Chemical Stability and Fate of the Cytostatic Drug Ifosfamide and Its N-Dechloroethylated Metabolites in Acidic Aqueous Solutions†

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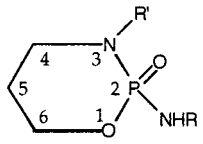
^{31}P NMR spectroscopy was used to study the products of the decomposition of the antitumor drug ifosfamide (IF, **1d**) and its N-dechloroethylated metabolites, namely, 2,3-didechloroethylIF (**1a**) and 2- (**1b**) and 3-dechloroethylIF (**1c**), in buffered solutions at acidic pH. The first stage of acid hydrolysis of these four oxazaphosphorines is a P−N bond cleavage of the six-membered ring leading to the phosphoramidic acid monoesters (**2a**−**d**) of type R′HN$(CH_2)_3$OP(O)(OH)-NHR, with R and/or R′ = H or $(CH_2)_2$Cl. The electron-withdrawing chloroethyl group at the endocyclic and/or exocyclic nitrogens counteracts the endocyclic P−N bond hydrolysis. This effect is even more marked when the *N*-chloroethyl group is in the exocyclic position since the order of stability is **1d** > **1c** > **1b** > **1a**. In the second stage of hydrolysis, the remaining P−N bond is cleaved together with an intramolecular attack at the phosphorus atom by the non-P-linked nitrogen of the compounds **2a**−**d**. This leads to the formation of a 2-hydroxyoxazaphosphorine ring with R = H (**3a** coming from compounds **2a**,**c**) or $(CH_2)_2$Cl (**3b** coming from compounds **2b**,**d**) and to the release of ammonia or chloroethylamine. The third step is the P−N ring opening of the oxazaphosphorines **3a,b** leading to the phosphoric acid monoesters, $H_2N(CH_2)_3OP(O)(OH)_2$ (**4a**) and $Cl(CH_2)_2HN(CH_2)_3OP(O)(OH)_2$ (**4b-1**), respectively. For the latter compound, the chloroethyl group is partially (at pH 5.5) or totally (at pH 7.0) cyclized into aziridine (**4b-2**), which is then progressively hydrolyzed into an *N*-hydroxyethyl group (**4b-3**). Compounds **3a,b** are transient intermediates, which in strongly acidic medium are not observed with ^{31}P NMR. In this case, cleavage of the P−N bond of the type **2** phosphoramidic acid monoesters leads directly to the type **4** phosphoric acid monoesters. The phosphate anion, derived from P−O bond cleavage of these latter compounds, is only observed at low levels after a long period of hydrolysis. Compounds **1a**−**c** and some of their hydrolytic degradation products (**4b-1**, **4b-2**, diphosphoric diester $[Cl(CH_2)_2NH(CH_2)_3OP(O)(OH)]_2O$ (**5**), and chloroethylamine) did not exhibit, as expected, any antitumor efficacy in vivo against P388 leukemia. ^{31}P NMR determination of the N-dechloroethylated metabolites of IF or its structural isomer, cyclophosphamide (CP), and their degradation compounds could provide an indirect and accurate estimation of chloroacetaldehyde amounts formed from CP or IF.

Introduction

The oxazaphosphorine ifosfamide (IF, **1d**) (Figure 1) is an alkylating antitumor agent employed in the chemotherapy of several tumors, including lung, cervical, and testicular cancers, Ewing's sarcoma, neuroblastoma, and soft-tissue sarcomas. In common with its structural isomer, cyclophosphamide (CP), IF is a prodrug that is entirely dependent on metabolism for its activity.[1−6] Apart from the activation pathway, which leads among other metabolites to the formation of the alkylating species isophosphoramide mustard (IPM) and the release of urotoxic and nephrotoxic acrolein, there is a competitive deactivation pathway via oxidation of the chloroethyl group.[3,5,6] This reaction results in the loss of one or both of the *N*-chloroethyl side chains of IF leading to the formation of 2-dechloroethylifosfamide (2DCIF, **1b**), 3-dechloroethylifosfamide (3DCIF, **1c**),[3,5−7] or 2,3-didechloroethylifosfamide (DDCIF, **1a**)[8−10] (Figure 1) and liberation of equimolar (or 2-fold molar for DDCIF) amounts of chloroacetaldehyde (CAA), a compound that may be responsible for the oxazaphosphorine-induced neurotoxicity, urotoxicity, and cardiotoxicity.[11−13]



1a 2,3 didechloroethylifosfamide (DDCIF) R=R'=H

1b 2-dechloroethylifosfamide (2DCIF) R=H, R'=CH_2CH_2Cl

1c 3-dechloroethylifosfamide (3DCIF) R=CH_2CH_2Cl, R'=H

1d Ifosfamide (IF) R=R'=CH_2CH_2Cl

Figure 1. Structures of ifosfamide and its N-dechloroethylated metabolites.

Knowledge of the amounts of CAA formed are thus of clinical interest. Although specific methods for analysis of CAA in blood or plasma have been validated, formaldehyde must be added to the blood samples at the bedside to prevent rapid degradation of CAA (half-life in whole blood ranging from 6 to 30 min[14,15]). Due to the rapid in vivo conversion of CAA into chloroacetate which is extensively metabolized mainly to *S*-carboxy-

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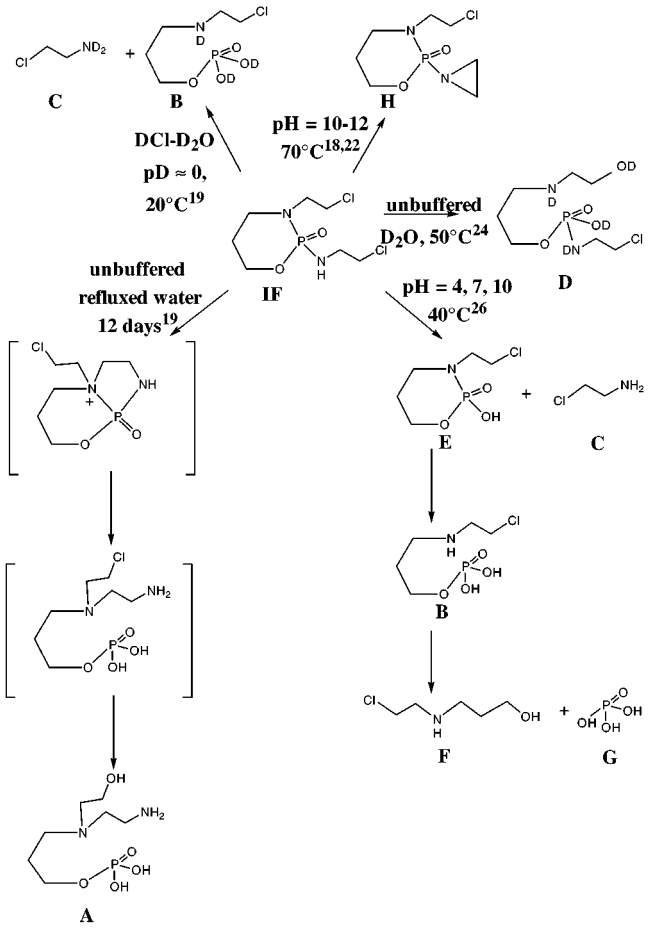
Scheme 1. Hydrolytic Pathways of Ifosfamide Reported in the Literature[a]



[a] All the compounds are represented in neutral forms.

methylcysteine and thiodiglycolic acid,[5,7] low CAA concentrations relative to those of 3DCIF and 2DCIF have been detected in plasma samples from patients treated with IF.[14,15] Assay of 2DCIF, 3DCIF, and DDCIF in biofluids of patients would therefore give a better estimate of amounts of CAA formed. Various methods have been reported for the determination of the dechloroethylated IF metabolites (up to 1992, they are reviewed in ref 16; see also refs 9, 10, 14, and 17). However, before concluding that the amount of dechloroethyl derivatives of IF in the urine of patients corresponds to the amount of CAA liberated, we wanted to determine their aqueous stability. If unstable in aqueous solution, we also needed to identify their degradation products.

The chemical stability in aqueous solutions of DDCIF has not been reported, while a study by HPLC and GC chromatography failed to detect any degradation products of 2DCIF or 3DCIF.[18] On the other hand, numerous studies have been devoted to the aqueous stability of their parent compound IF.[19−27] The hydrolytic cleavage of IF was first studied by Zon et al.[19] From an unbuffered solution of IF (starting pD ≈ 6.0) refluxed for 12 days, only one compound was isolated in low yield (8% as determined from data reported in the paper) and its structure determined from elemental analysis and proton NMR as that of compound A (Scheme 1). The formation of this compound may be accounted for by the reaction sequence shown in Scheme 1 which is akin to the Friedman mechanism for hydrolysis of CP.[28] Under strongly acidic conditions (pD ≈ 0) at 20 °C, they obtained an equimolecular mixture of B and C (Scheme 1) (as evidenced by proton NMR) resulting from the cleavage of the P−N bonds. In an unbuffered D_2O solution of IF maintained at 50 °C, Muñoz et al.[24] observed the disappearance of IF after 21 days and the formation of two final products, the major one having the structure D from ^1H and ^{13}C NMR spectroscopy. From hydrolysis of IF at 40 °C and pH 4, 7, or 10, Higley et al.[26] identified, using GC−MS, the methyl esters of oxazaphosphorine E and phosphoric acid (G) after derivatization by diazomethane and, after derivatization by trifluoroacetic anhydride, the trifluoroacetylated derivatives of chloroethylamine (CEA) (C) and 2-(chloroethyl)-3-hydroxypropylamine (F). They proposed that IF is hydrolyzed according to the sequential pathway of Scheme 1. Several degradation products were detected (up to nine after 48-h degradation at 75 °C in distilled water) by micellar electrokinetic chromatography, but only chloride ion was identified.[27] In alkaline solutions (pH 10−12), the degradation product detected by GC or HPLC was not formally identified,[22] but the aziridine structure H (Scheme 1) was proposed from its molecular weight of 224.[18] This was consistent with the well-documented cyclization of primary and secondary β-haloamines into aziridines.[29] Up to now, the behavior of IF toward hydrolysis has not been fully determined.

In an attempt to determine the stability and the behavior of IF and its dechloroethylated metabolites 2DCIF, 3DCIF, and DDCIF in urine, we investigated the time course of their hydrolysis in buffered solutions at neutral and various acidic pH by ^{31}P NMR. The degradation compounds were isolated and characterized by NMR and mass spectrometry. The toxicity and antitumor activity of IF, its dechloroethylated metabolites, and some of their degradation compounds were also evaluated.

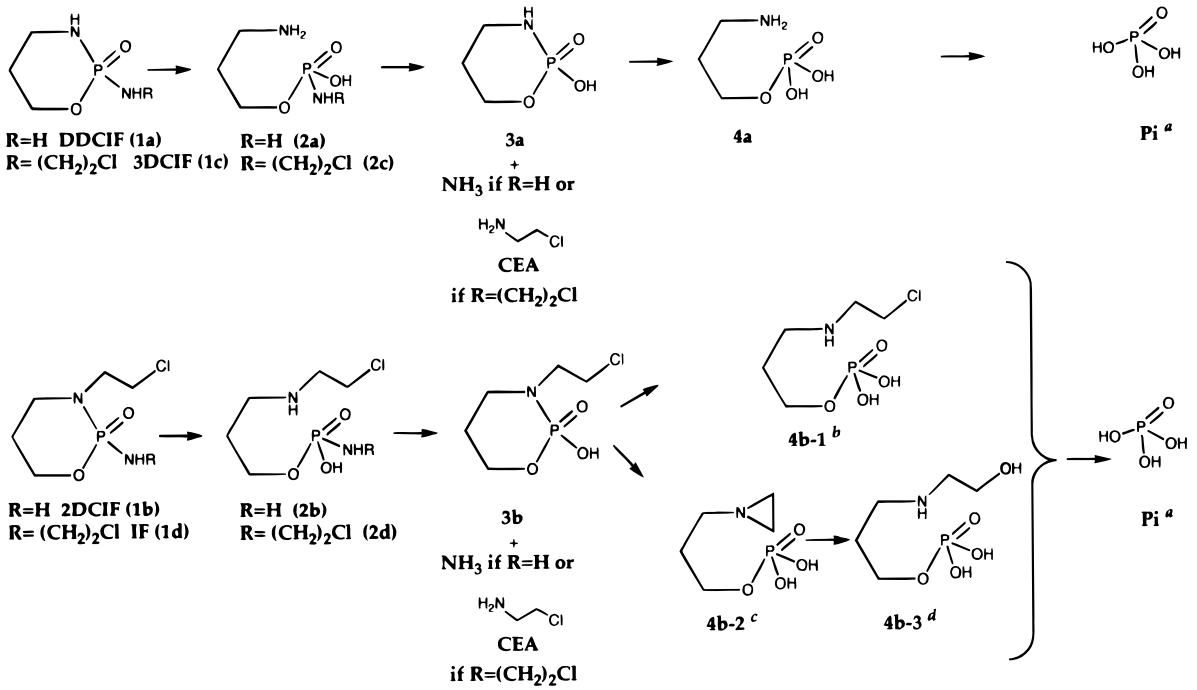
Results

For more clarity, the data supported by the experiments reported below are summarized in Scheme 2.

Hydrolysis of DDCIF. 1. ^{31}P NMR-Derived Time Courses for Hydrolytic Breakdown. DDCIF is not stable in aqueous solution, and its rate of degradation was higher at acidic pH than at neutral pH. Indeed, its half-life was 33 min at pH 5.4 and 1.6 h at pH 6.1 versus 6.8 h at pH 6.8 (Table 1).

Three phosphorylated hydrolysis products were detected with ^{31}P NMR (Figure 2A). The concentration−time plots, which depend on pH, are shown in Figure 2B,C. Their chemical shifts, which are also a function of pH in the range 5.5−7 for two of them,[30] are listed in Table 2. On the basis of the formation in time and on the temporal patterns of the degradation compounds of DDCIF (Figures 2), compound **2a** at 10.58 ppm is the first intermediate which is transformed into **3a** (δ = 6.04−6.31 ppm) and then in turn into compound **4a**, the final hydrolysis product (δ = 2.58−4.61 ppm). The second intermediate **3a** is much more stable at neutral pH than at acidic pH: the ratio of concentrations of **3a** and **4a** is 7 at pH 6.8 versus 0.6 at pH 6.1 and 0.3 at pH 5.4 after hydrolysis of DDCIF for ≈2 days.

