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FULL PAPER

Novel Amino Acid-Based β -Phosphorylated Nitroxides for Probing Acidic pH in Biological Systems by EPR Spectroscopy

Sophie Th  tiot-Laurent,^[a] Ga  lle Gosset,^[a] Jean-Louis Cl  ment,^[a] Mathieu Cassien,^[a] Anne Mercier,^[a] Didier Siri,^[a] Anouk Gaudel-Siri,^[a] Antal Rockenbauer,^[b] Marcel Culcasi,^{[a]*} and Sylvia Pietri^[a]

This article is dedicated to the memory of our colleague Fran  ois Le Moigne

Abstract: There is increasing attraction of measuring pH in biological studies using nitroxides having pH-dependent electron paramagnetic resonance (EPR) spectra. Aiming to improve the spectral sensitivity, Δa_X , of these probes (i.e. the difference between the EPR hyperfine splittings (hfs) in their protonated and unprotonated forms), we present here a series of novel linear α -carboxy, α' -diethoxyphosphoryl nitroxides constructed on an amino acid core and featuring a (α or α')-C-H bond. In buffers the three main hfs (a_N , a_H and a_P) of their EPR spectra vary reversibly with pH and, when it is inferred from a_P or a_H titration curves, a 2–4-fold increase in sensitivity is achieved vs reference imidazoline or imidazolidine nitroxides. The crystallized carboxylate **10b** ($pK_a \approx 3.6$), which demonstrated low cytotoxicity and good resistance to bioreduction, was applied to probe the stomach acidity in rats. The results pave the way to a novel generation of highly sensitive EPR pH markers.

Introduction

In living organisms the pH value is a fundamental parameter whose thorough regulation is essential for maintaining the structure of proteins and physiological functions, e.g., by sustaining the active conformation of biologically active macromolecules.^[1] Although normal extracellular and cytosolic acid-base homeostasis is associated with a pH range of 7.0–7.3, a local, substantially acidic intracellular pH (pH_i) occurs at pH 3–6 in certain cellular compartments such as the endoplasmic reticulum, the trans-Golgi complex or secretory vesicles, e.g., to allow cell internalization pathways (endosome-lysosome system).^[2] Besides, abnormal low pHs are associated with cellular dysfunctions and pathologies such as ischemia, oxidative stress,^[3] apoptosis^[4] or tumor size progression.^[5] As non-invasive alternatives to pH microelectrodes, optical and spectroscopic methods to assess pH in vivo have been the focus

of intense research in the last decades. To sense pH_i this usually involves introducing in tissue or cells weak acids displaying pH-dependent fluorescence (indicator dyes)^[6,7] or ^{31}P NMR parameters (i.e., chemical shift and/or coupling constants).^[8] Recently, structural modifications around the α - and β -aminophosphonate scaffold (cyclic or linear) have provided a series of non toxic pH markers showing finely tunable subcellular permeation and pK_a value, with up to fourfold increased ^{31}P NMR sensitivity compared to endogenous inorganic phosphate (P_i).^[9] Some of these compounds having a $pK_a < 6$ were successfully applied to probe acidic compartments in isolated rat liver, amoeba cultures and plant cells (Figure 1A).^[10]

The above spectroscopic techniques having their own limitations such as relative lack of sensitivity or requirement of nonabsorbing samples, interest in exogenous ionizable paramagnetic probes arose because much lower concentrations can be detected with short acquisition times using continuous wave X-band (~9.8 GHz) EPR spectroscopy. Up to now synthetic efforts have established imidazoline (Im)- and imidazolidine (In)-based nitroxides as the most utilized pH spin probes in biological studies, a model compound being 2,2,3,4,5,5-hexamethylimidazolidine-1-yloxy (In-1; Figure 1B).^[6] These free radicals show low toxicity, rather good resistance to reduction into EPR-silent species, and substitution can be varied as to offer modulation of their lipophilicity, cell permeability and targeting.^[11] In both Im and In families protonation occurs at N-3, leading to a reversible variation of the two main apparent EPR parameters, the nitrogen hyperfine splitting (hfs) a_N and g -factor. Furthermore, a wide range of pK_a s is accessible upon varying the electronic effects of substituents of the five-membered ring backbone.^[12] Combining low-field (< 1.2 GHz) EPR techniques with appropriately tailored Im- or In-type pH reporters (i.e., with $pK_a < 5$) allowed monitoring drug-induced changes in the gastric acidity of rats.^[13]

