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Synthesis and ^{31}P NMR Characterization of New Low Toxic Highly Sensitive pH Probes Designed for In Vivo Acidic pH Studies

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Abstract—With the aim to provide sensitive ^{31}P NMR probes of intra- and extracellular pH gradients that may reach cellular acidic compartments in biological systems, new α-aminophosphonates were designed to meet basic requirements such as a low pK_as and a great chemical difference ($\Delta\delta_{ab}$) between the limiting ^{31}P NMR chemical shifts in acidic (δ_a) and basic (δ_b) media. A series of six phosphorylated pyrrolidines and linear aminophosphonates were synthesized using aminophosphorylation reactions and were screened for cytotoxicity on cultured Müller cells. Among the compounds not being toxic under these conditions, three molecules were selected since they displayed the best in vitro (in several phosphate buffers and in a cytosol-like solution) properties as ^{31}P NMR acidic pH markers, that is **3**, **5** and **9**, having the pK_a values of 3.63, 5.89 and 5.66, respectively. The $\Delta\delta_{ab}$ values of these pH markers were at least 3 times larger than that of standard ^{31}P NMR probes, with a low sensitivity to ionic strength changes. From these data, it was proposed that **3**, **5** and **9** could be used as reporting probes of subtle proton movements in acidic compartments, an area that still remains poorly investigated using non invasive ^{31}P NMR methods. © 2002 Elsevier Science Ltd. All rights reserved.

Introduction

Much work has been performed on the characterization of the various intracellular regulatory systems that maintain cytosolic pH homeostasis (i.e., pH 7.0–7.3 in normal conditions) during the normal or pathologic metabolism. In various cell types the existence of very acidic ($4 < pH < 5.5$) cytoplasmic vesicles (i.e., endocytic and secretory vesicles, lysosomes, portions of the trans-Golgi complex, endoplasmic reticulum) that participate in cytosolic pH regulation has been recently demonstrated.[1,2] Among the experimental techniques that can be used to monitor the intracellular pH in vivo, non-invasive ^{31}P NMR spectroscopy has been extensively employed due to the presence in the cytosol of all cells of inorganic phosphate P_i (i.e., $H_2PO_4^-/HPO_4^{2-}$) the chemical shift (δ) of which being accurately correlated with pH in the range $6.0 < pH < 7.3$.[3,4] However, use of P_i as a dynamic ^{31}P NMR probe in biological pH studies is associated with several drawbacks,[4,5] for example, (i) the relatively weak difference $\Delta\delta_{ab}$ between the chemical shifts of the protonated (δ_a) and unprotonated (δ_b) forms (only of 2.6–2.7 ppm),[4–6] (ii) its varying content during cell metabolism that sometimes impairs accurate spectral detection, and (iii) the strong sensitivity of its pK_a to ionic strength, a parameter that can considerably vary in many cell compartments.[7–9] Due to these strong limitations, P_i cannot be used to probe (i) the pH of intracellular compartments having a pH less than 6, and (ii) variations between cytosolic and extracellular pH of less than 0.2 pH units.

Recently, we have described a new series of α- and β-phosphorylated amines and pyrrolidines (Scheme 1) that can greatly improve ^{31}P NMR pH probing by being 4-fold more sensitive than P_i ($\Delta\delta_{ab} = 9$–10 ppm) and by allowing a wide range of pK_a modulation upon changing the substituents connected to the C_1 and C_2 carbons (Scheme 1) bonded to the nitrogen atom.[10] When

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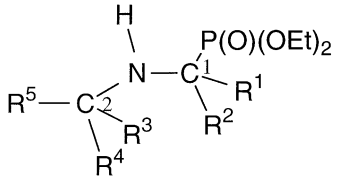
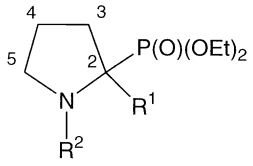
added in the perfusion medium of isolated ischemic/reperfused rat hearts or livers, diethyl(2-methylpyrrolidin-2-yl)phosphonate (**1**; see Scheme 2) allowed the first direct ^{31}P NMR observation[11] of simultaneous cytosolic and extracellular compartments that could not be investigated using other exogenously introduced alkyl derivatives of phosphonic acid that proved to be poorly sensitive ($\Delta\delta_{ab} = 2$–3 ppm) and weakly permeable to cells.[12–15] In the particular case of rat livers, acidic vesicles of pH 5.2–5.6 could be evidenced due to the outstanding NMR sensitivity of **1** ($\Delta\delta_{ab} = 9.8$ ppm) and its excellent cell penetration.[11]

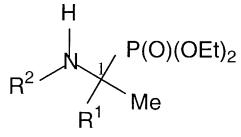
However, since the pK_a of **1** is close to 7,[10] the maximum accuracy for the determination of a pH under biological conditions using this pyrrolidine can be estimated to lie in the range 5.5–8.5 and, thus, even more acidic domains could escape from analysis. Such very acidic compartments ($4 < pH < 5$) have been evidenced in normal or ischemic hepatocytes but, due to the tedious invasive biochemical procedures that were employed in these studies,[16] a dynamic picture of the events associated with the removal or release of protons into the cytosol could not be obtained. In the present study, we sought to prepare new derivatives of **1** or linear aminophosphonates with enhanced properties as ^{31}P NMR pH markers of acidic compartments in biological systems, that is with lower pK_as and greater $\Delta\delta_{ab}$ values than **1**. Using the semi-empirical model that was established to correlate the experimental pK_a and ^{31}P NMR chemical shift values with the two-dimensional structure of α- and β-aminophosphonates,[10] we report here on the synthesis of six new compounds having the structures depicted in Scheme 2, on their potential as ^{31}P NMR pH markers in several biologically-relevant media, and on their toxicity behaviour in a standard model of mammalian cells.



