred to as the "Friedman mechanism" was thus proposed (Scheme **1**, D).[5,6] An initial intramolecular alkylation of **1** took place leading to **7** and was followed by subsequent hydrolysis of P–N and then P–O bonds giving compounds **9**–**11**. However, attempts to isolate the postulated bicyclic intermediate **7** had been unsuccessful. This hydrolytic pathway was then confirmed with high-resolution NMR spectroscopy by Zon et al.[7] who synthesized **7** from **1** under anhydrous conditions and demonstrated that, in the presence of **1** equiv of DCl, **7** was hydrolyzed into **9** (Scheme **1**, E). Pankiewicz et al.[8] showed that **7** dissolved in water gave the intermediate **12** which, in the presence of 1 N HCl, further hydrolyzed to **9** (Scheme **1**, F). It was also reported that maintenance of **1** under strongly acidic conditions (pH 0.25) at ambient temperature for 24 h released **2** and **3** as hydrochlorides (Scheme **1**, G).[7]

In an attempt to clarify the hydrolytic pathways of **1**, we investigated the time course of its hydrolysis in buffered solutions at various pH's by ^{31}P NMR. The degradation compounds were isolated and characterized by NMR and mass spectrometry. They were also independently synthesized to confirm unambiguously the assigned structures. Their antitumor activity and toxicity were also evaluated.

Results

Identification of Cyclophosphamide Hydrolysis Products. Hydrolysis of **1** at 20 °C and pH 1.2 led to one phosphorated compound with a ^{31}P NMR chemical shift of 0.78 ppm (from 85% H_3PO_4 as external reference) (Figure 1A). The mass spectrometry data and the ^{13}C NMR characteristics of the hydrolysis mixture at day 7 showed that the two compounds **2** and **3** (as

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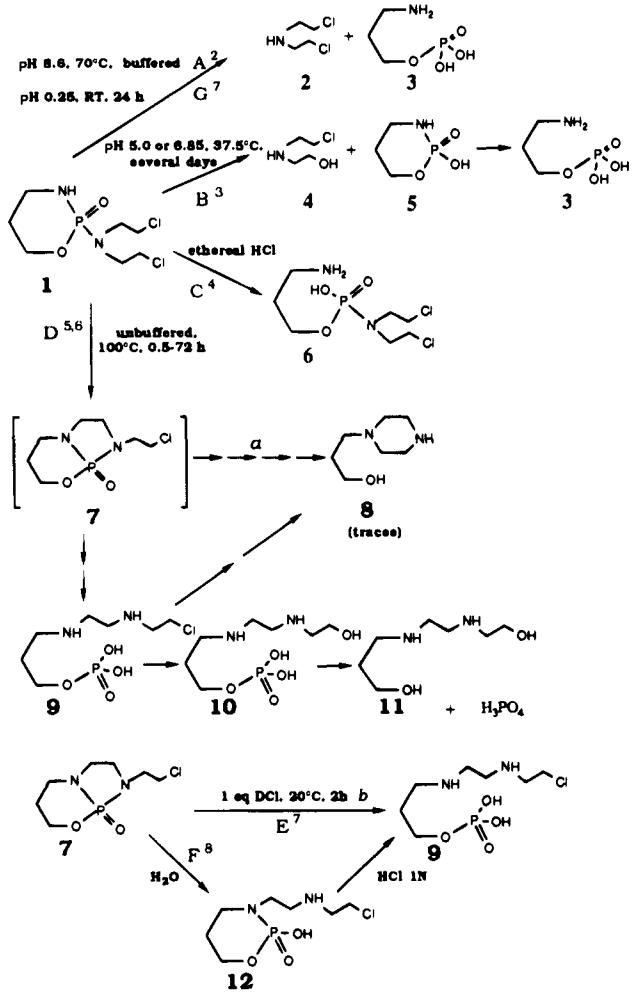
Scheme 1. Hydrolysis Pathways of Cyclophosphamide (1) Reported in the Literature (All the compounds are represented in neutral forms)[a]



[a] Conditions: (a) The number of arrows between compounds **7** and **8** was that indicated in the original article.[6] (b) In the obtained compound **9** (pathway E), mobile protons are replaced by deuterium.[7]

hydrochlorides) were formed (Scheme 2). This was confirmed by independent synthesis of **3** (Scheme 3). Its ^{13}C NMR characteristics were identical to those of the phosphorylated compound obtained by hydrolysis of **1** at pH 1.2. The presence of bis(2-chloroethyl)amine hydrochloride **2** in the hydrolysis mixture was confirmed by comparing its ^{13}C NMR characteristics with those of an authentic sample. Mass spectrometry and NMR data are listed in Table 1.

At pH 5.4, 6.9$_5$, 7.4, and 8.6, only one ^{31}P NMR signal (except the signal of **1**) located at 8.9 ppm could be detected during the hydrolysis (Figure 1D). From the observed ^{31}P NMR chemical shift, we assumed that this compound still possessed a P–N bond. Indeed, we noticed that, at these pH's, compounds with the structural pattern $P(O)(OR)(NR_1R_2)_2$, i.e., with two P–N bonds, lead to ^{31}P NMR chemical shifts >13 ppm, whereas compounds with no P–N bond, i.e., with the structural pattern $P(O)(OR)(OH)_2$, have low ^{31}P NMR chemical shifts (<5 ppm). From a consideration of the Friedman mechanism,[5-8] we assumed that this compound had structure **12** or **13** (Scheme 2). It was isolated from the hydrolysis mixture at pH 5.4 and characterized. The FAB mass spectrum in positive

