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Identification of new aqueous chemical degradation products of isophosphoramide mustard

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Abstract

NMR (^{31}P, ^{1}H and ^{13}C) spectroscopy was used to study the products of the degradation of isophosphoramide mustard (IPM) in buffered solutions at pH ranging from 1 to 13. At pH ≤ 1, the only degradation compounds detected were phosphate ion (Pi) and chloroethylammonium chloride (CEA–HCl), resulting from the breakdown of the two P–N bonds (pathway Ia). At pH 9.3 and 13, only the products of 1,3-cyclization of the *N*-chloroethyl group (monoaziridinylIPM (monoAzIPM) and a very low level of bisaziridinylIPM (bisAzIPM)) were found after ≈ 15 h of reaction (pathway II). At intermediate pH, the two pathways coexist. At pH 3.5 and 5.0, the P–N bond hydrolysis is the major pathway, but two final phosphorylated products were detected, Pi which represented 67% (pH 3.5) and 17% (pH 5.0) of all the IPM phosphorylated degradation products after ≈ 15 h of reaction, and phosphorylethanolamine (PEA) which represented 16% (pH 3.5) and 46% (pH 5.0) of the same sum. PEA formation can be explained by the 1,5-cyclization of a transient compound giving a 1,3,2-oxazaphospholidine intermediate whose P–N bond is exclusively cleaved in acidic medium. The presence of monohydroxyIPM (monoOHIPM) (whose percentage increases with pH from 5% (pH 3.5) to $\approx 28\%$ (pH 5.0) of all the IPM phosphorylated degradation compounds), probably coming from the alkylation by water of an aziridine/aziridinium intermediate, demonstrates the occurrence of pathway II. At pH 7.0 and 7.4, the pathway II is initiated first, leading to 1,3-cyclization(s), followed by water alkylation of the aziridines formed. The sequences are IPM 1 → monoAzIPM 5 → bisAzIPM 9; IPM 1 → monoAzIPM 5 → monoOHIPM 6 → monoAzIPM with a *N*-hydroxyethylchain (presumed structure) 7 → dihydroxyIPM 8. Nevertheless, PEA and Pi are the final products observed, which implies the P–N bond hydrolysis of products 5–9 as demonstrated by the presence in the medium of CEA, aziridine and ethanolamine. © 2001 Elsevier Science B.V. All rights reserved.

Abbreviations: PM, phosphoramide mustard; IPM, isophosphoramide mustard; monoAzIPM, monoaziridinylIPM; CEA–HCl, 2-chloroethyl ammonium salt; CEA, chloroethylamine; EtOA, ethanolamine; PEA, phosphorylethanolamine; Az, aziridine; bisAzIPM, bisaziridinylIPM; diOHIPM, dihydroxyIPM or *N*,*N*′-bis(2-hydroxyethyl)phosphorodiamidic acid; Pi, phosphate ion; monoOHIPM, monohydroxyIPM or *N*-(2-chloroethyl),*N*′-(2-hydroxyethyl) phosphoro-diamidic acid.

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1. Introduction

Like cyclophosphamide, its structural isomer ifosfamide is an important clinical alkylating anticancer agent with subclassification as oxazaphosphorines. Both compounds are prodrugs requiring hepatic oxidative activation to express their cytotoxicity [1,2]. Through a cascade of reactions, they give rise to phosphoramide mustard (PM) and isophosphoramide mustard (IPM), respectively, which are generally accepted as the alkylating agents of therapeutic consequence. PM and IPM are bifunctional alkylating agents of the nitrogen mustard type and they alkylate nucleophiles, primarily the bases of DNA, which results in inhibition of DNA synthesis and subsequent cell death, and also variety of endogenous nucleophilic species [1–3]. The alkylation pathway involves the consecutive formation of two aziridinium intermediates for PM [4] and two aziridinyl intermediates for IPM [5].

Alkylation chemistry of PM and IPM by strong and weak nucleophiles has been extensively studied for 20 years [3,5–12] but the concurrent P–N bond hydrolysis pathway is only well documented for PM [3,6–10]. The degradation of IPM has been essentially studied at pH 7.4 in the presence or absence of trapping agents [5,11–14] and the sole formation of the monoaziridinylIPM (monoAzIPM) [12,14] and alkylation products of the nitrogen mustard moiety by thiosulfate [5,11], mercaptoethanol [11], glutathione [12] thiocyanate [5] or water [12] has been reported. In studies at others pH or in media other than buffers as plasma or tissue culture medium, only the IPM degradation rates were evaluated [11,15]. A complete knowledge of the degradation pathway of IPM is necessary, as it has been recently proposed as an anticancer drug itself after preclinical studies in mice and rats [15,16].

