Synthesis and Biological Characterization of New Aminophosphonates for Mitochondrial pH Determination by $^{31}$P NMR Spectroscopy

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ABSTRACT: A series of mitochondria targeted α-aminophosphonates combining a diethoxyphosphoryl group and an alkyl chain-connected triphenylphosphonium bromide tail were designed and synthesized, and their pH-sensitive $^{31}$P NMR properties and biological activities in vitro and in vivo were evaluated. The results showed a number of these mitochondria phosphonates exhibiting $pK_a$ values fitting the mitochondrial pH range, short relaxation, and chemical shift parameters compatible with sensitive $^{31}$P NMR detection, and low cytotoxicity on green algae and murine fibroblasts cell cultures. Of these, two selected compounds demonstrated to distribute at NMR detectable levels within the cytosolic and mitochondrial sites following their perfusion to isolated rat livers, with no detrimental effects on cell energetics and aerobic respiration. This study provided a new molecular scaffold for further development of in situ spectroscopic real-time monitoring of mitochondrialcytosol pH gradients.

INTRODUCTION

Intracellular pH (pH$_i$) is an essential marker of a variety of physiological processes and the cellular response to external stimuli. In normal cells, pH$_i$ homeostasis is controlled within a narrow range of less than ±0.2 units by the high buffering capacity of cytosol and through the activity of transport systems located into the plasma membrane, such as the Na$^+/H^+$ exchangers and the Na$^+/HCO_3^-$ cotransporter. Their regulatory role strongly depends on cell type and function and varies in response to pathological situations. Although the rapid acidosis occurring when cells are exposed to apoptotic stimuli, during tumor size progression, ischemic episodes, oxidative stress, or starvation has been largely documented, the mechanisms of pH$_i$ decrease and the cellular targets of released protons are still not fully understood.

Beside the central role of plasma membrane regulating systems in pH$_i$ control, local specific pHs are maintained within several cellular organelles that are crucial in processes such as the transport of various metabolites and ions over membranes, enzyme structure and activity or the cellular redox state. Thus acidic pHs < 5 exist in many cytoplasmic organelles, including endocytic and secretory vesicles, lysosomes, or portions of the endoplasmic reticulum or trans-Golgi complex, in which the proton gradients are mediated by a ATP-dependent H$^+$ pump. Conversely, pHs up to 8 were found in the mitochondrial matrix of microbes and bacteria, as well as in mammalian cells, as a result of the respiratory activity which pumps protons from the inner matrix and generates a proton-motive force driving ATP synthesis in parallel with metabolite and ions uptake.

The need for a noninvasive, dynamic pH monitoring at the subcellular level has led to extensive research into the development of biocompatible weak acids or bases displaying pH-dependent spectroscopic properties mainly relying on fluorescence or nuclear magnetic resonance (NMR). From the known pH-dependency of the $^{31}$P NMR chemical shift ($\delta$) of cytosolic inorganic phosphate (Pi) and metabolites, such as ATP or glucose 6-phosphate, many synthetic alkyl- and aminoaalkylphosphonic acids, and alkylphosphonates have been proposed as pH markers. However most of the pH-sensitive compounds used in the pioneering studies (see ref 11 and references cited therein) showed limitations because of (i) their relatively low NMR spread $\Delta$$\delta_{ab}$ (defined as the mean difference between the chemical shifts of their protonated ($\delta_a$) and unprotonated ($\delta_b$) forms), which can be considered as an index of sensitivity, (ii) their poor cytosolic penetration and (iii) their lack or slow internalization or distribution in cell organelles.

Previously, we have reported about nontoxic, linear, and cyclic α- and β-aminophosphonates as pH markers having a three to 4-fold larger $^{31}$P NMR sensitivity as compared to...
However these endogenous mitochondrial pH probes become irrelevant under normothermic conditions because of the superimposition of the cytosolic and mitochondrial P$_i$ resonances. The P$_i$ NMR signal was detected in isolated mitochondria from rat heart$^{19}$ or liver$^{20}$ but its intensity was too dependent on the ATP-ADP turnover to allow accurate pH determination. Consequently, there is still a need for developing novel pH reporters that can specifically accumulate within the mitochondrial site.

Successful strategies for targeting the mitochondria have included the preparation of lipophilic cations, such as triphenylphosphonium, which actively enter the mitochondria driven by the large membrane potential generated during oxidative phosphorylation.$^{21,22}$ This property has allowed the design of vectorized antioxidants, cardioprotective or anticancer drugs, and analytical tools for mitochondrial energetics measurements.$^{21,28}$

In this study, we have combined the concepts of mitochondria targeting and enhanced $^{31}$P NMR pH probing to synthesize and biologically evaluate a new class of $\alpha$-aminophosphonates bearing a triphenylphosphonium cation. Their design should cope with (i) the known mitochondrial pH of 7–8, (ii) a final pK$_a$ range of 6.5–7.0 with $\Delta\delta_0 \approx 10$, and (iii) the balance between the expected pK$_a$ decreasing effect of the electron withdrawing P$_3$ nature and the potential cytotoxicity that may increase with the length of a lipophilic spacer introduced to counterbalance this former effect. On the basis of these criteria, we report on eleven mito-aminophosphonates having linear structures (Figure 1c and d) derived from DPP. In addition, we evaluated their toxicity in two cellular models and assessed their cytosolic and mitochondrial permeation, innocuity and resistance to biodegradation on the normothermic isolated perfused rat liver.

### RESULTS AND DISCUSSION

#### Chemistry

Most of the mitochondria targeted pH markers prepared in this study were built around linear secondary amines bearing an $\alpha$-diethoxyphosphoryl group as the pH-sensitive $^{31}$P NMR probe, and a triphenylphosphonium salt linked to the amino function by a hydrocarbon chain of different length as the mitochondria vector. In most of the syntheses, the key step was a Kabachnik–Fields one-pot aminophosphorylation with ammonia and diethylphosphate using a protocol previously optimized to prepare linear $\alpha$-aminophosphonates pH markers.$^{14}$ To obtain a uniform bromide counterion all phosphoryl salts were ultimately transformed into their protonated ammonium form. This was explored and modeled on a series of compounds that may increase with the length of a lipophilic spacer substituents are (c) R$_1$ = H or alkyl, R$_2$ = alkyl, spacer = (CH$_2$)$_n$ with n = 6, 8, or 12 and (d) R$_1$ = alkyl, R$_2$ = H or alkyl, spacer = (CH$_2$)$_3$. Inset: Structures of DPP and DEPMPP.

![Diagram](image.png)

**Figure 1.** General structures of linear (a) and cyclic (b) $\alpha$- or $\beta$-aminophosphonates, and (c, d) new mitochondria targeted $\alpha$-aminophosphonates designed as sensitive $^{31}$P NMR pH indicators for biological studies. In previous studies,$^{13,14}$ substituents in (a, b) were R = H or Me; R$_1$ and R$_2$ = alkyl; R$_3$ = alkyl or phosphoryl group; spacer = (CH$_2$)$_n$ with n = 0, 1. In the target structures of mito-aminophosphonates substituents are (c) R$_1$ = H or alkyl, R$_2$ = alkyl, spacer = (CH$_2$)$_n$ with n = 6, 8, or 12 and (d) R$_1$ = alkyl, R$_2$ = H or alkyl, spacer = (CH$_2$)$_3$. Inset: Structures of DPP and DEPMPP.
submitted to an exchange using saturated aqueous sodium bromide as previously described.26

In a first approach to obtain compounds bearing an alkyl spacer consisting of \( n = 6 \) carbons, the aminophosphorylation occurred after the triphenylphosphonium moiety was introduced (Scheme 1). Hence, the amine functionality of commercially available 6-aminohexan-1-ol was protected with Boc2O in methanol to yield compound 1 quantitatively. Then, conversion of the hydroxyl group of 1 to the corresponding mesylate followed by treatment with sodium iodide afforded the iodide 2,10 which reacted with triphenylphosphine in dioxane to give the triphenylphosphonium iodide 3.31 After deprotection of the Boc group as described in ref 31, the unprotected amine was subjected to aminophosphorylation14 in the presence of either isobutyraldehyde or pivaldehyde to give rise to mito-aminophosphonates 4a or 4c, respectively. Use of acetone instead of an aldehyde failed to give the expected 4b possibly because of the lack of reactivity of the imine intermediate caused by steric effects.