Scheme 1. General formula used in the predictive model of pK_a variation with substitution for α-aminophosphonates.



1 R^1 = Me, R^2 = H
2 R^1 = R^2 = Me
3 R^1 = P(O)(OEt)$_2$, R^2 = H
4 R^1 = P(O)(OEt)$_2$, R^2 = Me



5 R^1 = Ph, R^2 = n-Pr
6 R^1 = Ph, R^2 = Bn
7 R^1 = H, R^2 = n-Bu
8 R^1 = H, R^2 = Bu^t
9 R^1 = H, R^2 = CMe_2-CH_2OH

Scheme 2. Structures of the synthesized aminophosphonates.

Results and Discussion

To design new amines or pyrrolidines bearing an α-diethoxyphosphoryl group and that should have a pK_a value lower than that of **1**, we used our previously developed semi-empirical linear predictive model,[10] in which the pK_a can be calculated from the respective electronic a_i and steric contributions of the $(EtO)_2P(O)$ moiety and the substituents R^1–R^5 (Scheme 1) according to eq 1:

$$pK_a = a_0 + (\Sigma a_i.n_i) + a_{gem}.n_{gem} \quad (1)$$

where n_i represents the number of each substituent of type i, a_0 is a constant value, and $a_{gem}.n_{gem}$ corresponds to the nonlinear steric contribution of a bulky substituent [e.g., $(EtO)_2P(O)$] linked in *gem* position to the carbon substituted by the $(EtO)_2P(O)$ group (i.e., R^1 or R^2 in Scheme 1). With the hypothesis that the target molecules will display $\Delta\delta_{ab}$ values in the range of that found for the pH markers we previously described (i.e., $7.6 < \Delta\delta_{ab} < 10.9$),[10] a good requirement for their targeted pK_a range was estimated to be 5.5–6.5 to cope with an improved sensitivity in the acidic zone (i.e., a maximal precision for pH 4.5–6.5) and a parallel accurate information on the pH of the cytosol and the extracellular milieu. Owing to the reported[10] values of coefficients a_i (see footnotes to Table 1), a good strategy was to introduce, either at C_1 or C_2 (Scheme 1), electron withdrawing substituents and/or to replace alkyl groups by hydrogen atoms, leading to a first set of target molecules **5**–**8** shown in Scheme 2. In Scheme 2, the second set of target molecules **2** and the previously described[17,18] **4** was chosen as tertiary pyrrolidines obtained by methyl substitution should have lower pK_as than their parent secondary compounds **1** and **3**, respectively. An additional molecule **9** was selected to test whether an OH group may be an efficient alternative to a phenyl-containing derivative **5** in a further lowering of the pK_a value (Scheme 2).

The synthesis of α-aminophosphonates has been widely explored in relation with their biological activities[19,20] and their extensive applications in organic synthesis.[21–25]

The linear aminophosphonates **7**–**9** were obtained, in relatively good yields, by a one-pot Kabachnik–Fields aminophosphorylation reaction[26–28] involving diethylphosphite, acetaldehyde and the corresponding primary amine. The phenylated compounds **5** and **6**, which could not be obtained using this latter procedure, were prepared in moderate yields by a modified procedure[29] involving reaction of diethylphosphite with the imine formed by in situ condensation of acetophenone and the corresponding primary amine. The *N*-methyl pyrrolidine **2** was obtained in a satisfactory yield by a modification of the method of Fréjaville et al.[30]

Acquisition of ^{31}P NMR spectra at various pH values in a buffer representative of the extracellular milieu (i.e., a Krebs–Henseleit medium) allowed to determine the experimental pK_a values for the new compounds **2**, **4**–**9** (Table 1). Table 1 also shows, in the case of compounds **1**, **3** and **6**–**8** having substituents types such as already described in ref 10, a quite satisfactory agreement between the experimental pK_a values and the values calculated using our previously published semi-empirical model.[10] Although the differences between the predicted and experimental pK_as did not exceed 5%, this difference was of 8% for **7** possibly due to the existence in this molecule of less steric interactions between substituents linked on the two sp^3 carbons linked on the amino group than in any other compound in the present and previously[10] studied series of pH markers. As expected,[31,32] the tertiary compounds **2** and **4** displayed significantly lower pK_as than their secondary analogues **1** and **2**, respectively (Table 1), reflecting the higher stability of tertiary ammonium salts (the conjugate acids of tertiary amines) over secondary ammonium salts. The dramatic decrease in the pK_a observed for **9** with respect to compound **8** (Table 1) could be attributed to the attractive effect of the OH group on the nitrogen lone pair that diminishes the basic character of **9**. Such an effect has already been computed for ethanolamine which has a pK_a value lower than that of ethylamine.[32]