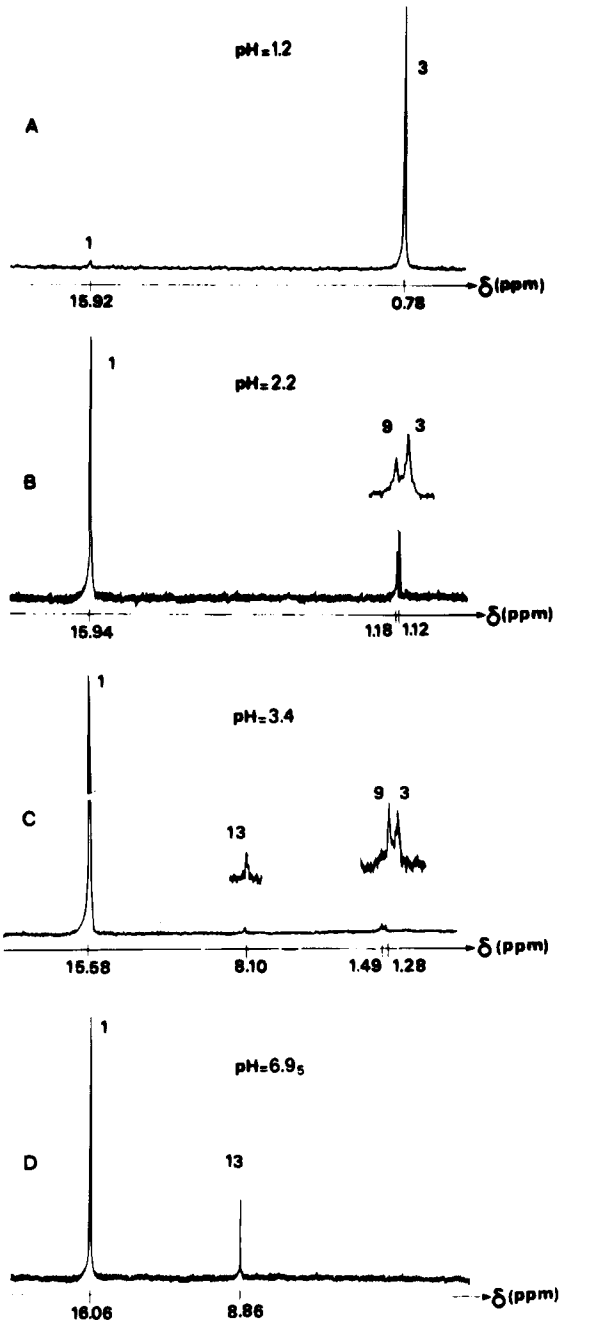
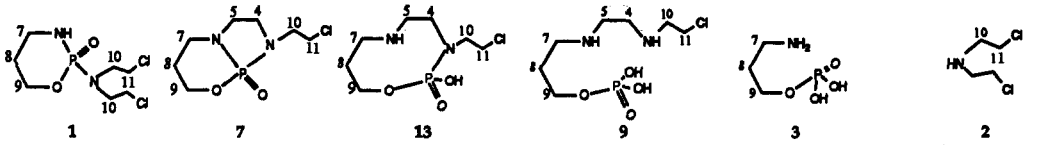


Figure 1. ^{31}P NMR spectra of hydrolysis mixtures of cyclophosphamide (1) at 20 °C: (A) pH 1.2, after 7 days; (B) pH 2.2, after 7 days; (C) pH 3.4, after 17 days; and (D) pH 6.9$_5$, after 17 days.

mode showed two peaks at 265 and 267 ($[MNa]^+$) that could be attributed to either compound **12** or **13** (Table 1).

The NMR data, however, indicated that this compound was **13**. Homonuclear and heteronuclear 2D experiments as well as decoupling techniques were employed to assign the ^1H and ^{13}C NMR spectra of **13**. The complete NMR data of **13** are given in Table I. The H,H-COSY spectrum of **13** showed three separate spin systems which were assigned to the substructures $>NCH_2(4)CH_2(5)N<$, $>NCH_2(7)CH_2(8)CH_2(9)O$-, and $>NCH_2(10)CH_2(11)Cl$. The assignment of the CH_2 groups (8), (9), and (11) was already clear from their characteristic chemical shifts in the 1D ^1H NMR spectrum. Consequently, using the COSY spectrum, the assignment of CH_2 groups (7) and (10) was straightfor-

Table 1. Mass Spectrometry and NMR Data of the Compounds Formed during the Hydrolysis of Cyclophosphamide (1)