This report describes the preliminary results of an investigation on the degradation of IPM in buffered solutions at various pH, and focuses on the identification of the products formed.

2. Experimental

2.1. Chemicals

ASTA Medica AG (Frankfurt, Germany) generously supplied IPM. 2-chloroethylammonium chloride (CEA–HCl) and ethanolamine (EtOA) were purchased from Aldrich, *O*-phosphorylethanolamine (PEA) from Sigma (Sigma-Aldrich, Saint-Quentin Fallavier, France). Aziridine (Az) was synthesized from CEA–HCl as described by Le Roux et al. [17]. All other chemicals used were of highest purity obtainable.

2.1.1. Mono- and bisaziridinyl derivatives of IPM: monoAzIPM 5 and bisAzIPM 9

IPM (11.65 mg, 0.053 mmol) was dissolved in 2.5 ml of D_2O. The pD of the solution was adjusted to ≈ 12 and maintained at this value for 48 h at ambient temperature by periodic addition of NaOD 1 or 0.1 M. ^{31}P NMR spectrum of the resulting mixture (pD 12.1) showed the presence of a major compound at 18.2 ppm representing $\approx 75\%$ of all the phosphorylated compounds detected. It was identified from its ^{1}H and ^{13}C NMR characteristics as monoAzIPM 5 (Table 1). The hydrolytic mixture contained two other phosphorylated compounds with chemical shifts (δ) of 25.3 and 9.8 ppm representing ≈ 17 and 8%, respectively of the total phosphorus content. The first one was identified as bisAzIPM 9 from its ^{1}H and ^{13}C NMR data (Table 1).

2.1.2. N,N′-bis(2-hydroxyethyl)phosphorodiamidic acid (diOHIPM 8)

IPM (13.85 mg, 0.063 mmol) was dissolved in 2.5 ml of deuterated sodium cacodylate buffer 0.2 M at pD 7.4 and mixed with basic anion-exchanger resin Amberlite IR-67 (50 mg). After heating at 50°C for 3 h, the solution (pD 7.3) was analyzed by NMR. The ^{31}P NMR spectrum contained three major signals. The chemical shifts, relative intensities to the sum of all the phosphorus-containing compounds detected, and attribution were as follows: 15.4 ppm, 31%, diOHIPM 8;

Table 1
^{1}H and ^{13}C NMR data of IPM and its hydrolytic products[a]

Compound[a]	IPM 1		monoOHIPM 6		diOHIPM 8		monoAzIPM 5		bisAzIPM 9		CEA		EtOA		PEA 4		Az	
NMR data	D_2O pD = 7.6[b]		D_2O pD = 7.6		D_2O pD = 7.3		D_2O pD = 11.1[g]		D_2O pD = 11.1[g]		D_2O pD = 7.6		D_2O pD = 7.6		D_2O pD = 7.6		D_2O pD = 7.6	
δ (ppm) multiplicity J (Hz)	^{1}H[c]	^{13}C[c]	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C		
Position of C atoms or their protons: 1	3.14 td 6.0, 10.0[d]	45.8 s	e	e			3.23 td 6.0, 9.4[d]	46.1 s			3.40 t 5.5	43.8 s			3.21 t 5.0	43.2 d 6.5		
2	3.65 t 6.1	48.7 d 6.7	e	e			3.64 t 6.0	48.7 d 5.6			3.87 t 5.5	43.5 s			3.98 td 5.1, 6.4[d]	63.1 d 4.3		
3			2.95 td 5.7, 9.2[d]	45.7 s	2.94 td 5.6, 9.4[d]	45.85 s							3.13 t 5.2	43.9 s				
4			3.61[f]	65.0 d 7.1	3.62 t 5.6	65.3 d 7.8							3.80 t 5.2	60.3 s				
5							1.92 d 13.9[d]	25.8 d 5.9	2.05 d 13.9[d]	26.0 d 6.2							2.63 s	24.2 s

[a] All the compounds are represented in neutral form; [b]pD values are not corrected for kinetic isotope effect and correspond to pH readings; [c] ^{1}H assignments were obtained from ^{31}P-decoupled ^{1}H spectra and COSY correlations, ^{13}C assignments from HETCOR or HMQC correlations; [d] $^{3}J_{H-P}$; [e] the resonances of the protons and carbons of the *N*-chloroethylchain of monoOHIPM 6 are beneath those of the starting material IPM 1, which is still present in the hydrolysis mixture analyzed. No specific value can therefore be reported; [f]since this resonance appears as a shoulder upfield that of the CH_2Cl group of IPM 1 and monoOHIPM 6, its multiplicity and coupling constant cannot be determined; [g]no modification of δ and J of monoAzIPM 5 and bisAzIPM 9 were observed when the pD of the solution was adjusted to 7.6.