Table 2 shows that all newly synthesized compounds display a 3–4-fold better sensitivity than the commonly used P_i, as assessed by $\Delta\delta_{ab}$ values which are in the range of that previously obtained for phosphorylated pyrrolidines and amines.[10] Overall, the data of Tables 1 and 2 indicate that compounds **3**, **5**, **6** and **9** should be considered as improved ^{31}P NMR probes to assess the pH of very acidic cellular compartments ($3.5 < pK_a < 5.5$) with a very good sensitivity. However, an important prerequisite for an exogenous compound to be used as a ^{31}P NMR pH marker in a biological system is that it should have a relatively short spin lattice relaxation time (T_1) value to allow the maximum spectra to be accumulated in the timescale of expected biological variations of parameters such as pH and phosphorylated metabolites concentrations, with acceptable saturation conditions. Typically, in perfused isolated organs the accumulation duration for each block of ^{31}P NMR spectra should not exceed 3–5 min and it is accepted that to achieve the best spectral resolution, free induction decay transients should be collected with short interpulse delays (< to 3 s).[11–15] It is known that the T_1 values of phosphorylated metabolites varies in different experimental conditions[33] and are considerably shorter in the intracellular milieu than in the perfusate.[12–15,34] In the particular case of P_i, strong T_1 variations have been evidenced between extracellular medium (10–12 s) and intracellular environment where T_1 values as small as 0.5–1.5 s have been measured[12–15,34] and in such conditions the T_1 value for any exogenously added probe should be as short as possible and relatively insensitive to cellular localization to allow easier quantitative measurements. This is not the case for the commonly used alkyl derivatives of phosphonic acid such as phenylphosphonate, methylphosphonate or dimethyl methylphosphonate which

Table 1. Experimental[a] and calculated pK_a values of aminophosphonates **1**–**9** at 20 °C

Compd	pK_a		n_i value (i = 1–4)[b]
	Expt.	Calcd	
1	6.98±0.05	6.69[c]	(1, 2, 0, 1)
2	6.47±0.05	NP[c]	—
3	3.63±0.02	3.65[c]	(0, 2, 0, 2)
4	2.76±0.02	NP[c]	—
5	5.89±0.01	5.60	(2, 2, 1, 1)
6	4.75±0.04	4.74	(1, 2, 2, 1)
7	6.92±0.02	6.37	(2, 3, 0, 1)
8	7.01±0.01	6.55	(4, 1, 0, 1)
9	5.66±0.03	NP[c]	—

NP, cannot be predicted by the model of ref 4.
[a]Obtained by iteratively fitting the ^{31}P NMR chemical shifts obtained at given pH values using the Henderson–Hasselbach relationship (see Experimental).
[b]Each number under brackets represent, in the given order, the number of substituents of the following types: [alkyl, H, Ph, P(O)(OEt)$_2$]. For each of the four substituent types, the corresponding a_i value (Results and Discussion) has been previously determined[4] to be, in the same given order: −0.118, −0.207, −0.977 and −4.911 (with the constant a_0 = 12.137); in the case of **3** and **4**, the additive steric increment[4] a_{gem} is equal to 1.745.
[c]From ref 4.

Table 2. ^{31}P NMR parameters of aminophosphonates (5 mM) and P_i (1.2 mM) at 20 °C

Compd	Chemical shift (ppm)[a]			T_1 (s)	
	δ_a	δ_b	$\Delta\delta_{ab}$	Krebs buffer	Heart homogenate
KH_2PO_4 (P_i)	−0.1±0.0	2.5±0.0	2.6±0.0	11.5	3.1
1	23.1±0.1	32.9±0.1	9.8±0.2	6.3	5.4
2	21.7±0.3	31.7±0.3	10.0±0.2	NM[b]	—
3	16.5±0.1	24.1±0.1	7.6±0.1	3.3	2.4
4	15.9±0.1	24.0±0.1	8.2±0.1	3.1	—
5	19.8±0.1	28.0±0.1	8.2±0.1	6.3	4.9
6	19.5±0.2	27.6±0.1	8.2±0.2	3.0	—
7	21.0±0.1	31.1±0.1	10.1±0.2	4.0	—
8	22.2±0.1	32.5±0.1	10.3±0.2	4.6	—
9	21.5±0.1	31.5±0.2	10.0±0.3	4.3	3.2

The composition of Krebs–Henseleit buffer, the preparation of heart homogenate and the protocol for titrations and T_1 measurements were described in Experimental. Chemical shifts are determined at 161.9 MHz relative to 85% H_3PO_4 in D_2O as external reference and are expressed as means±standard deviations from three independent measurements.
[a]δ_a, limiting chemical shift in acidic medium; δ_b, limiting chemical shift in basic medium; $\Delta\delta_{ab} = \delta_a - \delta_b$.
[b]Not measurable.

were found to be very sensitive to compartment localization with respect to their T_1 values (ranging 9–14 s extracellularly and 2–6 s intracellularly),[12–15] implying to operate with high interpulse delays to allow complete relaxation. Table 2 shows that for the new prepared aminophosphonates, T_1 values measured in Krebs buffer, considered close to T_1 extracellular values, should be compatible with biological applications being considerably shorter (by 2–9 s) than that of endogenous phosphorylated metabolites such as P_i, ATP or creatine phosphate.[12–15,33,34] A moderate shortening of T_1 (by 1–2 s) was observed in the case of the new aminophosphonates having the greatest potential as in vivo acidic pH markers (i.e., compounds **3**, **5** and **9**; see below) when the measurements were performed in a heart homogenate taken as a typical cytosolic medium (Table 2).