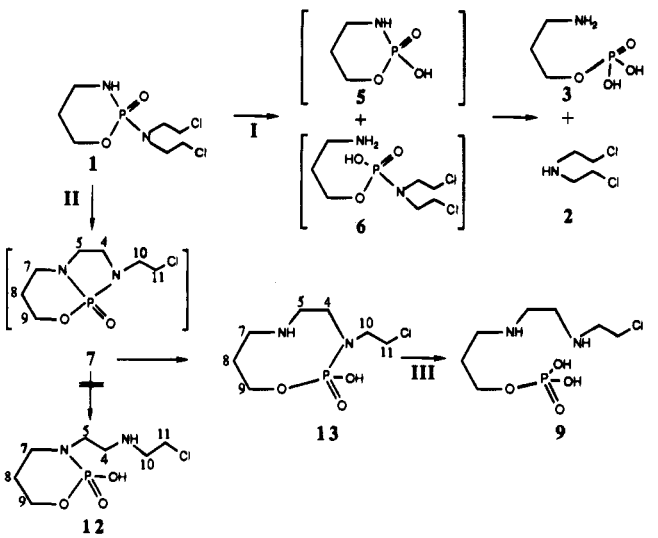


1 7 13 9 3 2

FAB positive mass spectral data (m/z)		[MH]$^+$ 225,227 (1Cl)	[MNa]$^+$ 265,267 (1 Cl)	[MH]$^+$ 261,263 (1 Cl)	[MH]$^+$ 156	[MH]$^+$ 142,144 (2 Cl)
^{31}P NMR δ^a (ppm)	16.06 (pH = 6.8)	24.50 ($CDCl_3$)	8.86 (pH = 6.8)	1.18 (pH = 2.3)	1.12 (pH = 2.3)	
^{13}C NMR δ^b (ppm) (multiplicity^c, J_{CP} (Hz))	pH = 7.0 (D_2O)	($CDCl_3$)^d	pH = 5.0 (D_2O)^d	pH = 5.5 (D_2O)^d	pH = 5.0 (H_2O)	pH = 5.0 (H_2O)
C7	43.3 (d, 2.6)	44.4 (s)	45.4 (s)	48.7 (s)	40.5 (s)	
C8	27.5 (d, 6.6)	21.2 (d, 7.2)	27.7 (d, 4.1)	29.1 (d, 5.7)	30.6 (d, 6.6)	
C9	71.8 (d, 6.7)	66.4 (d, 5.8)	65.4 (d, 6.4)	64.8 (d, 5.8)	65.8 (d, 5.3)	
C10	49.7 (d, 4.9)	48.0 (d, 4.7)	51.0 (d, 3.7)	51.8 (s)		51.7 (s)
C11	44.4 (d, 1.4)	43.2 (d, 3.5)	44.8 (s)	42.3 (s)		42.0 (s)
C4		46.1 (d, 15.3)	43.4 (d, 4.6)	46.15 (s), 45.9 (s)		
C5		44.4 (d, 9.8)	46.5 (d, 2.1)			
^{1}H NMR δ^e (ppm) (multiplicity^c, ^3J (Hz))	pH = 8.25 (D_2O)	($CDCl_3$)^d	pH = 6.8^d	pH = 5.5^d	pH = 5.1	
CH_2 7	3.35 (m)	3.17, 3.57 (m)	3.34 (t, 5.6)	3.18 (t, 6.4)	3.08 (t, 7.0)	
CH_2 8	1.97 (m)	1.60, 2.12 (m)	2.03 (quin, 5.6)	1.95 (app quin^h, 6.4, 5.4)	1.92 (app quin^h, 5.7, 7.0)	
CH_2 9	4.42 (m)	4.20, 4.40 (m)	4.12 (td, 5.6, 9.7^g)	3.89 (td, 5.4, 7.6^g)	3.90 (app q, 5.7, 6.7^g)	
CH_2 10	3.49 (td, 6.4, 11.6^g)	3.11, 3.59 (m)	3.38 (td, 6.6, 12.7^g)	3.39 (m)		
CH_2 11	3.75 (t, 6.4)	3.64 (m)	3.71 (t, 6.6)	3.81 (m)		
CH_2 4		3.24, 3.43 (m)	3.42 (td, 5.3, 9.3^g)	3.36 (m)		
CH_2 5		3.28 (m)	3.25 (t, 5.3)			

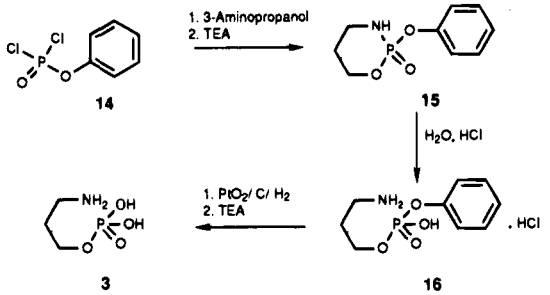
[a] Chemical shifts are related to external 85% H_3PO_4. Spectra were recorded at 25 °C. [b] Chemical shifts are related to external 3-(trimethylsilyl)propanesulfonic acid sodium salt (TMPS) or tetramethylsilane (TMS) for compound **7**. Spectra were recorded at 25 °C except for compound **7** (30 °C). [c] s = singlet, d = doublet, t = triplet, q = quadruplet, quin = quintuplet, m = multiplet, app = apparent. [d] ^{1}H and ^{13}C signals were assigned from 2D, H,H- and H,C-COSY experiments. For compound **7**, a revised assignment of the literature[7] is given. [e] Chemical shifts are related to external TMPS except for compounds **13** (internal 3-(trimethylsilyl)propionic acid sodium salt (TSP)) and **7** (internal TMS). Spectra were recorded in D_2O at 25 °C except for compounds **13** and **7** (30 °C). [f] Multiplicity observed in the ^{31}P-decoupled ^{1}H NMR spectrum. [g] $^3J_{HP}$. [h] Coupling constants were determined from homonuclear spin decoupling experiments.

Scheme 2. Hydrolysis Pathways of Cyclophosphamide (1) from Our Data[a]



[a] The reactions occur at (I) pH 1.2, 2.2, and 3.4; (II) pH 2.2, 3.4, 5.4, 6.9$_5$, 7.4, and 8.6; and (III) pH 2.2 and 3.4. All the compounds are represented in neutral forms.

Scheme 3. Synthetic Pathway for Compound **3**



TEA = Triethylamine

ward. However, the NCH_2 groups (4) and (5) had similar chemical shifts and could not *a priori* be distinguished. The six-ring **12** and nine-ring **13** structures were discriminated from the P couplings in the ^{1}H and ^{13}C NMR spectra. In the 1D ^{31}P-decoupled ^{1}H NMR spectrum, three complex signals were simplified into triplets, CH_2(9), CH_2(10), and that corresponding to either CH_2(4) or CH_2(5). The simplification of the multiplet corresponding to CH_2(10), whose attribution was unambiguous from the H,H-COSY spectrum, ruled out the six-ring structure **12** since, in this case, CH_2(10) would not exhibit H−P coupling. Moreover, the fact that CH_2(7) was not coupled to phosphorus also eliminated structure **12** for which this coupling would be observed. It was then possible to attribute the signals of CH_2 groups (4) and (5) in the ^{1}H NMR spectrum, since with structure **13** only the signals of CH_2(4) would be simplified in the ^{31}P-decoupled ^{1}H NMR spectrum. Couplings to phosphorus were thus observed in the ^{1}H NMR spectrum for CH_2 groups (4), (9), and (10). This was confirmed by the fact that only the cross-peaks of these protons showed an in-phase contribution from the H−P coupling in the phase-sensitive H,H-COSY spectrum.

The ^{13}C NMR spectrum of **13** was assigned simply by transducing the ^{1}H assignment information to ^{13}C via H,C-COSY. In the ^{13}C NMR spectrum, the signals of C4, C5, C8, C9, and C10 exhibited splittings due to couplings to phosphorus. In all the six-membered ring compounds of the oxazaphosphorinane family (e.g., **1**, ifosfamide, 2-dechloroethylifosfamide), a small coupling of C7 to phosphorus was observed. Moreover, the chemical shift of C9 was always more deshielded in the six-membered ring compounds (≈70 ppm) than in the linear compounds (≈65 ppm) (see, for example, the C9 chemical shifts of **1** and **3** in Table 1). In conclusion, the lack of H–P and C–P couplings between $CH_2(7)$ and P, the C9 chemical shift at 65.4 ppm, and especially the observation of couplings between $CH_2(10)$ and phosphorus in both the ^{1}H and ^{13}C NMR spectra ruled out the six-membered ring structure **12**.