Scheme 2. Hydrolytic Pathways of Ifosfamide and Its Dechloroethylated Metabolites from Our Data



[a] Observed at low levels at pH 5.5 after hydrolysis for ≥7 days (from 2DCIF) or ≥17 days (from 3DCIF) and at pH 6.8 after hydrolysis for ≈40 days from 2DCIF. [b] Observed from hydrolysis of IF at pH ≈0 and 2.0, from hydrolysis of 2DCIF and IF at pH 3.0 and 5.5, and from hydrolysis of compounds **2b,d** at pH 6.0. [c] Observed from hydrolysis of 2DCIF and IF at pH 5.5, from hydrolysis of compounds **2b,d** at pH 6.0, and from hydrolysis of 2DCIF at pH 6.8. [d] Observed at pH 6.8 for >20 days of hydrolysis (from 2DCIF). All the compounds are represented in neutral forms.

Table 1. ^{31}P NMR-Derived Kinetic Data for Hydrolysis Reactions of DDCIF, 2DCIF, 3DCIF, and IF as Well as Their First Intermediates at Various pH and 25 °C (unless otherwise specified)

		degradation rate constants and half-life[a]					
pH	compd	k_1	$t_{1/2}$ (**1**)	k_2	$t_{1/2}$ (**2**)	k_3	$t_{1/2}$ (**3**)
6.8	DDCIF (**1a**)	1.7×10^{-3} min^{-1}	6.8 h				
6.1	DDCIF (**1a**)	7.1×10^{-3} min^{-1}	1.6 h	2.4×10^{-4} min^{-1}	2.0 days	7.0×10^{-4} min^{-1}	16.4 h
5.4	DDCIF (**1a**)	2.1×10^{-2} min^{-1}	33 min				
6.8	2DCIF (**1b**)	1.3×10^{-5} min^{-1} (≈20 °C)	36 days				
5.5	2DCIF (**1b**)	1.3×10^{-4} min^{-1} (≈20 °C)	3.8 days				
3.0	2DCIF (**1b**)	2.8×10^{-2} min^{-1}	25 min				
6.0	**2b**			8.6×10^{-4} min^{-1}	13.5 h	5.0×10^{-3} min^{-1}	2.3 h
5.5	3DCIF (**1c**)	1.0×10^{-5} min^{-1} (≈20 °C)[b]	49 days				
3.0	3DCIF (**1c**)	4.1×10^{-3} min^{-1}[b]	2.8 h				
6.0	**2c**			3.9×10^{-4} min^{-1}	1.2 days	9.3×10^{-4} min^{-1}	12.4 h
3.0	IF (**1d**)	1.1×10^{-4} min^{-1} (≈20 °C)	4.3 days				
2.0	IF (**1d**)	2.2×10^{-3} min^{-1}[b]	5.3 h				
≈0	IF (**1d**)	0.16 min^{-1}	4.4 min				
6.0	**2d**			2.7×10^{-3} min^{-1}	4.3 h	7.0×10^{-3} min^{-1}	1.6 h

[a] k_1, k_2, and k_3 are the rate constants for the disappearance of compounds **1**, **2**, and **3**, respectively, and their derived half-lives are $t_{1/2}$(**1**), $t_{1/2}$(**2**), and $t_{1/2}$(**3**). [b] Since the pH of the solution drifted more than 0.2 pH unit (0.25−0.35 unit), this rate constant can only be viewed as an estimate.

We did not observe any formation of phosphate anion (Pi) over hydrolysis for 4 days (pH 5.4 and 6.1) or 5 days (pH 6.8) as verified by spiking with authentic standard.

2. Identification of Hydrolysis Products. The three hydrolysis products of DDCIF (**2a**−**4a**) were separately obtained in aqueous solutions in purities ranging from 80% to 100% as determined by ^{31}P NMR by acting on pH conditions and duration of hydrolysis. Their structures could thus be determined from their mass spectrometric and NMR characteristics (Table 3).

The carbon and proton signals from DDCIF were readily attributed from their characteristic chemical shifts. The three carbon atoms in DDCIF are coupled to the phosphorus atom, and disappearance of coupling of C4 in compounds **2a** and **4a** is indicative of breakdown of the endocyclic P−N bond in DDCIF. So the first degradation intermediate of DDCIF, compound **2a** with a molecular mass of 154 Da (18 Da higher than that of DDCIF (136 Da)), comes from hydrolysis of the endocyclic P−N bond of DDCIF and corresponds to the phosphoramidic acid monoester shown in Scheme 2 and Table 3.

Compound **3a**, in which the C4 has a C−P coupling constant, must have a cyclic structure. The molecular weight of 137 Da of this compound is 1 Da above that of DDCIF and 17 Da less than that of **2a**. This indicates that the amino group in DDCIF is replaced by a hydroxyl group. Compound **3a** is thus a 2-hydroxyoxazaphosphorine (Scheme 2 and Table 3).

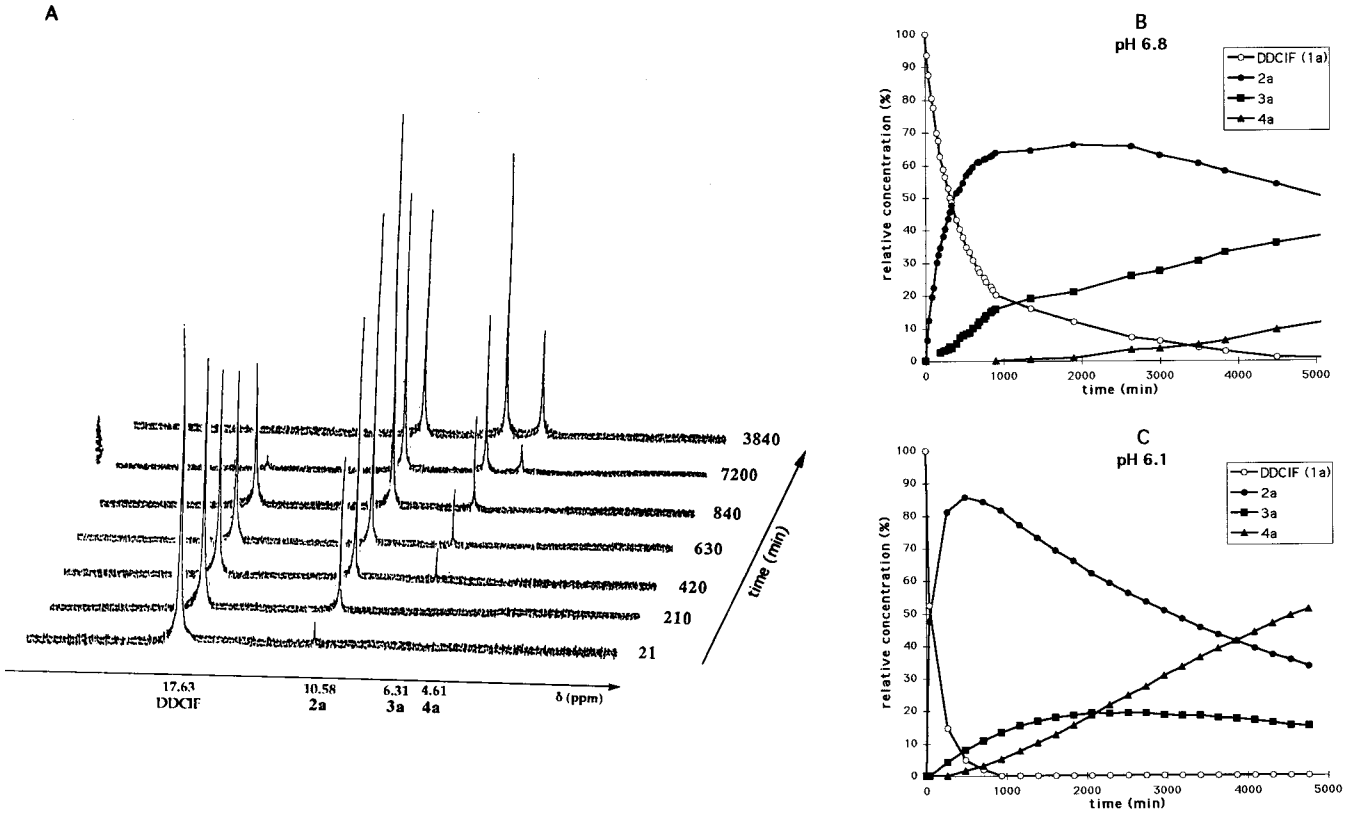


Figure 2. Stack plot of ^{31}P NMR spectra recorded during DDCIF (**1a**) hydrolysis in 0.1 M cacodylate buffer at pH 6.8, 25 °C (A). ^{31}P NMR time course at pH 6.8 (B) and 6.1 (C).

Table 2. ^{31}P NMR Chemical Shifts of Hydrolysis Products of IF and Its N-Dechloroethylated Metabolites

	^{31}P chemical shifts [a]													
	starting compounds [b]				hydrolysis products									
pH	DDCIF (**1a**)	2DCIF (**1b**)	3DCIF (**1c**)	IF (**1d**)	**2a**	**2b**	**2c**	**2d**	**3a**	**3b**	**4a**	**4 b-1**	**4 b-2**	**4 b-3**	Pi
7	17.63				10.58				6.31		4.61				
6.0					10.58				6.24		3.46				
5.5					10.58				6.04		2.58				
7		17.68				10.64							4.78	4.85	2.70
6.0[c]						10.64				7.83		3.76	3.66		
5.5						10.64				7.83		2.76	2.68		1.10
3						8.91						1.31			
7			15.72								4.61				
6.0[c]							9.49		6.24		3.46				
5.5							9.49		6.04		2.58				1.10
3.0							9.37		−6.28		1.31				
6.0[c]								9.54		7.83		3.76	3.66		
5.5				15.75								2.76	2.68		
3.0								9.37				1.31			
2.0								8.25				1.27			
≈0								5.68				0.53			

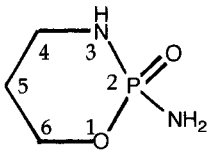
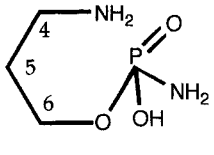
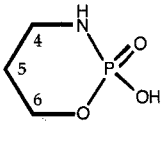
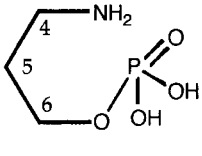
[a] Chemical shifts (δ) are expressed in ppm relative to external 85% H_3PO_4. [b] Chemical shifts of starting compounds are insensitive to pH. [c] Chemical shifts of compounds obtained on hydrolysis of first degradation intermediates of 2DCIF (**2b**), 3DCIF (**2c**), or IF (**2d**).

The final hydrolysis product, **4a**, has a molar mass of 155 Da, 1 Da above that of **2a**. This is indicative of a substitution of an amino group of **2a** by a hydroxyl group. Compound **4a** thus corresponds to the phosphoric acid monoester (Scheme 2 and Table 3), the structure of which was confirmed by independent synthesis of compound.[28]

It can be seen from Table 3 that the ^{13}C chemical shifts of the cyclic compounds (DDCIF and **3a**) are similar, as are those of the linear compounds **2a** and **4a**. On the other hand, the C4 and C6 are more shielded (3−5 and 5−7 ppm, respectively) in the linear compounds than in the cyclic ones. Moreover, a strong shielding of the ^{31}P chemical shift was observed (≈11−12 ppm for the oxazaphosphorine derivatives and ≈6−8 ppm for the linear compounds at pH 5.5−7) when the 2-amino group was replaced by a 2-hydroxyl group (Table 2). These observations helped determine the structures of the hydrolysis products of 2DCIF, 3DCIF, and IF.

Hydrolysis of 2DCIF. 1. ^{31}P NMR-Derived Time Courses for Hydrolytic Breakdown. The rate of hydrolysis of 2DCIF was lower than that of DDCIF but increased with decrease in pH. The half-life of 2DCIF

Table 3. Mass Spectrometry and NMR Data of DDCIF (**1a**) and Its Phosphorus-Containing Hydrolysis Compounds

	 **1a**	 **2a**	 **3a**	 **4a**	
FAB $^+$ data (m/z)		[MH]$^+$ 155 [MNa]$^+$ 177	[MH]$^+$ 138 [MNa]$^+$ 160	[MH]$^+$ 156	
NMR data δ (ppm), multiplicity, J(Hz)	^{13}C H_2O (pH=7.5)	^{1}H D_2O (pD=6.5)[a]	^{13}C H_2O (pH=6.9)	^{13}C H_2O (pH=7.1)	^{13}C H_2O (pH=4.6)	^{1}H D_2O (pD=5.1)[a]
position of C 4	43.6	3.23	40.4	45.0	40.2	3.08
atoms or their	d, 2.9	m	s	d, 4.4	s	t, 7.0
protons 5	28.4	1.81	30.4	29.8	30.5	1.92
	d, 7.1	m	d, 7.2	d, 6.7	d, 7.0	app. quin[b] 5.7, 7.0
6	71.9	4.33	65.1	70.8	66.0	3.90
	d, 6.3	m	d, 5.1	d, 5.5	d, 5.4	app. q[b] 5.7, 6.7[c]

[a] Observed pH readings, not corrected for kinetic isotope effect. [b] Coupling constants determined from homo- or heteronuclear spin-decoupling experiments. [c] $^3J_{H-P}$.