To assess whether newly synthesized compounds **3**, **5**–**7** and **9** could be used safely as in vivo pH probes, toxicity measurements were performed on Müller cells cultures. These neuronal cells are known to be highly sensitive to the toxic effects of xenobiotics and may provide a realistic picture of the toxicity of aminophosphonates on mammalian cells. Compound **4** was considered too acidic to be used for biological applications and the stability of compound **2** in aqueous medium did not exceed a few minutes. Figure 1 illustrates the cytotoxicity of aminophosphonates as assessed by lactate dehydrogenase (LDH) release, a standard index of cell injury. At physiologic-relevant concentrations (1 and 5 mM), none of the tested compounds was toxic, with the exception of the more lipophilic **6**. A higher concentration was further tested (15 mM) to cope with a more comfortable NMR detection in in vivo pH measurement experiments and, under these conditions, **7** and in a lesser extent **5** exerted a very weak toxic effect (Fig. 1). In our previous report,[11] **1** was found not toxic when perfused up to 10 mM on isolated rat hearts and livers.

In a last series of experiments, the sensitivity of pK_a to ionic strength and to cytosolic hydrosoluble molecules was investigated for the non toxic and highly sensitive pH markers **3**, **5** and **9**. Considering the Debye–Hückel equation, the apparent pK_a will depend on the activity coefficients of the acidic and basic forms as well as on temperature and ionic strength of the solution.[7–9] Figure 2 shows that the pK_a of tested aminophosphonates remains relatively constant (varying by only +0.09–0.12 pH units versus −0.31 pH units for P_i) in a wide ionic strength range. As was already reported for P_i,[8,9] the pK_as of the three tested compounds were found to vary linearly with ionic strength (Fig. 2). In a second set of experiments, the magnitude of the effect of solutes contained into the cytosol (including divalent cations such as Mg^{2+} and Ca^{2+}, and soluble proteins) was studied by comparing the pK_a values of P_i and of compounds **3**, **5** and **9** measured in a KCl (125 mM) solution and in a KCl (125 mM)-supplemented extract of heart homogenate. The changes in apparent pK_a when the aminophosphonates were placed in a cytosol-like environment was in the moderate range of 0.08–0.09 pH units (data not shown). Taken together, the results above show that the newly synthesized aminophosphonates also fit with an another important prerequisite of pH probes, that is, a low variation of their pK_a with changes in ionic strength that do occur between two cellular compartments.

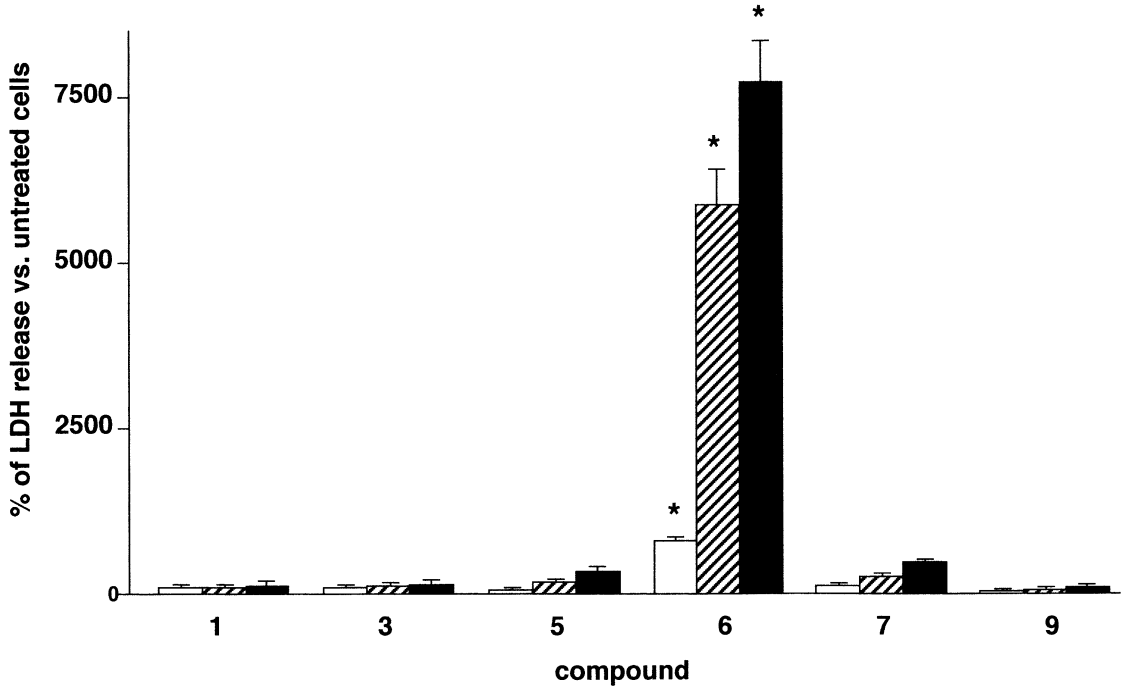


Figure 1. Effect of aminophosphonates on lactate dehydrogenase (LDH) release of cultured rat Müller cells. Cells were exposed to compounds at 1 mM (□), 5 mM (▨) or 15 mM (■) for 3 h before LDH assay. Data represent means ± SD from four distinct experiments made in triplicate, and are expressed as percent of LDH amount found in cells incubated for 3 h with medium alone (control experiments). Statistics [one-way ANOVA ($p < 0.01$) followed by Duncan t-test]: * $p < 0.01$ versus control.