Besides the necessary innocuity and stability conditions for a good EPR probe, high accuracy of pH measurement requires the largest sensitivity Δa_X for a given coupling nucleus X to be achieved, where $\Delta a_X = |(a_X)_b - (a_X)_a|$ is the absolute difference of the coupling constants in the unprotonated basic and protonated acid forms of the radical. Since generally $a_{14-N} \approx 15$ G in nitroxides and the reported Δa_N values in the Im and In families did not exceed 1.25 G so far,^[12a] our goal was to develop new ionizable β -phosphorylated nitroxides on the basis of their known large a_P hfs, e.g., they are often > 40 G in the 1-pyrrolidinyloxy series.^[14,15] Despite this advantage of getting increased sensitivity only two studies have addressed EPR pH effects of such five membered ring nitroxides substituted by a β -P(O)(OH)₂ group, reporting total

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a_P variations > 3 G in the whole pH range.^[15] Yet none of these radicals (one of them being a spin adduct^[15b]) were further evaluated in vivo and it is noteworthy that, because of the presence of two ionizable sites in the phosphono group, the useful Δa_P value for a single acidity may be actually lower. Hence, for a total variation of 3.8 G in the biphasic titration curve of Ref.^[15b], the useful Δa_P is only ≈ 2 G around pK_a 7.5. Such observation supports our earlier strategy in ^{31}P NMR studies^[9a] that a better sensitivity for spectral pH measurement is obtained when the ionizable site is remote to the NMR resonant (or EPR coupling) phosphorus.

With this concept in mind, our target EPR probes for acidic pH were α -carboxy, α' -diethoxyphosphoryl nitroxides where (i) ionization occurs at the CO_2H function and (ii) the scaffold (Figure 1C) is close to that of linear α -aminophosphonates we developed as ^{31}P NMR pH probes.^[9] Many fully alkylated members of this family, such as *N-tert-butyl-N*-[1-diethylphosphono(2,2-dimethylpropyl)] nitroxide (DEPN; Figure 1C), have become popular in nitroxide-mediated living polymerization^[16] and more recently a small set of α -substituted acyl derivatives has been used to model solvent-related EPR hfs.^[17] Although they bear one α -hydrogen, all these nitroxides are very persistent in solvents and water because their disproportionation into diamagnetic species can be prevented or slowed by steric crowding at the remaining α, α' positions,^[18] a substitution pattern we adopted in our target nitroxides by using (Y, Y') = Ph, *i*Pr or *t*Bu groups (Figure 1C). In this paper we report on our first foray into the EPR characteristics and biological in vitro activities of novel non-cyclic amino acid-based β -phosphorylated nitroxides. The lead isolable compound that emerged from this series was successfully applied to determine by X-band EPR the stomach acidity of living rats, in comparison with a structurally related α -aminoester especially developed for ^{31}P NMR.

Results and Discussion

Synthesis of EPR pH probes

All syntheses of target amino acid-derived β -diethoxyphosphoryl nitroxides proceeded via the formation of their α -aminophosphonate precursors, whose esters were isolated to evaluate their pH-dependent ^{31}P NMR properties in comparison to other probes of the same class (Figure 1A).^[9, 10]

The key step was a classical Kabachnik-Fields reaction with diethylphosphite and the appropriate carbonyl compound and α -amino acid, using either a one-pot procedure or a pathway where the intermediate imine was isolated. Desired nitroxides were derived by oxidation of α -aminophosphonates with 3-chloroperoxybenzoic acid (*m*CPBA). The syntheses of a series of α -aminophosphonates constructed from α -amino acids have already been described.^[19]

