Novel Sterically Crowded and Conformationally Constrained α-Aminophosphonates with a Near-Neutral pKₐ as Highly Accurate ³¹P NMR pH Probes. Application to Subtle pH Gradients Determination in Dictyostelium discoideum Cells

Caroline Delehedde, Marcel Culcasi, Emilie Ricquebourg, Mathieu Cassien, Didier Siri, Bruno Blaive, Sylvia Pietri and Sophie Thétiot-Laurent

Abstract: In order to discover new ³¹P NMR markers for probing subtle pH changes (<0.2 pH unit) in biological environments, fifteen new conformationally constrained or sterically hindered α-aminophosphonates derived from diethyl(2-methylpyrrolidin-2-yl)phosphonate were synthesized and tested for their pH reporting and cytotoxic properties in vitro. All compounds showed near-neutral pKₐ (ranging 6.28–6.97), chemical shifts not overlapping those of phosphorus metabolites, and spectroscopic sensitivities (i.e., chemical shifts variation Δδ between the acidic and basic forms) ranging from 9.2–10.7 ppm, being fourfold larger than conventional endogenous markers such as inorganic phosphate. X-ray crystallographic studies combined with predictive empirical relationships and ab initio calculations addressed the inductive and stereochemical effects of substituents linked to the protonated amine function. Satisfactory correlations were established between pKₐs and both the 2D structure and pyramidalization at phosphorus, showing that steric crowding around the phosphorus is crucial for modulating Δδ. Finally, the hit ³¹P NMR pH probe 1b bearing an unsubstituted 1,3,2-dioxaphosphorinane ring, which is moderately lipophilic, nontoxic on A549 and NHLF cells, and showing pKₐ = 6.45 with Δδ = 10.64 ppm, allowed the first clear-cut evidence of trans-sarcolemmal pH gradients in normoxic Dictyostelium discoideum cells with an accuracy of <0.05 pH units.

Keywords: α-aminophosphonates; pH probes; ³¹P NMR; phosphorus pyramidalization angle; X-ray crystallography; trans-sarcolemmal pH gradients; Dictyostelium discoideum cells

1. Introduction

Intracellular (cytosolic) pH (pHᵢ) is a fundamental physiological parameter whose changes affect nearly all metabolic and growth/proliferation cellular processes, and can also occur in response to exogenous agents. Maintaining pH homeostasis in the cytosol, extracellular milieu (pHₑ) and subcellular compartments (such as lysosomes, mitochondria, and endosomal vesicles) is crucial for all living organisms. Among the mechanisms of pHᵢ regulation, the Na⁺/H⁺ antiporter represents a major pathway for the exit of protons from cells during acidification and the entry of Na⁺ from the extracellular medium to balance the net charge movement [1]. Indeed, pHᵢ and pHₑ are in a dynamic steady state and, in normal mammalian cells, pHᵢ is close to neutrality (~7.2) while pHₑ is slightly alkaline (ranging 7.3–7.5) [1]. Therefore, experimentally assessing pH regulation in pathological situations requires both pHᵢ and pHₑ to be monitored simultaneously and accurately.
Since Moon and Richard’s first report [2], $^{31}$P nuclear magnetic resonance (NMR) has become a powerful and non-invasive technique for determining pH$_i$ on the basis of the variation of pH, with the chemical shift of endogenous inorganic phosphate (PO$_4^{3-}$; P$_i$) [3–8]. This technique has been applied to a variety of biological systems, e.g., for monitoring bioenergetics and/or the metabolism of various organs [9–15]. $^{31}$P NMR is also routinely used in the study of food chemistry, quality, and safety control [16,17] or for monitoring the production of everyday products [18], to name but a few. Despite P$_i$ being ubiquitous in cells, its use as an ‘universal’ $^{31}$P NMR pH sensor in biological studies has, however, many drawbacks that may impair spectroscopic detection. First, given that the absolute value of the chemical shift difference between the protonated acidic ($\delta_a$) and unprotonated basic ($\delta_b$) forms of P$_i$ ($\Delta\delta_{ab} = |\delta_a - \delta_b|$) is only ~2.6 ppm, with a pK$_a$ ~6.75 (for its second acidity [19]), it cannot sense pH$_i$/pH$_e$ differences of less than 0.3 pH units [2]. Moreover, the fluctuations of P$_i$ concentration during metabolism and its low availability in the extracellular space and many organelles considerably hinder direct access to local pH measurements using this probe [20]. Ideally, the structural requirements for improved $^{31}$P NMR pH markers would both involve (i) increasing $\Delta\delta_{ab}$ as to get pH titration curves with the steepest slope around the pK$_a$ value and (ii) modulating the pK$_a$ value(s) to fine tune extracellular/intracellular pH domains of interest.

In order to minimize the possible cytotoxic effects, the first synthetic attempts towards these above conditions focused on modifications around the P$_i$ structure to get alkylated phosphonic acids RP (O)(OH)$_2$ (Figure 1A), which were found to exhibit slightly increased $\Delta\delta_{ab}$ values and pK$_a$s of 7.0 ± 0.5 [21]. To illustrate, the methylphosphonate pK$_a$ = 7.36, with $\Delta\delta_{ab} = 3.88$ ppm at 20 °C in a Krebs–Henseleit (KH) buffer is currently used in organ perfusion experiments [19]. Further targets were α-, β- and γ-substituted linear aminophosphonic acids (Figure 1B) whose $\Delta\delta_{ab}$ values, however, did not exceed 3.4 ppm [22], but showed relatively weak toxicity and had lower pK$_a$s suitable for probing the acidic domains in cells, tumors, and perfused organs [23–26]. Since in all these above compounds, protonation always occurs at the phosphorus first, it was then speculated that scaffolds bearing a non-ionizable P-atom (e.g., a phosphonate) and a protonable α-amino group (typically, a secondary amine) would offer considerable advantages in terms of $\Delta\delta_{ab}$ and pK$_a$ modulation due to different inductive and steric effects. This approach led to the development of two families of uncharged pH probes, including cyclic pyrrolidine-based α-aminophosphonates (Figure 1C) and linear polysubstituted α-aminophosphonates (Figure 1D) [19,27–31]. For all these compounds, it was found that protonation, which gives the ammonium salt as the acidic species, resulted in lower pK$_a$s and gave $\Delta\delta_{ab}$ values up to fourfold greater than that of P$_i$ or phosphonic acids. Thus, the reported pH titration curves for the α-aminophosphonate having the structure shown in Figure 1D, with R$_1$ = R$_2$ = R$_3$ = Me; R$_4$ = Et; R$_5$ = Me; R$_6$ = H afforded $\Delta\delta_{ab} = 10.3$ ppm and pK$_a$ = 7.01 in KH buffer [28]. Later, the successful mitochondrial targeting of selected compounds shown in Figure 1D was achieved by grafting a triphenylphosphonium cation (Figure 1E), and most of the resulting structures retained the good pH-probing performance of their parent compounds [32,33]. To illustrate, using a nontoxic micromolar concentration of the triphenylphosphonium bromide having the structure shown in Figure 1E (right part) with R$_1$ = R$_2$ = H; R$_3$ = R$_4$ = Me; n = 7, for which $\Delta\delta_{ab} = 10.3$ ppm and pK$_a$ = 6.99, allowed the first $^{31}$P NMR assessment of acidic and cytosolic mitochondrial compartments of the green alga *Chlamydomonas reinhardtii* [33].
Figure 1. (A–E) Families of phosphorylated derivatives used as exogenous $^{31}$P NMR pH probes. (F) Conformationally constrained CyDEPMPHs pH probes (current study). (G) Sterically crowded DEPMPH-derived pH probes (current study). * $pK_a$ and $\Delta \delta_{ab}$ values of the second OH of phosphonic group.

The knowledge of small trans-sarcolemmal pH gradients is one of the most important pieces of dynamic information to be obtained using very sensitive pH probes, especially when the external $P_i$ peak is hardly or not detectable [34]. This was addressed in normoxic perfused rat hearts either by $^{31}$P NMR with the cell impermeable phenylphosphonic acid (Figure 1A; $R = \text{Ph}$) [34] or by $^{19}$F NMR using a fluorinated pyridine derivative [35], and in these early studies, reachable trans-sarcolemmal gradients were found to be 0.24 and 0.38 pH units, respectively. Based on their significantly larger $\Delta \delta_{ab}$s when compared to, e.g., phenylphosphonic acid (having $pK_a = 7.00$ and $\Delta \delta_{ab} = 2.10$ ppm; Figure 1A [21]), many newer $^{31}$P NMR pH probes displayed in Figure 1C,D could be much better alternatives in probing more subtle pH changes (<0.2 pH units) occurring in acidotic cells. Actually, such an improvement was obtained using diethyl(2-methylpyrrolidin-2-yl)phosphonate $1a$ (DEPMPH, having $pK_a = 7.01$ and $\Delta \delta_{ab} = 9.77$ ppm [19]; see Figure 1 inset) during ischemia of the rat isolated heart or liver [27]. However, despite the given advantage to probe the intra, extra and acidic regions simultaneously, $1a$ was found to be less accurate than $P_i$ for cytosolic pH determination [27]. The line width broadening at pH $\approx pK_a$ impairs any accurate pH determination [29], therefore excluding probes having a $pK_a$ close to the pH region of interest. Moreover, the strictly neutral $pK_a$ of $1a$ was a drawback for assessing accurately the pH variations in acidic compartments [27]. It can therefore be inferred that, besides a $\Delta \delta_{ab}$ value $> 10$ ppm, a better differentiation between the intracellular vs. extracellular NMR peaks would demand shifting of the $pK_a$ of the probe downwards to, e.g., 6.2–6.8.