To modulate the length of the alkyl spacer still using commercially available starting material, we further employed a chemical pathway in which Kabachnik–Fields aminophosphor-ylation was the initial step (Scheme 2). Thus \( \alpha \)-aminophosphonates 5a and 5b were first prepared from the appropriate carbonyl compounds and these compounds were directly converted into the corresponding mito-aminophosphonates having a \( n = 4 \) spacer 8a or 8b by treatment with 4-iodobutyltriphenylphosphonium iodide (IBTP).35 To increase the length of the spacer up to \( n = 8 \), 5a and 5b were first condensed with 1,8-dibromo-octane to afford \( \alpha \)-aminophosphonate bromides 6a and 6b and the phosphonium functionality was introduced in the last step by treatment with triphenylphosphine to give the corresponding mito-aminophosphonates 7a and 7b.

In a third approach to prepare mito-aminophosphonates having a \( n = 12 \) hydrocarbon chain spacer, intermediates bearing separately the triphenylphosphonium and the \( \alpha \)-aminophosphonate functionalities were prepared and coupled. In the synthetic strategy (Scheme 3), which also allowed to obtain compound 4b (\( n = 6 \)), monobromo alcohols 11 and 12 were first prepared by selective bromination of commercially available diols 9 and 10 as described13 and were then converted into the corresponding hydroxalkylphosphonium salts 13 and 14 by reaction with triphenylphosphine in acetonitrile, according to earlier procedures.34,35 These compounds were then reacted with HBr in toluene under reflux as described in36 to afford the corresponding bromoalkyltriphenylphosphonium bromides 15 and 16, and the aminophosphonate functionality was finally introduced by condensation with 5a or 5b to yield the desired mito-aminophosphonates 4b (\( n = 6 \)) or 18a and b (\( n = 12 \)).

The series of mito-aminophosphonates was finally completed by preparing two compounds having the shortest hydrocarbon chain spacer (\( n = 3 \)). The synthetic pathway (Scheme 4) involved two steps starting from the iodo-ketone 19, which was then prepared from commercially available 5-iiodopentan-2-one as described21 and reported.36 Compound 19 was then converted into its triphenylphosphonium iodide 20, which was then subjected to an aminophosphorylation with ammonia or isobutylamine in the presence of diethylphosphite to afford the desired 222-224 aminophosphonates 21 or 22, respectively, with rather good yields. It is noteworthy that 21 is the only mito-aminophospho-
Scheme 4. Synthesis of Mito-aminophosphonates 21 and 22

Reagents and conditions: (a) Ph₃P, toluene, reflux, 12 h (70%); (b) dry NH₃ gas, room temp., 1 h; (c) HP(O)(OEt)₂, room temp., 1 h then 65 °C, 4 h, satd. NaBr; (d) 2-methylpropan-1-amine, room temp., 1 h, satd. NaBr.

afforded monophasic titration curves, which could be fitted to the equation of a single experimental curve suggesting that protonation always occurred at the nitrogen atom as found earlier for DPP and its analogues. The fits allowed the calculation of the pKₐ, δ and Δδ values which are reported in Table 1 together with data for P₃ and DPP, which both were within the range reported in our earlier study.11,12

Previously, we found the basicity of linear α-aminophosphonates having the structure shown in Figure 1a to be mostly affected by inductive effects of substituents R₁–R₃. Thus pKₐ increased upon substitution by electron-donating groups, such as alkyls, and decreased by withdrawing substituents, such as dialkoxypyrophosphonyl, phenyl, or benzyl, this latter effect being weakened by intercalation of one methylene spacer around the amine protonation site.11,14,15 On this basis it was obvious that the pKₐ-decreasing, electron-withdrawing effect of Ph₃P⁺ may represent a drawback to maintain pKₐ values of mito-aminophosphonates near neutrality. We therefore prepared a first group of nine mito-aminophosphonates inspired by the structure of the neutral DPP, and having a n = 4–12 spacer to progressively move the Ph₃P⁺ group away from the amine function (structure A in Table 1, entries 3–11). As could be predicted from our former semiempirical model,11 the experimental pKₐ increased (i) with alkyl substitution at α-C for a given n value (see Table 1, entries 3 and 4 versus 7 and 8, respectively), and (ii) with increasing n for the same (R₁, R₂) set (see Table 1, entries 3–6 and 7–10). The sensitivity Δδ of pH probing of all structure A compounds was very high and similar to that of DPP and for this parameter n = 8 was the optimal value for the spacer chain length (see Table 1, entries 5 and 9 versus 6 and 10, respectively).

For the future purpose of developing vectorized mito-aminophosphonates, we prepared two compounds where both the phosphonyl and phosphonium groups are branched to the same α-C, leaving one amino substituent ready for suitable substitution. Because of the short n = 3 spacer present in these structure B compounds, pKₐ values were expectedly the lowest measured while Δδ values were still within an acceptable range (Table 1, entries 12 and 13).

Finally, regarding their NMR spectroscopic properties all prepared mito-aminophosphonates had very short spin–lattice relaxation times T₁, thus allowing easier quantitative determinations than when using P₃ (Table 1). Altogether from our initial requirements for optimized mitochondrial ³¹P NMR pH probing, compounds 4b, 7b (Figure 2), and 18b (almost neutral) or the slightly more sensitive 7a and 18a (rather acidic) appeared as the best candidates to probe the alcaline mitochondrial pH domain.

Cytotoxicity Studies. Since millimolar intracellular levels of phosphorylated molecules are at least needed to achieve a satisfactory ³¹P NMR sensitivity, the toxicity of the new mito-aminophosphonates was first assessed in vivo against algae (Chlamydomonas reinhardtii) and in vitro in mammalian (3T3 murine fibroblasts) cells by determining viabilities in a large concentration range and the data were compared to that of DEPMPH, DPP, the spin trap α-phenyl-N-tet-butylnitronate (PBN) and its mitochondria targeted derivative mitoPBN.26 The results are summarized in Table 2 as lethal doses (LD) and lactate dehydrogenase (LDH) leakage values, following a 1 and 3 h (algae) or 3 h (fibroblasts) incubation, and are examined together with predicted lipophilicities (clogD).