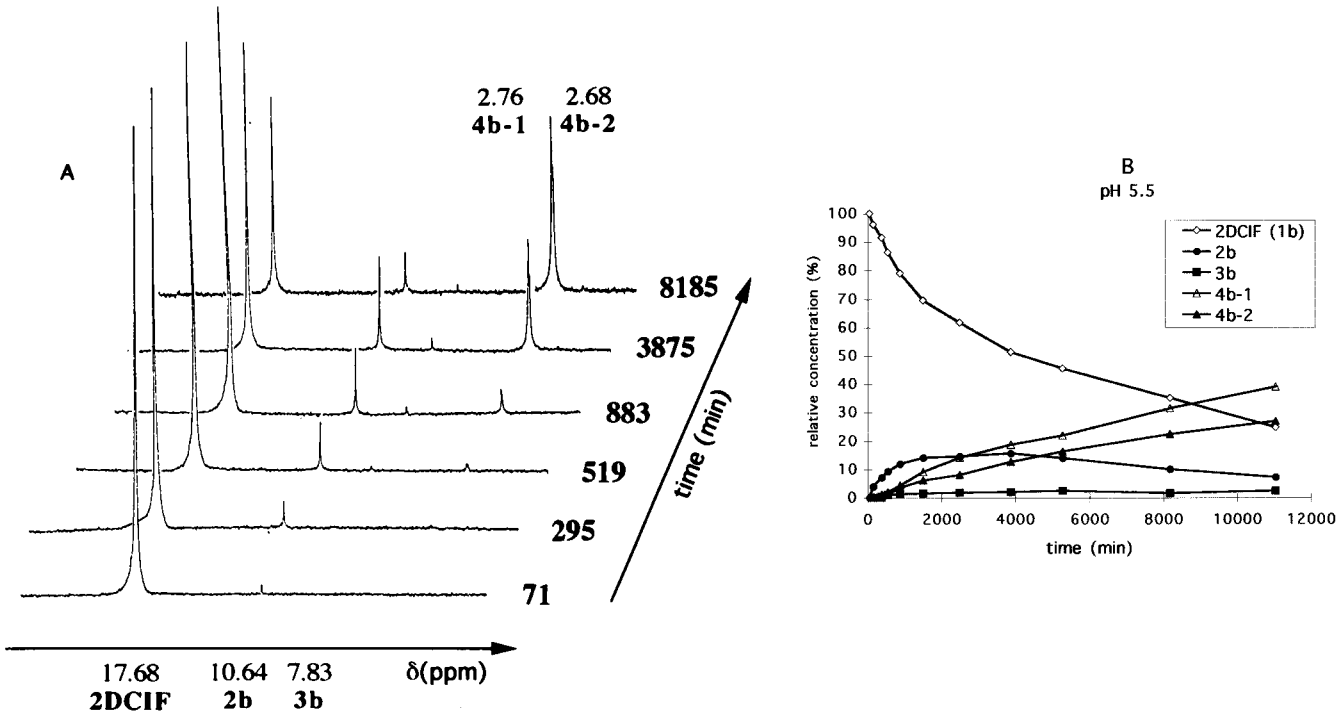


Figure 3. Stack plot of ^{31}P NMR spectra recorded during 2DCIF (**1b**) hydrolysis in 0.1 M cacodylate buffer at pH 5.5, 25 °C (A). Corresponding ^{31}P NMR time course (B).

fell from 36 days at pH 6.8, to 3.8 days at pH 5.5, to 25 min at pH 3.0 (Table 1).

Six different phosphorylated degradation products were observed depending on the pH of the medium and the duration of hydrolysis. Compound **2b** giving a signal at 8.91−10.64 ppm[30] (Table 2) is the first intermediate in the hydrolytic pathway (Figure 3). It was detected at all values of pH, but at pH 6.8 it never made up more than 2% of all phosphorylated compounds and was only detected between the 2nd and 11th days of hydrolysis. Compound **3b** (δ = 7.83 ppm) was only observed in small amounts (<3% of all ^{31}P NMR signals) and only after hydrolysis at pH 5.5. After hydrolysis for 6 h at pH 5.5, compounds **4b-1** and **4b-2** giving signals at 2.76 and 2.68 ppm, respectively (Figure 3A), were detected, and their signal intensities in an ≈60/40 ratio increased with time (Figure 3B). Compound **4b-1** was the sole detected one at pH 3.0 (δ = 1.31 ppm) and compound

4b-2 the sole one at pH 6.8 (δ = 4.78 ppm). The signal from Pi (δ = 1.10 ppm), identified by spiking with standard, was detected after 7 days of hydrolysis at pH 5.5, and its intensity increased with time to make up ≈2% of the hydrolysis products of 2DCIF after 7 days and ≈6% after 28 days of hydrolysis.

At pH 6.8, after hydrolysis for more than 20 days, we observed another signal at 4.85 ppm (compound **4b-3**) whose intensity increased with time and to a greater extent than that of compound **4b-2**. At this pH, we observed traces of Pi (δ = 2.70 ppm) after hydrolysis for ≈40 days.

2. Identification of Hydrolysis Products. Compounds **2b**, **4b-1**, and **4b-2** were separately obtained from 2DCIF in aqueous solutions at ≈100% purity as determined by ^{31}P NMR by acting on pH conditions and duration of hydrolysis. Compound **4b-3** was analyzed in a 64/20/16 mixture with compounds **4b-2** and starting 2DCIF (**1b**), respectively. Compound **3b** could not be isolated due to its instability, and its structure was only established from its ^{13}C NMR features on monitoring the degradation of compound **2b** at pH 6.0. The mass spectrometry data and the NMR characteristics (^{13}C and in some cases ^{1}H) of 2DCIF and all its degradation compounds are listed in Table 4.

The proton and carbon-13 signals of 2DCIF were attributed from ^{1}H spectra with ^{31}P decoupling and COSY, HETCOR, and HMBC correlations. The endocyclic C4 did not exhibit an observable $^{2}J_{C-P}$ coupling constant in contrast to the exocyclic C7. The $^{3}J_{C8-P}$ coupling constant was higher than the $^{2}J_{C7-P}$ one.

The FAB mass spectra of compound **2b** showed that it contained a chlorine atom and had a molar mass 18 units (216, 218 Da) above that of 2DCIF (198, 200 Da). Together with the disappearance of the C−P couplings on the two carbons C7 and C8 of the chloroethyl chain, this indicated that compound **2b** was the phosphoramidic acid monoester formed by hydrolysis of the endocyclic P−N bond of 2DCIF (Scheme 2 and Table 4).

The ^{13}C NMR characteristics of compound **3b** resembled those of 2DCIF where the two carbons atoms in the *N*-chloroethyl chain exhibit coupling with the phosphorus atom. Compound **3b** thus has the same carbon skeleton as 2DCIF. The ^{31}P chemical shift (Table 2) was close to that of compound **3a** indicating that the immediate neighborhood of the P atom was the same as that in **3a**. Moreover, the high shielding (≈10 ppm) of its ^{31}P chemical shift with respect to that of 2DCIF is comparable to the ^{31}P $\Delta\delta$ between compound **3a** and DDCIF (Table 2). Compound **3b** is thus a 2-hydroxyoxazaphosphorine (Scheme 2 and Table 4).

The molar mass of compound **4b-1** indicated that it contained a chlorine atom and, in comparison with compound **2b**, an amino group replaced by a hydroxyl group (Table 4). The protons and carbons were attributed from the COSY and HETCOR correlations, showing that compound **4b-1** was the phosphoric acid monoester shown in Scheme 2 and Table 4. Its structure was confirmed by obtaining this compound from IF hydrolysis for 24 h in 1 M HCl.

Compound **4b-2** harbored an aziridine ring as evidenced by the loss of the ^{1}H and ^{13}C signals from the *N*-chloroethyl chain of compound **4b-1** and the appearance of signals highly shielded by the strain of the three-membered ring: in ^{1}H NMR, two doublets at δ = 2.02 and 1.68 ppm corresponding to the two protons of the aziridine ring in trans and cis positions with respect to the third substituent on N3[31] and in ^{13}C NMR, one singlet whose intensity corresponds to two carbon atoms.

The mass spectrum of compound **4b-3** showed that it did not contain a chlorine atom and that relative to compound **4b-1**, this chlorine had been replaced by a hydroxyl group (Table 4). The ^{13}C NMR data showing the presence of two CH_2-O groups and the fact that this compound was always found together with compound **4b-2** were evidence that compound **4b-3** was the phosphoric acid monoester resulting from hydrolysis of the aziridine ring of compound **4b-2** (Scheme 2 and Table 4).

Hydrolysis of 3DCIF. 1. ^{31}P NMR-Derived Time Courses for Hydrolytic Breakdown. 3DCIF is more stable than either DDCIF or 2DCIF. We did not observe any degradation products after 4 days of hydrolysis at pH 6.8 and only a single product (≈2% degradation) after 28 days under the same conditions. But as for DDCIF and 2DCIF, the rate of degradation of 3DCIF was inversely proportional to pH: half-life of 49 days at pH 5.5 and 2.8 h at pH 3.0 (Table 1). Since the pH variations in these experiments respectively reached 0.25 and 0.3 pH unit, these values can only be considered as an estimate.

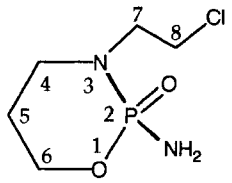
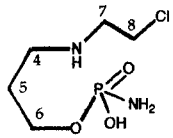
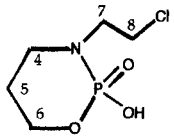
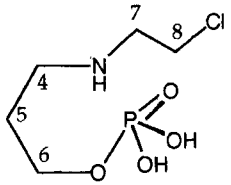
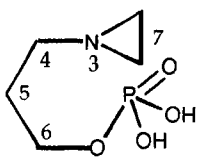
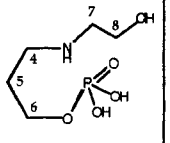
Three phosphorylated degradation compounds were formed (Figure 4A). Compound **2c** (δ = 9.37−9.49 ppm)[30] (Table 2) was the first intermediate in the 3DCIF degradation pathway (Figure 4B), but it was not observed at pH 6.8 and only detected in small amounts at pH 5.5 (≈2% of all ^{31}P NMR signals) due to the high stability of 3DCIF at these values of pH. A second compound whose chemical shift lays between −6.28 and +6.04 ppm was detected at pH 3.0 and 5.5, but it never exceeded 3% and 1%, respectively, of all the phosphorylated products. The third compound at δ = 1.31−4.61 ppm was the final hydrolysis product as its concentration increased with time (Figure 4B). It was the only degradation product observed at pH 6.8 and by far the major (≥80%) product detected after 17 days of hydrolysis at pH 5.5.

The signal of Pi was observed after 17 days of hydrolysis at pH 5.5, with an intensity that increased with time. However, after 31 days of hydrolysis, it only made up <10% of the degradation products. It was not detected after 28 days of hydrolysis at pH 6.8.

2. Identification of Hydrolysis Products. Compounds **2c** and **4a** were separately obtained from 3DCIF (**1c**) at ≈100% purity as determined by ^{31}P NMR by acting on pH conditions and duration of hydrolysis, and after purification by HPLC for **2c**. Compound **3a** was obtained with a "phosphorus purity" of ≈75% from aqueous degradation of **2c** at pH 8.

The proton and carbon-13 signals of 3DCIF were attributed from the ^{1}H spectra with phosphorus decoupling and the COSY, HETCOR, HETCOR-LR, and HMBC correlations (Table 5). The ^{13}C (δ and *J*) characteristics of the carbon atoms in the oxazaphosphorine ring resembled those of DDCIF with a $^{2}J_{C4-P}$ coupling observable, contrarily to 2DCIF.

Table 4. Mass Spectrometry and NMR Data of 2DCIF (**1b**) and Its Phosphorus-Containing Hydrolysis Products

	 **1b**		 **2b**	 **3b**	 **4b-1**		 **4b-2**		 **4b-3**
FAB + data (m/z)			[MH]+ 217, 219 [MNa]+ 239, 241		[MH]+ 218, 220				[MH]+ 200 [MNa]+ 222
NMR data	^{13}C [a]	^{1}H [b]	^{13}C	^{13}C	^{13}C [e]	^{1}H [e]	^{13}C	^{1}H	^{13}C
δ (ppm)	D_2O	D_2O	H_2O	H_2O	D_2O	D_2O	H_2O	D_2O	H_2O
multiplicity, J (Hz)	(pD=6.1) [c]	(pD=6.1) [c]	pH=6.95	pH=6.0	(pD=5.85) [c]	(pD=5.85) [c]	pH=5.1	pD=9.1 [c]	pH=6.65
position of C atoms 4	49.3	3.23	48.3	50.7	48.2	3.23	54.5	2.54	48.6
or their protons	s	m	s	s	s	t, 7.0	s	t, 6.8	s
5	28.4	1.94	29.3	28.2	29.2	2.00	30.0	1.82	29.3
	d, 4.7	m	d, 6.5	d, 4.2	d, 5.8	app. quin, 6.2	d, 6.9	app. quin, 6.6	d, 6.3
6	71.1	4.29	64.9	70.2	64.8	3.91	65.1	3.78	64.7
	d, 6.5	td, 12.3 [d], 5.4	d, 5.0	d, 5.6	d, 4.9	app q, 6.1	d, 5.6	app. q, 6.2	d, 4.6
7	51.9	3.23 (1H) and 3.35 (1H)	51.7	52.5	51.4	3.44		2.02 (2H, trans N-CH_2)	52.0
	d, 3.2	m m	s	d, 0.9	s	t, 5.6	32.8	d, 4.5	s
8	44.7	3.66	42.1	45.0	41.9	3.87	s	1.68 (2H, cis N-CH_2)	59.6
	d, 4.3	m	s	d, 6.4	s	t, 5.6		d, 4.5	s

[a] Assignments obtained from HETCOR and HMBC correlations. [b] Assignments obtained from ^{31}P-decoupled ^{1}H NMR spectra and COSY correlation. [c] Observed pH readings, not corrected for kinetic isotope effect. [d] $^{3}J_{H-P}$. [e] Attribution of the ^{1}H and ^{13}C signals from COSY and HETCOR correlations.