Pankiewicz et al.[8] have reported the synthesis of the bicyclic compound **7** which was found to give **12** in water. We replicated this experiment. Anhydrous **1** was intramolecularly alkylated by sodium hydride in tetrahydrofuran to the bicyclic compound **7**, which can only be characterized under anhydrous conditions (Table 1), since it is not sufficiently stable in H_2O to allow its ^{13}C NMR characterization (see below). In the presence of water, compound **7** was hydrolyzed exothermally to one compound, which had identical NMR characteristics to those described above for the compound isolated from the kinetic hydrolysis mixture and to which we attributed the nine-membered ring structure **13**. We therefore concluded that the hydrolysis product of **1** at pH 5.4, 6.9_5, 7.4, and 8.6 was compound **13**. The structure **12** proposed by Pankiewicz et al.[8] must therefore be excluded.

At pH 2.2, the signal of compound **13** was not detected, although two ^{31}P NMR signals appeared at high field (1.18 and 1.12 ppm) after 4 days (Figure 1B). Several experiments were carried out to determine their structures. The ^{13}C NMR spectrum of the kinetic mixture at pH 2.2 after 17 days at 20 °C and the elimination of residual **1** by extraction with chloroform showed the signals of compounds **2** and **3** as well as other carbons. To confirm the presence of **3**, we spiked the hydrolysis mixture with authentic **3**. The ^{31}P NMR signal at 1.12 ppm was increased. The structure **9** (Scheme 2) was attributed to the other phosphorylated compound resonating at 1.18 ppm. Its ^{31}P NMR chemical shift was compatible with a structure with no P–N bond. Its ^{13}C NMR spectrum was identical to that reported by Zon et al.[7] for compound **9** obtained by hydrolysis of an unbuffered aqueous solution of **1** at 100 °C. Moreover, the isolated bicyclic compound **7** and the nine-membered ring compound **13**, treated for 24 h in 1 N HCl (or DCl for ^{1}H NMR spectra), both led to only one phosphorylated compound with a molecular weight of 260. The ^{1}H and ^{13}C NMR spectra of this compound were consistent with structure **9** and identical to those reported by Zon et al.[7] (Table 1). To confirm the presence of **9**, we spiked the hydrolysis mixture at pH 2.2 with authentic **9**. The ^{31}P NMR signal at 1.18 ppm was increased.

At pH 3.4, a small amount of compound **13** was observed after 7 days and, by day 17, compounds **9** and **3** were also formed (Figure 1C). All these compounds were identified by spiking the hydrolysis mixture with the authentic standards, leading to corresponding increases in the ^{31}P NMR signals at 8.10, 1.49, and 1.28 ppm, respectively.

Table 2. Proportions of Phosphorylated Compounds Formed during the Hydrolysis of Cyclophosphamide (1) at 20 °C

pH buffer	day	percentage of compounds (%) 1[a]	13	9	3
1.2	2	37			63
KCl-HCl, 0.5 M	4	13			87
	7	3			97
2.2	4	89		3.5	7.5
KCl-HCl, 0.5 M	7	78		10	12
	17[b]	60		17.5	19.5
3.4	4	100			
$CH_3COONa-CH_3COOH$, 0.4 M	7	99	1		
	17	93	1	3	3
5.4	4	98	2		
Na cacodylate, 0.1 M	7	95	5		
	17	81	19		
6.9_5	4	97	3		
Na cacodylate, 0.1 M	7	95	5		
	17	80	20		
7.4	4	97	3		
Na cacodylate, 0.1 M	6	95	5		
8.6	4	98	2		
Tris, 0.5 M	7	95	5		
	17	86	14		

[a] The initial concentration of cyclophosphamide (1) was 26.8 mM. [b] 3% of inorganic phosphate was also formed.

Scheme 2 summarizes our data. At pH 1.2, **1** undergoes breakdown of the two P–N bonds, giving compounds **2** and **3** (probably via compounds **5** and **6**, which were never detected). Only compound **13** was obtained at pH's between 5.4 and 8.6 after 17 days of hydrolysis at 20 °C. This means that the intramolecular alkylation of **1** giving **7** (never detected) is followed by breakdown of the P–N6 bond. At pH 2.2 and 3.4, both mechanisms occur since compounds **2** and **3** are formed in addition to compound **9** resulting from the breakdown of the P–N3 bond in intermediate **13**.

Chemical Stability of Aqueous Solutions of Cyclophosphamide as a Function of pH. The results of the experiments on the stability of buffered solutions of **1** at 20 °C and various pH's as a function of time are listed in Table 2. The stability of **1** was high and independent of pH between 3.4 and 8.6. After 7 days at 20 °C, ≥95% of initial **1** was still present. **1** was degraded more rapidly at pH 2.2. At pH 1.2, nearly all of **1** was hydrolyzed after 7 days. We also compared the behavior of **1** at 20 and 37 °C over 6 days at physiological pH. As expected, **1** was significantly more stable in solutions kept at 20 °C (Figure 2), and only a small amount of compound **13** was formed (Table 2). After 6 days at 37 °C, 45% of **1** was hydrolyzed leading to 30% of compound **13** and 15% of eight unknown phosphorylated compounds.

Since at acidic pH (1.2 and 2.2) there was a large concentration of chloride ion due to the buffer (0.5 M KCl-HCl) with respect to the concentration of 1 (26.8 mM), we checked if the presence of this ion could affect the hydrolysis of **1**. We therefore compared data of experiments at pH 2.2 (pH at which the two hydrolysis pathways of **1** coexist (Table 2)) and 24 °C in 0.5 M KCl-HCl buffer and in 0.5 M *p*-toluenesulfonic acid–sodium *p*-toluenesulfonate buffer. The results, reported in Table 3, showed that there were no significant differences in the rates of hydrolysis of **1** ($t_{1/2}$ = 18.9 and 19.9 days in KCl-HCl and *p*-toluenesulfonic acid–sodium

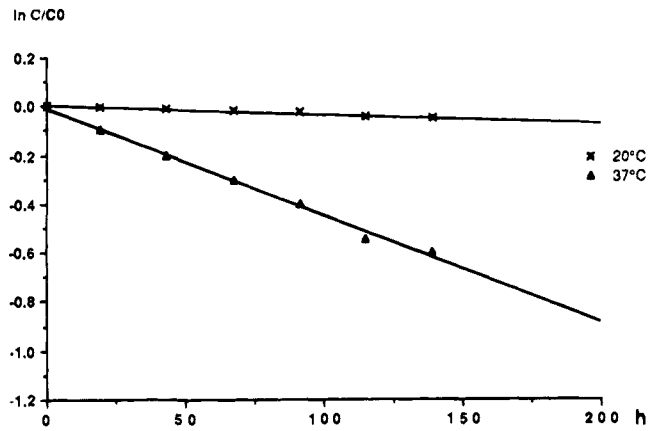


Figure 2. Plot of the time dependence of the change in cyclophosphamide concentration ($\ln[\text{cyclophosphamide}]_t/[\text{cyclophosphamide}]_0$) under hydrolytic conditions (pH = 7.4, 20 or 37 °C).