Of particular pertinence to the study in C. reinhardtii is the fact that in plants or algae there is a high interest in determining the pH inside chloroplasts and mitochondria, pHₐ (i.e., inside the lumen and the stroma of the chloroplasts) varies largely during the light period (i.e., the photosynthetic active period) as compared to night (respiratory period). In addition, the cytosolic pH can change drastically depending on the physiological conditions, like for example when cells are kept anaerobic.42 If in those studies using ³¹P NMR, the intracellular pH signal efficiently probed the chloroplastic and cytosolic compartments (pH 6.9–7.4) or the acidic vacuoles (pH ~ 5.7), there was no information gained on the mitochondria.38,39

Here compounds, such as DEPMPH and PBN, which are moderately lipophilic and have no mitochondria targeting properties, were not very active on photosynthetic and respiratory activities compared to mitoPBN (Table 2, entries 2–4). This background cytotoxicity of compounds containing...
the Ph3P+ group, which has been documented in mitoPBN and other mitochondria targeted antioxidants derived from vitamin E or coenzyme Q, is generally not considered as a drawback since applying very low nontoxic concentrations (e.g., micro- or nanomolar) leads to local millimolar mitochondrial levels still enabling NMR detection.21,22,27

In structure A, mito-aminophosphonates either increasing the length of the spacer from \( n = 4 \) to 12, or replacing a methyl by an isopropyl group in the diethoxyphosphonyl arm for a given \( n \) value expectedly resulted both in an increase of \( c \log D \) and a decrease of LD50 values (see Table 2, entries 5−8 versus 9−12). Overall, structure A compounds 8a, 4a, 8b, 4b, and 7b together with Structure B compound 22 having a short \( n = 3 \) spacer demonstrated the better innocuity in C. reinhardtii and thus may have the best potential as mitochondrial pH reporters.31

Regarding toxicity 3T3 fibroblasts were more resistant than green algae and it was found that their viability and LDH release values still paralleled lipophilicity in either structure A compounds having the same R1 substituent (Table 2, entries 5−8 versus 9−12) or when carbon substitution of the R1 group increased for the same \( n = 6 \) spacer length (Table 2, entries 8, 4, and 11). Of interest was the observation of a significant cell necrosis when mitoPBN (but not PBN itself) or only 3 of the 10 novel mito-aminophosphonates tested were added to the culture medium at a concentration that preserved 90% cell viability. This allowed to select the more biocompatible 4b and 7b in the next perfusion experiments, which are best able to preserve cellular function and integrity, and showed \( \text{pK}_a \) values compatible with the more accurate pH determination.21,22,27

Table 1. 31P NMR Titration and Relaxation Parameters of Mito-aminophosphonates in Comparison with Inorganic Phosphate (P) and DPP

<table>
<thead>
<tr>
<th>entry</th>
<th>compd</th>
<th>R1</th>
<th>R2</th>
<th>( n )</th>
<th>( \text{pK}_a )</th>
<th>( \delta_a )</th>
<th>( \delta_b )</th>
<th>( \Delta\delta )</th>
<th>( T_1 ) (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>P</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6.78 ± 0.03</td>
<td>0.82 ± 0.02</td>
<td>3.38 ± 0.04</td>
<td>2.56 ± 0.06</td>
<td>10.3</td>
</tr>
<tr>
<td>2</td>
<td>DPP</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>6.99 ± 0.02</td>
<td>22.85 ± 0.07</td>
<td>33.02 ± 0.08</td>
<td>10.17 ± 0.10</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 1. 31P NMR Titration\(^a\) and Relaxation\(^b\) Parameters of Mito-aminophosphonates in Comparison with Inorganic Phosphate (P)\(^c\) and DPP

\(^a\)\( \delta_a \) and \( \delta_b \), limiting 31P NMR chemical shift in acidic and basic medium, respectively; \( \Delta\delta = \delta_a - \delta_b \), data were determined at 25 °C in Krebs−Henseleit buffer and are given as means ± SEM from three repeated experiments. \(^b\)\( T_1 \) = spin−lattice relaxation time determined at \( \text{pH} \approx \text{pK}_a \).

\(^c\)Refers to the second acidity of the \( \text{H}_2\text{PO}_4^-/\text{HPO}_4^{2-} \) couple. \(^d\)nd: not determined.

Regarding toxicity 3T3 fibroblasts were more resistant than green algae and it was found that their viability and LDH release values still paralleled lipophilicity in either structure A compounds having the same R1 substituent (Table 2, entries 5−8 versus 9−12) or when carbon substitution of the R1 group increased for the same \( n = 6 \) spacer length (Table 2, entries 8, 4, and 11). Of interest was the observation of a significant cell necrosis when mitoPBN (but not PBN itself) or only 3 of the 10 novel mito-aminophosphonates tested were added to the culture medium at a concentration that preserved 90% cell viability. This allowed to select the more biocompatible 4b and 7b in the next perfusion experiments, which are best able to preserve cellular function and integrity, and showed \( \text{pK}_a \) values compatible with the more accurate pH determination.21,22,27
As expected, the mitochondrial penetration of PBN within the nuclear and mitochondrial fractions of isolated perfused rat hearts is very low. Compared to the more lipophilic PBN, the relatively low cytosolic levels of DPP seen here could be a consequence of a release into the KH buffer used during washout by passive membrane diffusion, a mechanism that was evidenced for uncharged aminophosphonates. To illustrate the different patterns of cytosolic versus mitochondrial distribution of Table 3, homogenates recovered in each fraction from the same liver perfused with 7b or mitoPBN were extracted with dichloromethane, evaporated to dryness and redissolved in 1 mL CDCl3. Individual 31P NMR spectra were then recorded (Figure 3) showing for both compounds no new NMR peaks indicating significant metabolic degradation, and the unique property of 7b to indicate simultaneously mitochondrial (Figure 3a) and cytosolic (Figure 3b) pH values unlike the nitrone, which only accumulates within the mitochondria, giving a pH-insensitive signal (see Figure 3c and arrow in Figure 3d).

### Table 2. Toxicity and Distribution Coefficients of Mito-aminophosphonates and Reference Compounds in *Chlamydomonas reinhardtii* and 3T3 Murine Fibroblasts

<table>
<thead>
<tr>
<th>entry</th>
<th>compd</th>
<th>clogD&lt;sup&gt;*&lt;/sup&gt;</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;(μM)</th>
<th>LD&lt;sub&gt;10&lt;/sub&gt;(μM)</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;(mM)</th>
<th>LD&lt;sub&gt;10&lt;/sub&gt;(mM)</th>
<th>LDH release (UI/mg prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DPP</td>
<td>0.86</td>
<td>nd&lt;sup&gt;±&lt;/sup&gt;</td>
<td>nd</td>
<td>76</td>
<td>100</td>
<td>11.9 ± 1.9</td>
</tr>
<tr>
<td>2</td>
<td>DEPMPH</td>
<td>0.43</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>78</td>
<td>102</td>
<td>13.1 ± 1.4</td>
</tr>
<tr>
<td>3</td>
<td>PBN</td>
<td>1.32</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>4.1</td>
<td>7.2</td>
<td>14.2 ± 1.9</td>
</tr>
<tr>
<td>4</td>
<td>mitoPBN</td>
<td>4.61</td>
<td>100</td>
<td>50</td>
<td>0.05</td>
<td>0.2</td>
<td>49.7 ± 2.8&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>8a</td>
<td>2.42</td>
<td>250</td>
<td>150</td>
<td>0.3</td>
<td>0.6</td>
<td>10.7 ± 1.8</td>
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<tr>
<td>6</td>
<td>4a</td>
<td>3.21</td>
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<td>130</td>
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<td>0.5</td>
<td>15.9 ± 1.9</td>
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<tr>
<td>7</td>
<td>7a</td>
<td>4.18</td>
<td>25</td>
<td>25</td>
<td>0.05</td>
<td>0.3</td>
<td>49.4 ± 1.4&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>18a</td>
<td>5.71</td>
<td>&lt;10</td>
<td>&lt;10</td>
<td>0.01</td>
<td>0.02</td>
<td>52.7 ± 3.8&lt;sup&gt;±&lt;/sup&gt;</td>
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<tr>
<td>9</td>
<td>8b</td>
<td>2.15</td>
<td>500</td>
<td>400</td>
<td>0.4</td>
<td>0.7</td>
<td>12.2 ± 1.2</td>
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<tr>
<td>10</td>
<td>4b</td>
<td>3.12</td>
<td>250</td>
<td>100</td>
<td>0.2</td>
<td>0.6</td>
<td>14.9 ± 2.1</td>
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<tr>
<td>11</td>
<td>7b</td>
<td>3.85</td>
<td>100</td>
<td>75</td>
<td>0.15</td>
<td>0.3</td>
<td>17.7 ± 1.9</td>
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<tr>
<td>12</td>
<td>18b</td>
<td>5.45</td>
<td>&lt;25</td>
<td>&lt;25</td>
<td>0.05</td>
<td>0.1</td>
<td>44.7 ± 3.1&lt;sup&gt;±&lt;/sup&gt;</td>
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<tr>
<td>13</td>
<td>4c</td>
<td>3.71</td>
<td>nd</td>
<td>nd</td>
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<td>0.3</td>
<td>16.7 ± 1.1</td>
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<tr>
<td>14</td>
<td>22</td>
<td>2.92</td>
<td>300</td>
<td>300</td>
<td>0.5</td>
<td>0.7</td>
<td>12.9 ± 2.2</td>
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</table>