Table 2
^{31}P NMR chemical shifts (δ) of hydrolysis products of IPM and their fractional distribution after ≈ 15 h of reaction time

PH	Buffer	T (°C)	BisAzIPM 9		Unknown U2 7		MonoAzIPM 5		DiOHIPM 8		MonoOHIPM 6		IPM 1		Unknown U1 2		PEA 4		Pi 3	
			δ [a]	% [b]	δ	%	δ	%	δ	%	δ	%	δ	%	δ	%	δ	%	δ	%
1.1	KCl-HCl 0.5 M	25											4.83	20					0.69	80
3.5	Na phtalate 0.2 M	25									6.05	3	6.83	40	2.31	7	1.03	10	0.89	40
5.1	Na cacodylate 0.2 M	25									12.55	14	13.15	50	2.56	3	1.92	23	0.94	8
7.0	Na cacodylate 0.2 M	25	25.30	1	18.44	1	17.85	26	15.47	2	14.58	26	13.78	34			4.36	5	2.22	2
7.4	Na cacodylate 0.2 M	37	25.21	2	18.61	1	17.97	0	15.31	14	14.50	2	13.66	0			4.49	67	2.65	12
9.3	Na cacodylate 0.2 M	25	25.34	2			18.23	63					13.83	34						
≈13	KOH 0.1 M	25	25.31	3			18.19	67					13.78	30						

[a] Chemical shifts (δ) are expressed in ppm relative to external 85% H_3PO_4.
[b] The sum of fractional distribution of all the phosphorus-containing compounds does not reach necessarily 100% since the minor degradation products are not reported in this table.