Previously a semi-empirical linear model was established for predicting the $pK_a$ of $1a$ and derivatives based on their 2D geometries and the additive inductive effect of substituents in $\beta$ position of the nitrogen (e.g., $R_1$–$R_5$ and the phosphonyl group in Figure 1D) [19]. In the present study the DEPMPH pyrrolidine scaffold was used to elaborate novel derivatives in which the $P$-bound alkoxy groups were either constrained as a 2-oxo-1,3,2-dioxaphospharinane ring (CyDEPMPHs family $1b$–f; Figure 1F) or induced steric crowding around the $P$-atom (crowded family $1g$–p; Figure 1G). Compounds were investigated for their $^{31}$P NMR relaxation properties and pH reporting behavior in vitro. The collected experimental $\Delta \delta_{ab}$ values and $pK_a$s were found to be consistent inputs to consolidate the empirical relationships of [19]. Additional density functional theory (DFT) calculations were performed on the [C–P–O] pyramidalization angle of compounds $1a$–p,
pooled with a large series of structurally related compounds. The results, together with structural complements using X-ray structures of those crystallized CyDEPMPHs, established a relationship between pyramidalization around P and $\Delta \delta_{ab}$. From the above parameters, experimentally determined cytotoxicities, and calculated lipophilicities, the hit compound 1b was selected and its ability to simultaneously probe the intra, extra and acidic regions was checked in normoxic Dictyostelium discoideum cells. For the first time, a trans-membrane pH gradient with an estimated precision of $<0.05$ pH units was found in full accordance with the pH values given by the cytosolic and external P$_i$ peaks, suggesting that 1b could be a useful tool in a pathological situation where P$_i$ peaks fall below the NMR detection limit.

2. Results and Discussion

2.1. Chemistry and X-ray Crystallography

Aminophosphonates 1a–p were synthesized according to the general sequence outlined in Scheme 1, involving the reaction of the corresponding dialkyl H-phosphonate 2a–p with 2-methyl-1-pyrroline. Starting compounds dialkyl H-phosphonates 2a, 2g, 2i, and 2j are commercially available, while the other precursors 2b–f, h, k–p were synthesized according to [36] with slight modifications. Briefly, H-phosphonic bis(dimethylamide) was prepared in situ by the addition of one equivalent of water to hexamethylphosphorous triamide (HMPT) in tetrahydrofuran (THF), and then alcohol was added to give the corresponding compounds 2b–f, h, k–p. Reactions were monitored directly by $^{31}$P NMR by following the disappearance of HMPT peak ($\delta = 122.24$ ppm) followed by the formation of bis(dimethylamino)phosphoric acid peak ($\delta = 24.0$ ppm), then by the disappearance of this latter and the formation of the corresponding dialkyl H-phosphonate peak. Dialkyl (2-methylpyrrolidin-2-yl) phosphonates 1a–p were obtained by the nucleophilic addition of dialkyl H-phosphonates 2a–p onto 2-methyl-1-pyrroline in toluene [37] in good yields (49–73%).

![Scheme 1](image)

Scheme 1. General synthesis of aminophosphonates 1a–p. Reagents and conditions: (i) 1 equiv H$_2$O, 3 h, 70 °C, THF; (ii) 1 equiv diol (series b–f), 6 h, 70 °C, THF or 2 equiv alcohol (series h,k–p), 3 h, 70 °C, THF; (iii) 1.1 equiv 2-methyl-1-pyrroline, 2 h–10 d, rt, toluene.

Single crystals suitable for X-ray diffraction analysis were obtained for the five compounds 1b, trans-1c, and 1d–f by recrystallization from a dichloromethane:tert-butylmethylether (TBME) mixture (Figure 2). The pyrroline rings were found to adopt puckered conformations which can be either the E + 4 ($\phi = 72^\circ$) envelope conformation (1b), the T1 ($\phi = 18^\circ$) twist conformation (trans-1c) or an intermediate situation between the T1 and E + 4 conformations (1d–f) according to the classification of [38]. The C–P bond is pseudo-axial in compounds 1e and 1f and pseudo-equatorial in compounds 1b, trans-1c, and 1d (Table 1), as referred to by the sign of the C(5)–N(1)–C(2)–C(3) dihedral angle in nitrones analogues of CyDEPMPHs [39].
Figure 2. ORTEP drawings of CyDEPMPHs (A) 1b; (B) trans-1c; (C) 1d; (D) 1e; (E) 1f showing the labeling of atoms (see also Table 1 heading) and their displacement ellipsoids at the 40% probability level. For clarity, H-atoms have been omitted.

2.2. pH Dependent $^{31}$P NMR Properties of New $\alpha$-Aminophosphonates

The pH dependence of the $^{31}$P NMR chemical shift of 1a–p was investigated at 22 °C in KH buffer (pH 7.35). Monophasic acid–base titration curves reflecting protonation on nitrogen were obtained for all the derivatives (see examples in Figure 3) and their fitting to the Henderson–Hasselbalch equation (see Equation (2) in Section 3) allowed the calculation of $pK_a$s, the $^{31}$P NMR parameters ($\delta_a$, $\delta_b$, and $\Delta\delta_{ab}$), and the spin lattice relaxation time ($T_1$) (Table 2).

Figure 3. pH Dependence of the $^{31}$P chemical shift of CyDEPMPHs 1b, cis-1c, and 1e compared to $P_i$. Titrations were performed at 22 °C in a modified KH buffer (pH 7.35) and each curve was obtained by the nonlinear fitting of 3–10 independent experiments. Outlined: limiting acidic ($\delta_a$) and basic ($\delta_b$) chemical shifts, and their difference ($\Delta\delta_{ab}$) for $P_i$. 
### Table 1. General Structure, Atoms Numbering and Selected X-Ray Crystallographic Data of CyDEPMPHs \(^a\).

| Bond length (Å)      | 1b | trans-1c | 1d | 1e | 1f |
|----------------------|----|----------|----|----|----|
| N(1)–H              | 0.941(0) | 1.058(0) | 1.053(0) | 1.004(0) | 0.990(0) |
| N(1)–C(2)           | 1.481(2) | 1.482(3) | 1.485(3) | 1.482(3) | 1.486(3) |
| N(1)–C(5)           | 1.463(4) | 1.452(3) | 1.478(3) | 1.480(3) | 1.461(3) |
| C(2)–P(6)           | 1.810(2) | 1.813(2) | 1.814(2) | 1.826(2) | 1.815(2) |
| P(6)–O(7)           | 1.466(2) | 1.465(2) | 1.462(2) | 1.466(2) | 1.465(2) |
| P(6)–O(8)           | 1.570(2) | 1.579(2) | 1.573(1) | 1.578(2) | 1.575(1) |
| P(6)–O(12)          | 1.576(2) | 1.571(1) | 1.579(1) | 1.581(2) | 1.561(2) |

| Distance length (Å) | 1b | trans-1c | 1d | 1e | 1f |
|---------------------|----|----------|----|----|----|
| H–O(7)              | 4.559(1) | 4.560(2) | 4.589(0) | 4.644(1) | 4.658(1) |

| Bond angle (°)      | 1b | trans-1c | 1d | 1e | 1f |
|---------------------|----|----------|----|----|----|
| C(5)–N(1)–C(2)     | 108.1(2) | 108.3(2) | 106.4(2) | 109.0(2) | 106.7(2) |
| N(1)–C(2)–C(3)     | 105.7(2) | 105.2(2) | 106.7(1) | 105.0(2) | 106.1(2) |
| N(1)–C(2)–P(6)     | 117.9(2) | 110.9(1) | 108.6(1) | 110.0(2) | 110.6(2) |
| N(1)–C(2)–C(13)    | 112.9(2) | 112.0(1) | 110.5(1) | 111.7(2) | 110.5(1) |
| C(2)–P(6)–O(7)     | 107.5(9) | 107.1(9) | 107.4(8) | 107.7(9) | 109.1(1) |
| C(2)–P(6)–O(12)    | 106.9(9) | 108.2(1) | 108.0(1) | 107.7(9) | 105.1(9) |
| C(13)–C(2)–P(6)    | 109.4(1) | 110.0(2) | 108.8(1) | 108.4(1) | 108.7(2) |

| Dihedral angle (°) | 1b | trans-1c | 1d | 1e | 1f |
|--------------------|----|----------|----|----|----|
| C(5)–N(1)–C(2)–C(3) | −0.5(2) | −1.0(2) | −9.0(2) | 0.4(2) | 10.9(2) |
| C(5)–N(1)–C(2)–P(6) | 117.9(2) | 117.9(2) | 109.3(2) | −117.8(2) | 106.7(2) |
| N(1)–C(2)–C(3)–C(4) | 5.6(0) | 24.1(2) | −12.3(2) | −20.2(2) | 12.9(2) |
| H–N(1)–C(2)–P(6)   | −122.6(2) | 127.9(0) | −132.6(1) | 138.8(1) | 119.7(1) |
| N(1)–C(2)–P(6)–O(7) | −174.1(1) | 175.8(1) | −175.8(1) | 177.2(1) | 178.1(1) |
| N(1)–C(2)–P(6)–O(12) | −92.2(0) | 53.2(2) | −51.6(1) | 53.9(1) | 46.9(2) |
| N(1)–C(2)–P(6)–O(8) | 7.87(1) | −60.4(2) | 61.8(1) | −58.9(1) | −66.5(2) |
| C(13)–C(2)–P(6)–O(7) | 64.2(2) | −61.5(2) | 63.9(1) | −61.3(2) | −68.0(2) |

\(^a\) Values in parentheses are estimated standard deviations.
pH-dependent variations of electron density around the protonation site; that is, pH
probing (see Introduction), exhibiting near-neutral pH variations for linear versus cyclic aminophosphonates (e.g., being 10 min for improved acidosis [40] and 15–20 min following alkalinization [41]), T1 does not therefore appear to be a decisive parameter to discriminate between probes having similar chemical structures.

All the new derivatives fulfilled both the desired conditions for more accurate pH probing (see Introduction), exhibiting near-neutral pH values (ranging 6.28–6.97) and very high ∆δab values. Indeed, the average 31P NMR sensitivities obtained here could compete with those of the best linear aminophosphonates available so far (Table S1). Interestingly, the compounds having no constrained phosphorus bound alkoxy groups such as 1a and almost all elements from the crowded family showed slightly more basic pKa values and lower 31P sensitivities when compared to compounds from the CyDEPMPHs family, for which ∆δab > 10 ppm. The only exception was compound 1f, which retained a high sensitivity despite ∆δab = 9.35 ppm and a pKa value in the range of that of the crowded DEPMPHs (Table 2).