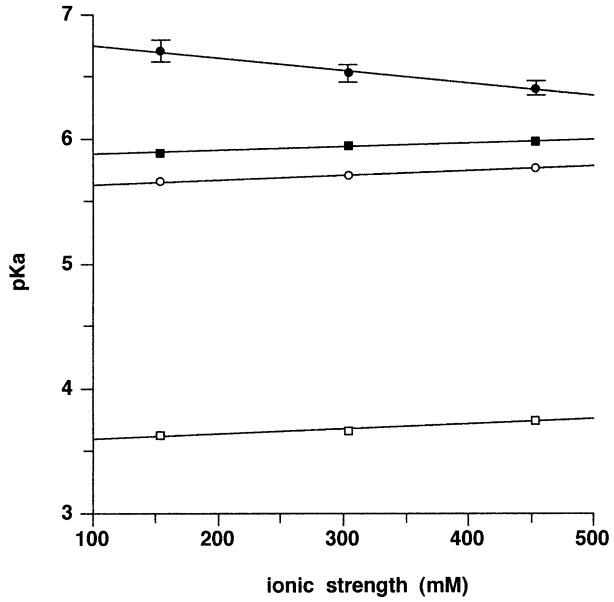


Figure 2. Comparative effect of ionic strength on the pK_a of highly sensitive acidic pH markers (**3**, □; 5, ■ and **9**, ○) at 5 mM and of P_i (●; 1.2 mM). ^{31}P NMR experiments were performed at 20 °C in a Krebs buffer supplemented with 118.5, 268.5 or 418.5 mM of NaCl. Data represent means ± SD ($n = 3$). The variations were found linear with correlations ranging from 0.982 to 0.999.

Conclusion

In conclusion, different aminophosphonates were designed and prepared to be used as auxiliary ^{31}P NMR pH markers of acidic cellular compartments that were not accessible using conventional exogenous probes.[12–15] Among the series of new compounds described in the present paper, a diphosphorylated cyclic pyrrolidine and two linear aminophosphonates were found to meet the main criteria for in vivo use as ^{31}P NMR pH markers since they (i) have a pK_a value in the pH range of acidic vesicles, (ii) exhibit a great pH-induced chemical shift variation and relatively short T_1 values and, (iii) are not toxic at concentrations compatible with in vivo NMR experiments. The assessment by ^{31}P NMR of acidic compartments has been performed in a few studies[35–37] using phosphonates probes that were far to possess the remarkable properties shared by most of the compounds presently described in terms of sensitivity ($\Delta\delta_{ab} = 1$–3 ppm). It was found that some cellular types such as *Dictyostelium discoideum* possess both an highly acidic endolysosomal and a less acidic post-lysosomal compartments.[35,36] With to aim to precisely follow the intracellular and vacuolar pH gradients in such complex systems, the new probes developed in this study, particularly compounds **3**, **5** and **9**, should provide new insights into the dynamics of intercompartment proton exchanges.

Experimental

Solvents, starting materials and reagents used for titration experiments were of the highest grade available from Aldrich Chimie (Saint Quentin Fallavier, France). Column chromatography was performed on Merck silicagel 60, 60–230 or 230–400 mesh for flash chromatography. Melting points were measured on a Büchi B510 apparatus and are uncorrected. NMR spectra were obtained on Bruker AC 100, AC 200, AC 300 or AMX 400 spectrometers. Chemical shifts (δ) are expressed in ppm to internal TMS (^{1}H and ^{13}C) or to 85% external H_3PO_4 (^{31}P) and J values are given in Hz. Doubly distilled deionized water was used in all titration experiments and test solutions were filtered through a 0.2 μm Millipore filter prior to use.

Ultrapure **1** was obtained by a modification[38] of the method of Fréjaville et al.,[30] by using commercial 2-methylpyrroline and diethyl phosphite as starting material. Tetraethyl(pyrrolidin-2,2-diyl)biphosphonate **3** and tetraethyl(*N*-methyl-pyrrolidin-2,2-diyl)biphosphonate **4** were prepared according to published procedures.[17,18,39,40]

Synthesis of diethyl(*N*-methyl-1-2-methyl pyrrolidin-2-yl)phosphonate (2). 5-Chloropentan-2-one (6.0 g, 40.8 mmol) in EtOH (15 mL) was added during 30 min at room temperature to a mixture of ethanolic methylamine (8.03 M, 20 mL) and diethylphosphite (10.4 g, 75.4 mmol). After stirring 12 h at 25 °C, ethanol was removed under vacuum and the resulting mixture was poured into water (100 mL), acidified to pH 1 with 11 N hydrochloric acid and extracted with CH_2Cl_2 (4×20 mL). The aqueous layer was treated with NaOH to reach pH 12, saturated with NaCl and then extracted with Et_2O (4×20 mL). The combined organic phases were dried over Na_2SO_4 and concentrated under vacuum to yield a yellow oil which was distilled under vacuum to yield **2** (6.0 g, 62%), bp 62 °C at 6×10^{-2} mbar (found: C, 50.65; H, 9.16; N, 5.71; P, 13.23. $C_{10}H_{22}NO_3P$ requires C, 50.80; H, 9.32; N, 5.93; P, 13.56); δ_P (161.9 MHz; $CDCl_3$) 30.01; δ_H (400 MHz; $CDCl_3$) 3.95–4.10 (4H, m, CH_2–O), 2.90 (1H, m, 5-H), 2.54 (1H, m, 5-H), 2.39 (3H, s, CH_3-N), 2.30 (1H, m, 3-H), 1.45–1.80 (3H, m, 3-H, 4-H), 1.23 (3H, t, $J = 7.04$, $\underline{CH_3}$–CH_2O), 1.22 (3H, t, $J = 7.14$, $\underline{CH_3}$–CH_2O), 1.15 (3H, d, $J_{HP} = 15.84$, 2-Me); δ_C (75 MHz; $CDCl_3$) 62.35 (d, $J_{CP} = 7.5$, CH_2–O), 61.52 (d, $J_{CP} = 7.4$, CH_2–O), 61.09 (d, $J_{CP} = 165.2$, 2-C), 55.07 (s, 5-C), 54.92 (s, N-CH_3), 36.64 (s, 4-C), 36.06 (s, 3-C), 22.79 (d, $J_{CP} = 6.1$, 1-C), 17.70 (d, $J_{CP} = 9.0$, $\underline{CH_3}$–CH_2O), 16.90 (d, $J_{CP} = 5.8$, $\underline{CH_3}$–CH_2O).