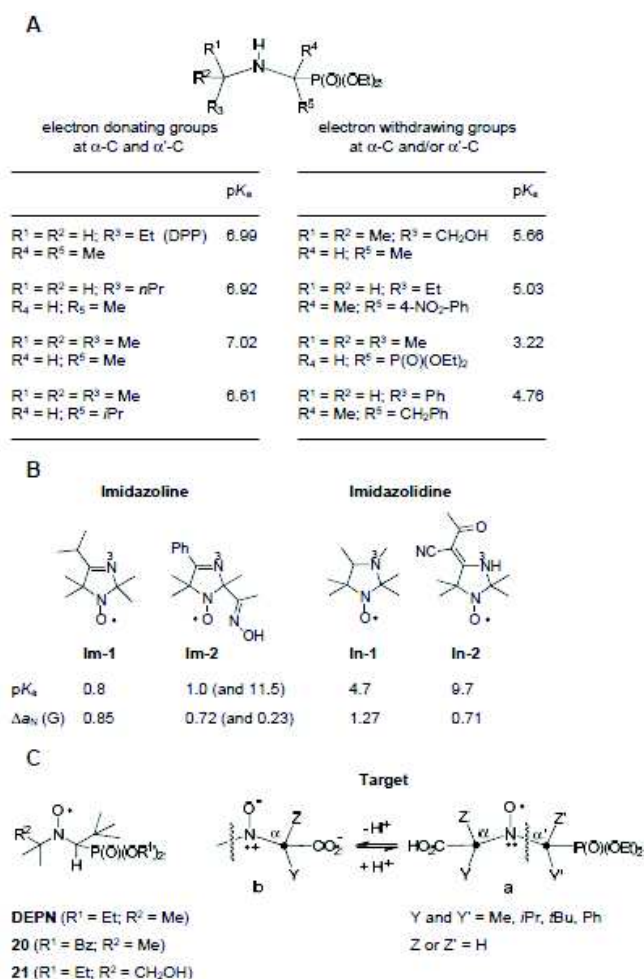
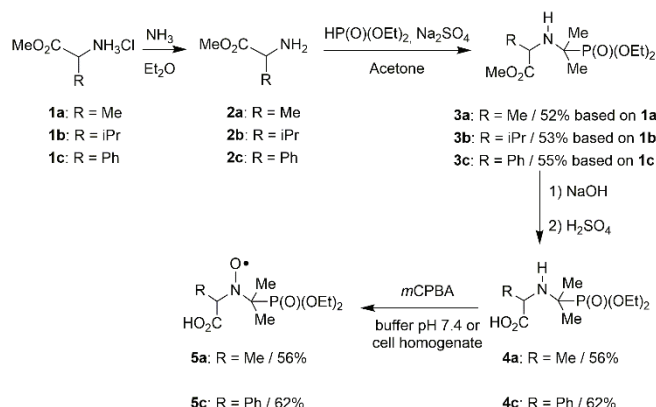


Figure 1. (A) ^{31}P NMR pH probes and their pK_a s in Krebs-Henseleit buffer.^[9a,9b,10b] (B) EPR pH probes with their reported pK_a s and sensitivities in Tris-HCl buffer.^[12,13a] (C) Right panel: general structure of target carboxylic EPR pH probes showing preferred resonance forms (a: non-polar form; b: dipolar form) of ionization states (right structure: protonated form; left structure: unprotonated form). Left panel: structurally related pH insensitive DEPN and crystallized derivatives.

The one-pot sequence was applied to diethylphosphite and acetone to derive the methyl esters **3a–c** of alanine, valine and phenylglycine, respectively, starting from the corresponding free amino acid esters **2a–c** (Scheme 1). Saponification of **3a** and **3c** yielded the α' -diethoxyphosphoryl carboxylic acids **4a** and **4c** in good yields. Intriguingly, attempts to hydrolyze **3b** by varying base, solvent, temperature and reaction time were unsuccessful. This appears consistent with the reported impossibility of saponifying alkyl esters in α -position of an *i*Pr substituent.^[20] Finally, target nitroxides **5a** and **5c** (for which Y' = Z' = Me; Figure 1C) were obtained by oxidation of **4a** and **4c** with aqueous *m*CPBA. Buffered solutions of **5a** and **5c** were EPR stable for at least 3 h at ambient temperature.