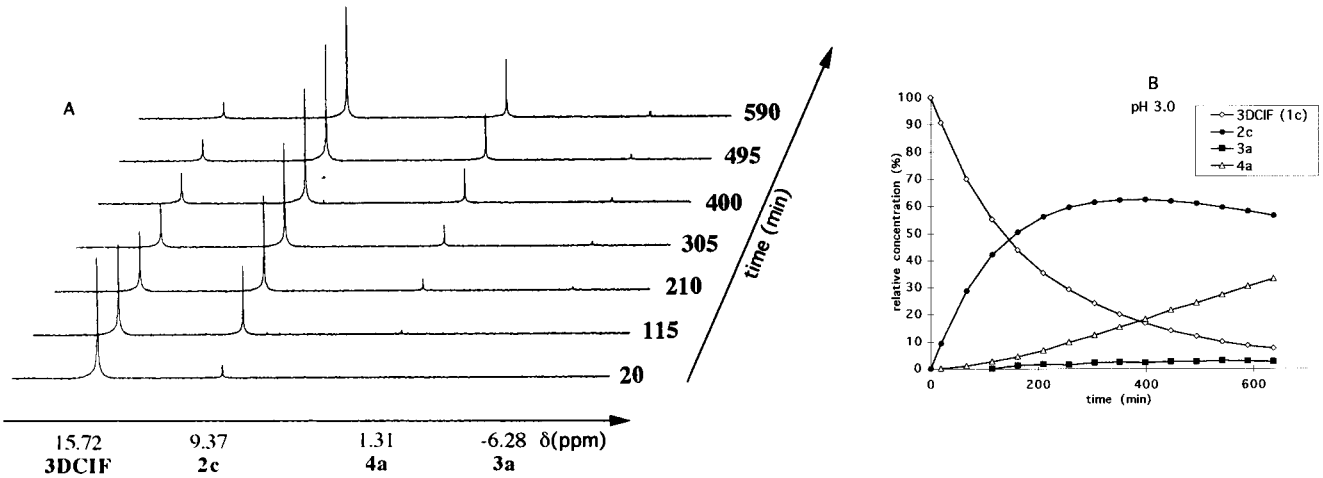


Figure 4. Stack plot of ^{31}P NMR spectra recorded during 3DCIF (**1c**) hydrolysis in 0.2 M phthalate buffer at pH 3.0, 25 °C (A). Corresponding ^{31}P NMR time course (B).

The structure of compound **2c** was established by comparison of its ^{13}C NMR and MS data with those of 3DCIF. The ^{13}C characteristics of compound **2c** were close to those of 3DCIF for the carbon atoms in the chloroethyl chain with a $^{3}J_{C10-P}$ coupling and to those of compound **2a** for the other carbons with no $^{2}J_{C4-P}$ coupling. Moreover, C4 and C6 were shielded by ≈3 and ≈7 ppm, respectively, with respect to those of 3DCIF, as were those of compound **2a** with respect to those of DDCIF (Tables 5 and 3). This indicated that compound **2c** derived from hydrolysis of the endocyclic P−N bond of 3DCIF like compound **2a** from DDCIF. The mass spectral data of compound **2c** confirmed that it contained a chlorine atom ([MH]$^+$ ions at m/z = 217 and 219 in a 3/1 ratio) and had a molar mass 18 Da more than that of 3DCIF (Table 5). Compound **2c** was thus the phosphoramidic acid monoester shown in Scheme 2 and Table 5.

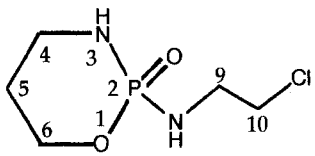
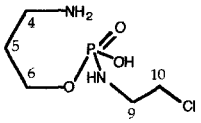
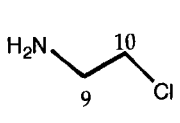
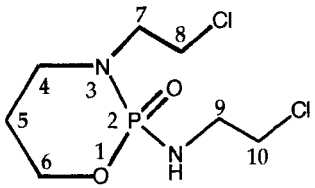
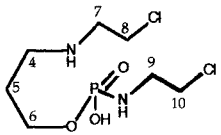
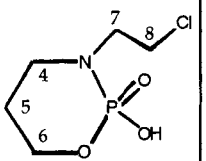
The ^{31}P chemical shifts of the second (δ = −6.28−+6.04 ppm) and final (δ = 1.31−4.61 ppm) hydrolysis products of 3DCIF were identical to those of compounds **3a** and **4a** resulting from hydrolysis of DDCIF. This was demonstrated by spiking the hydrolytic mixture of 3DCIF with that of DDCIF. Their ^{13}C NMR and mass spectrometric characteristics confirmed their structures as being those of compounds **3a** and **4a** (no chlorine atom or *N*-chloroethyl chain). This indicated that at this stage of hydrolysis of 3DCIF, CEA had been liberated. No signal at ≈25 ppm characteristic of free aziridine was detected. By spiking the samples with authentic material, signals from CEA were detected in the ^{13}C NMR spectra of compounds **3a** (from hydrolysis of compound **2c** at pH ≈8) and **4a** (from hydrolysis of 3DCIF in 1 M HCl) and hydrolytic mixtures of 3DCIF at pH 5.5 or 3.0 (Table 5). The ^{13}C signals of CEA were attributed from HETCOR correlations of CEA solutions in D_2O at pD in the range 6−13. The direction of the titration shift is opposite from ^{1}H and ^{13}C nuclei: downfield for ^{1}H, upfield for ^{13}C with decreasing pH. Throughout the pH range studied, the ^{1}H chemical shift of the CH_2Cl group is downfield from that of the CH_2N group. The same finding was observed for the ^{13}C chemical shifts at basic pH. Protonation of the amino group causes a larger upfield shift in the resonance of the β-carbon (−6.9 ppm for the CH_2Cl group) than the directly bonded α-carbon (−1.3 ppm). The CH_2Cl group is thus more shielded than the CH_2N group at acidic pH, the chemical shift of the two groups being the same at pD 7.7.

Hydrolysis of IF. 1. ^{31}P NMR-Derived Time Courses for Hydrolytic Breakdown. IF is more stable than its N-dechloroethylated metabolites. At pH 6.8 and 5.5, no degradation product was detected after hydrolysis for 1 month. At pH 5.5, after 49 days of hydrolysis, the two degradation products detected in almost equal amounts at δ = 2.76 and 2.68 ppm only made up around 4% of all the phosphorylated compounds. The rate of degradation of IF was inversely related to the pH of the medium, with a half-life of 4.3 days at pH 3.0, 5.3 h at pH 2.0 (estimated value since the pH of the solution drifted 0.35 pH unit during the experiment), and 4.4 min in 1 M HCl (Table 1). At these pH, hydrolysis gave rise to a degradation intermediate (compound **2d**) at δ between 9.37 and 5.68 ppm[30] (Table 2) whose rate of formation and level were inversely related to pH: ≈3% after 21 h of hydrolysis at pH 3.0, ≈8% after 2 h of hydrolysis at pH 2.0, and 27% after ≈4 min in 1 M HCl. This intermediate was transformed into the final hydrolysis product (δ = 0.53−1.31 ppm) identical to the one obtained at pH 5.5 and resonating at 2.76 ppm. We failed to detect Pi after 2−3 days of hydrolysis at pH ranging from ≈0 to 3.0 or 30−49 days at pH 6.8 and 5.5.

2. Identification of Hydrolysis Products. The proton and carbon-13 signals of IF were attributed from similar NMR analyses to those conducted on 3DCIF. The ^{13}C characteristics of IF resembled those of 3DCIF for C9 and C10 and those of 2DCIF for the other carbon atoms, although in contrast to 2DCIF, a C4−P coupling was observed for IF (Tables 4 and 5).

Pure compound **2d** was obtained after Sep-Pak chromatography of the hydrolytic mixture after 30 min of IF hydrolysis in 1 M HCl. The FAB positive mass spectrometry data showed that compound **2d** contained two chlorine atoms and had a molar mass 18 Da above that of IF (Table 5), which indicated that, in common with the other compounds **2a**−**c**, the endocyclic P−N bond of IF had been hydrolyzed. Compound **2d** was thus the phosphoramidic acid monoester shown in Scheme 2 and Table 5. The ^{13}C NMR data showing the disap-

Table 5. Mass Spectrometry and NMR Data of 3DCIF (**1c**), IF (**1d**), and Their Hydrolysis Compounds[a]

		 **1c**		 **2c**	 **CEA**	 **1d**		 **2d**	 **3b**
FAB $^+$ data (m/z)				[MH]$^+$ 217, 219	[MH]$^+$ 80, 82 [MH + glycerol]$^+$ 172, 174			[MH]$^+$ 279, 281, 283	[MH]$^+$ 200, 202
NMR data		$^{13}C^b$ D_2O (pD=6.7)[c]	$^1H^d$ D_2O (pD=5.7)[c]	^{13}C H_2O (pH=7.5)	^{13}C H_2O (pH=4.1)	$^{13}C^b$ D_2O (pD=6.6)[c]	$^1H^d$ D_2O (pD=6.8)[c]	^{13}C D_2O (pD=6.0)[c]	^{13}C D_2O (pD=6.0)[c]
δ (ppm)									
multiplicity, J (Hz)	4	43.3	3.23	40.3		49.3	3.26	47.9	50.7
		d, 2.8	m	s		d, 1.1	m	s	s
	5	28.1	1.82	30.4		28.2	2.00	29.0	28.2
		d, 6.9	m	d, 7.4		d, 5.2	m	d, 7.1	d, 4.2
	6	71.7	4.37	64.9		71.1	4.35	64.5	70.2
position of C atoms		d, 6.6	td, 5.6, 11.3[e]	d, 5.0		d, 6.9	td, 5.6, 12.3[e]	d, 5.3	d, 5.6
or their protons	7					51.7	3.26	51.3	52.5
						d, 3.4	m	s	d, 0.9
	8					44.4	3.73[g]	41.8	45.0
						d, 4.2	m	s	d, 6.4
	9	44.9	3.23	46.0	44.3[f]	45.1	3.26	45.7	
		s	m	s	s	s	m	s	
	10	48.0	3.60	48.5	43.8	48.0	3.66[g]	48.3	
		d, 4.5	t, 5.9	d, 5.8	s	d, 5.0	t, 5.9	d, 6.1	

[a] The MS and NMR data of the other hydrolysis products of 3DCIF (**1c**) (compounds **3a** and **4a**) and IF (**1d**) (compounds **4b-1** and **4b-2** as well as CEA) are only mentioned in the Experimental Section since they are identical to those listed in Tables 3−5 for the same compounds. [b] Attributions from HETCOR, HETCOR-LR, and HMBC correlations. [c] Observed pH readings, not corrected for kinetic isotope effect. [d] Assignments obtained from ^{31}P-decoupled ^1H NMR spectra and COSY correlation. [e] $^3J_{H-P}$. [f] Signals attributed from HETCOR correlation of CEA solution in D_2O at pD 6.5. [g] Attribution on the assumption that the CH_2Cl protons (8) resonate in the form of m as in 2DCIF (**1b**), while those of CH_2Cl (10) resonate as t as in 3DCIF (**2c**).

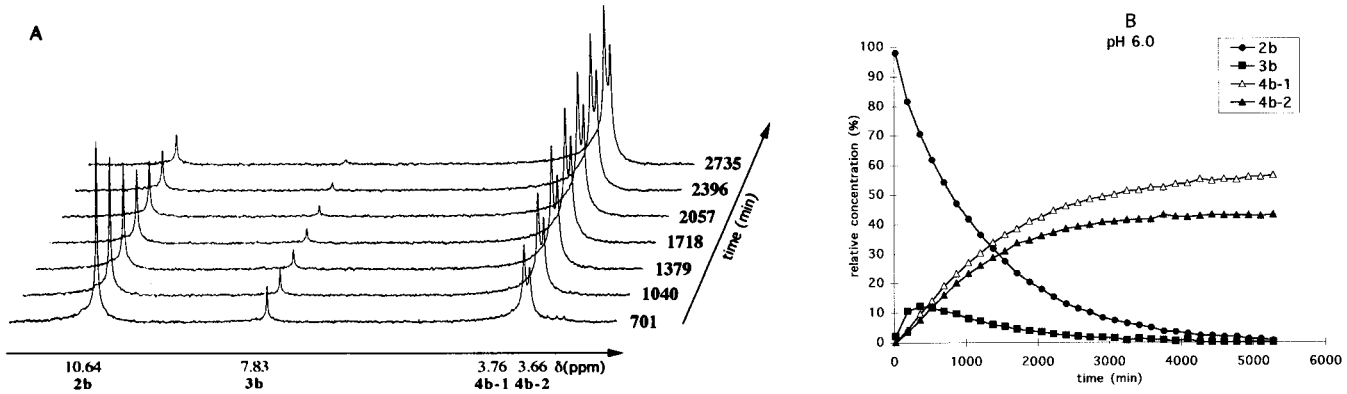


Figure 5. Stack plot of ^{31}P NMR spectra recorded during hydrolysis of the phophoramidic acid monoester **2b** in cacodylate buffer at pH 6.0, 25 °C (A). Corresponding ^{31}P NMR time course (B).

pearance of the couplings between P and C4, C7, and C8 were consistent with this structure.

The compounds at $\delta = 0.53-2.76$ ppm (pH $\approx 0-5.5$) and 2.68 ppm (pH 5.5) had the same ^{31}P chemical shifts as compounds **4b-1** and **4b-2**, respectively, as demonstrated by spiking hydrolytic mixtures of IF with standards. The mass spectra and ^{13}C NMR characteristics of these two compounds obtained with a "phosphorus purity" of 100% from IF hydrolysis for 24 h in 1 M HCl for the first one followed with alkalinization at pH $\approx$ 13 for 24 h for the second one confirmed that they were compounds **4b-1** and **4b-2**. This means that hydrolysis liberated CEA, which was detected in the ^{13}C spectrum of a reaction mixture from hydrolysis of IF at pH $\approx$ 0.

Hydrolysis of the Phosphoramidic Acid Monoesters 2a−d. The hydrolysis at pH 6 of the first intermediates **2a−d** confirmed the pathways shown in Scheme 2. Compound **2a**, the first intermediate in the hydrolytic pathway of DDCIF, is relatively stable at pH 6.1, with a half-life of ≈2 days (Table 1). The breakdown of compound **2a** led to the formation of compounds **3a** (δ = 6.24 ppm) and **4a** (δ = 3.46 ppm) (Table 2). Compound **3a** is an intermediate in the degradation process, which makes up a maximum of 19% of all the phosphorylated compounds between ≈34 and ≈46 h of hydrolysis. It is less stable than compound **2a** ($t_{1/2} \approx$ 0.7 day versus 2 days) (Table 1), and its hydrolysis leads to compound **4a**, the terminal degradation product (Scheme 2).