Table 3. Effect of Chloride Ion on the Proportions of Phosphorylated Compounds Formed during the Hydrolysis of Cyclophosphamide (1) at 24 °C and pH 2.2

		percentage of compounds[a] (%)		
day	buffer	1[b]	**9**	**3**
4	KCl-HCl, 0.5 M	85.6	5.7	8.7
	TSA–NaTS, 0.5 M[c]	83.4	6.9	9.6
7	KCl-HCl, 0.5 M	76.2	10.0	13.8
	TSA–NaTS, 0.5 M[c]	76.3	9.5	14.1
17	KCl-HCl, 0.5 M	55.1	24.1	20.8
	TSA–NaTs, 0.5 M[c]	53.0	25.1	21.9

[a] Average values for three experiments. [b] The initial concentration of cyclophosphamide (1) was 26.8 mM. [c] TSA–NaTS = *p*-toluenesulfonic acid–sodium *p*-toluenesulfonate buffer.

p-toluenesulfonate buffers, respectively) and in the proportions of compounds **3** and **9** thus formed, representative of the contribution of pathways I and II + III, respectively (Scheme 2).

Chemical Stability of Aqueous Solutions of Compound 13 as a Function of pH. The results of the experiments on the stability of buffered solutions of **13** at various pH's at 20 °C as a function of time are listed in Table 4. At pH ≤3.5, the rate of degradation of **13** was inversely proportional to pH ($t_{1/2}$ = 6.3 days at pH 3.5 and 20 °C, 32 min at pH 1.0 and 24 °C, and 9 or 7 min in 1 N HCl at 20 or 24 °C, respectively). Only compound **9** was obtained at pH ≤ 1, whereas at pH 3.5 a small amount of inorganic phosphate was also formed. The stability of **13** was high at pH 5.5 and 6.8_5 since >85% of initial **13** was still present after 15 days at 20 °C, although it was hydrolyzed more rapidly at pH 8.6. Hydrolysis of **13** did not lead to compound **9** at pH 6.8_5 and 8.6 and only produced a small amount of **9** at pH 5.5. Several unidentified phosphorylated compounds were obtained at pH's between 5.5 and 8.6.

Chemical Stability of Aqueous Solutions of Bicyclic Compound 7. Compound **7** was highly labile in aqueous solution. At pH 5.9 and 24 °C, a 37.0 mM solution of **7** in H_2O was hydrolyzed to **13** with a $t_{1/2}$ of 3 min as measured by ^{31}P NMR. Since Zon et al.[7] found a $t_{1/2}$ of 20 min for the hydrolysis of this compound into **9** under highly acidic conditions (pD ≈ 0) at 20 °C using ^1H NMR, we decided to monitor the hydrolysis of a 26.8 mM solution of **7** under similar conditions (1 N HCl, 24 °C) using ^{31}P NMR. In the first spectrum (obtained **11** min after dissolving **7** in this medium), the resonance of **7** was already undetectable and only the signals of compounds **13** and **9** were observed. The $t_{1/2}$ for the hydrolysis of **13** thus formed (which was only degraded into **3** under these conditions) was estimated to be 6 min.

Biological Activity of Compounds 3, 9, and 13. The biological activity of **3**, **9**, and **13** was investigated in three models: (i) acute toxicity (approximative LD_{50}) after a single ip injection in mice, (ii) direct cytotoxic activity *in vitro* from the inhibition of colony formation of murine leukemic L1210 cells, and (iii) antitumor efficacy *in vivo* against murine P388 leukemia.

The approximate LD_{50} of **3**, **9**, and **13** after a single ip injection in mice were above 1000, around 700, and around 1000 mg/kg, respectively. The corresponding toxicity value of **1** is around 620 mg/kg. Symptoms of toxicity were rather unspecific, i.e., retracted flanks, reduced locomotion, and piloerection. Death occurred 3–5 days after administration. No direct cytotoxic activity, i.e., no inhibition of colony formation of L1210 cells, was observed at concentrations ranging from 0.1 to 100 μg/mL of **3**, **9**, or **13**.

To determine the antitumor efficacy, **9** was administered with a single ip dose of 215 mg/kg or four ip doses of 68.1 mg/kg on consecutive days. There was no effect on the median survival time of tumor-bearing animals. Correspondingly, **13** was given in a single dose of 316 mg/kg or a fractionated dose of 4 × 100 mg/kg. No antitumor activity was observed. In view of the slight toxicity of **3** and the missing chloroethyl groups, antitumor efficacy was considered to be unlikely.

Discussion

The present study clarified mechanistic details regarding the hydrolytic behavior of **1** from pH 1.2 to 8.6. The "Friedman mechanism" involving the intramolecular N-alkylation of **1** as the first step of hydrolysis[5,6] was supported for pH's ranging from 2.2 to 8.6. Although we never observed the bicyclic compound **7**, we demonstrated formation of (i) compound **13** from the exothermal hydrolytic breakdown of the P–N6 bond of **7** and (ii) compound **9** resulting from the subsequent acid-catalyzed hydrolytic breakdown of the P–N3 bond. Chakrabarti and Friedman[6] and Zon et al.[7] have obtained compound **9** by treating unbuffered aqueous solutions of **1** at reflux for 30 min, but under these acid conditions, they did not observe the acid-labile intermediate **13**. After dissolving in water the bicyclic compound **7** obtained under anhydrous conditions by treating **1** with sodium hydride, Pankiewicz et al.[8] produced compound **13** to which they attributed the incorrect structure **12**. The preference for compound **13** is unexpected in view of the greater stability of the cyclohexane than the cyclononane ring. The reaction path leading to compound **13** depicted in Scheme 2 was supported by quantum mechanical and force field calculations on structure **7**. We calculated the strain energy of the MNDO[9]-optimized structure **7** by means of the force field program PIMM[10] and displayed the results graphically on a ball and stick model using the modeling program MOLCALD[11] (Figure 3). In this figure, the color code runs from violet to red corresponding to an increase in strain energy. Thus, comparing the colors of the two nitrogen atoms, it can be seen that the strain energy on the N6 (deep yellow) atom is greater than that on the N3 (light yellow) atom, sug-