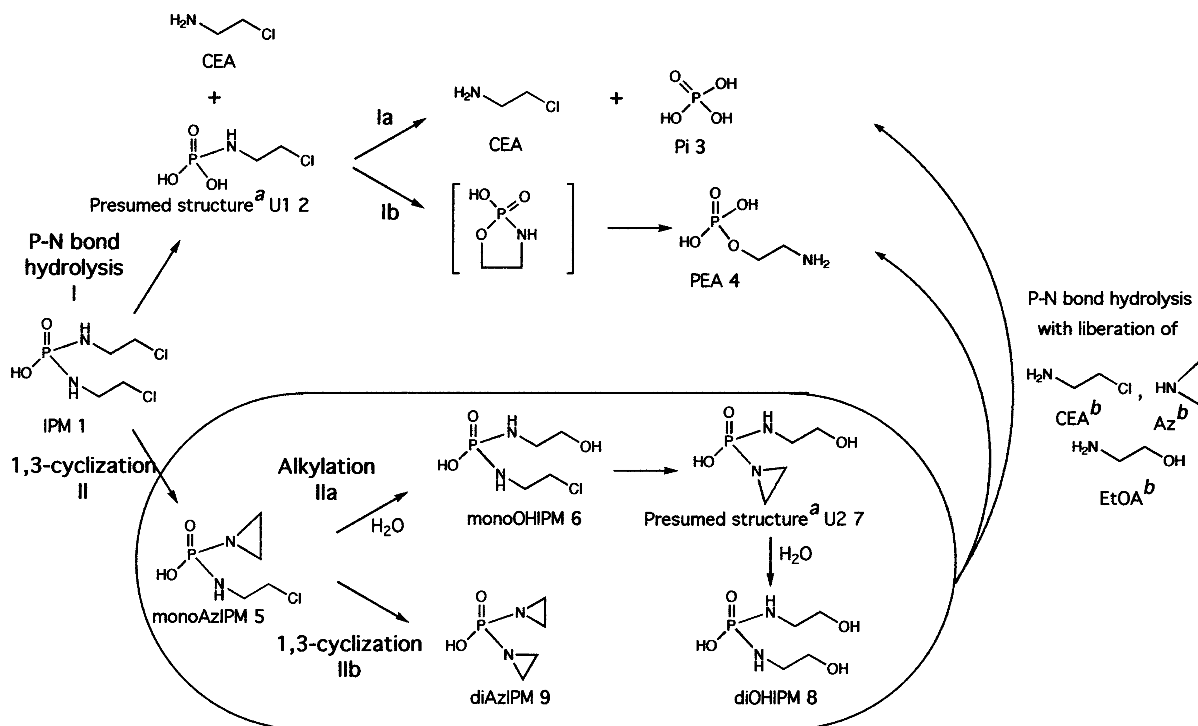

Fig. 1. Hydrolytic pathways of IPM from our data (all the compounds are represented in neutral forms).(a) U, unknown.(b) EtOA and Az detected in the hydrolytic mixture arise from P–N bond breakdown of the products 5–9 of pathway II. At pH ≥ 7.0, CEA also comes from the P–N bond breakdown of products 5–9 of pathway II as monoAzIPM 5 is the first product of IPM degradation at this pH; on the other hand, at pH 3.5 and 5.0, CEA could also result from IPM degradation according to pathway I.

4.7 ppm, 48%, PEA, 4; and 2.8 ppm, 11%, phosphate ion (Pi, 3). ^{1}H and ^{13}C spectra also confirmed the presence of signals corresponding to diOHIPM 8, PEA 4 and three other non-phosphorylated compounds: chloroethylamine (CEA), Az and EtOA. Spiking with authentic standards or/and by comparison of their chemical shifts and coupling constants with those of authentic standards identified all these compounds, except diOHIPM 8. The structure of diOHIPM 8 was established from its ^{1}H and ^{13}C NMR signals, whose attribution was done from $^{1}H\{^{31}P\}$ spectrum and ^{1}H–^{1}H and ^{1}H–^{13}C correlations (Table 1).

2.1.3. *N-(2-chloroethyl),N′-(2-hydroxyethyl) phosphorodiamidic acid (monoOHIPM 6)*

IPM (16.5 mg, 0.075 mmol) was dissolved in 2.5 ml of deuterated sodium cacodylate buffer 0.2 M. The solution (initial pD 6.7) was maintained at 25°C for 24 h. At that time (final pD 6.5), the solution was analyzed by ^{31}P NMR. The chemical shifts, relative intensities, and attribution of the major signals detected were as follows: 17.2 ppm, 3%, monoAzIPM 5;14.55 ppm, 33%, monoOHIPM 6; 13.9 ppm, 34%, IPM 1; 4.2 ppm, 16%, PEA 4; 1.7 ppm, 6%, Pi 3. ^{1}H and ^{13}C NMR spectra, recorded at pD 7.6, showed the signals of monoOHIPM 6, IPM 1, PEA 4, and three non-phosphorylated compounds, CEA, Az and EtOA. All these compounds, except monoOHIPM 6, were identified by spiking with authentic standards or by comparison of their NMR characteristics (^{1}H, ^{31}P, ^{13}C) with those of authentic standards in D_2O at similar pD. MonoOHIPM 6 was identified from NMR data obtained from ^{31}P, ^{1}H, $^{1}H\{^{31}P\}$ and ^{13}C NMR spectra as well as ^{1}H–^{1}H, ^{13}C–^{1}H and ^{31}P–^{1}H 2D experiments. The rationale of the signal assignments is discussed in the text and the complete NMR data are given in Table 1 and Table 2.

2.2. NMR spectroscopy

NMR spectra were recorded on Bruker WB-AM 300 or ARX 400 spectrometers. δ are reported in ppm, relative to 3-(trimethylsilyl)-propane sulfonic acid sodium salt as an internal standard for ^{1}H and ^{13}C NMR spectra, and to 85% H_3PO_4 as an external standard for ^{31}P NMR spectra.

NMR samples for ^{31}P NMR experiments were prepared immediately prior to use. IPM was dissolved in 2.5 ml of the appropriate buffer and the pH of the resulting solution was measured. The initial concentration ranged between 0.7×10^{-2} and 2×10^{-2} M. IPM degradation was continuously monitored in the NMR probe regulated at 25 or 37°C and the spectra acquired at varying intervals over 20–60 min.

Spectra were run using a 35° pulse of 5 μs (WB-AM 300) or 11 μs (ARX 400) and inverse-gated decoupling. FID were acquired into 32 K data points over a spectral width of 15–18 kHz and a total pulse repetition time of 1.9–2.1 s. The data were line-broadened by 1–3 Hz prior to Fourier transformation.