In a biological milieu where NMR peaks are usually broad, any added pH probe should exhibit protonated and non-protonated forms in fast exchange on the NMR timescale to provide a single sharp peak for every cytosolic/intracellular compartment. This implies that the T1 time of the probe should be as short as possible in order for the best resolved signals to be recorded within the timescale of expected variations of biological parameters, such as pH [28]. All new pH probes demonstrated T1 values similar to those found in other related aminophosphonates [28,30]. Expectedly, molecular motion restriction in the conformationally constrained CyDEPMPHs 1b–f versus the long-chain linear phosphonates 1l–p from the crowded series generally resulted in slightly increased T1 values (Table 2). It is worth noting that probe exchanges between cell compartments are slow on T1 and chemical shift NMR timescales, while diffusion kinetics may exhibit strong variations for linear versus cyclic aminophosphonates (e.g., being 10 min for 1a and 60 min for 2-aminoethylphosphonate (Figure 1B with R1−R3 = H and n = 2) [29]). Given that pH variations in vivo may occur in minutes (reported value were of ~10 min following acidosis [40] and 15–20 min following alkalinization [41]), T1 does not therefore appear to be a decisive parameter to discriminate between probes having similar chemical structures.

2.3. pKa Modeling as a Function of Substituents

Predicting pKa values by molecular modeling is of paramount importance in the design of improved 31P NMR pH markers. In this regard, a pertinent approach is to consider the pH-dependent variations of electron density around the protonation site; that is, pKa should
depend linearly on the sum of individual inductive and stereochemical contributions of substituents. This has been applied successfully, first to a series of alkylphosphonic acids as depicted in Figure 1A [42], second to the cyclic and linear α-aminophosphonates developed in the early 2000s (see Figure 1C,D). In these studies [19,28], pKₐs were computed using the semi-empirical linear model of Equation (1):

\[ \text{pK}_a = a_0 + \sum a_i \times n_i \]  

where, for a given structurally related family of compounds, \( a_0 \) is the pKₐ of the common parent chemical structure and parameters \( n_i \) (with maximum \( \Sigma n_i = 6 \)) and \( a_i \) (in pH units, with \( 1 < a_i < 10 \)) represent the number of each different kind of substituents of type \( i \) having the same structure and/or position relative to the nitrogen protonation site and the weight of its electronic effect relative to the pKₐ value \( a_0 \), respectively.

Starting from the general dialkylamino structure with up to seven surrounding substituents, a new refinement, including compounds 1a–p, of the previous \( a_i \) database collected from earlier studies afforded the updated Table S2 (see general structure in heading), where the selected reference structure bears a cycloalkyl substituent. The extended \( a_i \) database encompassed a total of 59 aminophosphonates, comprising 1a and the new analogues 1b–p, twenty-two linear (LAP-1–22) and ten cyclic (CAP-1–10) aminophosphonates, five aminophosphonic acid derivatives (APA-1–5), and six alkylamines (Tables S1 and S2) studied previously [19,22,28–31,39,43,44]. As shown in Figure 4, updating the \( a_i \) values still resulted in a good fit (\( R^2 = 0.9876 \)) between experimental and calculated pKₐs.

![Figure 4](image_url)

**Figure 4.** Correlation between experimental and calculated pKₐ values for a pooled database of 59 α-aminophosphonates using the updated linear predictive model of Equation (1) (see also Table S2). The red circle indicates the pKₐ domain of new compounds 1b–p.

In the computational pKₐs model of Equation (1), all compounds 1a–p share the same substitution pattern, i.e., one H atom on the nitrogen, two C(5)–H, three alkyls on C(1) and C(5), and one C(1)–Phosphonate. Having the same \( \{n_1–n_{10}\} \) set of \( \{1, 0, 2, 3, 0, 0, 0, 0, 1, 0\} \), compounds 1a–p obviously yielded the same calculated pKₐ = 6.475, while an
experimental range of $\Delta pK_a = 0.75$ was found (Table 2). When compared to this common $pK_a$ value, the experimental $pK_a$s of the more substituted compounds 1f and 1i were found to be strongly underestimated by 0.315 and 0.495 units, respectively (Table 2), suggesting that steric factors should be accounted for in the $pK_a$s predictive model.

2.4. [C–P–O] Pyramidalization Angle Calculations

Previously, electrostatic interactions such as internal $^1$NH$_2$···(O)P hydrogen bonding have been proposed to explain the shielding of the NMR peaks occurring upon protonation of $\alpha$-aminophosphonates (i.e., $\delta_b > \delta_a$), an effect that would decrease the pyramidalization of the angle [C–P–O] of the tetrahedral phosphorylated moiety [19]. In view of the lack of accuracy of the predictive model of Equation (1) for the most sterically hindered new pH probes, it was investigated whether additional conformationally derived effects may be implicated.

DFT-B3LYP calculations using the Gaussian16 package were carried out for optimizing [C–P–O] angle variations between acidic and basic forms ($\Delta[C–P–O]_{ab}$) for 1a–p, pooled with the previously studied compounds LAP-1–34, CAP-1–10, and APA-1–7 (Table S1) [19,22,28–32,39,43,45]. For this large set of compounds (corresponding to 78 pairs of acidic and basic chemical shifts) a moderately good linear correlation ($y = 2.907 x + 3.150$; $R^2 = 0.8816$) was obtained between $\Delta[C–P–O]_{ab}$ and $\Delta\delta_{ab}$ (Figure 5A), confirming that pyramidalization at phosphorus should have a notable impact on the $^3$P NMR sensitivity of aminophosphonates. For simplification, the [C–P–O] pyramidalization angle $\alpha$ here was defined as the average of the three angles between P–O$_{1,3}$ bonds and the p-plane orthogonal to the P–C bond (Figure 5B, inset); that is, $\alpha$ decreases as the pyramid is more flattened. Negative $\Delta[C–P–O]_{ab}$ variations associated to $\Delta\delta_{ab} < 0$ (i.e., $\delta_a > \delta_b$) were found for APA-1–6 aminophosphonic derivatives (Table S1) in the $pK_{a2}$ region of the second acidity (Figure 5A, region I, and Figure 5B, reactions I). A second group of molecules (Figure 5A, region II, and Figure 5B, reactions II) showed small changes in the pyramidalization angle ($0 < \Delta[C–P–O]_{ab} < 1.13^\circ$), associated with low $^3$P NMR sensitivities ($2.3 < \Delta\delta_{ab} < 5.3$ ppm). These latter structures bear at least two carbons between the N and P atoms of the phosphonic derivatives (LAP-23, APA-2,5,7; Table S1).

As anticipated behind the design of compounds 1b–p, Figure 5A (region III) and Figure 5B (reactions III) show that those molecules having the largest $\Delta[C–P–O]_{ab}$ (ranging 1.51–3.60°) also exhibited the greater $\Delta\delta_{ab}$ (6.48 < $\Delta\delta_{ab}$ < 10.94 ppm). Together with 1a–p, region III compounds in Figure 5A also included the derivatives APA-1, LAP-1–22,24–34, and CAP-1–10 (Table S1); that is, in all these molecules, the environment around P is moderately to strongly sterically crowded. Of interest, among the CyDEPMPHs, the apparently less constrained 1b (Figure 2A), which bears a less bulky unsubstituted 1,3,2-dioxaphosphorinane ring, demonstrated the highest protonation-induced $\Delta[C–P–O]_{ab}$ value of 2.10°.

2.5. In Vitro Cytotoxicity Studies

The cytotoxic activities of compounds 1a–p in A549 human lung carcinoma cells and in normal human lung fibroblasts (NHLF) were evaluated in 0.2% DMSO-supplemented Dulbecco’s modified Eagle’s medium (DMEM) and fibroblast basal medium (FBM), respectively. The methods used included the tetrazolium dye MTT reduction, the fluorometric microculture cytotoxicity (FMCA), and the intracellular ATP assays. The MTT and FMCA assays are standard colorimetric determinations of cell viability during in vitro drug treatment while the ATP assay is an index of metabolic activity.

The IC$_{50}$ values following 48 h incubation at 37 °C of the cells with varying concentrations of test compounds are reported in Table 3, together with the predicted lipophilicities based on ALOGP calculations. When the three above-cited endpoints were found to be affected by treatment with a test compound, the release of cytosolic lactate dehydrogenase (LDH) was assessed to check for cell necrosis.
Figure 5. (A) Plot of $^{31}$P NMR sensitivity against ab initio calculated pyramidalization angles at phosphorus between the acidic and basic forms of a series of phosphorylated pH probes. Circled regions I to III visualize increasing pH probing properties. (B) Proton exchange reactions on (I) the O atom of phosphonic or aminophosphonic acids, (II) the N atom of aminophosphonic compounds with at least two carbons between the N and P atoms and (III) the N atom of aminophosphonates or aminophosphonic acids with only one carbon between the N and P atoms. Inset: Pyramidalization angle $\alpha$.

In the supernatant of untreated A549 and NHLF cells, the baseline LDH value was found to be $10.2 \pm 1.5$ and $11.3 \pm 1.9$ UI/mg protein, respectively. When compared to the total LDH activity measured after 100% lysis of the cells, which was $690 \pm 47$ and $701 \pm 34$ UI/mg protein for A549 and NHLF, respectively, the baseline released LDH vs. total cellular LDH in untreated cells was found to be as low as 1–2%.

After 48 h incubation of the cells, no significant dispersion among assays was found with compounds 1b–e of the CyDEPMPHs family ($p > 0.05$ by one-way ANOVA), suggesting those aminophosphonates induced cell death by altering metabolic activity, enzymatic and mitochondrial functions rather than by causing membrane damage and/or cell necrosis. The more lipophilic 1d and 1e were found to be slightly more toxic (Table 3).
Table 3. Cytotoxicity of α-Aminophosphonates Against A549 Cells and Normal Human Lung Fibroblasts (NHLF), and their Calculated Lipophilicities.