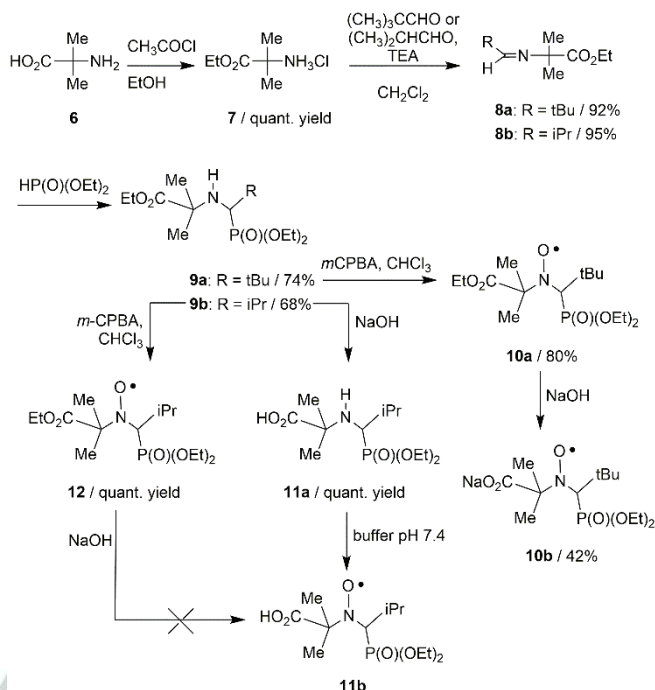
Scheme 1. Synthesis of α-aminophosphonates **3a-c**, **4a** and **4c**, and nitroxides **5a** and **5c**.

To synthesize target nitroxides for which Y = Z = Me, α,α-dimethylated amino acid based α'-diethoxyphosphoryl esters were prepared starting from 3-aminoisobutyric acid **6** which was esterified quantitatively to the ethyl ester hydrochloride **7**. Subsequent treatment of **7** by either pivaldehyde or isobutyraldehyde using a reported procedure^[21] afforded the corresponding imines **8a** and **8b** with good yields (Scheme 2). Microwave-assisted addition of diethylphosphite to **8a** and **8b** gave the 2-aminoisobutyric acid-derived α-aminophosphonates ethyl esters **9a** and **9b** in rather good yields. Oxidation of **9a** and **9b** with *m*CPBA using a slight modification of a reported procedure^[16a] quantitatively yielded the corresponding esters nitroxides **10a** and **12** which were isolated as oils. Saponification of **10a** with NaOH gave the target α,α-dimethylated β'-phosphorylated nitroxide (with an overall yield of 23% for the five steps), whose sodium salt **10b** afforded stable crystals. However, under the same base hydrolysis conditions the *i*Pr analog **12** failed to give the expected nitroxide **11b** as checked by EPR.

Nevertheless, nitroxide **11b** was obtained in two steps, i.e., saponification of **9b** to give the α-aminophosphonate α-carboxylic acid **11a** whose oxidation with aqueous *m*CPBA gave **11b**. When prepared in situ such solutions of **11b** exhibited EPR stable signals for at least 3 h but again attempts to isolate the nitroxide by organic solvent extraction resulted in degradation, EPR silent products.

Lastly, we came to prepare α-aminophosphonate **16** (Scheme 3) to assess whether increasing both electron donating property and steric hindrance at α-C may affect the pK_a value.

Commercially available (*S*)-methylvaline **13** was quantitatively converted to the ethyl ester hydrochloride **14**, whose subsequent reaction with pivaldehyde gave the corresponding imine **15** with a good yield. While microwave-assisted condensation of diethylphosphite on **15** only yielded degradation products, a 1:1 mixture of diastereomers of the desired compound **16** was obtained in moderate yield upon heating the imine at 55 °C for two weeks in a sealed tube.