The relative concentrations of the phosphorylated compounds were determined from their signal intensities (peak height or signal integration). To ensure that the relative concentrations measured under our experimental conditions were correct, we prepared a solution containing known concentrations (range 4–13 mM) of glucosylifosfamide (Glucose-*O*-P(O)($NHCH_2CH_2Cl)_2$), IPM 1, PEA 4 and Pi 3 at pH 6.7 in the following proportions, 11.9, 21.1, 30.7 and 36.3%. The proportions of these compounds determined from ^{31}P NMR in the conditions described above were 13.1% for glucosylifosfamide, 23.9% for IPM 1, 30.5% for PEA 4 and 32.6% for Pi 3 (mean of 5 experiments), indicating that the relative concentrations measured were correct.

3. Results and discussion

Using ^{31}P NMR spectroscopy, the degradation of IPM was studied in buffered aqueous solutions at 25°C and pH 1–13, and at 37°C and physiological pH (7.4).

The ^{31}P NMR chemical shifts (δ) of IPM and its phosphorylated degradation products at various pH as well as their fractional distribution after $\approx$15 h of reaction are reported in Table 2. The ^{1}H and ^{13}C NMR characteristics of IPM and some of its degradation compounds are reported in Table 1. Fig. 1 illustrates the set of spontaneous reactions available to IPM.

3.1. pH 1.1

The hydrolysis of IPM led to one phosphorylated compound with a ^{31}P NMR δ of 0.69 ppm identified as Pi by spiking with the authentic standard. After 15 h of hydrolysis, ^{13}C and ^{1}H (in D_2O solution) spectra showed, in addition to the signals of residual IPM, the resonances of CEA–HCl, as verified by spiking with the authentic standard. These findings are in accordance with the facile cleavage of the P–N bonds of phosphoramidates via the *N*-protonated species as the reactive form [18] (pathway Ia of Fig. 1).

3.2. pH 3.5

The degradation of IPM led to a mixture of phosphorylated compounds with ^{31}P NMR δ of 6.83 (IPM), 6.05, 2.31, 1.03 and 0.89 ppm (Table 2). By spiking with authentic standards, the compounds resonating at 1.03 and 0.89 ppm were identified as PEA 4 and Pi 3, respectively. Based on the formation in time and on the temporal pattern, the compound at 2.31 ppm was the first intermediate, which made up a maximum of 10% of all the phosphorylated compounds observed after $\approx$8 h of hydrolysis. The intensity of the signal at 6.05 ppm was always low reaching 3% of all the ^{31}P NMR signals between 13 and 18 h of hydrolysis. A rough pH titration of the hydrolysis mixture showed that this signal shifted downfield to 12.5_5 ppm at pH 5.1 and 14.6 ppm at pH 7.0, pH at which it was identified as monoOHIPM 6 from its ^{1}H and ^{13}C NMR characteristics (Table 1). ^{1}H and ^{13}C NMR spectra allowed the characterization of CEA–HCl and PEA 4, in addition to that of IPM. ^{1}H NMR monitoring of the evolution of a deuterated aqueous solution of IPM (pD $\approx$4.0) showed that CEA–HCl was the first degra-

dation compound to be detected. So, the P–N bond cleavage of IPM 1 (pathway I) could lead to the first phosphorylated intermediate 2 resonating at 2.31 ppm, for which the structure $OP(OH)_2(NHCH_2CH_2Cl)$ could be tentatively proposed. After cleavage of the P–N bond, this compound mainly gave Pi 3, which accounted for 67% of the sum of IPM phosphorylated degradation compounds after 15 h of hydrolysis (pathway Ia). But, via a 1,5-cyclization, it could also lead to a 1,3,2-oxazaphospholidine intermediate whose only P–N bond is cleaved at acidic pH [17] giving exclusively PEA 4 (representing 16% of the sum of IPM phosphorylated degradation compounds) (pathway Ib). As demonstrated by the presence of monoOHIPM 6 in the mixture, pathway II (1,3-cyclization of IPM leading to aziridine/aziridinium intermediate, which is alkylated by water) exists as a minor one although the pH of the medium (3.5) was lower than the pK_A of IPM (4.3) [14].