The hydrolysis rate of compound **2b** at pH 6.0 was faster than that of compound **2a** (half-life ≈ 0.6 day versus 2 days) (Table 1). The products formed were the same as those obtained during the hydrolysis of 2DCIF at pH 5.5. Compound **3b** appeared as an intermediate in the degradation process. Its ^{31}P signal reached a maximum between ≈6 and 9 h of hydrolysis and never exceeded ≈12% of all the phosphorylated compounds (Figure 5). Its degradation rate constant was much higher than its formation rate constant (5.0×10^{-3} min^{-1} versus 8.6×10^{-4} min^{-1}) (Table 1). The final products of hydrolysis were compounds **4b-1** and **4b-2**, whose signal intensities increased with time in a fairly fixed ratio of 60/40 (ratio of their formation rate constants = 1.32) (Figure 5B). After 30 h of hydrolysis, the Pi signal was detected, but it only made up 2% of all phosphorylated products observed after 4 days of hydrolysis. Since the amount of **4b-2** relative to **4b-1** increased with increase in pH (0% at pH 3.0, ≈40% at pH 5.5−6.0, and 100% at pH 7.0), a cyclization of the *N*-chloroethyl chain of compound **4b-1** into an aziridine may occur giving rise to compound **4b-2**. We therefore followed by ^{31}P NMR the behavior of compound **4b-1** at pH 5.5 and 7.0. At pH 5.5, no cyclization of the *N*-chloroethyl chain into an aziridine was observed. Compound **4b-1** was slowly hydrolyzed into Pi (≈20% after 14 days and ≈40% after 44 days). At pH 7.0, no transformation of compound **4b-1** into the aziridine **4b-2** was observed during hydrolysis for 3 days. On the other hand, after 15 days, the aziridine represented ≈30% of the initial **4b-1** and ≈40% after 23 days. We thus concluded that compound **4b-2** resulted from hydrolysis of compound **3b** and not from cyclization into an aziridine of the *N*-chloroethyl chain of compound **4b-1**. Similarly, compound **4b-1** was also derived from hydrolysis of the intermediate **3b** and not from opening of aziridine ring of **4b-2** by chloride ion since, at pH 5.8 (obtained by addition of HCl), ^{31}P and ^{13}C NMR failed to detect any trace of degradation of compound **4b-2** after 18 h at room temperature.

At pH 6.0, the hydrolysis rate of compound **2c** (half-life 1.2 days) lays between those of compounds **2a** (half-life 2.0 days) and **2b** (half-life 0.6 day) (Table 1). The products obtained were identical to those obtained from hydrolysis of compound **2a**, i.e., compound **3a**, an intermediate which peaked at ≈20% after 23−40 h hydrolysis, and compound **4a**, the final hydrolysis product.

At pH 6.0, the hydrolysis rate of compound **2d** was much faster than that of the three other phosphoramidic acid esters **2a−c** ($t_{1/2}$ 4.3 h) (Table 1). The degradation pathway leads to the formation of a transient compound at 7.83 ppm (Table 2), never observed during hydrolysis of IF and which made up a maximum of ≈18% of the phosphorylated compounds observed between ≈3 and 5 h of hydrolysis (Figure 6). The final hydrolysis products were compounds **4b-1** and **4b-2**, observed in a 60/40 ratio (Figure 6B) (ratio of their rate constants of formation: 1.33), already obtained on hydrolysis of the first intermediate (**2b**) in the 2DCIF hydrolytic pathway. The transient compound whose ^{31}P chemical shift was identical to that of compound **3b** could not be isolated. Its ^{13}C NMR characteristics, determined by monitoring the degradation of compound **2d** in the NMR probe, were identical to those of compound **3b** (Tables 4 and 5). Characteristic quasi-molecular ions $[MH]^+$ at *m/z*

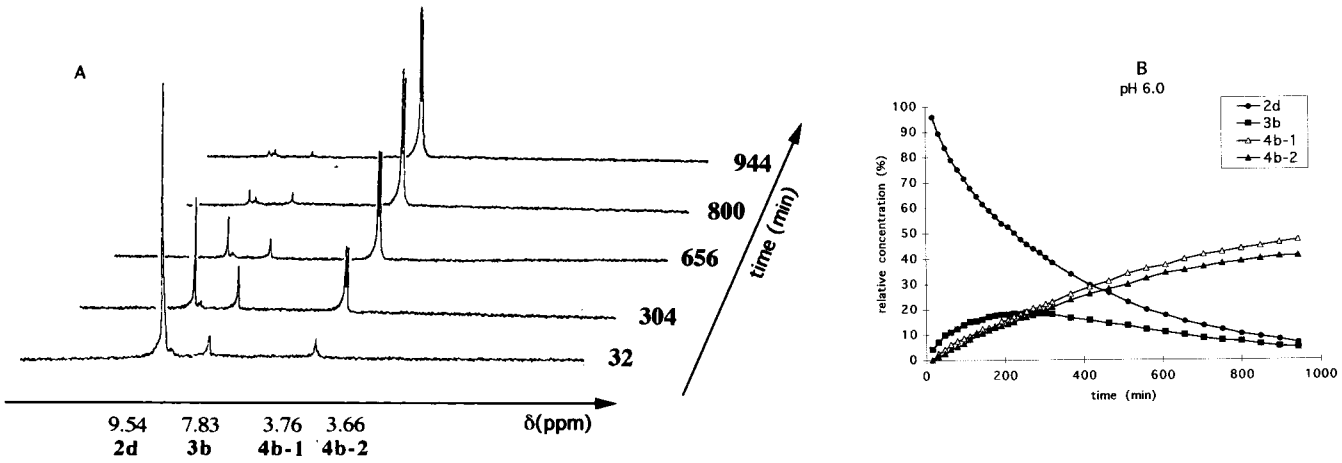


Figure 6. Stack plot of ^{31}P NMR spectra recorded during hydrolysis of the phophoramidic acid monoester **2d** in cacodylate buffer at pH 6.0, 25 °C (A). Corresponding ^{31}P NMR time course (B).

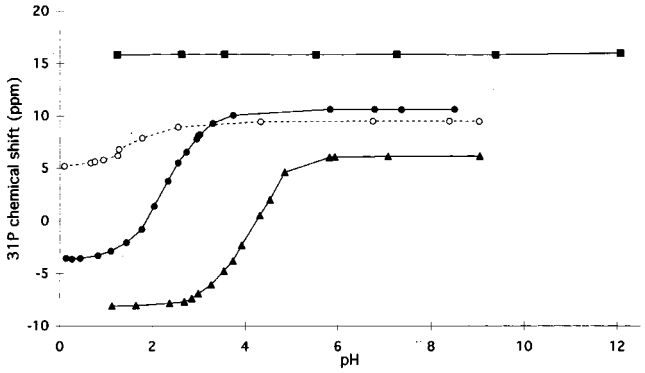


Figure 7. ^{31}P chemical shifts of IF (**1d**) (■), phosphoramidic acid monoesters **2b** (●) and **2d** (○), and 2-hydroxy-1,3,2-oxazaphosphorine (**3a**) (▲) as a function of pH at 25 °C.

200, 202 were observed in the FAB$^+$ mass spectra of degradation mixtures of compound **2d** after different hydrolysis times, along with those at *m*/*z* 80, 82 corresponding to the CEA released on transformation of compound **3b**, at *m*/*z* 218, 220 corresponding to the final hydrolysis products **4b-1**, and at *m*/*z* 182 corresponding to the other final hydrolysis product **4b-2**.

^{31}P Chemical Shifts as a Function of pH and pK_a of Hydrolysis Products of IF and Its Dechloroethylated Metabolites. To facilitate characterization by ^{31}P NMR of the hydrolysis products of DDCIF, 2DCIF, 3DCIF, and IF, the ^{31}P chemical shifts as a function of pH were determined for each type of structure: phosphoramidic acid monoester (compounds **2**), 2-hydroxyoxazaphosphorine (compounds **3**), and phosphoric acid monoester (compounds **4**) along with the starting compound (compounds **1**). The ^{31}P chemical shift for IF (**1d**) was insensitive to pH, as was that of phosphoric triamide $P(O)(NH_2)_3$, which, in common with IF, has no titratable oxygen bound to a phosphorus.[32] For the other compounds, the plots of ^{31}P chemical shifts versus pH followed a single sigmoid curve (compounds **2** and **3**) or a biphasic curve (compounds **4**). Selected data are presented in Figure 7.

Table 6 gives the values of pK_a calculated from curve fitting as well as the changes in ^{31}P chemical shift due to protonation. The pK_a ranged from 0.8 to 5.9 and corresponded to protonation of either oxygen or nitrogen for compounds **2** and **3** and oxygens for compounds **4**.

Table 6. Changes in ^{31}P Chemical Shifts Due to Protonation of Hydrolysis Products of IF and Its Dechloroethylated Metabolites

compd	range of pH studied	Δδ shift[a] (ppm)	pK_a
ifosfamide (**1d**)	12.1−1.2	≈0[b]	
phosphoramidic acid esters			
(**2b**)	$8.5-0.1_5$	−14.3	2.3
(**2c**)	9.1−0.1	−4.2	1.6
(**2d**)	9.1−0.1	−4.4	1.6
2-hydroxyoxazaphosphorine (**3a**)	$9.1-1.1_5$	−14.3	4.1
phosphoric acid esters			
(**4a**)	9.1−0	−3.65	5.9
		−0.8	0.8
(**4b-1**)	9.9−0	−3.7	5.9
		−0.8	0.8

[a] Upfield shifts are negative with decreasing pH. [b] A Δδ shift of 0.04 ppm was observed, but it was not correlated with pH.

These values are in agreement with those expected for compounds with this type of structure. Indeed, the first and the second pK_a's of compounds **4** (**4a** and **4b-1**), 0.8 and 5.9, are in line with those (1.5 and 6.3) of CH_3-OPO_3H_2 (which has a similar structure around the phosphorus atom to that of compounds **4**).[33] Moreover, the pK_a of **2b** (2.3) is very similar to that (2.5) of monomethylphosphoramidate.[34] The ΔpK_a of 0.7 between compounds **2b** and **2c,d** is due to the acid-strengthening effect of the *N*-chloroethyl group. This is in agreement with the difference observed (0.6) between the pK_a of phosphoramide mustard (protonation of P-NH_2 group) and IPM (protonation of P-NCH_2CH_2Cl group).[32,35]

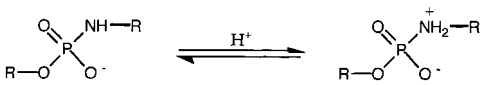
Apart from IF, the magnitude of the pH-induced shift of the ^{31}P resonance fell into three ranges: ≈−1 (i.e., upfield shift with decreasing pH), ≈−4, and ≈−14 ppm. The two smaller shifts correspond to O-protonation for the first and second P-OH ionization respectively, as demonstrated by the ^{31}P protonation shifts observed in compounds of type **4**. These values agree well with the upfield ^{31}P chemical shifts due to protonation of inorganic phosphate: −0.2, −2.5, and −2.7 ppm for the first, second, and third P-OH ionization, respectively.[32] Gamcsik et al. showed that a large pH-induced titration shift (−12 to −14 ppm) was indicative of an N-protonation site.[32,35] The ≈14 ppm upfield shift was thus assumed to stem from N-protonation. The phosphoramidic acid ester **2b** (and probably also **2a**) and the 2-hydroxy-1,3,2-

Table 7. Biological Activity of IF, Its Dechloroethylated Metabolites, and Some of Its Degradation Compounds

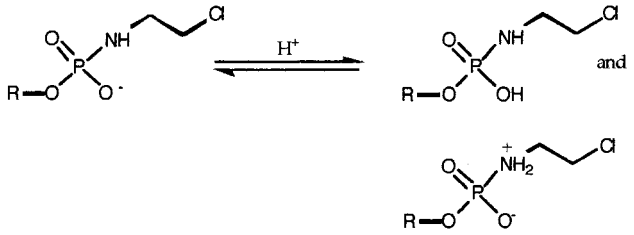
				antitumor activity[b]	
compd	species	route	LD_{50} (mg/kg)[a]	in vitro	in vivo
DDCIF (**1a**)			nd	−	nd
2DCIF (**1b**)	rat	iv	>1000	−	−
3DCIF (**1c**)	rat	iv	≈800	−	−
IF (**1d**)	rat	iv	300	−	+++
	mouse	ip	700		+++
4b-1	rat	iv	≈800	−	−
4b-2	mouse	ip	180	−	−
5	mouse	ip	>1000	−	−
CEA HCl	mouse	ip	2200	nd	nd

[a] nd, not determined. [b] +, effective; −, no activity.

oxazaphosphorine **3a** which bear a P-NH_2 or P-NH-alkyl group give rise to a zwitterion on protonation:



The large effect on pK_a produced by the *N*-alkyl substitution on going from compound **2b** (pK_a = 2.3) to compound **3a** (pK_a = 4.1) is also consistent with N-protonation in view of the marked difference in pK_a between the ammonium ion and its *N*-alkyl derivatives (9.2 versus 10.6−10.7).[33] On the other hand, the ≈−4 ppm shift observed for the phosphoramidic acid esters **2c**,**d** was much smaller than that observed for N-protonation but greater than the shifts normally observed for O-protonation of the first acidity of a phosphate group. This is consistent with the existence of both N- and O-protonated forms as already stated for IPM, a compound in which the phosphorus atom bears an anionic oxygen (or a hydroxyl group) and an *N*-chloroethyl group as in **2c**,**d**:[35]



Biological Activity of Compounds 1a−d, 4b-1, 4b-2, and 5. The biological activity of these compounds was investigated in three models: (i) acute toxicity (approximative LD_{50}) after a single ip or iv injection in mice or rats, (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 most predictive parameter of biological activity or toxic hazard seems to be the acute toxicity. The LD_{50} values and the antitumor activity are listed in Table 7. 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 in vitro, i.e., no inhibition of colony formation of L1210 cells, was observed at concentrations ranging from 0.1 to 100 μg/mL for all the compounds. Similarly, no antitumor efficacy in vivo was observed except for IF.