Synthesis of compounds 5 and 6

The general procedure involved the stirring at room temperature for 48 h of a mixture of acetophenone and the corresponding amine in the presence of Na_2SO_4 and 0.5 mL of 11 N HCl. After addition of diethylphosphite and an additional 48 h stirring at room temperature, the crude mixture was poured into 150 mL water, acidified to pH 1 with 11 N HCl, extracted with Et_2O (4×50 mL), treated with Na_2CO_3 and extracted with $CHCl_3$ (3×50 mL). The combined organic phases were dried over $MgSO_4$ and concentrated under vacuum to lead to the aminophosphonate.

Diethyl (1-phenyl-1-propylaminoeth-1-yl) phosphonate (5). From acetophenone (10.4 g, 86.7 mmol), *n*-propylamine (8.4 g, 142.4 mmol), Na_2SO_4 (4.0 g, 28.2 mmol) and diethylhosphite (14.6 g, 105.8 mmol), yielded **5** (7.6 g, 29%), bp 111 °C at 9×10^{-2} mbar (Found: C, 60.69; H, 8.61; N, 3.88, P, 9.55. $C_{15}H_{26}NO_3P$ requires C, 60.80; H, 8.69 N, 4.68; P, 10.7); δ_P (121.47 MHz; $CDCl_3$) 26.9; δ_H (300 MHz; $CDCl_3$) 7.6 (2H, m, CH_{ar}), 7.20–7.40 (3H, m, CH_{ar}), 3.75–4.05 (4H, m, CH_2–O), 2.45 (1H, dt, $J=11.0$, $J=7.5$, CH_2–N), 2.25 (1H, dt, $J=11.0$, $J=7.5$, CH_2–N), 1.85 (1H, br s, NH), 1.77 (3H, d, $J_{HP}=21.0$, 1-Me), 1.50 (2H, sextet, $J=7.5$, CH_2), 1.25 (3H, t, $J=6.0$, $\underline{CH_3}$–CH_2O), 1.15 (3H, t, $J=6.0$, $\underline{CH_3}$–CH_2O), 0.87 (3H, t, $J=6.0$, CH_3); δ_C (75 MHz; $CDCl_3$) 139.52, (s, C_{ar}), 127.92 (s, 4×CH_{ar}), 127.0 (s, CH_{ar}), 62.98 (s, CH_2–O), 62.84. (d, $J_{CP}=7.4$, CH_2–O), 59.89 (d, $J_{CP}=152.6$, 1-C), 43.43 (d, $J_{CP}=13.8$, CH_2–N), 23.78 (s, 1-Me), 20.64 (s, CH_2), 16.28 (s, $\underline{CH_3}$–CH_2O), 16.20 (s, $\underline{CH_3}$–CH_2O), 11.82 (s, CH_3).

Diethyl (1-phenyl-1-benzylaminoeth-1-yl) phosphonate (6). From acetophenone (14.7 g, 122.5 mmol), benzylamine (10.0 g, 93.3 mmol), Na_2SO_4 (10.0 g, 70.4 mmol) and diethylphosphite (14.1 g, 102.2 mmol), yielded **6** (14.0 g, 43%), mp 43 °C [(from pentane) found: C, 65.93; H, 7.50; N, 4.05; P, 9.11. $C_{19}H_{26}NO_3P$ requires C, 65.58; H, 7.47; N, 4.02; P, 9.19]; δ_P (40.48 MHz; $CDCl_3$) 26.96; δ_H (400 MHz; $CDCl_3$) 7.63 (1H, d, $J=9.3$, CH_{ar}), 7.10–7.40 (9H, m, CH_{ar}), 3.80–4.10 (4H, m, CH_2–O), 3.64 (1H, d, $J=12.5$, CH_2–Ph), 3.50 (1H, d, $J=12.5$, CH_2–Ph), 2.30 (1H, br s, NH), 1.85 (3H, d, $J_{HP}=16.2$, 1-Me), 1.25 (3H, t, $J=7.0$, $\underline{CH_3}$–CH_2O), 1.16 (3H, t, $J=7.0$, $\underline{CH_3}$–CH_2O); δ_C (75 MHz; $CDCl_3$) 140.49 (s, C_{ar}), 139.28 (d, $J_{CP}=3.1$, C_{ar}), 128.32 (s, CH_{ar}), 128.13, (s, CH_{ar}), 128.08 (s, CH_{ar}), 128.04 (s, CH_{ar}), 127.97 (s, CH_{ar}), 127.22 (d, $J_{CP}=2.9$, CH_{ar}), 126.88 (s, CH_{ar}), 63.21 (d, $J_{CP}=7.4$, CH_2–O), 62.95 (d, $J_{CP}=7.5$, CH_2–O), 60.27 (d, $J_{CP}=152.6$, 1-C), 46.06 (d, $J_{CP}=14.3$, CH_2–N), 21.0 (s, 1-Me), 16.40 (d, $J_{CP}=5.7$, $\underline{CH_3}$–CH_2O), 16.33 (d, $J_{CP}=7.5$, $\underline{CH_3}$–CH_2O).