3.3. pH 5.1

The same IPM degradation compounds than those observed at pH 3.5 were detected during the hydrolytic pathway (Table 2). Pi 3 represented only $\approx 17\%$ of all IPM degradation phosphorylated compounds, monoOHIPM 6 $\approx 28\%$ and PEA 4 $\approx 46\%$ after 15 h of reaction. ^{1}H and ^{13}C NMR spectra showed the presence of IPM, monoOHIPM 6, PEA 4, CEA, EtOA and Az. All these compounds (except monoOHIPM) were identified by comparing their NMR characteristics to those of authentic standards at the same pH. By comparison with the δ and J of monoOHIPM ^{1}H and ^{13}C resonances obtained at p*D* 7.6 (Table 1), the resonances of the protons of the $-NHCH_2CH_2OH$ chain and the carbon of the $-CH_2OH$ moiety were observed in the hydrolysis mixture. At this pH, pathway Ib is the major one, much more important than pathway Ia (PEA and Pi representing respectively ≈ 46 and 17% of the sum of IPM phosphorylated degradation compounds after 15 h of reaction). Moreover, compared to the hydrolytic degradation of IPM at pH 3.5, pathway II is enhanced as monoOHIPM represented $\approx 28\%$ of all the phosphorylated degradation compounds after 15 h of reaction versus 5% at pH 3.5. The contribution of pathway II is underestimated as the detection of EtOA and Az implies that a part of monoOHIPM or its precursor monoAzIPM 5 (not detected) is transformed into compounds bearing one or no P–N bond coming from pathway I (Fig. 1).

3.4. pH 9.3 and 13 (KOH 0.1 M)

IPM led primarily and essentially to monoAzIPM 5 (63 and 67%, respectively after 15 h of reaction) (Table 2), identified from its NMR characteristics (Table 1 and Table 2). A very low level (2–3%) of bisAzIPM 9 was also formed after 15 h of reaction. It results from the 1,3-cyclization of the remaining *N*-chloroethyl chain of monoAzIPM 5. Its structure was established from the characteristic ^{1}H and ^{13}C resonances of the aziridine moiety: (a) an upfield doublet at 2.05 ppm due to equivalent protons of the rapidly inverting aziridine ring bound to the phosphorus atom with $^{3}J_{H-P} = 13.9$ Hz, which disappears in the $^{1}H\{^{31}P\}$ spectrum; (b) an upfield carbon doublet at 26.0 ppm with $^{2}J_{C-P} = 6.2$ Hz (Table 1). To our knowledge, bisAzIPM 9 has never been identified in aqueous solution of IPM, even if Kwon and Borch [19] reported its formation at pH 12 but without any structural spectroscopic characteristics. In contrast, monoAzIPM 5 has already been observed [12,14] but the complete assignment of ^{1}H and ^{13}C NMR resonances from $^{1}H\{^{31}P\}$ spectra and 2D correlations (^{1}H–^{1}H and ^{1}H–^{13}C) as reported in Table 1, has never been described. After 15 h of reaction at these pH, only the 1,3-cyclization of IPM leading to mono- and bisAzIPM 5 and 9 (pathway IIb) is operative.

3.5. pH 7.0 and 7.4

Several IPM phosphorus-containing degradation compounds were detected, the ^{31}P δ of the major ones being listed in Table 2. The identification of most of them is straightforward from addition of authentic materials (PEA 4 and Pi 3), time frame for signal disappearance (IPM), or characteristic ^{31}P δ (monoAzIPM 5 and bisAzIPM 9).

The compound with a ^{31}P δ of 14.58 ppm was identified as monoOHIPM 6 from its ^{31}P, ^{1}H and ^{13}C NMR characteristics in the hydrolytic degradation mixture obtained after maintaining IPM at pD ≈ 7 for ≈ 24 h at 25°C. This mixture mainly contains monoOHIPM 6 and IPM, each accounting for $\approx 1/3$ of the sum of the phosphorylated compounds observed, and to a lesser extent PEA 4 and Pi 3. CEA, Az and EtOA (in a low amount) are the others non-phosphorus-containing compounds detected in the ^{1}H and ^{13}C spectra. All these compounds, except monoOHIPM 6, were identified by comparison of their NMR characteristics to those of authentic standards, or by spiking with equimolar amounts of the compound of interest.

Even if the ^{1}H and ^{13}C resonances of the –$NHCH_2CH_2Cl$ chain of monoOHIPM 6 and IPM were not distinct, the presence of monoOHIPM 6 was deduced from two considerations: (a) the observation of ^{1}H and ^{13}C signals of the –$NHCH_2CH_2OH$ chain in the medium