| Compounds   | IC50 (mM) b | AlogP c |
|-------------|-------------|---------|
|             | A549 NHLF | A549 NHLF | ATP NHLF |
| CyDEPMPHs family |         |         |         |         |
| 1b          | 95 ± 8     | 89 ± 5   | 92 ± 8   | 79 ± 6 | 85 ± 9 | 70 ± 7 | 0.26     |
| trans-1c    | 80 ± 5     | 72 ± 7   | 76 ± 5   | 70 ± 3 | 69 ± 7 | 62 ± 6 | 0.54     |
| cis-1c      | 77 ± 5     | 70 ± 5   | 78 ± 7   | 71 ± 9 | 67 ± 5 | 61 ± 5 | 0.54     |
| 1d          | 74 ± 9     | 69 ± 6   | 62 ± 5 * | 58 ± 8 * | 63 ± 5 * | 56 ± 8 | 0.77     |
| 1e          | 70 ± 7 *   | 67 ± 8 * | 65 ± 6 * | 67 ± 7 * | 62 ± 5 * | 55 ± 5 * | 1.51     |
| 1f          | 44 ± 6 *‡  | 35 ± 7 *‡ | 52 ± 9 *‡ | 45 ± 4 *‡ | 53 ± 4 *‡ | 47 ± 6 *‡ | 1.73     |
| Crowded family |         |         |         |         |
| 1g          | 118 ± 9    | 104 ± 6  | 111 ± 3  | 97 ± 4 | 107 ± 9 | 92 ± 9 | 0.25     |
| 1h          | 101 ± 4    | 95 ± 7   | 97 ± 4   | 89 ± 2 | 98 ± 5 | 87 ± 5 | 1.65     |
| 1i          | 103 ± 3    | 98 ± 3   | 95 ± 4   | 90 ± 3 | 96 ± 4 | 89 ± 6 | 1.46     |
| 1j          | 18 ± 7 §5  | 16 ± 7 §8 | 30 ± 8 §5 | 21 ± 7 §5 | 31 ± 7 §8 | 20 ± 8 §5 | 2.31 §5 |
| 1k          | 17 ± 6 §5  | 14 ± 6 §5 | 39 ± 5 §5 | 29 ± 8 §5 | 29 ± 7 §8 | 14 ± 8 §5 | 2.20 §5 |
| 1l          | 11 ± 8 §5  | 7 ± 6 §5  | 21 ± 7 §5 | 14 ± 5 §5 | 23 ± 7 §8 | 12 ± 8 §5 | 3.31 §5 |
| 1m          | 10 ± 4 §5  | 8 ± 7 §5  | 15 ± 4 §5 | 11 ± 2 §5 | 14 ± 3 §8 | 13 ± 4 §5 | 3.03 §5 |
| 1n          | 12 ± 3 §5  | 9 ± 4 §5  | 16 ± 2 §5 | 10 ± 2 §5 | 17 ± 7 §8 | 13 ± 6 §5 | 2.92 §5 |
| 1o          | 11 ± 5 §5  | 7 ± 4 §5  | 14 ± 3 §5 | 11 ± 2 §5 | 16 ± 3 §8 | 12 ± 4 §5 | 0.90 §5 |
| 1p          | 13 ± 3 §5  | 10 ± 4 §5 | 16 ± 2 §5 | 11 ± 2 §5 | 20 ± 4 §8 | 17 ± 4 §5 | 0.25 §5 |

* Cells seeded at 2.5 × 10^4 cells/well in DMEM (A549) or FBM (NHLF) until confluence were then incubated in triplicate for at least four concentrations.

b IC50 defined as the concentration of compound resulting in 50% loss of cell viability after 48 h, calculated from concentration–response curves.

c Calculated using the ALOGPS 2.1 software (available at Virtual Computational Chemistry Laboratory: www.vcclab.org/lab/alogsps/ accessed on 25 July 2021).

Regarding the three viability assays, the more lipophilic compound of the CyDEPMPHs family, 1f, was associated with the strongest global decrease of IC50 values, which was of 42–50% (p < 0.05) as compared to 1b, and reached 50–60% (p < 0.05) as compared to 1a. In parallel, the application of 1f resulted in a significant LDH leakage (>45% of total baseline LDH content; p < 0.01 vs. untreated cells and 1a, 1b) showing it additionally caused cell necrosis.

In the crowded family of pH probes, the decrease in viability globally paralleled the increase of lipophilicity, except for 1e and 1p. Hence, IC50 values for 1j–p decreased by 60–80% (p < 0.05) as compared to the hydrophilic 1g in the MTT and ATP assays, while an even greater impact was found for these lipophilic compounds in the FMCA assay (i.e., a 80–90% decrease; p < 0.05 vs. 1g), suggesting they induced membrane damage at concentrations > 10 mM (Table 3). Consistently, application of 1j–p to both types of cells resulted in a strong LDH leakage at IC50 values (60–80% of total baseline intracellular content).

Altogether, the cytotoxicity data of Table 3 demonstrated that most of the new pH probes, i.e., 1b–e and 1g–4, could be added safely in cell medium at a concentration window (up to 1–20 mM) compatible with NMR applications. Due to its innocuity and optimal 31P NMR titration parameters (pKab = 6.45; ∆δab = 10.64 ppm), compound 1b was considered ideal for probing small pH gradients, since it can be used at 4 mM, a concentration at which >98% cell viability was preserved.

2.6. Application of the 31P NMR pH Probes 1b vs. 1a in Dictyostelium discoideum Amoebae

Dictyostelium discoideum, a widely used cell model for investigating acidic organelles, has been found to be particularly suitable to monitor by 31P NMR the kinetics of anoxia-
and reoxygenation-induced pH variations in various endosomal acidic vesicles [23]. In
the search for improved resonance tools for assessing trans-sarcolemmal pH gradients,
i.e., \(\text{pH}_i/\text{pH}_e\) differences, \(D.\text{discoideum}\) was also found to be a reliable model, since in this
case endogenous and external \(\text{P}^{31}\) NMR \(\text{P}_i\) peaks are easily distinguishable [21] and may
allow a direct comparison with the expected resonances arising from the probe, provided
it could significantly accumulate within the cytosolic and external compartments. Such
properties were not reported in other models, including the rat heart [46] and liver [47]
due, among other reasons, to the low and/or varying concentrations of \(\text{P}_i\), the large line
widths of cellular \(\text{P}^{31}\) NMR peaks, and the low \(\text{pH}_i/\text{pH}_e\) difference < 0.5 pH units.

Figure 6 shows representative \(\text{P}^{31}\) NMR spectra obtained during incubation at 20 °C
of normoxic amoebae in the presence of 4 mM of \(1a\) (lower trace, deshielded region) or
\(1b\) (upper trace). In the more deshielded spectral region (20–30 ppm) of the experiment
with \(1b\), prominent resonances were apparent, likely arising from the probe internalized
in cells and located in three distinct intracellular compartments. From the calibration
curve of \(1b\) (Figure 3), the corresponding pH values were calculated as ~5.6 (for the more
acidic compartment at 22.6 ppm) and 6.20 and 7.27 for the lowfield peaks at 25.4 and
29.9 ppm, respectively. These two latter compartments probed by \(1b\) likely correspond
to the extracellular and cytosolic environments, in full accordance with the pH values
determined by the two \(\text{P}_i\) peaks, \(\text{pH}_i\sim7.24\) (cytosol) and \(\text{pH}_e\sim6.32\) (extracellular milieu).

When \(1a\) was used instead of \(1b\), acidic compartments were similarly probed at
23.6 ppm (pH~5.7) while the cytosolic and extracellular regions exhibited broader lines and
higher apparent pH values of \(\text{pH}_i\) 7.57 (resonance at 30.5 ppm) and \(\text{pH}_e\sim6.56\) (broad reson-
ance at ~26.5 ppm), respectively. This showed that \(1a\) accumulated mainly in the external
space vs. the cytosol and confirmed the previous observations [29] of (i) a line broadening
at the resonance corresponding to the cytosolic pH, and (ii) a slight alkalinization of the
cytosolic and extracellular compartments because of the higher \(\text{pK}_a\) of the probe, which
behaved as a weak base.

The innocuity of \(1b\) (4 mM) in the system was confirmed by its lack of effect on the
endogenous intracellular phosphorylated compounds including nucleotides, inositol hex-
akisphosphate and phosphomonoesters, as compared to control spectra (data not shown).
As already reported with other series of cyclic and linear \(\alpha\)-aminophosphonates [29], \(1b\)
was internalized in amoebae in less than 5 min (data not shown) both in the cytosol, extra-
cellular region and acidic vesicles. To explain the rapid rate of diffusion and intracellular
distribution observed for \(1b\) and other aminophosphonates [27,29], it was supposed that
these compounds are membrane permeable under their unprotonated amine form, and
can accumulate by passive diffusion [29]. This general property confers a great advantage
to \(\alpha\)-aminophosphonates as compared to the commonly used alkylated phosphonic acids
derivatives, which were shown to internalize slowly, at least during 90 min, within the
cells, involving energy requiring processes such as continuous \(\text{H}^+\) pumping by vacuolar
\(\text{H}^+\text{--ATPase},\) leading to NTP depletion [48].

To stress on the specific \(\text{P}^{31}\) NMR pH reporting properties, the biocompatible \(1b\)
showed a ~3.2 better potential accuracy than \(\text{P}_i\) (Figure 6). This may allow us to reliably
address pH differences as low as 0.05 pH units, corresponding to narrow peaks separated
by ~0.25 ppm. As anticipated in the design of the novel pH probes, this property results
from their improved \(\Delta\delta_{ab}\) values, giving a steepest slope around \(\text{pK}_a\)s close to that of \(\text{P}_i\).
Figure 6. $^{31}$P NMR spectra from D. discoideum cells ($3 \times 10^8$ cells/mL) aerobically incubated at 20 °C in the presence of 4 mM of 1b (upper trace) or 1a (green trace, only deshielded region shown). The signals correspond to a 15-min accumulation (900 scans) starting 15 min after addition of the probe. Using the corresponding titration curves allowed for the simultaneous calculation of all pH values, i.e., intracellular (pH$_i$), extracellular (pH$_e$), and that of acidic compartments (cpts). Cytosolic and extracellular inorganic phosphate (P$_i$) pH values (pH$_i$ ∼7.24 and pH$_e$ ∼6.32) were drawn from the 1b titration curve (Figure 3). Shielded resonances in the upper trace are from phosphomonoesters (PME); inositol hexaphosphate (IP6); γ-, α-, and β-nucleoside triphosphates (NTPs) [21,29]. Chemical shifts are given in ppm with respect to 85% external H$_3$PO$_4$ at 0 ppm and to 50 mM methylene diphosphonate (pH 8.9) placed in a capillary and used as an additional standard at 16.4 ppm. Insets: a schematic representation for comparison between 1b and P$_i$ pH probing accuracies around their pK$_a$ values.