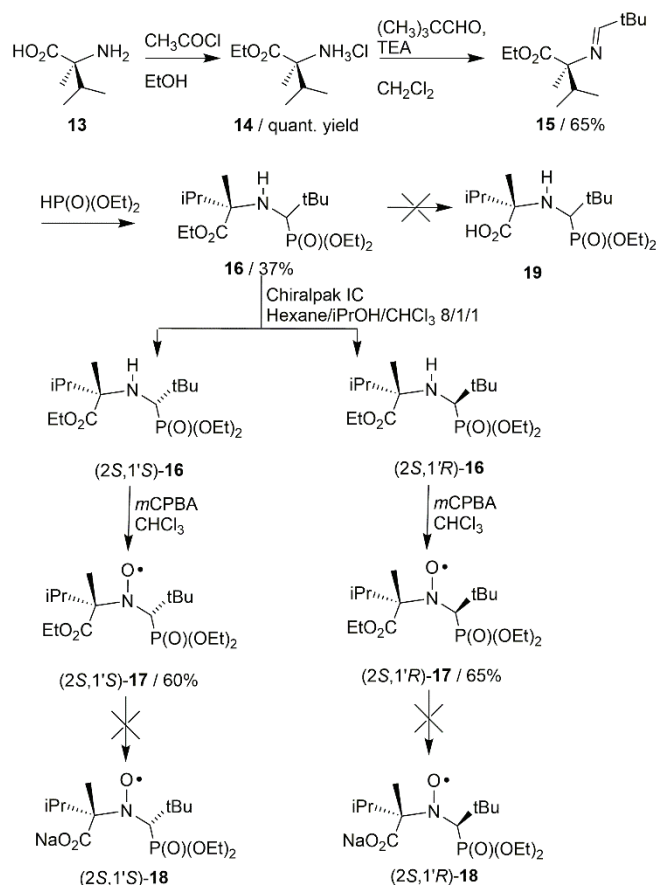


Scheme 2. Synthesis of α-aminophosphonates **9a**, **9b** and **11a**, and related nitroxides **10a**, **10b**, **11b** and **12**.

After separation by chiral preparative HPLC (Chiralpak IC eluting with hexane/isopropanol/chloroform (8/1/1)), the stereochemistry of the two diastereomers was determined by NMR studies including ¹H-¹H 2D ROESY experiments. For the first eluting diastereomer at *t*_R = 4.87 min one ROE correlation was observed between the isopropyl proton (δ 1.82 ppm) and the α'-C-H (δ 2.73 ppm), demonstrating a (2*S*,1'*R*)-**16** configuration (Figure S1a, Supporting Information). For the second eluting diastereomer at *t*_R = 6.05 min one ROE correlation was observed between the α'-C-H (δ 2.95 ppm) and the protons of the α-C-Me (δ 1.11 ppm), demonstrating (2*S*,1'*S*)-**16** configuration (Figure S1b, Supporting Information). Subsequent oxidation by *m*CPBA of (2*S*,1'*R*)-**16** and (2*S*,1'*S*)-**16** afforded the corresponding nitroxides (2*S*,1'*R*)-**17** and (2*S*,1'*S*)-**17** in good yields. Disappointingly, in relation to compound **3b** all diastereomeric α-*i*Pr substituted esters **16** and **17** resisted hydrolysis (that should have yielded compounds **18** and **19**) and when drastic conditions (strong base, high temperature, long reaction time) were used, only decomposition products were recovered.

³¹P NMR pH-calibration of α-aminophosphonates esters

Besides the principal goal of this study, we investigated the pH dependence at 22 °C of the ³¹P NMR chemical shift of the new alkyl ester derivatives **3a-c**, **9a** and **9b** in various physiologically relevant media, including Krebs-Henseleit buffer (KH) as well as KCl (125 mM)-supplemented cytosolic homogenates from rat heart (CytM_h) or liver (CytM_l).



Scheme 3. Synthesis of diastereomeric α -aminophosphonates esters **16** and related nitroxides esters **17**.