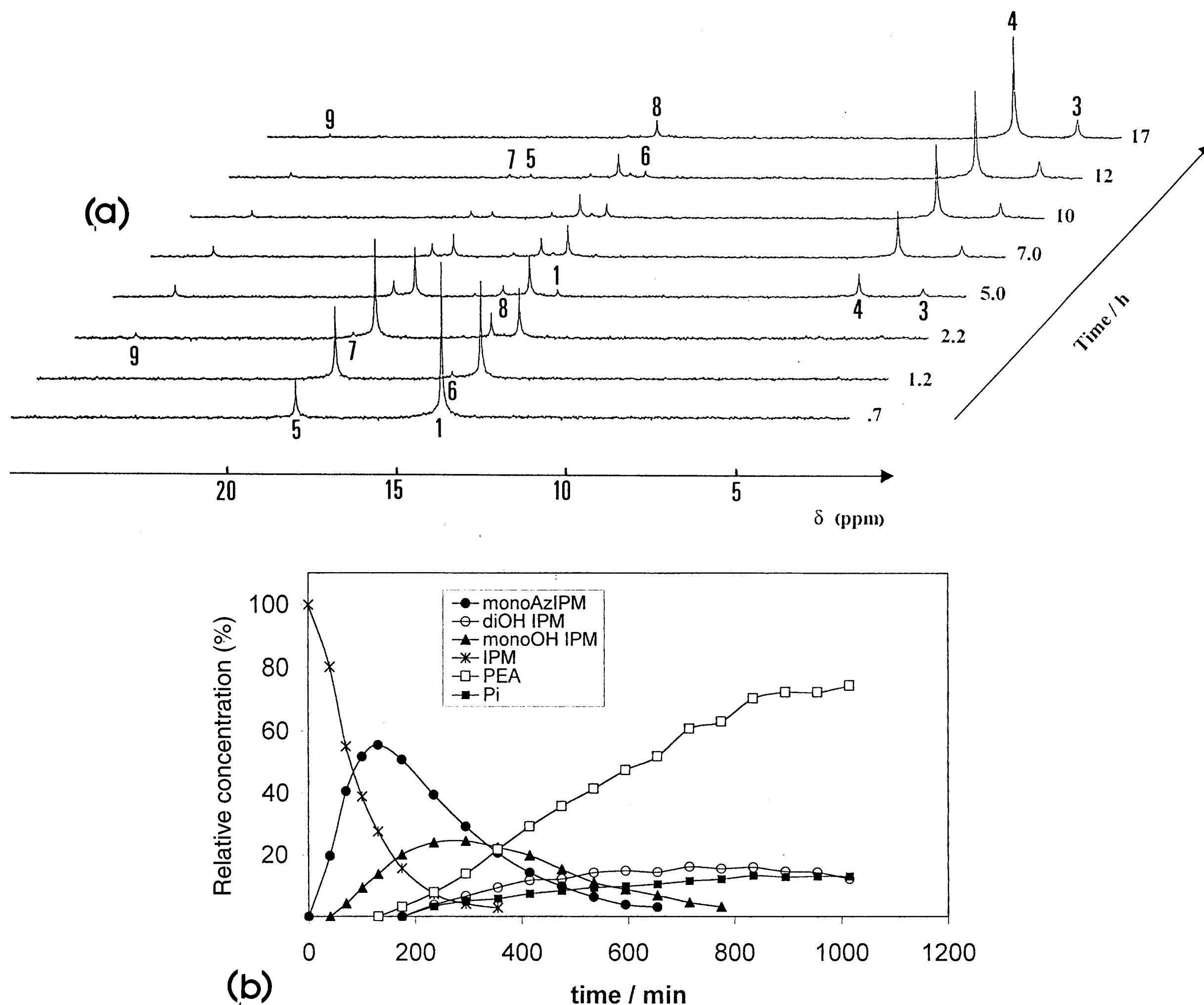

Fig. 2. Stack plot of ^{31}P NMR spectra during IPM hydrolysis in 0.2 M cacodylate buffer at pH 7.4 and 37°C: (a). Corresponding ^{31}P NMR time course of compounds which made up a maximum $>10\%$ of all the phosphorus-containing products detected (bisAzIPM 9 and U2 7 peaking at ≈ 7 and 10%, respectively are not represented) (b).

1, IPM; 3, Pi; 4, PEA; 5, monoAzIPM; 6, monoOHIPM; 7, U2; 8, diOHIPM; 9, bisAzIPM.

(Table 1); and (b) the demonstration that the phosphorus atom with a ^{31}P peak at 14.6 ppm (δ characteristic of a phosphorodiamidic acid structure) is bound both to *N*-chloroethyl and *N*-hydroxyethyl chains. ^{1}H–^{31}P correlation shows cross peaks between the ^{31}P signal at 14.6 ppm and ^{1}H signals of the N–CH_2 groups at 2.95 and 3.14 ppm belonging to *N*-hydroxyethyl and *N*-chloroethyl groups, respectively. Moreover, the N–CH_2 protons of the –$NHCH_2CH_2Cl$ group (3.14 ppm) are also correlated with the ^{31}P signal of IPM at 13.8 ppm, thus confirming that the resonances of the –$NHCH_2CH_2Cl$ chains of IPM and monoOHIPM 6 are not distinct.