Synthesis of compounds 7–9. The general procedure involved the stirring at room temperature for 40 h of acetaldehyde with a mixture of diethylphosphite and the corresponding amine or aminoalcool. After vacuum removal of the excess of unreacted acetaldehyde and amino compound, the mixture was poured into 150 mL water, treated with 11 N HCl until pH 1 and extracted with 3×30 mL CH_2Cl_2. The remaining aqueous phase was treated with aqueous NaOH to reach pH 12, saturated with NaCl and extracted with 3×20 mL CH_2Cl_2. The combined organic phases were dried over Na_2SO_4 and concentrated under vacuum to yield the pure aminophosphonates **7** and **8** as yellow oils. In the case of **9**, purification was achieved by distillation.

Diethyl (1-butylaminoeth-1-yl) phosphonate (7). From acetaldehyde (2.2 g, 50.2 mmol), *n*-butylamine (3.65 g, 50.0 mmol) and diethylphosphite (6.9 g, 50.0 mmol), yielded **7** (8.3 g, 70%), (found: C, 50.24; H, 10.11; N, 6.18; P, 12.96. $C_{10}H_{24}NO_3P$ requires C, 50.62; H, 5.90; N, 10.19; P, 13.05); δ_P (121.47 MHz; $CDCl_3$) 28.93; δ_H (300 MHz; $CDCl_3$) 4.05–4.20 (4H, m, CH_2–O), 2.93 (1H, dq, $J=7.0$, $J_{HP}=12.8$, 1-H), 2.70 (1H, m, CH_2–N), 2.61 (1H, m, CH_2–N), 1.41 (2H, m, $\underline{CH_2}$–CH_2–N), 1.31 (2H, m, CH_3–$\underline{CH_2}$), 1.29 (6H, t, $J=7.05$, $\underline{CH_3}$–CH_2O), 1.28 (3H, dd, $J=7.1$, $J_{HP}=17.7$, 1-Me), 0.87 (3H, t, $J=7.3$, CH_3); δ_C (75 MHz; $CDCl_3$) 61.91 (d, $J_{CP}=7.4$, CH_2–O), 61.53 (d, $J_{CP}=7.5$, CH_2–O), 50.24 (d, $J_{CP}=154.3$, 1-C), 47.59 (d, $J_{CP}=10.9$, CH_2–N), 32.08 (s, $\underline{CH_2}$–CH_2–N), 20.1 (s, CH_3–$\underline{CH_2}$), 16.32 (d, $J_{CP}=5.8$, $\underline{CH_3}$–CH_2O), 16.30 (d, $J_{CP}=5.8$, $\underline{CH_3}$–CH_2O), 14.98 (s, 1-Me), 13.72 (s, CH_3).

Diethyl(1-(*t*-butylamino)-eth-1-yl)phosphonate (8). From acetaldehyde (7.0 g, 159.0 mmol), *t*-butylamine (9.0 g, 123.3 mmol) and diethylphosphite (13.8 g, 100.0 mmol), yielded **8** (17.0 g, 72%), (found: C, 47.96; H, 9.87; N, 5.39, P, 13.60. $C_{10}H_{24}NO_3P$ requires C, 50.62; H, 10.19; N, 5.90; P, 13.05); δ_P (121.47 MHz; $CDCl_3$) 29.14; δ_H (300 MHz; $CDCl_3$) 4.69 (1H, br s, NH), 4.00–4.20 (4H, m, CH_2–O), 2.99 (1H, dq, $J=7.1$, $J_{HP}=10.3$, 1-H), 1.34 (3H, dd, $J=7.2$, $J_{HP}=17.7$, 1-Me), 1.33 (3H, t, $J=7.3$, $\underline{CH_3}$–CH_2O), 1.32 (3H, t, $J=7.1$, $\underline{CH_3}$–CH_2O), 1.03 (9H, s, *t*-Bu); δ_C (75 MHz; $CDCl_3$) 62.85 (d, $J_{CP}=7.4$, CH_2–O), 61.50 (d, $J_{CP}=7.5$, CH_2–O), 51.46 (d, $J_{CP}=12.0$, C, *t*-Bu), 44.8 (d, $J_{CP}=164.6$, 1-C), 29.61 (s, CH_3, *t*-Bu), 18.97 (d, $J_{CP}=2.3$, 1-Me), 16.35 (d, $J_{CP}=6.5$, $\underline{CH_3}$–CH_2O), 16.27 (d, $J_{CP}=6.5$, $\underline{CH_3}$–CH_2O).