3. Materials and Methods

3.1. Chemistry

All reagents and solvents were of the highest grade available from Sigma-Aldrich, Fluka, SDS, or Acros, and were used without further purification, except for 2-methyl-1-pyrroline (from Acros Organics, Halluin, France), which was distilled under reduced pressure (20 mm Hg, bp: 50 °C) before use. Diethyl H-phosphonate (2a), dimethyl H-phosphonate (2g), di(isopropyl) H-phosphonate (2i), and di-n-butyl H-phosphonate (2j) were from Acros. The dialkyl H-phosphonates 2b–f and the aminophosphonates 1b–f belonging to the CyDEPMPHs family were synthesized and purified as previously described [39]. DEPMPH (1a) [19], and its analogue 1i [49], and dialkyl H-phosphonates 2h and 2n, and aminophosphonates 1g, 1h, and 1n [29] were synthesized and purified.
as previously described. Reactions were followed by thin layer chromatography (TLC) on Merck-Kieselgel 60 F254 precoated silica gel plates, and the spots were visualized by staining with phosphomolybdic acid. NMR spectra (1H NMR at 300 MHz, 13C NMR at 75.5 MHz and 31P NMR at 121.5 MHz) were recorded on a Bruker AV 300 spectrometer. Chemical shifts (δ) are expressed in ppm (parts per million) relative to internal tetramethylsilane (1H and 13C) or external 85% H3PO4 (31P), and coupling constants J are given in Hz. The abbreviations s, d, t, m and q refer to singlet, doublet, triplet, multiplet and quartet signals, respectively. Melting points were determined using a B-540 Buchi apparatus and are uncorrected. Elemental analyses were performed using a Thermo Finnigan EA-1112 analyzer and were within 0.2% of theoretical values. Column chromatography was performed on Merck silica gel 60 (230–400 mesh).

3.1.1. Synthesis of Dialkyl H-Phosphonates 2k–m, 2o, and 2p

Water (1.1 g, 61 mmol) was added to HMPT (10 g, 61 mmol) in refluxing anhydrous THF (20 mL) under argon atmosphere and the mixture was stirred at reflux for 2 h. Then the corresponding alcohol (122 mmol) was added and the mixture was refluxed for another 2 h. Afterwards, the mixture was concentrated under reduced pressure to give the corresponding dialkyl H-phosphonate 2k–m, 2o, and 2p in high yields (95–98%) and a purity of >95% determined by 31P NMR. 31P NMR (121.5 MHz, CDCl3) δ 2k, 8.42; 2l, 8.05; 2m, 7.80; 2o, 9.39; 2p, 9.53.

3.1.2. General Procedure for Synthesis of Compounds 1j–m, 1o, and 1p

A mixture of 2-methyl-1-pyrroline (1.1 eq) and the corresponding dialkyl H-phosphonate (1 eq) was stirred at room temperature until completion. TLC or 31P NMR were used to follow the reaction. The mixture was poured into water (aminophosphonate final concentration, ~1.8 M) and slowly acidified to pH 3 with concentrated HCl. The aqueous layer was extracted with TBME (3 × volume of water), basified with NaOH pellets to pH 9–10, and extracted with CH2Cl2 (4 × volumes of water). The combined organic phases were dried over MgSO4, filtered and concentrated under reduced pressure. The residue was purified by SiO2 column chromatography with a CH2Cl2:EtOH mixture as eluent to yield the expected aminophosphonates.

Di-n-butyl (2-methylpyrrolidin-2-yl)phosphonate (1j). Title compound was obtained by stirring 2-methyl-1-pyrroline (10 g, 120 mmol) with commercially available di-n-butyl H-phosphonate (21.1 g, 109 mmol) for 10 days. Flash chromatography using a CH2Cl2:EtOH, 90:10, v:v as eluent yielded 1j as a yellow oil (22.3 g, 67%); 1H NMR (300 MHz, CDCl3) δ 4.06–3.97 (m, 4H, 2 × O-CH2), 3.04–2.88 (m, 2H, 5-CH2), 2.22–2.10 (m, 1H, 3-C(H)H), 1.83–1.70 (m, 4H, NH, 3-C(H)H, 4-CH2), 1.64–1.51 (m, 5H, 2 × O-CH2-CH2 and 3-C(H)H), 1.37–1.28 (q, J = 6.0 Hz, 4H, 2 × CH2-CH3), 1.27 (d, J = 15.0 Hz, 3H, 2-C(CH3)), 0.87 (t, J = 6.0 Hz, 6H, O-(CH2)3-CH3); 13C NMR (75.5 MHz, CDCl3) δ 66.0 (d, J = 8.2 Hz, O-CH2), 65.8 (d, J = 8.2 Hz, O-CH2), 59.2 (d, J = 163.8 Hz, 2-C), 47.0 (d, J = 7.5 Hz, 5-C), 34.3 (d, J = 3.0 Hz, 3-C), 32.7 (d, J = 2.5 Hz, O-CH2-CH2), 32.6 (d, J = 2.5 Hz, O-CH2-CH2), 25.6 (d, J = 4.5 Hz, 4-C), 24.2 (d, J = 6.0 Hz, 2-C(CH3)), 18.7 (2C, CH2-CH3), 13.5 (2 × O-(CH2)2-CH3); 31P NMR (121.5 MHz, CDCl3) δ 31.03. Elemental analysis calcd (%) for C13H28NO3P: C 56.50, H 10.18, N 5.05; found: C 54.72, H 10.10, N 5.01.

Disobutyl (2-methylpyrrolidin-2-yl)phosphonate (1k). Title compound was obtained by stirring 2-methyl-1-pyrroline (5.5 g, 66 mmol) with commercially available disobutyl H-phosphonate 2k (11.8 g, 60 mmol) for 7 days. Flash chromatography using a CH2Cl2:EtOH, 90:10, v:v as eluent yielded 1k as a yellow oil (11.8 g, 70%); 1H NMR (300 MHz, CDCl3) δ 3.54–3.43 (m, 4H, 2 × O-CH2), 2.73–2.57 (m, 2H, 5-CH2), 1.95–1.88 (m, 1H, 3-C(H)H), 1.63–1.24 (m, 6H, NH, 3-C(H)H, 4-CH2, 2 × CH(CH3)2), 0.98 (d, J = 15.0 Hz, 3H, 2-C(CH3)), 0.60–0.57 (d, J = 6.0 Hz, 12H, 2 × CH(CH3)2); 13C NMR (75.5 MHz, CDCl3) δ 71.4 (d, J = 7.5 Hz, O-CH2), 71.1 (d, J = 7.5 Hz, O-CH2), 58.8 (d, J = 165.3 Hz, 2-C), 46.2 (d, J = 7.5 Hz, 5-C), 32.9 (d, J = 2.5 Hz, 3-C), 28.4 (2 × CH(CH3)2), 24.5 (d, J = 4.5 Hz, 4-C), 23.5 (d, J = 6.0 Hz, 2-C(CH3)), 17.8 (2 × CH(CH3)2); 31P NMR (121.5 MHz, CDCl3) δ 31.17.
Elemental analysis calcd (%) for C_{13}H_{28}NO_{3}P: C 56.30, H 10.18, N 5.05; found: C 55.06, H 10.56, N 4.96.

**Dipentyl (2-methylpyrrolidin-2-yl)phosphonate (II)**. Title compound was obtained by stirring 2-methyl-1-pyrroline (2.5 g, 30 mmol) with dipentyl H-phosphonate II (6 g, 27.3 mmol) for 7 days. Flash chromatography using a CH_{2}Cl_{2}:EtOH, 90:10, v:v as eluent yielded II as a yellow oil (4.2 g, 49%); \(^{1}\)H NMR (300 MHz, CDCl_{3}) \(\delta\) 4.07–3.99 (m, 4H, 2 × O-CH_{2}), 3.07–2.91 (m, 2H, 5-CH_{2}), 2.22–2.18 (m, 1H, 3-(C(H)H)), 1.90–1.52 (m, 8H, 2 × OCH_{2}CH_{2}NH, 3-(C(H)H)CH_{2}, 1.30 (d, J = 15.0 Hz, 3H, 2-(C(CH_{3}))), 0.86 (t, J = 9.0 Hz, 6H, 2 × CH_{2}CH_{3}). \(^{31}\)P NMR (121.5 MHz, CDCl_{3}) \(\delta\) 66.4. 1m). Title compound was obtained by stirring 2-methyl-1-pyrroline (4.4 g, 53 mmol) with diisopentyl H-phosphonate 2m (10.8 g, 48.2 mmol) for 7 days. Flash chromatography using a CH_{2}Cl_{2}:EtOH, 90:10, v:v as eluent yielded 1m as a yellow oil (4.2 g, 49%); \(^{1}\)H NMR (300 MHz, CDCl_{3}) \(\delta\) 4.07–3.99 (m, 4H, 2 × O-CH_{2}), 3.07–2.91 (m, 2H, 5-CH_{2}), 2.22–2.18 (m, 1H, 3-(C(H)H)), 1.90–1.52 (m, 8H, 2 × OCH_{2}CH_{2}NH, 3-(C(H)H)CH_{2}, 1.30 (d, J = 15.0 Hz, 3H, 2-(C(CH_{3}))), 0.86 (t, J = 9.0 Hz, 6H, 2 × CH_{2}CH_{3}). \(^{31}\)P NMR (121.5 MHz, CDCl_{3}) \(\delta\) 66.4. 1o). Title compound was obtained by stirring 2-methyl-1-pyrroline (2.5 g, 30 mmol) with diethoxyethyl H-phosphonate 2o (6.2 g, 27.3 mmol) for 1 day. Flash chromatography using a CH_{2}Cl_{2}:EtOH, 96:4, v:v as eluent yielded 1o as a yellow oil (6.1 g, 73%); \(^{1}\)H NMR (300 MHz, CDCl_{3}) \(\delta\) 4.23–4.11 (m, 4H, 2 × P-O-CH_{2}), 3.66–3.62 (t, J = 6.0 Hz, 4H, 2 × CH_{2}OCH_{2}CH_{2}), 3.57–3.50 (q, J = 6.0 Hz, 4H, 2 × CH_{2}CH_{3}), 3.02–2.90 (m, 2H, 5-CH_{2}), 2.25–2.17 (m, 1H, 3-(C(H)H)), 1.90–1.53 (m, 3H, 3-(C(H)H)CH_{2}, NH, 1.31 (d, J = 15.0 Hz, 2-(C(CH_{3}))), 1.13 (t, J = 9.0 Hz, 6H, 2 × CH_{2}CH_{3}); \(^{31}\)P NMR (75.5 MHz, CDCl_{3}) \(\delta\) 69.7. 2p (3.2 g, 6.8 mmol) with di(2,5,8,11-tetraoxatridecan-13-yl)phosphonate 1p (0.6 g, 7.5 mmol) for 1 day. Flash chromatography using a CH_{2}Cl_{2}:EtOH, 96:4, v:v as eluent yielded 1p as a yellow oil (2.7 g, 71%); \(^{1}\)H NMR (300 MHz, CDCl_{3}) \(\delta\) 4.30–4.12 (m, 4H, 2 × POCH_{2}), 3.71–3.65 (m, 4H, 2 × OCH_{2}), 3.65–3.58 (m, 20H, 10 × OCH_{2}), 3.55–3.49 (m, 4H, 2 × OCH_{2}), 3.33 (6H, 2 × OCH_{3}), 3.10–2.91 (m, 2H, 5-CH_{2}), 2.28–2.17 (m, 1H, 3-(C(H)H)), 1.90–1.52 (m, 4H, 3-(C(H)H)CH_{2}, NH, 1.32 (d, J = 15.0 Hz, 2-(C(CH_{3}))); \(^{31}\)C NMR (75.5 MHz, CDCl_{3}) \(\delta\) 72.7. 0.5 (OCH_{2}), 70.4 (OCH_{2}), 70.3 (OCH_{2}), 70.1 (OCH_{2}), 65.3 (d, J = 7.5 Hz, POCH_{2}), 65.2 (d, J = 7.5 Hz, POCH_{2}), 59.7 (d, J = 164.6 Hz, 2-C), 58.9 (2C, OCH_{2}), 46.9 (d, J = 7.5 Hz, 5-C), 34.5 (d, J = 2.3 Hz 3-C), 25.5 (d, J = 4.5 Hz, 4-C), 23.0 (d, J = 6.8 Hz, 2-(C(CH_{3}))); \(^{31}\)P NMR (121.5 MHz, CDCl_{3}) \(\delta\) 29.52. Elemental analysis calcd (%) for C_{23}H_{48}NO_{11}P: C 50.63, H 8.87, N 2.57; found: C 47.67, H 8.84, N 2.37.
3.2. X-ray Crystallography