The attribution of the ^{31}P signal at 15.47 ppm to diOHIPM 8 was based on its ^{1}H and ^{13}C NMR characteristics in the mixture resulting from IPM hydrolysis in D_2O at pD ≈ 7 for 3 h at 50°C, in which only two other phosphorus-containing compounds (PEA 4 and Pi 3) are present in significant amounts. Since the ^{31}P signals of IPM, monoAzIPM 5 and monoOHIPM 6 are no longer detected in this mixture, the ^{1}H and ^{13}C resonances observed can be unambiguously attributed to diOHIPM 8 without any possible confusion with those, very close or even identical, of the three compounds cited above (Table 1).

The downfield shift of diOHIPM 8 relative to IPM (1.7 ppm) is in keeping with that observed between dihydroxyPM (H_2N–P(O)(OH)–N(CH_2-$CH_2OH)_2$) and PM (2.0 ppm) [3]. The attribution by Dirven et al. [12] of a signal downfield shifted by only 0.3 ppm to diOHIPM 8 is certainly erroneous. More likely, this signal can be tentatively assigned to a product resulting from the concurrent alkylation of monoAzIPM 5 by the phosphate buffer present in $\approx$ 10-fold excess relative to IPM. Alkylation reactions with phosphate have been previously reported for the alkylating agent melphalan [20]. In addition to the signals of the phosphorus-containing compounds already described, ^{1}H and ^{13}C NMR analysis of IPM hydrolysis mixtures at pH (pD) $\approx$ 7.0–7.4 show those of Az, CEA and EtOA.

Moreover, based on the formation in time and on the temporal pattern of the ^{31}P signals detected during IPM degradation kinetics at pH 7.0 (25°C) and pH 7.4 (37°C) (Fig. 2A and B), the following conclusions can be drawn:

1. The first step of the hydrolysis pathway is the formation of monoAzIPM 5, a transient compound which makes up a maximum of $\approx$ 30% at 25°C and $\approx$ 55% at 37°C of all the phosphorylated compounds detected after 9–11 h and $\approx$ 2 h of reaction, respectively.
2. The disappearance of monoAzIPM 5 is linked primarily to the formation of monoOHIPM 6 through alkylation by water, and to a minor extent of bisAzIPM 9.
3. MonoOHIPM 6 is also a transient compound which leads to diOHIPM 8 via the compound resonating at 18.61 ppm and peaking at $\approx$ 10% of all the ^{31}P observable products after 5–6 h of reaction at pH 7.4 and 37°C. The structure of this compound whose δ is characteristic of a phosphorodiamidic acid with a P–N aziridine group was tentatively assumed to be 7.
4. DiOHIPM 8 is a reasonably stable product as it represents 14–16% of all the ^{31}P observable products between 9 and 16 h after the beginning of IPM hydrolysis at 37°C and pH 7.4.
5. Concurrently with the alkylation pathway of monoAzIPM 5, P–N bond hydrolysis reactions occur and lead to PEA 4 and Pi 3 that are the final products of IPM hydrolysis. Since Az, CEA and EtOA are detected in the medium, the P–N bond breakdown of compounds 5–9 can be involved (Fig. 1). As suggested by one referee, PEA may be formed from hydrolysis of the 1,3,2-oxazaphospholidine intermediate already evoked in the acid degradation pathway. This intermediate may be obtained through a 1,5-cyclization of compound 7 tentatively identified as monohydroxyAzIPM, involving the internal displacement of the aziridine moiety by the hydroxyethylamino group. This mechanism could also explain the presence of Az in the reaction mixture. Nevertheless, the comparison of the relative percentages of IPM phosphorylated degradation products after 26 h of degradation at 37°C and pH 7.4 (PEA 4/Pi 3/diOHIPM 8: 78%/10%/12%) and 42 h (PEA/Pi/diOHIPM: 86%/10%/4%) demonstrates that diOHIPM is transformed into PEA.

Investigations on the identification and the distribution of the IPM degradation products in presence of two important endogenous nucleophiles, glutathione and phosphate ion are under current work.

In conclusion, the present study clarified the hydrolytic behavior of IPM. Three of the degradation products identified (PEA 4, monoAzIPM 5, and monoOHIPM 6) were detected in urine of rats treated with ifosfamide at a dose of 40 mg/kg b.w. [21].

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