Diethyl(1-(1,1-dimethyl-2-hydroxyethylamino)-eth-1-yl)-phosphonate (9). From acetaldehyde (4 g, 90.9 mmol), 2-amino-2-methylpropan-1-ol (4.5 g, 50.6 mmol) and diethylphosphite (6.9 g, 50.0 mmol), yielded **9** (7.6 g, 60%), bp 108 °C at 5×10^{-2} mbar (found: C, 46.01; H, 9.41; N, 5.85, P, 12.01. $C_{10}H_{24}NO_4P$ requires C, 47.42; H, 9.55; N, 5.53; P, 12.23); δ_P (121.47 MHz; $CDCl_3$) 29.59; δ_H (300 MHz; $CDCl_3$) 4.0–4.25 (4H, m, CH_2–O), 3.40 (1H, d, $J=11.7$, CH_2–OH), 3.10 (1H, d, $J=11.7$, CH_2–OH), 2.99 (1H, dq, $J=7.1$, $J_{HP}=16.1$, 1-H), 1.34 (3H, t, $J=6.9$, $\underline{CH_3}$–CH_2O), 1.33 (3H, t, $J=7.0$, $\underline{CH_3}$–CH_2O), 1.27 (3H, dd, $J=7.1$, $J_{HP}=17.3$, 1–Me), 1.08 (3H, s, CH_3), 0.97 (3H, s, CH_3); δ_C (75 MHz; $CDCl_3$) 67.63 (s, CH_2–OH), 62.87 (d, $J_{CP}=7.5$, CH_2–O), 61.91 (d, $J_{CP}=7.4$, CH_2–O), 54.74 (d, $J_{CP}=8.6$, C), 44.06 (d, $J_{CP}=164.7$, 1-H), 26.29 (s, CH_3), 22.86 (s, CH_3), 19.06 (s, 1-Me), 16.37 (d, $J_{CP}=5.7$, $\underline{CH_3}$–CH_2O), 16.28 (d, $J_{CP}=6.3$, $\underline{CH_3}$–CH_2O).

^{31}P NMR titrations

For all aminophosphonates (final concentration 5 mM), the effect at 20 °C of pH on the chemical shift of the ^{31}P NMR resonance peak was tested in a standard Krebs–Henseleit perfusion medium containing NaCl (118.5 mM), KCl (4.8 mM), $MgSO_4$ (1.2 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (1.2 mM), and having a ionic strength representative of the extracellular milieu (i.e., 0.154). For three selected aminophosphonates **3**, **5** and **9**, this experiment was repeated in the same Krebs–Henseleit milieu containing higher NaCl concentrations in order to reach ionic strengths of 0.304 and 0.454. For the same three aminophosphonates as above, the effect of pH on the ^{31}P NMR chemical shift was also tested either in an aqueous KCl (125 mM) solution of physi-

ological intracellular ionic strength (i.e., 0.125) or in a KCl (125 mM)-supplemented rat heart homogenate (prepared from freshly excised rat hearts as previously described[11]) taken as a complete intracellular medium. For all the previously described experiments, the pH was adjusted to 15–20 different values in the range 1.0–12.0 with 6 N solutions of HCl or NaOH and a sample was transferred into a 10 mm NMR tube for ^{31}P NMR analysis (161.9 MHz) at 20 °C. The pK_a values were calculated by iterative fitting of the δ and pH data according to the standard Henderson–Hasselbalch equation:

$$pH = pK_a + \log [(\delta - \delta_a)/(\delta_b - \delta)]$$

using a nonlinear regression.

T_1 was measured at $pH = pK_a$ using the inversion/recovery (180°–τ–90°) TR pulse-sequence with a 180° composite pulse. The repetition time (TR) was always 6–7 times the value of T_1 and the number of scans were 64.

Toxicity experiments on cultured Müller cells

Eyes from 9–11 days Sprague–Dawley rats (CERJ, France) were stored overnight in Dulbecco's modified Eagle's Medium (DMEM) at room temperature in the dark according to Hicks and Courtois.[41] Retinas were isolated in fresh DMEM, then enzymatically dissociated in phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+}, with trypsine/EDTA 0.05% and DNase (80U, Sigma Aldrich Chimie, France) for 25 min at 37 °C. Cells were then mechanically dissociated with a sterile Pasteur pipette in culture medium (i.e., DMEM containing 1 g/L glucose, 10% fetal calf serum and penicillin [100 U/mL)/streptomycin (100 μg/mL)]. DMEM, PBS, trypsine/EDTA and fetal calf serum were obtained from Life Technologies, France. After centrifugation, retinal cells were suspended in culture medium and seeded in 3-cm culture dishes at 11 million cells/dish and maintained in a 5% CO_2/humidified atmosphere at 37 °C. Medium was removed 3 days after plating and replenished every 2–3 days until confluency was achieved. Müller glial cells were trypsinized and subcultivated one time (P_1) in 24-well dishes. Cell purity was assessed by immunocytochemical characterization of glial fibrillary acid protein-containing glial cells.

Cellular injury was assessed by LDH release in the cell culture. Confluent cells at P_1 were incubated 3 h at 37 °C in DMEM without phenol red containing 1 g/L glucose in the presence of increasing concentrations of aminophosphonates **1**, **3**, **5**–**9** (i.e., 1, 5 or 15 mM). The LDH activity was assayed spectrophotometrically using a commercial kit (Roche Diagnostic, GmbH, Germany). For each aminophosphonate concentration, the results were obtained from four distinct experiments made in triplicate and were expressed as percent of their respective controls. Evaluation of statistical significance was conducted by one-way analysis of variance (ANOVA) followed by Duncan *t*-test.

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