CyDEPMPHs were dissolved in the minimum of CH₂Cl₂, recrystallized from TBME, and dried in a vacuum to give single crystals suitable for X-ray diffraction studies. Intensities were collected at 293 K on a Nonius Kappa CCD diffractometer (Bruker) using graphite-monochromated MoKα radiation (λ = 0.71073 Å).

Crystal data for 1b: C₈H₁₆NO₅P M = 205.20, monoclinic space group P-21 c, Hall group -P 2ybc, a = 12.3308(2), b = 13.7792(2), c = 15.2243(2) Å, β = 126.3761(7)°, V = 2082.69(5) Å³, Z = 4.

Crystal data for trans-1c: C₈H₁₆NO₅P M = 219.21, monoclinic space group P-21 c, Hall group -P 2ybc, a = 6.6716(1), b = 12.6291(2), c = 13.6892(3) Å, β = 92.9872(8)°, V = 1151.83(4) Å³, Z = 4.

Crystal data for 1d: C₁₀H₂₀NO₅P M = 233.24, monoclinic space group P-21 c, Hall group -P 2ybc, a = 10.5502(5), b = 11.1665(4), c = 11.4207(2) Å, V = 1214.42(9) Å³, Z = 4.

Crystal data for 1e: C₁₂H₉₂NO₅P M = 261.29, orthorhombic space group Pbcn, Hall group -P 2ac 2ab, a = 13.3114(2), b = 10.4734(2), c = 20.0050(2) Å, V = 2789.01(9) Å³, Z = 8.

Crystal data for 1f: C₁₂H₉₄NO₅P M = 261.29, triclinic space group P-1, Hall group -P 1, a = 6.5802(5), b = 7.9200(9), c = 14.228(1) Å, V = 699.37(11) Å³, Z = 2.

CCDC 794184, 794158, 794208, 794102, 793993 contains the supplementary crystallographic data of 1b, trans-1c, 1d–f, respectively. These data are provided free of charge by The Cambridge Crystallographic Data Centre, 12 Union Road, Cambridge, UK (fax: +44 1223 336033; e-mail: deposit@ccdc.cam.ac.uk).

3.3. P NMR pH-Titration of Aminophosphonates 1a-p

All titrations were carried out at 22 °C in modified KH medium (pH 7.35), consisting of (in mM): KH₂PO₄, 1.2; MgSO₄, 1.2; NaCl, 118.5; KCl, 4.8; NaHCO₃, 25, and EDTA, 0.55 dissolved in doubly distilled deionized water. Spectra were acquired (32 scans) at 22 °C on a Bruker AMX 400 spectrometer with a 9.4 Tesla wide-bore magnet at a phosphorus frequency of 161.98 MHz. Samples were placed in 10-mm tubes. ³¹P₂H₂O in a small capillary was used as a lock signal for the spectrometer. Chemical shifts are given in ppm with respect to 85% external H₃PO₄ at 0 ppm and were plotted against pH to fit the Henderson-Hasselbach Equation (2) for NMR using nonlinear regression (Prism, GraphPad Software Inc., San-Diego, CA, USA):

\[
pH = pK_a + \log \left( \frac{\delta - \delta_b}{\delta_a - \delta} \right)
\]  

(2)

where δ is the experimental ³¹P chemical shift and the δ_a and δ_b correspond to the limiting chemical shift values of protonated and unprotonated amines forms, respectively. Calculated titration data (pK_a, δ_a, δ_b and their difference (ΔK_ab)) are expressed as mean ± SD.

3.4. Computational Methods for [C–P–O] Angle Calculations

[C–P–O] angle calculations were performed for both acidic and basic forms of the aminophosphonates. To improve the accuracy of the calculations for compounds 1a–p, compounds LAP-1–34, CAP-1–10, APA-1–5 (Table S1), and 6 amines (i.e., dimethylamine, diethylamine, diisopropylamine, piperidine, pyrrolidine, tetramethylpiperidine) were included to the pooled molecules. The angle calculations were performed using Gaussian16 software [50] after geometry optimization at B3LYP/6-31G(d) level of theory. To obtain the most stable conformation of each molecule before DFT optimization, a simulated annealing calculation at the AM1 level with the Ampac 11 [51] software package was performed. The optimizations were achieved with a maximum time of 12 h. A check for the absence of imaginary frequencies and a gradient close to zero was performed. All calculations were performed in water solvent with the Polarizable Continuum Model (PCM) [52]. Data (in °) are expressed as the differences in [C–P–O] angles between the acidic and basic structures (Δ[C–P–O]lab).
3.5. Cell Culture, Cytotoxicity Assays, and pH Assessment in Amoebae

DMEM, GlutaMax™, and phosphate-buffered saline (PBS) were obtained from Gibco Life Technologies Inc. (Thermo Fisher Scientific, Illkirch, France). FBM and growth factors (FGM; Clonetics FGM-2 Bullet Kit) were from Lonza (Amboise, France). A549 (ATCC CCL-185; LGC Standards, Molsheim, France) and NHLF (Lonza) cells were routinely maintained in [DMEM + GlutaMax] and [FBM + FGM], respectively, as described previously [53,54]. After reaching 90% confluence, cells were harvested for subculture. Cells were trypsinized, seeded in 96-well microplates (density, $2.5 \times 10^4$ cells/well) and incubated at 37 °C in a humidified atmosphere with 5% CO₂ to reach ~80% confluence in the appropriate medium. The medium was renewed and cells were exposed for 48 h to the tested compounds (1–1000 µg/mL) or DMSO (0.2%) added medium (control). Afterwards, cells were washed twice with PBS 1X (+/+)+ for cytotoxicity analysis. Supernatant medium samples were kept to evaluate cytosolic LDH release using a commercial kit (Biolabo, Maisy, France). To estimate the total LDH content, a control measurement was performed for each set of experiments by treating cells with 1% Triton X-100 to induce a total LDH release in the supernatant and induce a 100% loss of viability.

The FMCA and MTT assays were carried out as described [55]. Intracellular ATP content was assayed using a luciferin–luciferase reagent (Biofax A®; Yelen Analytics, Marseille, France; http://www.yelen-analytics.com (accessed on 10 January 2021)) according to [55]. IC₅₀ values, defined as the concentration of test compounds resulting in 50% cell viability after 48 h, were calculated from concentration–response curves (PrismSoftware) and are expressed as mean ± SD (standard deviation relative to the mean from 3–10 independent experiments).

3.6. Dictyostelium Discoideum Cells Cultures and $^{31}$P NMR

*Dictyostelium discoideum* amoebae, axenic strain (ATCC 24397), were cultured aerobically as previously described [21,29]. Briefly, cells were harvested in their exponential growth phase, washed with ice-cold 20 mM 2-morpholinoethanesulfonic acid sodium salt (MES-Na) buffer and then suspended ($3 \times 10^8$ cells/mL) at 20 °C under O₂ bubbling in MES-Na (20 mM), 6% $^2$H₂O and 5 µL of Antifoam 289 (Sigma-Aldrich, Saint Quentin Fallavier, France) to a final volume of 20 mL (pH 6.5). Afterwards, 1a or 1b were added (4 mM final concentration) to the cell medium and incubation was prolonged for up to 30 min at 20 °C.

Incubation samples were then placed in 25-mm NMR tubes and an $^2$H₂O sample, placed in a small capillary, was used as a lock signal for the NMR spectrometer. $^{31}$P NMR spectra were routinely recorded during cell incubation at 20 °C on a Bruker AMX 400 spectrometer with a 9.4 T wide-bore magnet at a phosphorus frequency of 161.98 MHz. Chemical shifts are given in ppm with respect to 85% external H₃PO₄ at 0 ppm and to 50 mM methylene diphosphonate (pH 8.9), placed in a capillary and used as an additional standard at 16.4 ppm. Spectral acquisitions were carried out using a 60° (10 µs) pulse width, a 0.28 s acquisition time, a 0.72 s repetition delay, and gated Watz proton decoupling [21]. Data were stored during 120 min as 300-scan (5 min) or 900-scan (15 min) blocks. Gaussian line broadening (GB = 0.05, LB = –5 Hz) was applied prior to Fourier transformation. The extracellular and intracellular distribution of the probe was determined by the respective areas of the corresponding resonances.

3.7. Statistics

Titration and biological values are expressed as mean ± SD. Differences were analyzed using a one-way analysis of variance (ANOVA) followed by a posteriori Newman–Keuls test. Intergroup differences were considered to be significant at $p < 0.05$.