<sup>a</sup>Data are means of at least three independent experiments. <sup>b</sup>Data are means ± SEM of 3–6 independent experiments. <sup>c</sup>P < 0.05 vs untreated cells, by one-way ANOVA followed by Duncan test. <sup>d</sup>Calculated at pH 7.4. <sup>e</sup>Concentration causing 50% loss of photosynthetic or respiratory activity after 1 h exposure at 25 °C, as compared to control. <sup>f</sup>LD<sub>10</sub> and LD<sub>50</sub> = compound concentrations at which 90% or 50% of the cells were viable, respectively, after 3 h incubation at 37 °C. <sup>g</sup>Measured after 1 h exposure at the concentration corresponding to LD<sub>50</sub>. In untreated cells the baseline value was of 9.7 ± 2.5 UI/mg prot. and the total LDH activity was 680 ± 12.5 UI/mg prot. (n = 6).<sup>h</sup>Not determined.

### Table 3. Intracellular Distribution, Effect on Tissue ATP, and Mitochondrial Function of Spin Traps and pH Markers (0.1 mM) after 1 h Normothermic Perfusion in the Isolated Perfused Rat Liver

<table>
<thead>
<tr>
<th>compd</th>
<th>cytosol&lt;sup&gt;c&lt;/sup&gt; (μM/g)</th>
<th>mitochondria&lt;sup&gt;c&lt;/sup&gt; (μM/g)</th>
<th>tissue ATP&lt;sup&gt;b&lt;/sup&gt; (μM ATP/g)</th>
<th>membrane potential&lt;sup&gt;e&lt;/sup&gt; (mV)</th>
<th>respiratory control ratio&lt;sup&gt;cd&lt;/sup&gt;</th>
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<tr>
<td>none</td>
<td>2.38 ± 0.21</td>
<td>180.9 ± 3.4</td>
<td>6.2 ± 1.3</td>
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<tr>
<td>PBN</td>
<td>0.88 ± 0.11</td>
<td>&lt;0.05&lt;sup&gt;±&lt;/sup&gt;</td>
<td>2.11 ± 0.22</td>
<td>178.9 ± 3.2</td>
<td>5.5 ± 0.5</td>
</tr>
<tr>
<td>mitoPBN</td>
<td>0.12 ± 0.04</td>
<td>1.95 ± 0.17</td>
<td>1.95 ± 0.11&lt;sup&gt;±&lt;/sup&gt;</td>
<td>170.7 ± 3.2</td>
<td>4.7 ± 1.1&lt;sup&gt;±&lt;/sup&gt;</td>
</tr>
<tr>
<td>DPP</td>
<td>0.56 ± 0.12</td>
<td>&lt;0.05&lt;sup&gt;±&lt;/sup&gt;</td>
<td>2.33 ± 0.12</td>
<td>181.3 ± 1.9</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td>4b</td>
<td>0.93 ± 0.15</td>
<td>1.55 ± 0.28</td>
<td>2.29 ± 0.29</td>
<td>174.2 ± 2.9</td>
<td>5.7 ± 0.7</td>
</tr>
<tr>
<td>7b</td>
<td>0.64 ± 0.13</td>
<td>1.32 ± 0.23</td>
<td>2.08 ± 0.30</td>
<td>171.9 ± 3.0</td>
<td>5.2 ± 0.7</td>
</tr>
</tbody>
</table>

<sup>a</sup>Data are means ± SD of 3–9 independent experiments. <sup>b</sup>P < 0.05 vs Krebs-Henseleit perfused livers, by one-way ANOVA followed by Duncan test. <sup>c</sup>Expressed per gram of fresh liver tissue. <sup>d</sup>From isolated mitochondria prepared at the end of perfusion. <sup>e</sup>Defined as the ratio of the oxidation rate under phosphorylating vs nonphosphorylating conditions. <sup>f</sup>Below the detection limit.

This could be in part related to the protective effect of α-aminophosphonates seen in isometric-reperfused isolated rat hearts and livers we previously assigned to an intrinsic effect of the diethoxyporphosphoryl group.

**Experiments on the Isolated Perfused Rat Liver and Mitochondrial Function.** To check for their intracellular uptake and mitochondria targeting properties in a highly active metabolizing environment, compounds 4b and 7b were compared to mitoPBN using isolated rat livers perfused for 1 h with a KH buffer containing 0.1 mM of test compound followed by a 10 min washout with plain cold KH. As compared to mitoPBN, the more hydrophilic compounds 4b and 7b exhibited (i) a good accumulation within the mitochondrial fraction and (ii) a significantly better recovery in the cytosolic fraction (i.e., the cytosolic distribution percentage was 7%, 33%, and 33% for mitoPBN, 7b and 4b, respectively; see Table 3). As expected, the mitochondrial content of the parent DPP and PBN was negligible (Table 3), this latter result being in line with a study showing that penetration of PBN within the nuclear and mitochondrial fractions of isolated perfused rat hearts is very low. Compared to the more lipophilic PBN, the relatively low cytosolic levels of PBN, the relatively low cytosolic levels of
extracellular concentration followed by a 100–6000-fold accumulation in mitochondria (versus cytosol), giving millimolar levels into the mitochondrial matrix.\textsuperscript{25,27} Table 3 shows that none of the tested compounds significantly altered control tissue ATP levels at the end of perfusion period, with the notable exception of mitoPBN.\textsuperscript{25,27} Current opinion associates excess uptake of lipophilic cations such as Ph$_3$P$^+$ in the mitochondria to disruption of ATP synthesis as a result of their adsorption on the surface of the inner membrane.\textsuperscript{25,27} Accordingly, mitochondria isolated from livers after the end of perfusion with mitoPBN demonstrated significant impairment of respiratory control ratio despite membrane potential (an index of inner mitochondrial membrane integrity) was maintained (Table 3). Compared to control values, these two parameters were maintained by 4b and 7b possibly because of their lower lipophilicity threshold and accumulation profile within the cytosol and the mitochondria (Figure 3).

\section*{CONCLUSIONS}

This work demonstrates the feasibility of triphenylphosphonium salts bearing an $\alpha$-aminophosphonate scaffold as nontoxic, poorly metabolizable, specific, and sensitive $^{31}$P NMR probes of mitochondrial pH. Modulating the spacer length between the two functional groups allowed a fine-tuning of their $pK_a$ value and cytosolic versus mitochondrial distribution, resulting in the two optimized bromides 4b and 7b which demonstrated a unique potential to finely monitor pH gradients at the subcellular level in plant and animal studies.