Table 4. Proportions of Phosphorylated Compounds Formed during the Hydrolysis of the Nine-Membered Ring Compound **13** at 20 °C

pH buffer	time	percentage of compounds (%)			
		13[a]	**9**	inorganic phosphate	unknown (number)
0 (1 N HCl)[b]	53 min	2	98		
1.0[b,c] KCl-HCl, 0.5 M	2 h	7	93		
3.5	1 d	91	9		
$CH_3COONa-CH_3COOH$, 0.4 M	2 d	82	18		
	4 d	62	36	2	
	7 d	46	52	2	
	17 d	15	80	5	
5.5	4 d	100			
Na cacodylate, 0.1 M	7 d	97	0.5		2.5 (2)
	17 d	86.5	2		11.5 (3)[d]
6.8_5	4 d	97			3 (4)
Na cacodylate, 0.1 M	7 d	96			4 (5)
	15 d	89		0.5	10.5 (5)[e]
8.6	4 d	92.5			7.5 (8)[f]
Tris, 0.5 M	7 d	84			16 (10)[f]
	17 d	63			37 (11)[f]

[a] The initial concentration of compound **13** was 26.8 mM. [b] The experiments were continuously monitored in the NMR probe. [c] The temperature was 24 °C. [d] One unknown phosphorylated compound resonating at $\delta = 3.86$ ppm was preponderant and represented 7.5% of total phosphorylated compounds. [e] Two unknown phosphorylated compounds resonating at $\delta = 4.81$ and 4.64 ppm were preponderant and represented respectively 4.7% and 3.9% of total phosphorylated compounds. [f] Two unknown phosphorylated compounds resonating at $\delta = 10.34$ and 8.56 ppm were preponderant and represented respectively 2.0% and 3.2% of total phosphorylated compounds after 4 days, 4.7% and 5.4% after 7 days, and 14.8% and 6.0% after 17 days.

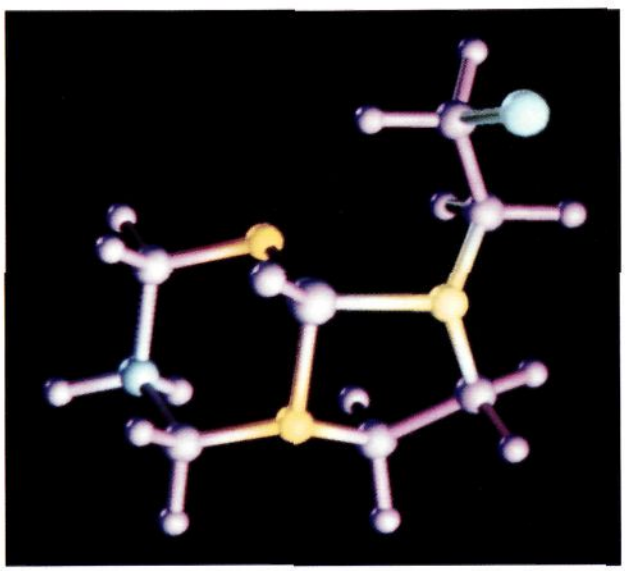


Figure 3. *Color-coded stick and ball model of structure* **7** *showing the differences in strain energy for the various atoms.* The color code runs from violet to red corresponding to an increase in strain energy. It can be seen that the strain energy of N6 (deep yellow) is slightly greater than that of N3 (light yellow).

gesting that there is a preferred position for bond cleavage. Although the difference in strain energy between the two nitrogen atoms is only around 0.3–0.4 kcal/mol, it is probably enough to explain the experimentally observed bond cleavage.

At pH 1.2, our study confirmed the occurrence of another hydrolysis mechanism involving direct breakdown of the two P–N bonds of **1**, described by Zon et al.[7] after treating **1** at room temperature and pH 0.25 for 24 h. We observed that both mechanisms occur at pH's between 2.2 and 3.4. Between pH 1.2 and 3.4, we failed to detect the intermediates **5** and **6** proposed by Arnold et al.[3,4] Their P–N bonds would appear to be too unstable.

Concerning the stability of **1**, our results agree with those of Hirata et al.[12] who demonstrated the independence of the hydrolysis rate of **1** on pH over the range ≈3–10. They are also comparable to those of Brooke et al.[13] who reported ≈10% and 30% decomposition of **1** dissolved in sterile water at ambient temperature (24–27 °C) after 4 and 14 days, respectively. The lower degradation we observed (2–5% after 4 days and 14–20% after 17 days at pH's ranging from 5.4 to 8.6 at 20 °C) can probably be accounted for by the lower temperature used in our experiments. Hirata et al.[12] demonstrated that the observed disappearance rate constant for **1** was subject to acidic catalysis at pH < 2. The rate constant we determined for hydrolysis of **1** at pH 1.2 and 20 °C (5.64×10^{-6} s^{-1}, $t_{1/2} = 1.4$ days) would be expected to be considerably lower than that measured by Zon et al.[7] in D_2O at 20 °C and pH 0.25 (4.35×10^{-5} s^{-1}, $t_{1/2} = 0.18$ day). The rate constant we obtained for degradation of **1** (1.22×10^{-6} s^{-1}, $t_{1/2} = 6.6$ days) in physiological conditions (37 °C, pH 7.4) was close to that

reported by Zon et al.[7] at 37.4 °C and ambient pD (1.43 × 10^{-6} s^{-1}, $t_{1/2}$ = 5.6 days). At pH 2.2, the presence of chloride ion did not affect the rate of hydrolysis of **1**. This result agrees with that of Zon et al.[7] who demonstrated that added chloride ion did not modify the kinetic disappearance of **1** at ambient pD and 37 °C ($t_{1/2}$ = 5.6 days without chloride ion vs 5.7 days with 2 equiv of NaCl). Moreover, chloride ion did not favor pathway I of cyclophosphamide hydrolysis over pathways II + III (Table 3, Scheme 2).