4. Conclusions

A novel series of fifteen α-aminophosphonates have been synthesized and screened for their $^{31}$P NMR properties in the probing of subtle pH changes (as low as 0.05 pH units) occurring in normal or acidic cells. The new compounds, obtained in only two
or three synthetic steps, showed the following enhanced properties: (i) chemical shifts in the 18–36 ppm range distinct from those of phosphorus metabolites; (ii) near-neutral pKₐs (6.3–7.0); (iii) a high NMR sensitivity (Δδ_ab ranging 9.2–10.6 ppm) that can be modulated by adjusting the substituents and steric effects around the phosphorus atom; (iv) no cytotoxic effect for most of them in the concentration range used for biological NMR applications (up to 20 mM); (v) the ability to penetrate the compartments of interest (i.e., intra- and extracellular media); (vi) protonated and non-protonated forms in fast exchange on the NMR timescale, thus providing a single signal for each compartment. The hit compound 1b was applied successfully to accurately measure a trans-sarcolemmal pH gradient in *D. discoideum*. It is believed this aminophosphonate may be widely used to study the proton exchange dynamics between cellular compartments.

**Supplementary Materials:** The following are available online at https://www.mdpi.com/article/10.3390/molecules27144506/s1, Table S1: pKₐ and Δδ_ab values in KH medium reported in the literature for various aminophosphorylated compounds; Table S2: Substituents types and Increments aᵢ for pKₐ calculation using Equation (1).

**Author Contributions:** Conceptualization, S.P.; methodology, E.R., B.B., D.S. and S.P.; validation, M.C. (Marcel Culcasi), S.P. and S.T.-L.; formal analysis, M.C. (Mathieu Cassien) and B.B.; data curation, D.S., B.B. M.C. (Marcel Culcasi), E.R., M.C. (Mathieu Cassien), S.P. and S.T.-L.; writing—original draft preparation, C.D. and E.R.; writing—review and editing, M.C. (Marcel Culcasi), S.P. and S.T.-L.; supervision, M.C. (Marcel Culcasi), S.P. and S.T.-L.; project administration, S.P.; funding acquisition, M.C. (Marcel Culcasi), S.P. and S.T.-L. All authors have read and agreed to the published version of the manuscript.

**Funding:** This work was funded by the MNERT (CNRS-UMR 7273, Institut de Chimie Radicalaire) grants program to C.D. Authors thank Yelen Analytics (Marseille, France) for financial support in cell culture studies. Part of this study (chemistry, NMR titrations, biological NMR) was supported by fundings of the Agence Nationale de la Recherche, France (ANR ROS Signal—N° ANR-09-BLAN-005-03). Other part of this study (pKₐ modeling and CPO angle calculations) was supported by fundings of the Agence Nationale de la Recherche, France (ANR JCJ C MitoDiaPM—N° ANR-17-CE34-0006-01).

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available on request from the authors.

**Acknowledgments:** Authors thank G. Gosset for her very valuable contribution to synthesis and titration experiments, M. Giorgi for his kind help in collection of crystallographic data and M. Satre (UMR 5092 CNRS CEA and University Joseph Fournier, Grenoble, France) for expert assistance in NMR experiments on *Dictyostelium discoideum* amoebae.

**Conflicts of Interest:** The authors declare no conflict of interest.

**Sample Availability:** Samples of the compounds are not available from the authors.

**References**

1. Casey, J.R.; Grinstein, S.; Orlowski, J. Sensors and regulators of intracellular pH. *Nat. Rev. Mol. Cell Biol.* 2010, 11, 50–61. [CrossRef] [PubMed]
2. Moon, R.B.; Richards, J.H. Determination of intracellular pH by 31P magnetic resonance. *J. Biol. Chem.* 1973, 248, 7276–7278. [CrossRef]
3. Cohen, S.M.; Ogawa, S.; Rottenberg, H.; Glynn, P.; Yamane, T.; Brown, T.R.; Shulman, R.G. 31P nuclear magnetic resonance studies of isolated rat liver cells. *Nature* 1978, 273, 554–556. [CrossRef]
4. Barton, J.K.; den Hollander, J.A.; Lee, T.M.; MacLaughlin, A.; Shulman, R.G. Measurement of the internal pH of yeast spores by 31P nuclear magnetic resonance. *Proc. Natl. Acad. Sci. USA* 1980, 77, 2470–2473. [CrossRef]
5. Adam, W.R.; Koretsky, A.P.; Weiner, M.W. 31P-NMR in vivo measurement of renal intracellular pH: Effects of acidosis and K⁺ depletion in rats. *Am. J. Physiol.* 1986, 251, F904–F910. [CrossRef]
6. Pietri, S.; Bernard, M.; Cozzone, P.J. Hydrodynamic and energetic aspects of exogenous free fatty acid perfusion in the isolated rat heart during high flow ischemia and reoxygenation: A 31P magnetic resonance study. *Cardiovasc. Res.* 1991, 25, 398–406. [CrossRef]
7. Durand, T.; Gallis, J.L.; Masson, S.; Cozzone, P.J.; Canioni, P. pH regulation in perfused rat liver: Respective role of Na(+)–H(+) exchanger and Na(+)–HCO(3)(-) cotransport. *Am. J. Physiol. 1993, 265*, G43–G50. [CrossRef]

8. Khramtsov, V.V. Biological imaging and spectroscopy of pH. *Curr. Org. Chem. 2005, 9*, 909–923. [CrossRef]

9. Sapega, A.A.; Sokolow, D.P.; Graham, T.J.; Chance, B. Phosphorus nuclear magnetic resonance: A non-invasive technique for the study of muscle bioenergetics during exercise. *Med. Sci. Sports Exerc. 1987, 19*, 410–420. [CrossRef]

10. Roden, M. Non-invasive studies of glycogen metabolism in human skeletal muscle using nuclear magnetic resonance spectroscopy. *Curr. Opin. Clin. Nutr. Metab. Care 2001, 4*, 261–266. [CrossRef]

11. Foxall, P.J.; Nicholson, J.K. Nuclear magnetic resonance spectroscopy: A non-invasive probe of kidney metabolism and function. *Exp. Nephrol. 1998, 6*, 409–414. [CrossRef] [PubMed]

12. Chapman, J.D. Measurement of tumor hypoxia by invasive and non-invasive procedures: A review of recent clinical studies. *Radiother. Oncol. 1991, 20*, 13–19. [CrossRef]

13. Mancuso, A.; Zhu, A.; Beardsley, N.J.; Gliskson, J.D.; Wehrli, S.; Pickup, S. Artificial tumor model suitable for monitoring 31P and 31C NMR spectroscopic changes during chemotherapy-induced apoptosis in human glioma cells. *Magn. Reson. Med. 2005, 54*, 67–78. [CrossRef] [PubMed]

14. Street, J.C.; Mahmood, U.; Ballon, D.; Alfieri, A.A.; Koutcher, J.A. 31C and 31P NMR investigation of effect of 6-amino- nicotinamidamide on metabolism of RIF-1 tumor cells in vitro. *J. Biol. Chem. 1996, 271*, 4114–4119. [CrossRef]

15. Bubnovskaya, L.; Mikhailenko, V.; Kovelskaya, A.; Osinsky, S. Bioenergetic status and hypoxia in Lewis lung carcinoma assessed by 31P NMR spectroscopy: Correlation with tumor progression. *Exp. Oncol. 2007, 29*, 207–211.

16. Fan, K.; Zhang, M. Recent developments in the food quality detected by non-invasive nuclear magnetic resonance technology. *Crit. Rev. Food Sci. Nutr. 2019, 59*, 2202–2213. [CrossRef]

17. Hatzakis, E. Nuclear magnetic resonance (NMR) spectroscopy in food sciences: A comprehensive review. *Compr. Rev. Food Sci. Food Saf. 2019, 18*, 189–220. [CrossRef]

18. Cheng, C.H.; Balsandorj, Z.; Hao, Z.; Pan, L. High-precision measurement of pH in the full toothpaste using NMR chemical shift. *J. Magn. Reson. 2020, 317*, 106771. [CrossRef]

19. Pietri, S.; Miollan, M.; Martel, S.; Le Moigne, F.; Blaive, B.; Culcasi, M. α- and β-Phosphorylated amines and pyrrolidines, a new class of low toxic highly sensitive 31P NMR pH indicators. *J. Biol. Chem. 2000, 275*, 19905–19912. [CrossRef]

20. Lundberg, P.; Harmsen, E.; Ho, C.; Vogel, H.J. Nuclear magnetic resonance studies of cellular metabolism. *Anal. Biochem. 1990, 191*, 193–222. [CrossRef]

21. Satre, M.; Martin, J.B.; Klein, G. Methionine phosphate as a 31P-NMR probe for intracellular pH measurements in *Dictyostelium discoideum*. *Biochimie 1989, 71*, 941–948. [CrossRef]

22. Robitaille, P.M.L.; Robitaille, P.A.; Brown, G.G., Jr.; Brown, G.G. An analysis of the pH-dependent chemical-shift behavior of phosphorus-containing metabolites. *J. Magn. Reson. 1991, 92*, 73–84. [CrossRef]

23. Brenot, F.; Aubry, L.; Martin, J.B.; Satre, M.; Klein, G. Kinetics of endosomal acidification in *Dictyostelium discoideum* amoebae. 31P-NMR evidence for a very acidic early endosomal compartment. *Biochimie 1992, 74*, 883–895. [CrossRef]

24. Raghunand, N.; Altbach, M.I.; van Sluis, R.; Baggett, B.; Taylor, C.W.; Bhujwalla, Z.M.; Gillies, R.J. Plasmaemmal pH-gradients in drug-sensitive and drug-resistant MCF-7 human breast carcinoma xenografts measured by 31P magnetic resonance spectroscopy. *Biochem. Pharmacol. 1999, 59*, 309–312. [CrossRef]

25. Lutz, N.W.; Le Fur, Y.; Chiche, J.; Pouysségur, J.; Cozzone, P.J. Quantitative in vivo characterization of intracellular and extracellular pH profiles in heterogeneous tumors: A novel method enabling multiparametric pH analysis. *Cancer Res. 2013, 73*, 4616–4628. [CrossRef]

26. Vidal, G.; Thiaudiere, E.; Canioni, P.; Gallis, J.L. Aminomethylphosphate and 2-aminoethylphosphonate as 31P-NMR pH markers for extracellular and cytosolic spaces in the isolated perfused rat liver. *NMR Biomed. 2000, 13*, 289–296. [CrossRef]