\section*{EXPERIMENTAL SECTION}

\textbf{Chemistry. General Methods.} Starting materials, reagents, solvents and the spin trap PBN were obtained from Aldrich Chemical Co. and were used without further purification. Analytical $^1$H NMR (operating at 300.1 MHz), $^{13}$C NMR (operating at 75.5 MHz), and $^{31}$P NMR (operating at 121.5 MHz) spectra were recorded in the indicated solvent on a Bruker AVL300 spectrometer. Chemical shifts were expressed as $\delta$ (ppm) relative to TMS. Coupling constants ($J$) were reported in Hertz (Hz) and abbreviations of multiplicity were as follows: s, singlet; d, doublet; t, triplet; m, multiplet; dd, doublet of doublets. Progress of the reaction was monitored by TLC on silica gel 60 aluminum plates with F254 as indicator (Merck) and products were purified by flash chromatography on silica gel 60 (230–400 mesh). Melting points were determined with a Stuart melting point apparatus SMP30 (Bibby Scientific, U.K.) and are not corrected. High resolution mass spectrometry (HRMS) in electron spray ionization (ESI) was performed at the Spectopole (Analytical Laboratory) at Campus St. Jérôme (Marseille, France) on a Q-STAR Elite instrument (Applied Biosystems, U.S.A.). The purity of key compounds was checked with a 1200 series HPLC apparatus (Agilent; Santa Clara, CA) using a Beckman Coulter C18 (250 $\times$ 10 mm, 5 $\mu$m) reverse phase column. The mobile phase A was 0.01% trifluoroacetic acid in water and the mobile phase B was acetonitrile, which was increased linearly from 10% to 100% over 21 min, and 100% over the next 8 min with a flow rate of 2.5 mL/min. Peaks were detected at 230 nm and showed $\geq$95% purity for all compounds in this system. The retention times $R_t$ of selected compounds are reported in minutes. Yields referred to purified products and were not optimized. Chiral compounds were...
isolated as racemates. The starting compounds and reagents: (6-54 hydrogenyl)-carboxylic acid 1,1-dimethyl ester (1),59 (6-54 hydroxyethyl)-carboxylic acid 1,1-dimethyl ester (2),60 6-bromohexy-
55 an-1-ol (11),51 12-bromobenzenec-1-ol (12)33 (for these two latter
56 compounds, the synthetic procedure is summarized in Scheme 3), 5-
57 iodopentan-2-one (19),56 IBTP,32 DPP,11 and [4-4-[[(1,1-
58 dimethylyl)oxidoiminomethyl]phenoxyl]butyl].
59 triphenylphosphonium bromide (mitoPBN)56 were synthesized
60 according to reported procedures.
61 Synthesis of 6-[(tert-Butyloxycarbonyl)amino]-
62 heptyltriphenylphosphonium iodide (3). A flask charged with 2 (3.0
63 g, 9.1 mmol) and triphenylphosphine (4.0 g, 15.26 mmol) in dioxane
64 (40 mL) was heated at 80 °C for 12 h in the dark. The mixture was
65 cooled and added dropwise to well-stirred EtO (200 mL). The
66 resulting white precipitate was separated by filtration, washed
67 in CHCl3 (10 mL), and added dropwise to well-stirred EtO (100 mL).
68 The resulting white solid was distilled under reduced pressure to give 3
69 (1.0 g, 1.69 mmol) in CHCl3 (10 mL) was stirred with 4 M HCl in
70 dioxane (1 mL) for 3 h at room temperature and concentrated under
71 reduced pressure. The resulting yellow solid was washed with Et2O,
72 dried under reduced pressure, dissolved in dry CH2Cl2 (15 mL) under
73 argon, and TEA was added slowly to reach a pH of 6.5–7.0. After the
74 mixture was stirred for 1 h at room temperature, 2.00 mmol of either
75 pivaldehyde (synthesis of 4a) or isobutyraldehyde (synthesis of 4c)
76 was added and the reaction mixture was stirred for 12 h at room
77 temperature. Diethyl phosphate (50 µL, 0.42 mmol) was then added
78 dropwise and the mixture was refluxed for 2 days under argon. After
79 removal of the precipitate by filtration, the mixture was added
80 dropwise to well-stirred EtO (50 mL). After cooling, the solvent layer
81 was decanted and the resulting pale yellow oil was dissolved in CH2Cl2
82 (5 mL) and added dropwise to well-stirred EtO (50 mL). The
83 resulting oil was dissolved in CHCl3 (15 mL), washed with water (15
84 mL), and washed twice with 15% aqueous NaBr solution. The organic
85 layer was dried with MgSO4 and evaporated to dryness in vacuo to
86 give the desired compound.
87 6-[(1-Diethylphosphoryl-2-methylpropyl)amino]-
88 heptyltriphenylphosphonium Bromide (4a). Sticky yellow oil (0.43 g,
89 43%).1H NMR (CDCl3) δ 0.84 (d, 3 J = 7.0 Hz, –CH3), 0.87 (d,
90 3 H, J = 7.0 Hz, –CH3), 1.25 (t, 6 H, J = 7.0 Hz, 2 × –OCH2CH3),
91 1.31–1.42 (m, 4 H), 1.50–1.65 (m, 4 H), 1.98–2.13 (m, 1 H,
92 –CH(CH3)2), 2.50–2.82 (m, 3 H), 3.55–3.70 (m, 2 H, –CH2–
93 PPh3), 4.01–4.12 (m, 4 H, 2 × –OCH2–), 7.60–7.70 (m, 15 H, 94
95 –PPh3); 31P NMR (CDCl3) δ 24.18, 28.73; 13C NMR (CDCl3) δ
96 16.38, 16.44 (2 × –OCH2CH3), 17.25 (d, J = 3.9 Hz, –CH2–),
97 20.50 (d, J = 14.2 Hz, –CH2–), 22.23 (d, J = 3.7 Hz, –CH2–PPh3),
98 22.52 (d, J = 50.6 Hz, –CH2–PPh3), 26.30 (–CH2–), 28.88 (d,
99 4.9 Hz, –CH(CH3)2), 29.62 (–CH2–), 30.01 (d, J = 16.9 Hz, –CH–
100 CH2–PPh3), 49.23 (d, J = 5.0 Hz, –CH–), 60.50 (d, J = 146.0 Hz,
101 CH2, 60.70, 60.22 (2 × –OCH2–), 117.15 (d, J = 85.8 Hz,
102 –PPh3 ipso), 130.17 (d, J = 12.6 Hz, –PPh3 meta), 133.16 (d, J =
103 9.9 Hz, –PPh3 para), 134.83 (d, J = 2.7 Hz, –PPh3 para).
104 HRMS-ESI: calcld for C33H39N3P3O3 [M+H+] 561.7403, found
105 561.7409.
106 6-[[Diethylphosphoryl-2,2-dimethylpropyl]amino]-
107 heptyltriphenylphosphonium Bromide (4c). Sticky yellow oil (0.52 g,
108 48%).1H NMR (CDCl3) δ 1.00 (s, 9 H, 3 × –CH3), 1.23–1.40 (m,
109 10 H), 1.55–1.70 (m, 4 H), 2.30–2.85 (m, 3 H), 3.60–3.72 (2 H,
110 –CH2–PPh3), 4.00–4.15 (m, 4 H, 2 × –OCH2–), 7.60–7.90 (m, 15
111 H, –PPh3); 31P NMR (CDCl3) δ 23.95, 28.28; 13C NMR (CDCl3) δ
112 16.09, 16.21 (2 × –OCH2CH3), 22.17 (d, J = 3.7 Hz, –CH2–
113 PPh3), 22.39 (d, J = 50.6 Hz, –CH2–PPh3), 26.38 (–CH2–), 27.15
114 (d, J = 6.6 Hz, 3 × –CH3), 29.81 (–CH–), 29.97 (d, J = 16.9 Hz,
115 –CH–CH2–PPh3), 31.6 (2 × –OCH2–), 32.60 (–CH2–), 45.67 (d, J =
116 27.8 Hz, –CH3), 140.0 (CH2), 117.76 (d, J = 86.2 Hz, –PPh3 ipso),
117 130.15 (d, J = 12.6 Hz, –PPh3 meta), 133.18 (d, J = 9.9 Hz, –PPh3 ortho), 134.71
118 (d, J = 2.7 Hz, –PPh3 para). HRMS-ESI: calcld for C32H37N3P3O3
119 569.3104, found 569.3101.
120
8-(1-Diethylphosphoryl-2-methylpropylamino)-octyltriphenylphosphonium Bromide (7a). Sticky yellow oil (0.24 g, 32%). $^1$H NMR (CDCl$_3$) $\delta$ 0.93 (d, 3 H, J = 6.8 Hz, $-CH(CH_3)_2$), 2.57–2.65 (m, 2 H, J = 4.4 Hz, CH$_2$), 1.50–1.65 (m, 4 H, CH$_2$), 1.8–2.13 (m, 1 H, $-CH_2CH(CH_3)$), 2.50–2.82 (m, 3 H, J = 3.3 Hz), 3.53–3.70 (m, 2 H, $-CH_2CH_2$), 4.98 (d, J = 149.7 Hz, CH$_2$), 118.19 (d, J = 85.8 Hz, $-PPh_3$ ipso), 130.46 (d, J = 12.5 Hz, $-PPh_3$ meta), 133.71 (d, J = 10.3 Hz, $-PPh_3$ ortho), 134.99 (d, J = 2.9 Hz, $-PPh_3$ para). HRMS-ESI: calcd for C$_{33}$H$_{48}$NO$_3$P$_2$ 524.2505, found 524.2505.