Our results demonstrate the dependence of the rate of hydrolysis of **13** on pH over the range 0–8.6 (Table 4). Furthermore, the nature of the hydrolysis products was also found to depend on pH since compound **9** was obtained at pH ≤ 3.5 whereas several phosphorylated compounds apart from **9** were formed at pH ≥ 5.5.

The marked lability of bicyclic compound **7** in aqueous solutions was supported by the low value of its $t_{1/2}$ at pH 5.9 (3 min) and the inability to detect its ^{31}P NMR signal in 1 N HCl. Under these highly acidic conditions, only the hydrolytic degradation of **13** into **9** could be monitored. The measured value of $t_{1/2}$ (≈6 min) was close to that obtained for the hydrolysis of compound **13** under the same conditions (7 min). From our ^{31}P NMR data, we presume that the $t_{1/2}$ value of 20 min reported by Zon et al.[7] for the hydrolysis of compound **7** at pD ≈ 0 and 20 °C is most likely to be that of the rate of hydrolysis of compound **13** into compound **9**. It should be borne in mind that the differences in chemical shifts of compounds **7**, **13**, and **9** are much higher in ^{31}P NMR than in ^{1}H NMR (Table 1). It is thus possible that these authors could not discriminate the ^{1}H NMR signals of compounds **7** and **13** in the hydrolysis mixture.

Evaluation of biological activity showed that the intra-alkylating process leading to **13** and **9** and the degradation to **3** result in a biological detoxification. All these compounds are less toxic than **1**. The approximate LD_{50} of parent compound **1** is 620 mg/kg vs 700, 1000, and >1000 mg/kg for compounds **9**, **13**, and **3**, respectively. In common with **1**, compounds **3**, **9**, and **13** had no direct cytotoxic activity. Although in contrast to **1**, **9** and **13** had no antitumor efficacy toward murine P388 leukemia.

Experimental Section

A. Chemistry. The pharmaceutical formulation Endoxan-Asta was used as a source of cyclophosphamide monohydrate. Melting points were determined on a capillary apparatus and are uncorrected. NMR spectra were recorded on a Bruker AM 300 WB or Bruker AMX 500 spectrometer. Unless otherwise noted, ^{1}H and ^{13}C NMR chemical shifts are reported in ppm from 3-(trimethylsilyl)propanesulfonic acid sodium salt as external reference. ^{31}P NMR chemical shifts are reported in ppm from 85% H_3PO_4 as external reference. Fast atom bombardment mass spectra in positive mode were obtained with a VG ZAB HS spectrometer.

1-(2-Chloroethyl)tetrahydro-1*H*,5*H*-[1,3,2]diazaphospholo[2,1-*b*][1,3,2]oxazaphosphorine 9-Oxide (7). The pure racemic compound was prepared by modifying the procedure for the racemic compound[7] and the enantiomeric forms.[8] To a solution of 20 g (77 mmol) of anhydrous 1 in 1.5 L of diethyl ether was added a suspension of 9.2 g (380 mmol) of sodium hydride in 100 mL of ether under cooling and stirring. After 30 min, the solution was stirred at room temperature for 2.5 h. The suspension was then decanted, the ether phase evaporated, and the residue recrystallized twice in ether to give **7** in 64% yield (11 g): mp 80–81 °C. Anal. ($C_7H_{14}N_2O_2PCl$) C, H, N. NMR and mass spectrometry data are reported in Table 1.

3-(2-Chloroethyl)-2-hydroxy-1,3,6,2-oxadiazaphosphonane 2-Oxide (13). Compound **7** (2.6 g, 12 mmol) was dissolved in water (20 mL). When the exothermal reaction ended, the solution was evaporated under reduced pressure. The crystals were treated with ether and dried to give **13** in 72% yield (2.0 g): mp 200–203 °C. Anal. ($C_7H_{16}N_2O_3PCl$) C, H, N. NMR and mass spectrometry data are reported in Table 1.

Extraction of 13 from a Hydrolysis Mixture of 1 at pH 5.4. Cyclophosphamide (1) (0.192 g, 0.69 mmol) was dissolved in 15 mL of sodium cacodylate buffer (0.1 M) and kept at 37 °C for 4 days. After the presence of **13** was verified with ^{31}P NMR, the reaction mixture was extracted with three 10 mL portions of chloroform. The aqueous phase, which contained **13** as the sole compound, was then lyophilized and resuspended in 2.5 mL of D_2O for NMR analysis.

1-Propanol, 3-[[2-[(2-Chloroethyl)amino]ethyl]amino], Dihydrogen Phosphate (Ester Dihydrochloride) (9). Compound **7** (2.6 g, 12 mmol) was dissolved in 100 mL of 1 N HCl. When the exothermal reaction ended, the solution was evaporated under reduced pressure at 70 °C for 1 h. The crystals were treated with ether and dried to give **9** as dihydrochloride in 65% yield (2.5 g): mp 121–127 °C (lit.[6] mp, for the monohydrochloride, 101–105 °C). Anal. ($C_7H_{20}Cl_3N_2O_4P$) C, H, N. NMR and mass spectrometry data are reported in Table 1.

1-Propanol, 3-Amino-, Dihydrogen Phosphate (Ester) (3). A solution of 11 g (51 mmol) of phosphoric acid phenyl ester dichloride (**14**)[14] in 50 mL of dichloromethane was added under stirring and cooling at 10 °C to a solution of 3.8 g (51 mmol) of 3-amino-1-propanol in 100 mL of dichloromethane. After addition of a solution of 10 g (102 mmol) of triethylamine in 100 mL of dichloromethane, the reaction mixture was stirred at room temperature for 1 h and washed with water. The organic solution was dried over sodium sulfate, the solvent evaporated, and the residue purified on a silica gel column with dichloromethane/methanol (95:5) as eluent to give compound **15** in 83% yield (9 g) (R_f = 0.6 for **15** and 0.9 for **14**): ^{1}H NMR ($CDCl_3$, 30 °C, δ from tetramethylsilane (TMS)) δ 7.35–7.05 (5H, m, Ar), 4.45–4.32 (2H, m, CH_2O), 4.23 (1H, m, NH), 3.34–3.22 (2H, m, CH_2N), 2.20–2.00 (1H, m, CCH_2C), 1.62 (1H, d, CCH_2C); ^{31}P NMR ($CDCl_3$, 30 °C) δ −0.58.