Discussion

Our findings are consistent with the facile cleavage of the P−N bond of phosphoramidates, phosphonamidates, or phosphinamidates in aqueous acidic solutions[36,37] since the P−N bond in IF and its dechloroethylated metabolites was cleaved at pH between 0 and 7. No P−O bond cleavage was observed.

The detailed investigations carried out by Modro and co-workers on the acidic cleavage of the P−N bond in acyclic and cyclic phosphoramidates have shown that it proceeds via the N-protonated species as a reactive form and that the amino group departs directly from the apical position of the trigonal-bipyramidal transition state or intermediate.[38] Indeed, in cyclic phosphorodiamidates for which a phosphorus atom and a nitrogen atom are incorporated into a five-membered ring, the endocyclic P−N bond is broken much more readily than the exocyclic P−N bond. Our results are in accordance with this behavior since the first compounds formed in the hydrolytic pathway of the four oxazaphosphorinanes studied are the phosphoramidic acid monoesters (**2a**−**d**) from cleavage of the P−N bond in the six-membered ring (Scheme 2). These compounds were detected on hydrolysis at pH 0−7 provided the degradation was not too slow. If it was slow, only the final hydrolysis products are observable by ^{31}P NMR as was found for hydrolysis of 3DCIF (**1c**) at pH 7.0 or IF (**1d**) at pH 5.5 (Table 2).

The decreasing order of stability to acid hydrolysis of the oxazaphosphorines studied (IF > 3DCIF > 2DCIF > DDCIF) showed that the presence of the chloroethyl electron-withdrawing group at the endocyclic and/or exocyclic nitrogens counteracted cleavage of the P−N bonds. Since only the endocyclic P−N bond is cleaved and this cleavage involves the N-protonated form, the key step governing the hydrolysis rate of **1a**−**d** is the protonation of the endocyclic nitrogen. The presence of an electron-withdrawing chloroethyl group on this nitrogen decreases its basicity, hindering N-protonation. The phosphorus atom is therefore less electrophilic and less reactive toward the weakly nucleophilic water.[39] This explains the reduced rate of P−N bond hydrolysis in 2DCIF (**1b**) relative to DDCIF (**1a**) by a factor of ≈150 at pH 6.5 and 5.5 and of IF (**1d**) relative to 3DCIF (**1c**) by a factor of ≈40 at pH 3 (Table 1), but the presence of a chloroethyl group on the exocyclic nitrogen decreases the reactivity of the endocyclic P−N bond by a much higher factor than on the endocyclic nitrogen. Indeed, the hydrolytic rate of 3DCIF (**1c**) relative to DDCIF (**1a**) is reduced by a factor of ≈2000 at pH 5.5 and that of IF (**1d**) relative to 2DCIF (**1b**) by a factor of ≈250 at pH 3 (Table 1). In other words, the hydrolysis rate of the P−N endocyclic bond was reduced by a factor of ≈10 by the presence of a chloroethyl group on the exocyclic rather than endocyclic nitrogen: the ratios of hydrolytic rate constants 2DCIF/3DCIF were 13 and 7 at pH 5.5 and 3.0, respectively (Table 1). These values are comparable to that of 9 obtained by Kaijser et al. for the ratio of 2DCIF and 3DCIF degradation rate constants in acidic aqueous medium at 70 °C.[18] These results can only be explained in terms of lower basicity of the endocyclic NH or NCH_2CH_2Cl group relative to the exocyclic nitrogen even when bearing a chloroethyl group. The possible reason is that the protonation has a more

unfavorable steric effect on the endocyclic than on the exocyclic nitrogen for which the P−N rotation minimizes the torsional strain at the tetrahedral protonated moiety. The strongly electronegative protonated exocyclic $^{+}NH_2CH_2CH_2Cl$ group increases the electrophilicity of the phosphorus atom and thus, by a compensating effect, decreases the basicity of the endocyclic nitrogen (more than the presence of a directly bonded chloroethyl group). The protonation is then much more difficult, and the cleavage of the P−N bond is slowed.

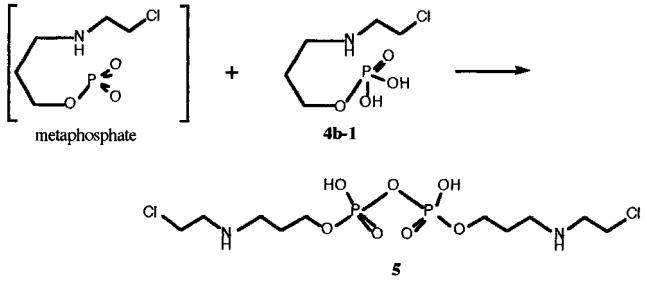
Our observations on the stability of aqueous solutions of IF (no degradation product detected after 1 month at pH 6.8 and two degradation products representing together only ≈4% of all the phosphorylated compounds after 49 days of hydrolysis at pH 5.5) are in line with its documented stability at room temperature close to neutral pH. IF has been shown to be stable for at least 1 week in sterile water for injection (pH ≈ 6.5) at room temperature and for 6 weeks under refrigeration (2−8 °C).[20] No evidence of IF decay in normal saline solution (0.9% NaCl solution) was observed after 9 days at room temperature or 27 °C, and a loss of only 7% was reported over a 9-day period at 37 °C in the same solutions.[21] Less than 0.6% degradation was observed in water for injection or 0.9% NaCl solution (pH ≈ 6.5) stored for 8 days in the disposable cassettes for portable iv pumps at 4, 25, or 35 °C.[23] A 3.2% loss of IF was found over a 7-day period at 37 °C in Ringer lactate buffer at pH 7.3.[25] The only determined IF half-life at 37 °C at natural pD (≈6) was 119 days.[19] Various other values for $t_{1/2}$ have been reported at higher temperatures: ≈12 days at 50 °C and natural pH,[24] 20 h at 70 °C in the pH range 5−9[22] (but, at the same temperature in normal saline solution (pH ≈ 6.5), another study claimed that, after 72 h, only 32−44% of IF was degraded[21]), and 6.5 h at 75 °C in water (pH ≈ 6.5).[27] From the observed rate constants for degradation of IF at various temperatures, Kaijser et al.[22] calculated a half-life of 254 days at 20 °C in the pH range 5−9 and Muñoz et al.[24] 619 days at 25 °C and pH 5.0. Since ≈4% of IF is degraded after 49 days at pH 5.5, we estimated a half-life at room temperature of 832 days, which is closer to the value reported by Muñoz et al.[24] Among the various studies on the stability of IF, only Highley et al.[26] reported a nonnegligible degradation of IF in aqueous solution at pH ranging from 4 to 10 at 37−40 °C. They detected significant amounts of CEA in the medium after 5−12 h of hydrolysis. However, acid hydrolysis during the derivatization procedure may have contributed to some of this degradation.[40] The rate constant we determined for hydrolysis of IF in 1 M HCl (0.16 min^{-1}, $t_{1/2}$ = 4.4 min) was close to that reported by Zon et al. at pD ≈0 and 20 °C (0.10 min^{-1}, $t_{1/2}$ = 6.7 min).[19]

At weakly acidic pH, cleavage of the P−N bond of compounds **2a**−**d** is accompanied by an intramolecular attack at the phosphorus atom by the non-P-linked nitrogen of the compounds **2a**−**d**. This leads to the formation of a new oxazaphosphorine ring with a 2-hydroxy substituent (instead of a 2-amino or 2-(2-chloroethyl)amino as in compounds **1a**−**d**) with the loss of ammonia or CEA moiety (compounds **3a,b**) (Scheme 2). Substitution on the non-P- or P-linked nitrogen by a chloroethyl group accelerates the formation of these compounds. Comparison of the degradation rate constants of compounds **2a**−**d** at pH 6.0 (Table 1) showed that (i) compound **3b** is formed faster than compound **3a** (by a factor of ≈4 if there is liberation of ammonia and by a factor of 7 if there is liberation of CEA), demonstrating that the nonprotonated form of the non-P-linked *N*-chloroethyl group is present at a higher concentration than that of the non-P-linked NH_2 moiety; (ii) compounds **3a**,**b** are formed 2−3 times more rapidly when the P-linked nitrogen bears the electron-withdrawing chloroethyl group which gives a better leaving ability.

The following stage in hydrolysis leads to the formation of type **4** phosphoric acid monoesters, the final hydrolysis products, by cleavage of the P−N bond of compounds **3a,b**. Compounds **3a,b** are transient reaction intermediates. Even at pH 6.0, their rate of degradation is faster than their rate of formation (Table 1). The P−N bond in the oxazaphosphorine ring is made even more labile by the chloroethyl group on the nitrogen: $t_{1/2}$ ≈ 2 h for **3b** versus ≈12−16 h for compound **3a** (Table 1). Provided that the precursors (**2a**−**d**) make up more than 2% of all ^{31}P NMR signals in the reaction medium, compound **3a** resulting from hydrolysis of DDCIF (**1a**) or 3DCIF (**1c**) is detected from pH 3.0 to 7.0, whereas compound **3b** resulting from the hydrolysis of 2DCIF (**1b**) or IF (**1d**) is only detected at pH between 5.5 and 7.0, but not at 3.0 (Table 1). At highly acidic pH, the non-P-linked nitrogen of compounds **2a**−**d** is totally protonated, so their P−N bonds are directly cleaved leading to phosphoric acid monoesters **4** without any transformation into the type **3** oxazaphosphorines. The breakdown of the P−O bond of compounds of type **4** leading to formation of Pi was only observed after long periods of hydrolysis (footnotes to Scheme 2).

It is likely that formation of compounds of type **3** or **4** from compounds **2a**−**d** occurs via a unimolecular mechanism (formation of a metaphosphate intermediate) with some nucleophilic participation. It should be borne in mind that prerequisites for the fragmentation of a substrate with the extrusion of a reactive metaphosphate derivative $X\text{-}PO_2$ are the accumulation of a negative charge on an atom directly bonded to phosphorus and a good leaving group.[41,42] For the compounds studied here, these criteria depend on the pH and the presence of a chloroethyl substituent on a nitrogen atom. For example, in weakly acidic medium, the P-NHR group of compounds **2a**−**d** is not protonated, and assistance by an incoming nucleophile (here the other nitrogen atom of the same molecule) is required for breaking the P−NHR bond and the formation of compounds **3** (Scheme 2). In strongly acidic medium, compounds **2a**−**d** will be in the P-NHR protonated form (at least in part), and the conditions for fragmentation via a transient metaphosphate are fulfilled. The presence, in the ^{31}P NMR spectrum of the hydrolytic mixture of 0.5 M IF (**1d**) at pH ≈ 0, of a singlet resonating at δ = −10.63 ppm (characteristic of a symmetrical diphosphate moiety[43]) is in favor of such a mechanistic pathway. Formal identification of this compound as the diphosphate **5** was obtained by spiking with authentic standard, which led to an increase in signal intensity. This results from the trapping of the metaphosphate

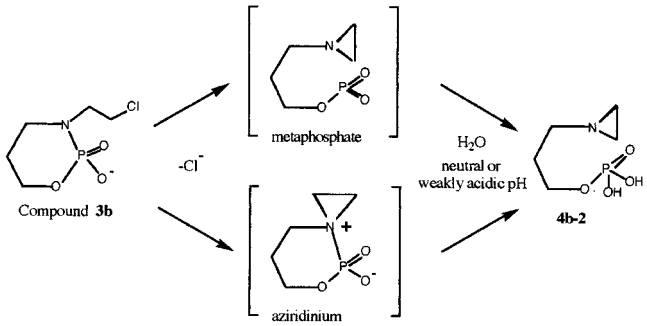
intermediate by the hydrolysis product **4b-1** as reported below:



(all the compounds are represented in their non-ionized form)

The amount of diphosphate **5** formed rises with increase in the molar ratio of the two available nucleophiles, **4b-1** and H_2O. In 2 mL of 1 M HCl, 4% of 1 mmol of IF (**1d**) was transformed into diphosphate **5** and 96% was transformed into compound **4b-1**, while 17% of the 0.7 mmol of further IF added to this mixture was transformed into diphosphate **5** and 83% into compound **4b-1**. Similarly, although 0.035 mmol of 2DCIF (**1b**) in acetonitrile in the presence of 0.1 mL of 12 M HCl was exclusively hydrolyzed into compound **4b-1**, 97% of the further 0.035 mmol of **1b** added to this mixture was also transformed into compound **4b-1** but 3% into diphosphate **5** identified by spiking with authentic standard.

Similarly, the hydrolytic decomposition of compound **3b** at neutral or weakly acidic pH leading to compound **4b-2** can proceed via the metaphosphate route. At such pH, compound **4b-2** cannot be formed by cyclization of the *N*-chloroethyl group of **4b-1** into an aziridine as demonstrated in the Results section. The formation of **4b-2** can be accounted for by a unimolecular fragmentation of compound **3b** akin to that of the N-phosphorylated nitrogen mustards described by Modro et al.[41,42] This fragmentation leads to a metaphosphate species which quickly reacts with water and to an aziridine moiety which is stable at neutral or weakly acidic pH, but an alternative mechanism involving an intermediate aziridinium zwitterion with a subsequent P−N bond hydrolysis might also be operative:[19]



With decrease in pH, cleavage of the P−N bond of the oxazaphosphorine ring in compounds **3a,b** probably proceeds via the N-protonated species, as for compounds **1a**−**d**, leading to compound **4a** or **4b-1**. At moderately acidic pH, compound **4b-1** cannot derive from opening of the aziridine ring of compound **4b-2** by chloride ion as ^{31}P NMR demonstrated that **4b-2** was stable at such pH (see Results section). On the other hand, the aziridine ring of **4b-2** reacts slowly with water at neutral pH to form an *N*-(2-hydroxyethyl) group giving rise to compound **4b-3**. This compound was observed after hydrolysis for more than 20 days.

Our results on the hydrolysis of IF are consistent in most respects with literature data. For example, the formation of compound **4b-1** and 2-chloroethylammonium ion on hydrolysis under highly acidic conditions is in line with the ^{1}H NMR study of Zon et al.[19] These authors detected an equimolecular mixture of these two compounds under similar experimental conditions (Scheme 1). The lack of detection of Pi by capillary electrophoresis among the nine degradation products of IF after hydrolysis for 48 h at 75 °C[27] is in agreement with our results and confirms the absence of P−O bond cleavage of the oxazaphosphorine ring in acidic medium. However, compound A identified by Zon et al.[19] in the degradation mixture obtained after refluxing an aqueous solution of IF (pH ≈ 6.5) for 12 days or its presumed precursors (Scheme 1) was not formed during hydrolysis of IF at room temperature at values of pH between 0 and 7.