27. Pietri, S.; Martel, S.; Culcasi, M.; Delmas-Beauvieux, M.C.; Canioni, P.; Gallis, J.L. Use of diethyl(2-methylpyrrolidin-2-yl)phosphonate as a highly sensitive extra- and intracellular 31P NMR pH indicator in isolated organs. *J. Biol. Chem. 2001, 276*, 750–758. [CrossRef]

28. Martel, S.; Clément, J.L.; Muller, A.; Culcasi, M.; Pietri, S. Synthesis and 31P NMR characterization of new low toxic highly sensitive pH probes designed for in vivo acidic pH studies. *Bioorg. Med. Chem. 2002, 10*, 1451–1458. [CrossRef]

29. Gosset, G.; Satre, M.; Blaive, B.; Clément, J.L.; Martin, J.B.; Culcasi, M.; Pietri, S. Investigation of subcellular acidic compartments using α-aminoephosphonate 31P magnetic nuclear resonance probes. *Anal. Biochem. 2008, 380*, 184–194. [CrossRef]

30. Gosset, G.; Martel, S.; Clément, J.L.; Blaive, B.; Olive, G.; Culcasi, M.; Rosas, R.; Thévand, A.; Pietri, S. Nouveaux marqueurs de pH utilisables en RMN du 31P. Détermination de la relaxation longitudinale en fonction de la structure chimique, de la température, du pH et du milieu biologique. *CR Chim. 2008, 11*, 541–552. [CrossRef]

31. Thétiot-Laurent, S.; Gosset, G.; Clément, J.-L.; Cassien, M.; Mercier, A.; Siri, D.; Gaudel-Siri, A.; Rockenbauer, A.; Culcasi, M.; Pietri, S. New amino-acid based β-phosphorylated nitroxides for probing acidic pH in biological systems by EPR spectroscopy. *ChemBioChem 2017, 18*, 300–315. [CrossRef] [PubMed]

32. Culcasi, M.; Casano, G.; Lucchesi, C.; Mercier, A.; Clément, J.L.; Pique, V.; Michelet, L.; Krieger-Liszkat, A.; Robin, M.; Pietri, S. Synthesis and biological characterization of new aminophosphonates for mitochondrial pH determination by 31P NMR spectroscopy. *J. Med. Chem. 2013, 56*, 2487–2499. [CrossRef] [PubMed]
33. Culcasi, M.; Thétiot-Laurent, S.; Atteia, A.; Pietri, S. Mitochondrial, acidic, and cytosolic pHs determination by 31P NMR spectroscopy: Design of new sensitive targeted pH probes. In Mitochondrial Medicine: Methods in Molecular Biology; Weissig, V., Edeas, M., Eds.; Human Press: New York, NY, USA, 2015; Volume 1265, pp. 135–147. [CrossRef]

34. Clarke, K.; Stewart, L.C.; Balshi, J.A.; Smith, T.W.; Ingwall, J.S.; Nédélec, J.F.; Humphrey, S.M.; Kleber, A.G.; Springer, C.S., Jr. Extracellular volume and transsarcomemmal proton movement during ischemia and reperfusion: A 31P NMR spectroscopic study of the isovolumic rat heart. NMR Biomed. 1993, 6, 278–286. [CrossRef][PubMed]

35. Hunjan, S.; Mason, R.P.; Mehta, V.; Kulkarni, P.; Aravind, S.; Arora, V.; Antich, P.P. Simultaneous intracellular and extracellular pH measurement by 19F NMR of 6-fluoropyridoxil. Magn. Reson. Med. 1998, 39, 551–556. [CrossRef][PubMed]

36. Page, P.; Mazieres, M.R.; Bellan, J.; Sanchez, M.; Chadref, B. A simple and convenient synthesis of 2-phosphonomethyl pyridines. Phosphorus Sulfur Silicon Relat. Elem. 1992, 70, 205–210. [CrossRef]

37. Ządoł-Dobrowolska, A.; Kłossowski, S.; Koszelewski, D.; Paprocki, D.; Ostaszewski, R. Enzymatic Ugi Reaction with Amines and Aldehydes. J. Chem. Soc. Perkin Trans. 27, 1992, 3473–3476. [CrossRef]

38. Chalier, F.; Tordo, P. 5-Diisopropoxyphosphoryl-5-methyl-1-pyrroline N-oxide: Syntheses, Etudes Physico-Chimiques, Mécanismes. Ph.D. Thesis, Université d’Aix-Marseille I, Marseille, France, 2000.

39. Jenecks, W.P.; Regenstein, J. Ionization constants of acids and bases. In Handbook of Biochemistry and Molecular Biology, 4th ed.; Lundblad, R.L., MacDonald, F.M., Eds.; CRC Press: Boca Raton, FL, USA, 2010; pp. 995–635. ISBN 978-0-8493-9168-2.

40. Huck, V.; Niemeyer, A.; George, T.; Schaerker, E.M.; Ossig, R.; Rogge, P.; Schneider, M.F.; Oberleithner, H.; Schneider, S.W. Delay of acute intracellular pH recovery after acidosis decreases endothelial cell activation. J. Cell Physiol. 2007, 211, 399–409. [CrossRef]

41. Simchowitz, L.; Davis, A.O. Intracellular pH recovery from alkalinization. Characterization of chloride and bicarbonate transport by the anion exchange system of human neutrophils. J. Gen. Physiol. 1990, 96, 1037–1059. [CrossRef]

42. Ohta, K. Prediction of pKa values of alkylphosphonic acids. Bull. Chem. Soc. Jpn. 1992, 65, 2543–2545. [CrossRef]

43. Lemercier, C. Nitroxydes D’Alcoxyamines D’Ugi. Ph.D. Thesis, Université d’Aix-Marseille I, Marseille, France, 2009.

44. Maurelli, E.; Culcasi, M.; Delmas-Beauvieux, M.C.; Miollan, M.; Gallis, J.L.; Toton, P.; Pietri, S. New perspectives on the cardioprotective phosphonate effect of the spin trap 5-(diethoxyphosphoryl)-5-methyl-1-pyridine N-oxide: An hemodynamic and 31P NMR study in rat hearts. Free Radic. Biol. Med. 1999, 27, 34–41. [CrossRef]

45. Delmas-Beauvieux, M.C.; Pietri, S.; Culcasi, M.; Leducq, N.; Valeins, H.; Liebgott, T.; Diolez, P.; Canioni, P.; Gallis, J.L. Use of spin-traps during warm ischemia-reperfusion in rat liver: Comparative effect on energetic metabolism studied using 31P nuclear magnetic resonance. MAGMA 1997, 5, 45–52. [CrossRef][PubMed]

46. Davies, L.; Farrar, N.A.; Satre, M.; Dottin, R.P.; Gross, J.D. Vacuolar H(+)-ATPase and weak base action in Dictyostelium. Mol. Microbiol. 1996, 22, 119–126. [CrossRef]

47. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Petersson, G.A.; Nakatsuji, H.; et al. Gaussian 16, Revision A.03; Gaussian Inc.: Wallingford, CT, USA, 2016.

48. AMPAC 11, 1992–2017 Semichem, Inc. 12456 W 62nd Terrace—Suite D, Shawnee, KS 66216. Available online: http://www.semicem.com/ (accessed on 15 September 2019).

49. Davies, L.; Farrar, N.A.; Satre, M.; Dottin, R.P.; Gross, J.D. Vectorial H(+)-ATPase and weak base action in Dictyostelium. Mol. Microbiol. 1996, 22, 119–126. [CrossRef]

50. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Petersson, G.A.; Nakatsuji, H.; et al. Gaussian 16, Revision A.03; Gaussian Inc.: Wallingford, CT, USA, 2016.

51. Ohta, K. Prediction of pKa values of alkylphosphonic acids. Bull. Chem. Soc. Jpn. 1992, 65, 2543–2545. [CrossRef]

52. Delmas-Beauvieux, M.C.; Pietri, S.; Culcasi, M.; Leducq, N.; Valeins, H.; Liebgott, T.; Diolez, P.; Canioni, P.; Gallis, J.L. Use of spin-traps during warm ischemia-reperfusion in rat liver: Comparative effect on energetic metabolism studied using 31P nuclear magnetic resonance. MAGMA 1997, 5, 45–52. [CrossRef][PubMed]

53. Chalier, F.; Tordo, P. 5-Diisopropoxyphosphoryl-5-methyl-1-pyridine N-oxide, DIPPMO: Synthesis and spin trapping properties. J. Chem. Soc. Perkin Trans. 2002, 2, 2110–2117. [CrossRef]

54. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Petersson, G.A.; Nakatsuji, H.; et al. Gaussian 16, Revision A.03; Gaussian Inc.: Wallingford, CT, USA, 2016.

55. AMPAC 11, 1992–2017 Semichem, Inc. 12456 W 62nd Terrace—Suite D, Shawnee, KS 66216. Available online: http://www.semicem.com/ (accessed on 15 September 2019).

56. Tomasi, J.; Mennucci, B.; Cammi, R. Quantum mechanical continuum solvation models. Chem. Rev. 2005, 105, 2999–3093. [CrossRef]

57. Chalier, F.; Tordo, P. 5-Diisopropoxyphosphoryl-5-methyl-1-pyridine N-oxide, DIPPMO: Synthesis and spin trapping properties. J. Chem. Soc. Perkin Trans. 2002, 2, 2110–2117. [CrossRef]

58. Frisch, M.J.; Trucks, G.W.; Schlegel, H.B.; Scuseria, G.E.; Robb, M.A.; Cheeseman, J.R.; Scalmani, G.; Barone, V.; Petersson, G.A.; Nakatsuji, H.; et al. Gaussian 16, Revision A.03; Gaussian Inc.: Wallingford, CT, USA, 2016.

59. AMPAC 11, 1992–2017 Semichem, Inc. 12456 W 62nd Terrace—Suite D, Shawnee, KS 66216. Available online: http://www.semicem.com/ (accessed on 15 September 2019).

60. Tomasi, J.; Mennucci, B.; Cammi, R. Quantum mechanical continuum solvation models. Chem. Rev. 2005, 105, 2999–3093. [CrossRef]

61. Chalier, F.; Tordo, P. 5-Diisopropoxyphosphoryl-5-methyl-1-pyridine N-oxide, DIPPMO: Synthesis and spin trapping properties. J. Chem. Soc. Perkin Trans. 2002, 2, 2110–2117. [CrossRef]