A solution of 9 g (42 mmol) of **15** in 150 mL of 1 N HCl was stirred for 2 h at 70 °C and then evaporated to dryness to give an oily residue of compound **16**: ^{1}H NMR (D_2O, 30 °C, δ from D_2O = 4.8 ppm) δ 7.50 (2H, t, Ar), 7.30 (3H, m, Ar), 4.20 (2H, m, CH_2O), 3.13 (2H, t, CH_2N), 2.10 (2H, m, CCH_2C); ^{31}P NMR (D_2O, 30 °C) δ −1.27.

The above residue was dissolved in 100 mL of methanol and hydrogenated with PtO_2 (1 g)/C/H_2 at room temperature under normal pressure. After filtration of the catalyst, the solvent was evaporated to dryness and the residue was dissolved in 150 mL of a solution of ethanol/water (1:1.2). After neutralization with triethylamine and stirring, the crystalline product was filtered off and washed with ether to give compound **3** in 38% yield (2.5 g) from compound **15**: mp 201–205 °C. Anal. ($C_3H_{10}NO_4P$) C, H, N. NMR and mass spectrometry data are reported in Table 1.

^{31}P NMR Recordings of Hydrolysis of Cyclophosphamide (1), Nine-Membered Ring Compound 13, and Bicyclic Compound 7. 1 (18.7 mg, 0.067 mmol) (or 16.3 mg (0.067 mmol) of **13**) was dissolved in 2.5 mL of the appropriate buffer, and the pH of the resulting solution was measured. After recording initial "zero-time" ^{31}P NMR spectra, the samples were maintained at 20 or 37 ± 0.1 °C in a circulating water bath and then periodically removed for ^{31}P NMR analysis. The degradation kinetics of compounds **13** at pH 1.0 or in 1 N HCl and **7** (15.0 mg (0.067 mmol) in 2.5 mL of 1 N HCl or 20.8 mg (0.093 mmol) in 2.5 mL of H_2O at pH 5.9) were continuously monitored in the NMR probe, and the spectra were acquired at varying intervals over 45–120 min. Time points for each spectrum were taken at the midpoint of data acquisition.

Spectra were run on the Bruker AM 300 spectrometer using 10 mm diameter NMR tubes under the following instrumental conditions: probe temperature, 24 or 20 °C; sweep width, 15151 Hz; 32 K data points zero-filled to 64 K; pulse width, 5 μs (i.e., flip angle $\approx$ 35°); repetition time, 2.08 s; FID spectra processed by exponential multiplication with a line broadening of 0.5–2 Hz; number of transients, 300–1000. Negligible reaction took place during the NMR recording ($\approx$10–30 min) of the samples analyzed over several days.

pH was measured before and after each NMR spectrum. A slight change in pH ($\leq$0.2 pH unit) was generally observed except for solutions of 1 at pH 2.2/20 °C (+0.4 pH unit after 17 days), pH 2.2/24 °C (+0.6 pH unit after 17 days), pH 8.6/20 °C (−0.3 pH unit after 17 days), and pH 7.4/37 °C (−0.6 pH unit after 6 days). Despite the changes in pH, the hydrolysis reaction of 1 at pH 7.4 and 37 °C or at pH 2.2 and 24 °C obeyed a pseudo-first-order rate law.

The relative concentrations of the phosphorylated compounds observed in the ^{31}P NMR spectra were determined after measuring the peak areas by cutting and weighing the expanded signals. To ensure that the relative concentrations measured under our experimental conditions were correct, we made up a solution containing known concentrations (around 10 mM) of the different cyclic and linear compounds, namely 1, **13**, **3**, and $NaH_2PO_4,2H_2O$, in cacodylate buffer at pH 6.8 in the following proportions respectively: 21.9%, 27.7%, 22.1%, and 28.3%. We recorded two ^{31}P NMR spectra of this solution under the conditions described above. The proportions of the various compounds determined from the peak areas were 20.2% for 1, 26.7% for **13**, 21.2% for **3**, and 31.9% for inorganic phosphate, indicating that the measured relative concentrations were correct.

Linear least-squares fits of pseudo-first-order plots of ln ([starting material]$_t$/[starting material]$_0$) vs time gave the values of the rate constants.

B. Biological Methods. Animals. Male CD2F1 mice were obtained at an age of 6–8 weeks from the Zentralinstitut für Versuchstierzucht, Hannover, Germany. They were kept under specific pathogen-free conditions, were fed with a standard pellet diet (Altromin 1324) *ad libitum*, and had an unrestricted supply of acidified water (pH 3).

Cell Lines. P388 and L1210 mouse leukemia were obtained from Dr. Atassi, Institut Jules Bordet, Brussels, Belgium.

Materials. The materials used included compounds 1, **3**, **9**, and **13**, RPMI-1640 (Gibco, D-7500 Karlsruhe, Germany), fetal calf serum (Seromed, D-1000 Berlin, Germany), Bacto-Agar (Difco, Detroit, MI), and phosphate-buffered saline (Seromed, D-1000 Berlin, Germany).

Acute Toxicity. Two male CD2F1 mice per dose group were injected ip with the test compound. The animals were observed for lethality and clinical signs of toxicity for 28 days. An approximate LD_{50} value was thus determined.

Colony Assays. *In vitro* assays for inhibition of colony growth in soft agar (colony assay) were performed according to the method of Hamburger and Salmon.[15] Briefly, tumor cells were incubated in RPMI-1640 medium containing 20% fetal calf serum and solidified with 0.3% agar in the presence of different concentrations of the cytostatic agent at 37 °C, 95% relative humidity, and 7.5% CO_2. The experiments were performed in triplicate. The incubation time was 6 days. Subsequently, the colonies of more than 50 cells were counted. The concentration of the cytostatic agent resulting in an inhibition of colony formation by 90% (EC_{90}) was determined graphically.

Experimental Tumors *in Vivo*. Six male CD2F1 mice per group were inoculated ip with 10^6 freshly harvested P388 cells in 0.2 mL of phosphate-buffered saline. The animals were given the test substances ip either once on the following day (day 1) or daily from day 1 to 4. Control animals were treated with solvent. Median survival times (MST) were determined for the respective groups, and an increase of life span (ILS) was determined as a percentage of the control group.

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