We are not in agreement with the structure of compound D (Scheme 1) attributed by Muñoz et al. to the major of the two final hydrolysis products of IF in unbuffered D_2O at 50 °C after 21 days.[24] Our results indicate that the phosphorylated final product of IF hydrolysis should have a phosphate structure rather than the phosphoramidate structure claimed by Muñoz et al.[24] The ^{31}P NMR characteristics of the hydrolytic IF mixture should have allowed these authors to propose a phosphate ester rather than a phosphoramidate structure since the chemical shift of the phosphorus atom in these two environments is different. Indeed, the extent of the downfield ^{31}P chemical shift was directly related to the number of nitrogens bonded to phosphorus atom (Table 2). The compounds with two P−N bonds of structure 2-amino-1,3,2-oxazaphosphorine 2-oxide (DDCIF (**1a**) and 2DCIF (**1b**)) exhibited a chemical shift of ≈18 ppm. The presence of a chloroethyl group on the exocyclic nitrogen induced a further upfield shift of ≈2 ppm, producing a ^{31}P chemical shift for 3DCIF (**1c**) and IF (**1d**) of ≈16 ppm. The first intermediates in the process of hydrolysis, the phosphoramidic acid monoesters (**2a**−**d**) (Scheme 2), only harbor a single P−N bond, and their ^{31}P chemical shifts were observed between 9.5 and 10.5 ppm (Table 3) at weakly acidic and neutral pH. Their chemical shifts were shifted upfield at pH 2−3 as they have a pK_a ≈ 2 (Table 6). For this type of compounds, the presence of a chloroethyl group on the nitrogen bound to the phosphorus leads to an upfield shift of ≈1 ppm (≈9.5 ppm for **2c,d** versus ≈10.5 ppm for **2a,b**). The second intermediates with a single P−N bond in a 1,3,2-oxazaphosphorine ring (**3a,b**) at pH 5−7 exhibited a ^{31}P chemical shift of ≈6−8 ppm, which is shifted upfield at more acidic pH. The final hydrolysis products (**4a** and **4b1**−**3**) are phosphoric monoesters with no P−N bond, and their ^{31}P chemical shifts are upfield (<5 ppm). Since they have a pK_a ≈ 6 (Table 6), their ^{31}P chemical shifts range from ≈1 to 5 ppm for values of pH ranging from ≈2 to 7 (Table 2).

In an attempt to identify the final hydrolysis products of IF (**1d**) at 50 °C, we replicated the experiment described by Muñoz et al.[24] After hydrolysis for 17 days

in unbuffered H_2O at 50 °C, ^{31}P NMR detected the presence of unchanged IF (**1d**) (16%) and five phosphorylated hydrolysis products with chemical shifts close to that of the external reference, H_3PO_4. This indicated that they were phosphates. After extraction of the residual IF with chloroform, ^{31}P NMR showed the presence of compound **4b-3** (55%) and four minor components. Three of these four compounds were identified by spiking with authentic standards (**4b-1** (15%), **4b-2** (4%), Pi (11%)). The unknown compound made up the remaining 15%. The ^{13}C NMR data allowed the identification of the two major products (**4b-3** and CEA) and two minor compounds (**4b-1** and 2-hydroxyethylamine). The mistaken identification of Muñoz et al.[24] for the major product of hydrolysis of IF at 50 °C from ^{13}C NMR data was due to the fact that (i) there are two major hydrolysis products (**4b-3** and CEA) rather than one and (ii) the *N*-chloroethyl group is no longer bound to the phosphorus atom but is found in the medium as CEA.

Some IF degradation products were also identified by GC−MS analysis of IF aqueous solutions at pH 4−10 heated at ≈37 °C for 5−12 h after treatment with either diazomethane or trifluoroacetic anhydride.[26] The methyl ester of phosphoric acid or the trifluoroacetylated derivative of 2-(chloroethyl)-3-hydroxypropylamine (compound F, Scheme 1) can be obtained only after cleavage of the P−O bond of IF, a mechanism that does not occur in water at any value of pH (vide supra). These findings were indicative of IF degradation during the derivatization procedure rather than in the aqueous medium. The other compounds detected, the methyl ester of 2-hydroxy-1,3,2-oxazaphosphorine 2-oxide (**3b**) and the trifluoroacetylated derivative of CEA, may have been produced by hydrolysis of IF at pH 4 but not at pH 7 or 10.[40] They could not have derived from rupture of the exocyclic P−N bond of IF as described by Highley et al.[26] Indeed, this cleavage was consistently observed after that of the endocyclic P−N bond (Scheme 2).

Evaluation of biological activity of IF, its dechloroethylated metabolites, and some of their hydrolytic degradation products showed that, except for IF, all these compounds have no antitumor efficacy toward murine P388 leukemia. Neutral or acidic hydrolysis of IF led to compounds that are less toxic than IF. IF dechloroethylated metabolites and their hydrolysis products are also less toxic than IF. The sole exception is the aziridino compound **4b-2**, but 2DCIF and IF which are the sources of **4b-2** are slowly or not hydrolyzed under physiological conditions.

In conclusion, the present study showed the value of ^{31}P NMR for determining the successive steps in the acidic hydrolytic pathways of IF and its dechloroethylated metabolites (DDCIF, 2DCIF, and 3DCIF). The combination of data from MS and ^1H, ^{13}C, and ^{31}P NMR led to the identification of all their degradation products in neutral or acidic aqueous solutions. Some of these compounds, **2a**−**c**, **3a**, and **4a**, were detected and quantified in urine of patients treated with CP or IF. Degradation compounds of DDCIF, **2a** and **3a**, and 2DCIF, **2b**, represented ≈10% of 2DCIF (DDCIF itself was not detected) excreted over 24 h in urine of patients receiving 3 g/m^2 IF as a 3-h infusion.[9] In a more recent and detailed study, degradation compounds of 3DCIF, **2c**, **3a**, and **4a**, represented ≈22% of 3DCIF excreted in urine samples at pH 5.8 from patients treated with CP at a dose of 60 mg/kg/day as a 3-h infusion. This percentage was markedly higher in urine at more acidic pH: 57% at pH 5.1−5.3.[44] ^{31}P NMR determination of the N-dechloroethylated metabolites of IF or CP and their degradation compounds provides an indirect and accurate estimation of CAA amounts formed from CP or IF.

Experimental Section

Analytical Methods. NMR spectra were recorded on Bruker WB-AM300 or Bruker ARX 400 spectrometers. Chemical shifts (δ) are reported in ppm, relative to 3-(trimethylsilyl)-propanesulfonic acid sodium salt (TMPS) as an internal standard for ^1H and ^{13}C NMR spectra and relative to 85% H_3PO_4 as an external standard for ^{31}P NMR spectra. Fast atom bombardment mass spectra in positive mode (FAB^+) were obtained with a VG ZAB HS spectrometer. A Waters (model 991) system equipped with a photodiode array detector was used for the HPLC analyses.

Chemicals. DDCIF (**1a**), 2DCIF (**1b**), and 3DCIF (**1c**) were generously supplied by ASTA Medica AG (Frankfurt, Germany). IF (**1d**) was obtained from ASTA Medica (Bordeaux, France). All other chemicals used were of highest purity obtainable.

Synthesis of 1-Propanol, 3-[(2-Chloroethyl)amino]-, Dihydrogen Phosphate (Ester) (4b-1). IF (10 g, 38.3 mmol) was dissolved in 500 mL of 1 M HCl. When the exothermal reaction ended, the solution was evaporated under reduced pressure (about 20 mbar). The residue was dissolved in 100 mL of chloroform:ethanol (1:1). After alkalinization with triethylamine, the solution was stored for crystallization. The crystalline product was filtered off, washed with ethanol, and recrystallized in water:ethanol to give compound **4b-1** in 57% yield (3.7 g): mp 190−191 °C. Anal. ($C_5H_{13}NO_4PCl$) C; H: calcd, 6.07; found, 6.23. N: calcd, 6.44; found, 6.40. NMR and mass spectrometry data are identical to those reported in Table 4.

Synthesis of Diphosphoric Acid *P,P′*-Bis[3-[(2-chloroethyl)amino]propyl] Ester ($[Cl(CH_2)_2NH(CH_2)_3OP(O)(OH)]_2O$, 5). 3-(Chloroethyl)-2-chlorooxazaphosphorinane 2-oxide (50 g, 230 mmol) synthesized as previously described[45] was dissolved in 400 mL of dry THF; 11.4 g (285 mmol) of crushed sodium hydroxide was added and the suspension stirred at room temperature and then refluxed for 10 h. The precipitate of sodium chloride was filtered off and the solution concentrated. The oily residue was resuspended in 300 mL of THF: water (1:1), stirred for 1 h after acidification with HCl, and then concentrated in vacuo. The residue was dissolved in 100 mL of water and 350 mL of ethanol. After alkalinization with triethylamine to pH 8, the solution was stirred for 2 h at 0 °C. The next day, the crystalline product was filtered off and washed with ethanol and diethyl ether. Recrystallization from acetone:water at 4 °C gave compound **5** in 29% yield (14 g): mp 193−194 °C. Anal. ($C_{10}H_{24}N_2O_7P_2Cl_2$) C, H; N: calcd, 6.72; found, 6.56. FAB^+ mass spectrometry: ions at *m/z* 417, 419, 421 [MH^+] (2 Cl). NMR (D_2O, pD 2.6): δ (^1H) 4.00 (4H, m as they are diastereotopic, CH_2O), 3.82 (4H, m, CH_2Cl), 3.40 (4H, t, 5.6 Hz, NCH_2 CH_2Cl), 3.22 (4H, t, 7.1 Hz, $NCH_2CH_2CH_2O$), 2.02 (4H, app quin, 6.3 Hz, $NCH_2CH_2CH_2O$); δ (^{13}C) 66.5 (broad signal, CH_2O), 51.6 (s, NCH_2 CH_2Cl), 48.2 (s, $NCH_2CH_2CH_2O$), 41.8 (s, CH_2Cl), 28.92 and 28.95 ($NCH_2CH_2CH_2OP$, the carbon of the two chains are magnetically nonequivalent, each giving a doublet with a $^3J_{CP}$ 3.8 Hz); δ (^{31}P) −10.0 (s). The ^1H and ^{13}C signals were attributed from COSY and HETCOR correlations.

Hydrolysis Products of DDCIF (1a). 1-Propanol, 3-amino-, hydrogen phosphoramidate (ester) (2a): DDCIF (**1a**) (0.012 g, 0.09 mmol) was dissolved in 3 mL of water, the pH of which was then adjusted to 2.3 with HCl. After 20 min at room temperature, the solution was neutralized with NaOH. ^{31}P NMR showed disappearance of the DDCIF (**1a**) signal and appearance of those of **2a** (>95%) and traces of **3a** and **4a**.

The NMR and mass spectrometric data for compound **2a** are listed in Table 3.

2-Hydroxy-2-oxo-1,3,2-oxazaphosphorine (3a): A solution of compound **2a**, whose pH was maintained at 8.5 by periodic addition of NaOH, was sequentially analyzed (once a day) by ^{31}P NMR. After 7 days, the compound **3a** represented 80% of all the ^{31}P signals. The solution was then neutralized, and the mass and ^{13}C NMR characteristics of compound **3a** were determined (Table 3).

1-Propanol, 3-amino-, dihydrogen phosphate (ester) (4a): DDCIF (**1a**) (0.013 g, 0.10 mmol) was dissolved in 3 mL of 1 M HCl. After 10 h at room temperature, the ^{31}P NMR spectrum showed the presence of a sole compound whose mass and, after raising the pH to ≈5, NMR spectrum (Table 3) were identical to that of compound **4a** obtained by an independent synthesis.[28]

Hydrolysis Products of 2DCIF (1b). 1-Propanol, 3-[(2-chloroethyl)amino]-, hydrogen phosphoramidate (ester) (2b): 2DCIF (**1b**) (0.008 g, 0.04 mmol) was dissolved in 2 mL of water. The pH of the solution was then adjusted to 2.4 with HCl. After 20 min at room temperature, the pH of the solution was neutralized with NaOH. The ^{31}P NMR spectrum, recorded at 4 °C, showed the presence of **2b** and traces of **4b-1** (≈3%). The degradation kinetics of compound **2b** were obtained after dissolving the freeze-dried solution in cacodylate buffer. The ^{13}C NMR and mass spectrometric data of compound **2b** are listed in Table 4.

1-Propanol, 3-[(2-chloroethyl)amino]-, dihydrogen phosphate (ester) (4b-1): 0.033 g (0.17 mmol) of 2DCIF (**1b**) was dissolved in 8 mL of 1 M $HClO_4$. After 15 h at room temperature, ^{31}P NMR showed the disappearance of the 2DCIF signal and the presence of a sole signal. Aliquots of this solution (solution A) were analyzed by mass spectrometry and, after raising the pH to 5.8 with 1 M KOH, centrifugation to remove precipitated $KClO_4$, freeze-drying, and redissolution in D_2O, by ^{1}H and ^{13}C NMR. The mass and NMR characteristics of the compound obtained (**4b-1**) are listed in Table 4.

1-Propanol, 3-aziridino-, dihydrogen phosphate (ester) (4b-2): The pH of the solution A remaining was adjusted to 13 by addition of 1 M KOH. After 24 h at room temperature, the solution was centrifuged and the supernatant, analyzed by ^{31}P NMR, showed the sole presence of compound **4b-2** whose structure was characterized by ^{1}H and ^{13}C NMR (Table 4). The high salt concentration of this solution excluded mass spectrometry, but the MS characteristics of compound **4b-2** (*m/z* ion $[MH]^+$ at 182) were obtained by analysis of the degradation mixture of compound **2d**.