Synthesis of Mito-aminophosphonates $\alpha$a and $\alpha$b. To a solution of 6.00 mmol of either $\alpha_A$ (synthesis of $\alpha_A$) or $\alpha_B$ (synthesis of $\alpha_B$) and K$_2$CO$_3$ (2.20 g, 15.94 mmol) in acetonitrile (25 mL) was added IBTP (3.37 g, 5.90 mmol) over 10 min. The reaction mixture was refluxed for 72 h in the dark under argon. After removal of the precipitate by filtration, the filtrate was evaporated to dryness in vacuo. The residue was dissolved in CH$_2$Cl$_2$ (10 mL) and added dropwise to well-stirred Et$_2$O (50 mL). After the mixture was cooled, the solvent layer was decanted and the resulting white solid was washed twice with 15% aqueous NaBr solution, and evaporated to dryness in vacuo to give the desired compound.

6-Hydroxytriphenylphosphonium Bromide (13). White solid (13.52 g, 92%): mp 133 °C; $^1$H NMR (CDCl$_3$) $\delta$ 1.40–1.44 (m, 4 H), 6.00–1.64 (m, 4 H), 3.50–3.54 (d, J = 7.0 Hz, HO–$-CH_2$), 3.58–3.65 (m, 2 H, $-CH_2CH_2$), 6.70–7.90 (m, 15 H, $-PPh_3$) $^3$P NMR (CDCl$_3$) $\delta$ 22.24 (d, J = 3.7 Hz, $-CH_2$, $-CH_2$), 2.62–24.69 (m, J = 9.9 Hz, $-CH_2$, $-CH_2$), 130.46 (d, J = 12.6 Hz, $-PPh_3$ meta), 133.54 (d, J = 9.9 Hz, $-PPh_3$ meta), 134.96 (d, J = 2.7 Hz, $-PPh_3$ para). HRMS-ESI: calcd for C$_{28}$H$_{30}$O$_3$P 363.1872, found 363.1873.

12-Hydroxydicyclohexyltriphenylphosphonium Bromide (14). White solid (15.91 g, 91%): mp 99 °C; $^1$H NMR (CDCl$_3$) $\delta$ 1.10–1.37 (m, 20 H), 1.50–1.65 (m, 4 H), 2.55–2.65 (t, 2 H, J = 6.8 Hz, $-CH_2$), 3.55–3.70 (m, 2 H, $-CH_2$), 4.01–4.12 (m, 4 H, 2 x $-OCH_2$), 6.70–7.90 (m, 15 H, $-PPh_3$) $^3$P NMR (CDCl$_3$) $\delta$ 16.41, 16.48 (2 x $-CH_2CH_2$), 22.42 (d, J = 5.06 Hz, $-CH_2CH_2$), 22.83 (d, J = 3.3 Hz, 2 x $-CH_2$), 26.96 (d, J = 1.80 Hz, $-CH_2$), 29.29 (7 x $-CH_2$), 61.77 (d, J = 14.0 Hz, CH$_3$), 87.2 (2 x $-OCH_2$), 118.14 (d, J = 85.6 Hz, $-PPh_3$ ipso), 130.36 (d, J = 12.6 Hz, $-PPh_3$ meta), 133.44 (d, J = 9.9 Hz, $-PPh_3$ ortho), 134.90 (d, J = 2.7 Hz, $-PPh_3$ para). HRMS-ESI: calcd for C$_{33}$H$_{48}$O$_3$P 547.2811, found 547.2808.

Synthesis of Compounds 15 and 16. To a solution of 3.38 mmol of either 13 (synthesis of 15) or 14 (synthesis of 16) in toluene (15 mL) was added a 48% aqueous HBr solution (565 µL, 5.00 mmol). The reaction mixture was heated at 85 °C for 12 h. After it was cooled, the reaction mixture was extracted with CH$_2$Cl$_2$ (5 mL), washed twice with 15% aqueous NaOH solution and extracted with CH$_2$Cl$_2$ (2 x 20 mL). The combined organic layers were washed twice with 15% aqueous NaBr solution, and evaporated to dryness in vacuo to give the desired compound.

6-Bromoethyltriphenylphosphonium Bromide (15). Brown oil (1.50 g, 88%): $^1$H NMR (CDCl$_3$) $\delta$ 1.32–1.40 (m, 2 H), 1.52–1.75 (m, 6 H), 3.25–3.30 (t, 2 H, J = 7.0 Hz, Br–$-CH_2$), 3.62–3.73 (m, 3 H, 2 x $-CH_2CH_2$), 6.70–7.90 (m, 15 H, $-PPh_3$) $^3$P NMR (CDCl$_3$) $\delta$ 14.64; $^1$C NMR (CDCl$_3$) $\delta$ 21.95 (d, J = 3.7 Hz, $-CH_2$, $-CH_2$), 22.55 (d, J = 5.06 Hz, $-CH_2$, $-CH_2$), 25.59–29.27 (m, J = 9.9 Hz, $-CH_2$, $-CH_2$), 32.62 (d, J = 9.57 Hz, $-CH_2$, $-CH_2$), 62.64 (d, J = 11.80 Hz, Br–$-CH_2$), 130.42 (d, J = 12.6 Hz, $-PPh_3$ meta), 134.54 (d, J = 9.9 Hz, $-PPh_3$ meta), 134.92 (d, J = 2.7 Hz, $-PPh_3$ para). HRMS-ESI: calcd for C$_{29}$H$_{38}$BrP 425.1028, found 425.1022.
803

Synthesis of Mito-aminophosphonates 21 and 22. The iodide salt of 5-mitopentan-2-one 20 was prepared from 10.0 g (47.17 mmol) of 5-iodopentan-2-one 19 in toluene (60 mL) by adding triphenylphosphine (13.1 g, 50.0 mmol) and refluxing the mixture for 12 h in the dark under argon. The reaction mixture was cooled, concentrated under reduced pressure, and the crude solid was dissolved in CH₂Cl₂ (20 mL) and added dropwise to well-stirred Et₂O (150 mL). After decantation, the resulting white solid was filtered and evaporated to dryness in vacuo to give 20. The H NMR and ³¹P NMR data of 20 were in accordance with the data reported in the literature for the corresponding bromide salt which was obtained by another chemical pathway.