1-Propanol, 3-[(2-hydroxyethyl)amino]-, dihydrogen phosphate (ester) (4b-3): 2DCIF (**1b**) (0.0085 g, 0.04 mmol) was dissolved in 2 mL of 0.1 M sodium cacodylate buffer at pH 6.9. After 58 days at room temperature, the ^{31}P NMR spectrum of the resulting mixture (pH 6.7) showed the presence of three strong signals at 17.68 (16%) (2DCIF, **1b**), 4.64 (64%) (**4b-3**) and 4.56 ppm (20%) (**4b-2**), the latter being identified by spiking with authentic standard. Mass spectrometry and ^{13}C NMR analysis of the reaction mixture also showed the presence of the same three compounds. The mass and ^{13}C NMR characteristics of compound **4b-3** are listed in Table 4.

Hydrolysis Products of 3DCIF (1c). 1-Propanol, 3-amino-, *N*-(2-chloroethyl)-, hydrogen phosphoramidate (ester) (2c): 3DCIF (0.015 g, 0.08 mmol) was dissolved in 2 mL of aqueous 0.1 M HCl. After a few minutes, the solution was neutralized with 1 M KOH. ^{31}P NMR showed that 3DCIF had been completely hydrolyzed into **2c** and a compound previously identified as **4a**. We analyzed the mixture by HPLC with a 5-μm Lichrosorb-RP Select B column (150 × 4 mm) under the following conditions: eluant, water/acetonitrile (98/2) at a flow rate of 0.7 mL/min, with UV detection at 200 nm. The retention time of **2c** was 5.6 min, whereas that of **4a** was 3.2 min. The fractions containing **2c** were immediately frozen in liquid nitrogen, then freeze-dried, and stored at −80 °C. The compound isolated was 98% pure as estimated from ^{31}P NMR. Its mass and ^{13}C NMR data are listed in Table 5.

2-Hydroxy-2-oxo-1,3,2-oxazaphosphorine (3a): This compound was not isolated, but it represented ≈75% of the reaction mixture (estimated by ^{31}P NMR) after keeping an aqueous solution of **2c** for 3 days at room temperature after adjusting the pH to 8 with 0.1 M NaOH. The structure of compound **3a** was characterized by positive-ion FAB mass spectrometry: ions at *m/z* 138 $[MH]^+$ and 160 $[MNa]^+$. ^{13}C NMR: δ C4 44.9 (d, 4.4 Hz), C5 29.8 (d, 6.7 Hz), C6 70.7 (d, 5.4 Hz) in H_2O at pH 7.9. Liberation of CEA in the medium was evidenced by the presence of a ^{13}C signal at 44.4 ppm corresponding to the CH_2Cl and CH_2N carbons as demonstrated by spiking the mixture with authentic standard.

1-Propanol, 3-amino-, dihydrogen phosphate (ester) (4a): 3DCIF (0.014 g, 0.07 mmol) was dissolved in 2 mL of 1 M HCl. After 24 h at room temperature, ^{31}P NMR showed the presence of the sole compound **4a**, the positive-ion FAB mass data (glycerol matrix) showed that of **4a** with ion at *m/z* 156 $[MH]^+$ and CEA with ions at *m/z* 80, 82 $[MH]^+$ and 172, 174 $[MH + \text{glycerol}]^+$, and the ^{13}C NMR spectrum showed also the presence of **4a** with signals at δ 40.5 (s, C4), 30.6 (d, 7.0 Hz, C5), and 65.8 (d, 5.1 Hz, C6), as well as that of CEA with signals at δ 43.8 (s, CH_2Cl) and 44.3 (s, CH_2N) in H_2O at pH 4.1.

Hydrolysis Products of IF (1d). 1-Propanol, 3-[(2-chloroethyl)amino]-, *N*-(2-chloroethyl)-, hydrogen phosphoramidate (ester) (2d): IF (0.1 g, 0.38 mmol) was dissolved in 10 mL of water, and the pH was adjusted to 0.9 with 1 M HCl. After stirring for 30 min at room temperature, the solution was neutralized with 1 M KOH and then extracted with $CHCl_3$ to remove unreacted IF. The aqueous phase was freeze-dried and the residue chromatographed on a SepPak C18 column and eluted with distilled water at pH 7. Fractions were collected and analyzed by HPLC under the following conditions: column, Lichrosorb-RP Select B 5 μm (150 × 4 mm); UV detection at 195 nm; eluant, water/acetonitrile (95/5) at a flow rate of 0.8 mL/min. The retention time of **2d** was 5.8 min, whereas that of **4b-1** (which was also formed in acidic medium) was 2.8 min. The fractions containing compound **2d** were immediately freeze-dried and stored at −80 °C. The mass and ^{13}C NMR data of **2d** are listed in Table 5.

2-Hydroxy-2-oxo-3-(2-chloroethyl)-1,3,2-oxazaphosphorine (3b): Compound **2d** in neutral and slightly acidic aqueous solutions is degraded to compound **3b**, which is unstable and is transformed into **4b-1** and/or **4b-2** depending on pH. Compound **3b** could not, therefore, be isolated, and its characteristics (mass and ^{13}C NMR) had to be obtained by monitoring the degradation of compound **2d**.

1-Propanol, 3-[(2-chloroethyl)amino]-, dihydrogen phosphate (ester) (4b-1) and 1-Propanol, 3-aziridino-, dihydrogen phosphate (ester) (4b-2): IF (0.04 g, 0.15 mmol) was dissolved in 2 mL of 1 M $HClO_4$. After 24 h at room temperature, the solution containing only one phosphorylated compound as evidenced by ^{31}P NMR was split into two equal parts.

The first part was analyzed by MS and, after raising the pH to 3.4 with 1 M NaOH, by ^{13}C NMR. FAB-positive MS showed the presence of compound **4b-1** with ions at *m/z* 218, 220 $[MH]^+$. ^{13}C NMR signals were characteristic of compound **4b-1** (δ 48.3 (s, C4), 29.3 (d, 6.9 Hz, C5), 65.6 (d, 5.2 Hz, C6), 51.8 (s, C9), 42.2 (s, C10)) and chloroethylamine (δ 43.8 (s, CH_2Cl), 44.3 (s, CH_2N)).

The pH of the second part was raised to 13 with 1 M KOH. After centrifugation to remove the precipitate of $KClO_4$, the solution was left for 24 h at room temperature. ^{31}P NMR showed the presence of the sole phosphorylated compound **4b-2**, confirmed by ^{13}C NMR, which also indicated the presence of the characteristic resonance of aziridine at δ = 20.3 ppm. The solution was freeze-fried to remove the highly volatile aziridine. After the pellet was dissolved in H_2O, the pH was adjusted to 7.5 with 1 M HCl. ^{13}C NMR showed the presence of the sole compound **4b-2** with δ = 55.5 (s, C4), 30.4 (d, 5.8 Hz, C5), 64.7 (d, 5.0 Hz, C6), 32.0 (s, C9/C10) of the aziridine ring.

Prolonged Hydrolysis of IF at 50 °C. IF (0.023 g, 0.05 mmol) was dissolved in 3 mL of water. The solution (initial pH 6.5) was maintained at 50 °C in a circulating water bath for 17 days (final pH 4.6). At this time, ^{31}P NMR showed the presence of unreacted IF (**1d**, ≈16% of all the ^{31}P NMR signals) along with five other signals with chemical shifts ranging from 1.4 to 0.8 ppm, characteristic of phosphate structures. This solution was then extracted with 2 × 1 mL of $CHCl_3$ to remove unreacted IF and the aqueous phase analyzed by ^{31}P and ^{13}C NMR.

The ^{31}P NMR spectrum recorded at pH 6.0 (pH giving the best discrimination) contained five signals of which four were attributed to **4b-1**, **4b-2**, and Pi by spiking with authentic standards and **4b-3** by comparison of its chemical shift with that of an authentic standard. The chemical shifts, relative intensities, and attribution were as follows: 3.76 ppm, 15%, **4b-1**; 3.71 ppm, 55%, **4b-3**; 3.69 ppm, 4%, **4b-2**; 1.76 ppm, 11%, Pi; 1.73 ppm, 15%, unknown.

The ^{13}C NMR spectrum recorded at pH 4.6 had major signals from compounds **4b-3** and CEA attributed by comparison with those of known standards. **4b-3**: δ 48.1 (s, C4), 29.3 (d, 7.0 Hz, C5), 65.4 (d, 5.2 Hz, C6), 52.1 (s, C7), 59.5 (s, C8). CEA: δ 44.2$_5$ (s, CH_2N), 43.8 (s, CH_2Cl).

Among the numerous minor signals, we identified those of **4b-1** and 2-hydroxyethylamine by comparison with those of authentic standards. **4b-1**: δ 48.2 (s, C4), 29.2 (d, 7.2 Hz, C5), 65.3 (d, 5.2 Hz, C6), 51.7 (s, C7), 42.0 (s, C8). 2-Hydroxyethylamine: δ 60.5 (s, CH_2OH), 44.3 (s, CH_2N).

The ratio of the signal intensities of **4b-3**/**4b-1** was ≈4 in common with that observed on ^{31}P NMR.

Hydrolysis Kinetics by ^{31}P NMR. Compounds to be hydrolyzed were dissolved in 2.5 mL of the appropriate buffer (0.1 or 0.2 M sodium cacodylate at pH 6.8, 6.0, and 5.5, 0.2 M sodium phthalate at pH 3.0, 0.5 M KCl−HCl at pH 2.0, and 1 M HCl at pH ≈ 0), and the pH of the resulting solutions was measured. The initial concentration of all these compounds was 2.8 × 10^{-2} M.

After recording initial "zero-time" ^{31}P NMR spectra, the samples of 2DCIF (**1b**) at pH 6.8 and 5.5, 3DCIF (**1c**) at pH 5.5, and IF (**1d**) at pH 3.0 whose degradation kinetics are very slow ($t_{1/2}$ ≈ 4 days) were maintained at room temperature near 20 °C and their ^{31}P NMR spectra then recorded at various times. The degradation kinetics of all the other compounds were continuously monitored in the NMR probe regulated at 25 °C, and the spectra were acquired at different intervals depending of their rate of hydrolysis. Time points for each spectrum were taken at the midpoint of data acquisition.

Spectra were run on a Bruker WB AM 300 or ARX 400 spectrometer using the inverse-gated decoupling technique under the instrumental conditions described elsewhere,[28,40,46] shortly a 35° pulse of 5 μs and a recycle time of 2.08 s. Negligible reaction took place during the NMR recording (≈10−30 min) of the samples analyzed over several days.

pH was measured before and after each NMR spectrum. Only a slight change in pH (0.2 pH unit) was generally observed during the hydrolysis, except for that of 3DCIF at pH 5.5 (0.25 pH unit) and pH 3.0 (0.3 pH unit) and IF at pH 2.0 (0.35 pH unit). All runs were followed over reaction periods >50%, except for degradation of 3DCIF at pH 5.5 and IF at pH 3.0 for which only ≈35% of the starting compounds were hydrolyzed. All kinetic runs were made in duplicate or triplicate, except for the very slow degradation reactions of 2DCIF at pH 6.8 and 3DCIF at pH 5.5 where only one run was made.

The relative concentrations of the phosphorylated compounds observed in the ^{31}P NMR spectra were determined from their integrated signal intensities (peak heights). In a previous study,[28] we found that this procedure gave an accurate estimate of the relative concentrations of CP (an isomer of IF) and its hydrolysis products, which have similar structures to the oxazaphosphorines **1** and their degradation products.

Linear least-squares fits of pseudo-first-order plots of ln-[starting materials]$_t$/[starting materials]$_{t0}$ (ln C/C_0) versus time gave the values for the rate constants, with in all cases $r \geq$ 0.990. C and C_0 refer to the percent of ^{31}P NMR signal intensities of the starting material relative to that of all observable ^{31}P signals. We checked for various degradation kinetics that the rate constants for the disappearance of the starting material were close to those obtained with values of the concentrations (C and C_0) of the starting materials measured by comparison of the integrated intensities of their ^{31}P NMR signals with that of the ^{31}P NMR signal of methylphosphonic acid, a quantification standard placed in a sealed coaxial capillary.

The rate constants for consecutive reactions were determined by a least-squares fit method using a computer program developed in the laboratory.[47] The program analyzed the measured time dependence of the relative concentrations of compounds involved (**1**−**4** or **2**−**4**) for kinetic equations describing a consecutive-irreversible two $\mathbf{2} \xrightarrow{k_2} \mathbf{3} \xrightarrow{k_3} \mathbf{4}$ or three $\mathbf{1} \xrightarrow{k_1} \mathbf{2} \xrightarrow{k_2} \mathbf{3} \xrightarrow{k_3} \mathbf{4}$ stage reaction. We assumed that all the reactions obeyed a first-order rate law, with k_1, k_2, and k_3 the rate constants for the disappearance of compounds **1**, **2**, and **3**, respectively. For **2b**,**d**, k_3 is the sum of the rate constants for the disappearance of **3** leading to simultaneous formation of **4b-1** and **4b-2**. The k_1 values differed little on fitting either the first step, the first and second steps, or the three steps of the hydrolytic reaction of **1a**. Similar values for k_2 were found by fitting either the first step or the two steps of the hydrolysis of **2b**−**d**. Half-lives were derived from the rate constants ($t_{1/2} = \ln 2/k$).

pK_a Determinations. For the determinations of pK_a, the compound of interest was added to aqueous 0.1 M NaCl to avoid variations in ionic strength during titration. The pH was measured before and after each ^{31}P NMR spectrum recorded at 25 °C and modified by the addition of NaOH or HCl. The pK_a values were calculated according to the equation reported by Lachmann and Schnackerz.[48]

Biological Methods. Animals: Female SD rats and male CD2F1 mice were obtained from the Zentralinstitut für Versuchstierzucht, Berlin, 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 **1a**−**d**, **4b-1**, **4b-2**, and **5**, CEA HCl, 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 female SD rats or male CD2F1 mice per dose group were injected iv or 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.[98] 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 female SD rats or 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 iv or ip either once on the following day (day 1) or daily from days 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.

Patients' urine: ^{31}P NMR analysis was carried out directly on urine without any extraction. For patients treated with IF, crude (i.e., nonconcentrated) urine samples were analyzed, whereas they were concentrated from 20 to 6 mL for patients treated with CP as these patients exhibited a marked diuresis (2.5−7.9 L/day).

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