5-(2-Oxo)pentaphenylphosphonium iodide (20). White solid (33.00 mmol, 15.7 g, 70%); mp 205 °C; ³¹P NMR (CDCl₃) δ 1.75–1.86 (m, 2 H; CH₂–P⁺Ph₃), 2.13 (3 J, 3 H, –CH₃), 3.03–3.07 (t, J = 6.05 Hz, 2 H, –CH₂–P⁺Ph₃), 7.60–7.90 (m, 15 H, –H–P⁺Ph₃); ¹³C NMR (CDCl₃) δ 24.07, 31.15; ¹³C NMR (CDCl₃) δ 16.60 (d, J = 2.8 Hz, –CH₂–), 21.68 (d, J = 0.56 Hz, CH₃–P⁺Ph₃), 30.19 (~CH₂), 42.49 (d, J = 17.1 Hz, –CH₂–), 117.87 (d, J = 8.64 Hz, –H–P⁺Ph₃), 130.35 (d, J = 12.7 Hz, –H–P⁺Ph₃ meta), 132.65 (d, J = 6.4 Hz, –H–P⁺Ph₃ ortho), 134.95 (d, J = 3.3 Hz, –H–P⁺Ph₃ para), 208.38 (C≡O). HRMS-ESI: calcd for C₇₅H₇₁OP⁺ 347.1559, found 347.1554.

To prepare compound 21, compound 20 (1.00 mmol) in ethanol (10 mL) was saturated with dry ammonia. After the mixture was stirred for 1 h, diethylphosphite (1.10 mol) was added slowly, and the mixture was first stirred and then heated at 65 °C for 4 h. After it was cooled, the mixture was washed in water (15 mL), acidified with 1 N HCl, and extracted with ethyl acetate (2 × 15 mL). The aqueous layer was basified with 10% aqueous NaOH solution, and extracted with CH₂Cl₂ (2 × 50 mL). The combined organic layers were washed twice with 15% aqueous NaBr solution, dried over MgSO₄ and evaporated to dryness in vacuo to give 21. Compound 22 was obtained using the same procedure as above except that 2-methylpropan-1-amine (1.00 mmol) was used instead of ammonia.

4-Amino-4-(diethylphosphoryl)pentaphenylphosphonium Bromide (21). Sticky yellow oil (0.42 g, 75%) ¹³C NMR (CDCl₃) δ 1.14–1.18 (d, J = 15.8 Hz, 3 H, –CH₃), 1.25 (t, J = 7.0 Hz, 2 H × 2-OCH₂CH₃), 1.70–1.85 (m, 4 H, 2 × –CH₂–), 3.63–3.85 (m, 2 H, –CH₂–P⁺Ph₃), 4.01–4.12 (m, 4 H, 2 × OCH₂–), 6.50–7.80 (m, 15 H, –H–P⁺Ph₃); ¹³C NMR (CDCl₃) δ 21.62, 26.62 (~CH₂–), 26.11 (d, J = 16.0 Hz, –CH₂–), 30.35 (~–CH₂–), 42.65 (d, J = 5.2 Hz, –CH₂–), 53.12 (d, J = 146.3 Hz, –C–), 62.03, 62.18 (~2 × –OCH₂–), 118.08 (d, J = 85.8 Hz, –H–P⁺Ph₃), 130.33 (d, J = 12.1 Hz, –H–P⁺Ph₃ meta), 133.48 (d, J = 9.9 Hz, –H–P⁺Ph₃ ortho), 134.93 (d, J = 3.3 Hz, –H–P⁺Ph₃ para). HRMS-ESI: calcd for C₇₅H₇₁NO₃P⁺ 540.2786; found 540.2786; R₂ 116.36.

12-1-(Diethylphosphoryl)-2-methylpropan-2-ylamino)pentaphenylphosphonium Bromide (22). Sticky yellow oil (0.43 g, 70%); ¹¹B NMR (CDCl₃) δ 0.77 (d, J = 6.7 Hz, 2 CH₂), 1.14–1.18 (d, J = 16.5 Hz, 3 H, –CH₃), 1.25 (t, J = 7.0 Hz, 2 H × 2-OCH₂CH₃), 1.70–1.85 (m, 4 H, 2 × –CH₂–), 3.22–3.29 (m, 2 H, –CH₂–), 3.50–3.68 (m, 2 H, –CH₂–P⁺Ph₃), 4.01–4.12 (m, 4 H, 2 × OCH₂–), 6.50–7.80 (m, 15 H, –H–P⁺Ph₃); ¹³C NMR (CDCl₃) δ 21.39, 26.45 (~CH₂–), 26.79 (d, J = 14.8 Hz, –CH₂–P⁺Ph₃), 49.15 (d, J = 4.9 Hz, –CH₃), 59.91 (d, J = 146.0 Hz, CH₂), 60.75, 60.94 (~2 × OCH₂–), 113.49 (d, J = 85.6 Hz, –H–P⁺Ph₃), 128.91 (d, J = 12.0 Hz, –H–P⁺Ph₃ meta), 137.45 (d, J = 9.9 Hz, –H–P⁺Ph₃ ortho), 137.45 (d, J = 27.8 Hz, –H–P⁺Ph₃ para). HRMS-ESI: calculated for C₇₅H₇₁NO₃P⁺ 584.2165, found 584.2169.

4-(Sobutylamino)4-(diethylphosphoryl)pentaphenylphosphonium Bromide (23). Sticky yellow oil (0.45 g, 73%) ¹³C NMR (CDCl₃) δ 1.14–1.18 (d, J = 15.8 Hz, 3 H, –CH₃), 1.25 (t, J = 7.0 Hz, 2 H × 2-OCH₂CH₃), 1.70–1.85 (m, 4 H, 2 × –CH₂–), 3.63–3.85 (m, 2 H, –CH₂–P⁺Ph₃), 4.01–4.12 (m, 4 H, 2 × OCH₂–), 6.50–7.80 (m, 15 H, –H–P⁺Ph₃); ¹³C NMR (CDCl₃) δ 16.62–16.74 (~CH₂ and 2 × –OCH₂CH₂), 22.54 (d, J = 2.2 Hz, –CH₂–P⁺Ph₃), 23.25 (d, J = 49.9 Hz, –CH₂–P⁺Ph₃), 37.45 (d, J = 16.9 Hz, –H–CH₂–), 51.89 (d, J = 148.2 Hz, C), 62.57, 62.64 (2 × OCH₂CH₂), 118.12 (d, J = 85.8 Hz, –H–P⁺Ph₃ iso), 130.47 (d, J = 12.5 Hz, –H–P⁺Ph₃ meta), 132.73 (d, J = 9.5 Hz, –H–P⁺Ph₃ ortho), 135.04 (d, J = 2.9 Hz, –H–P⁺Ph₃ para). HRMS-ESI: calculated for C₇₅H₇₁NO₃P⁺ 584.2165, found 584.2169.
acquired on a Bruker AMX 400 spectrometer at 161.98 MHz, with chemical shifts being referenced to external 85% H3PO4 at 0 ppm. A small capillary containing D2O was inserted in each sample as a lock reference during and perfusates were filtered through a 0.2-μm Millipore filter prior to use. Dulbecco’s modified Eagle’s medium (DMEM), Eagle’s minimal essential medium (MEM) and fetal calf serum (FCS) were obtained from Life Technologies Corp. (St Aubin, France). All other reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). Stock solutions of the test compounds in DMSO (0.1 M) were diluted in aqueous medium before use.

**Toxicity Tests on Chlamydomonas reinhardtii Cultures.** Cell wall-less green algae *Chlamydomonas reinhardtii* D66 were grown in Tris-acetate phosphate medium to densities of 1–5 × 10^6 cells per mL under fluorescent light (50 μmol quanta/m^2/s) at 25 °C. The cells were then incubated at 25 °C with aeration to achieve cultures that could be used to determine photosynthetic and respiratory activities. In the end of incubation, 1 mM NaHCO3 was added to the sample (to avoid any CO2 limitation) and the activities were measured against the corresponding control containing the same amount of DMSO (0.1% maximum) by using a Clark-type oxygen electrode. The toxicity of each test compound was expressed as LD50 (phot. and respectively), which corresponds to the concentration leading to the loss of 50% of photosynthetic or respiratory activity, respectively as compared to the control.

**Toxicity Tests on 3T3 Murine Fibroblasts.** Murine 3T3 fibroblasts (ATCC-LGC Promochem, Molsheim, France) were cultured at 37 °C in an atmosphere of 5% CO2 in DMEM containing 1% glucose and supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin as previously described. Cells were distributed into 24- well plates and the medium was replenished every 2–3 days until confluence, which was checked by microscopic observation and by measuring protein content in four over 24 randomly selected control wells by the method of Lowry et al. Each well of confluent cells was then filled with phenol red-free DMEM containing 1% glucose. Incubation of the cells at 37 °C for 3 h was then carried out by adding to the culture medium aliquots of the stock solution of each test compound to reach a final concentration ranging 0.01–1 mM and 0.1% for the test compound and DMSO, respectively, in a final volume equal to 0.5 mL/well. Following incubation the supernatants were sampled to measure cytoplasmic LDH release (considered as a marker of cell necrosis) using a commercial kit (Biolabo, Maizy, France), and the adherent cells were reincubated for 2 h in MEM containing neutral red (50 μg/mL) and 2% FCS, and cell viability was evaluated at 540 nm using a microplate reader (SAFAS, Monaco) as described.

The medium lethal dose (LD50) and the concentration reducing cell viability by 10% (LD10) were determined from appropriate dose–response curves. 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 total LDH release in the supernatant and 100% loss of viability. The total LDH amount is the sum of the enzymatic activity in the lysate and in the culture medium.

**Isolated Liver Experiments and Mitochondrial Preparation.** Sprague–Dawley male rats (120–150 g) used for liver perfusion and mitochondrial preparation were from CERJ (Le Genest St Isle, France) and fed ad libitum with a standard Teklad 2016 diet (Harlan Laboratories, Gannat, France). Animals were maintained in the local animal house under conventional conditions, in a room with controlled temperature (21–25 °C) and a reverse 12 h light/dark cycle. All animal procedures used were in strict accordance with the Directive 2010/63/EU of the European Parliament. The CNRS and Aix-Marseille University have currently valid licenses for animal experimentation (agreement C13-055-06) delivered by the French Government. Animals were deeply anesthetized by intraperitoneal injection of sodium pentobarbital (100 mg/kg; Ceva Santé Animale, Libourne, France), and the liver was excised and perfused as previously described. Briefly, after the opening of the abdominal wall, the portal vein was cannulated (anastomosis perfusion) and the liver was perfused at 37 °C in a nonrecirculating mode by a KH bicarbonate buffer containing (in mM): KH2PO4 (1.2), NaCl (119), KCl (4.8), MgSO4 (1.2), NaHCO3 (25), and CaCl2 (1.3) and bubbled with a gas mixture of 95% O2 and 5% CO2 (pH 7.35). Assuming that the liver weight represents about 4% of the body weight (i.e., 6–8 g), the starting perfusion rate was set at 20 mL/min. The excised liver was weighed, and the perfusion was adjusted to a flow rate of 3 mL/min/g of wet weight. After a 30 min equilibration period, an aliquot of the stock solution of each test compound in DMSO was added to the perfusion medium to achieve a final concentration of 0.1 mM, and the perfusion was extended for 1 h. The perfusate was then switched to an ice-cold KH buffer for 10 min to completely remove the test compound from the extracellular space, and the liver was homogenized in five volumes of a solution containing 0.25 M sucrose and 10 mM HEPES (pH 7.5). After filtration, the homogenate centrifuged at 750 × g for 10 min at 2 °C the mitochondrial and cytosolic fractions were isolated from the supernatant by differential centrifugation as previously described, and finally resuspended in 1 mL KH buffer. These samples were extracted at 20 °C with CH3Cl2 (2 × 5 mL), and the combined organic layers were concentrated at reduced pressure to give a dry residue, which was first weighed to determine the mitochondrial and cytosolic distribution of each test compound. Data are pooled from 3–4 livers/test compound and are given in μmoles per gram of fresh tissue. To check for any metabolic degradation of the perfused compound, each residue was subsequently redissolved in 1 mL of CDCl3 for 1H and 31P NMR analytical analysis as described above.

To further evaluate the toxicity of test compounds two additional sets of livers (3–6 livers per test compound) underwent the same perfusion protocol as described above. In the first set of livers the end of perfusion was followed by quickly freezing the organ in liquid nitrogen and analysis of tissue ATP content of perchloric extracts using an enzymatic standard procedure. In the second set of livers, mitochondrial and cytosolic fractions were isolated from homogenates in ice-cold sucrose (0.3 0.9 M) buffer by differential centrifugation and the simultaneous monitoring of respiration and phosphorylation rates, and membrane potential were carried out as previously described. In parallel the respiratory control ratio was determined by measuring the ratio of the respiratory rate in the presence of 0.5 mM ADP to that measured after the cessation of ADP phosphorylation, in a medium containing (in mM): KH2PO4 (5), EDTA (0.005), MOPS (20), MgCl2 (5), glutamate (20), and malate (1) maintained at 37 °C (pH 7.2).

**Statistics.** All experiments were run at least in triplicate. Data were presented as mean ± SD or SEM for the indicated number of independently performed experiments. Evaluation of statistical significance was conducted by one-way or two-way analysis of variance (ANOVA) followed, if significant (P < 0.05), by a Duncan test. Differences between groups were considered significant when P < 0.05.
The study was supported by the Agence Nationale pour la Recherche (ANR-09-BLAN-005-03 and ANR-09-BLAN-005-01-ROS Signal). The authors thank G. Gosset for her valuable help in NMR experiments and S. Lemaire (IBPC-CNRS, Paris) and C. Roumestand (CBS-CNRS, Montpellier) for their kind help.

ACKNOWLEDGMENTS

The Authors thank G. Gosset for her valuable help in NMR experiments and S. Lemaire (IBPC-CNRS, Paris) and C. Roumestand (CBS-CNRS, Montpellier) for their kind help.

ABBREVIATIONS USED

pH, intracellular pH; P, inorganic phosphate; DEPMPH, diethyl(2-methylpyrrolidin-2-yl)phosphonate; DFP, diethyl(2-propylaminoprop-2-yl)phosphonate; PBN, α-phenyl-N-tert-butylnitrite; IBTP, 4-iodobutyltriphenylphosphonium iodide; KH, Krebs–Henseleit; LDH, lactate dehydrogenase; mitoPBN, [4-4-[[1,1-dimethylethyl]oxidoimino]methyl]phenoxymethyltriphenylphosphonium bromide; DMEM, Dulbecco’s modified Eagle’s medium; MEM, Eagle’s minimal essential medium; FCS, fetal calf serum.

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