All the esters, except diastereomers of **16**, gave monophasic acid-base titration curves reflecting protonation at nitrogen and their fitting to the Henderson-Hasselbalch equation (see Experimental Section and Figure S2, Supporting Information) allowed calculation of the pK_a s, the limiting chemical shifts of the protonated ammonium (δ_a) and unprotonated amine (δ_b) forms, and the ^{31}P NMR sensitivities $\Delta\delta_{ab} = \delta_a - \delta_b$ around the pK_a region (Table 1). The five titrated compounds yielded acidic pK_a s ~ 3 with only $< 2\%$ variation with medium (i.e., ± 0.04 – 0.12 pH units). Owing to the strong electron withdrawing effect of ester groups, these quite low pK_a s for **3a–c**, **9a** and **9b** confirm our previous findings^[9,10] that, in β -diethoxyphosphoryl aminophosphonates, introducing inductive attractors (e.g., phenyl or benzyl) at C_α and/or $C_{\alpha'}$ results in more acidic pK_a values (Figure 1A). Among our reported ^{31}P NMR pH indicators, only a few cyclic (i.e., β -phosphorylated pyrrolidines) or linear (e.g., compound with $R^5 = \text{P}(\text{O})(\text{OEt})_2$ in Figure 1A) derivatives featuring two P-containing groups demonstrated pK_a values < 3.5 .^[9a,10b]

Regarding the main characteristics of ^{31}P chemical shift pH titration, compounds **3a–c**, **9a** and **9b** showed very high $\Delta\delta_{ab}$ values of ~ 10 ppm with $< 1\%$ changes with medium (i.e., ± 0.15 – 0.73 ppm) and much shorter longitudinal relaxation times T_1

than the poorly sensible P_i .^[22] Moreover, in KH the NMR sensitivities found for all tested esters were similar to that of DPP (i.e., ~ 10.2 ppm at 25°C).^[9c] a highly sensitive alkylated α -aminophosphonate pH sensor (Figure 1A) in the 5–8 pH range.^[9a] In the case of diastereomers of **16** for which no sigmoidal acid-base curves could be obtained (i.e., $\Delta\delta_{ab} \approx 0$), longer T_1 values were yet measured at pH 4 (Table 1). In principle, the homogeneous low pK_a s found here for all aminophosphonates esters, giving a useful pH range of 1.5–4.5 with great NMR dynamic range, would encourage their use to probe subcellular acidic compartments or study physiological conditions such as gastric acidity.

Crystal structure of nitroxide **10b** and molecular dynamics study

The sodium carboxylate **10b** crystallizes as a dimer pooling two Na^+ , with each cation being linked to six oxygen atoms (from two phosphonyl groups, the nitroxyl oxygen and carboxylate anion, and two water molecules), plus μ^2 -bridging performed by the two $\text{P}=\text{O}$ functions and one water molecule. The structure appears similar to a square bipyramid centered on Na^+ , the tops being occupied by a $\text{P}=\text{O}$ group and a water molecule. Chiral HPLC analysis revealed that the synthesis of **10b**, which is chiral at α' -C (Figure 1C), produced a racemate.

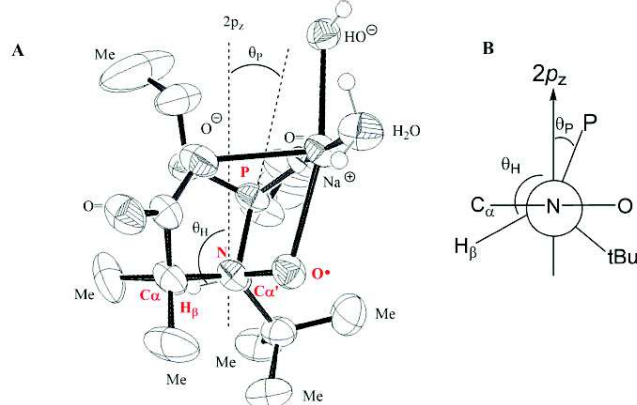


Figure 2. (A) ORTEP view of **10b** showing the nitroxide function and the atoms and dihedral angles implicated in the Heller-McConnell relation. Thermal ellipsoids represent 50% equiprobability envelopes. (B) Newman projection along the $\text{N}-\text{C}_\alpha$ axis.

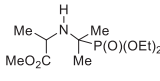
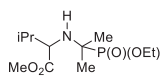
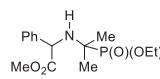
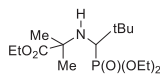
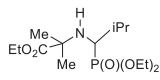
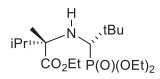
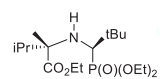
Figure 2A depicts an ORTEP view of one molecular unit of **10b** in the dimeric aggregate, in which (α, α') -C, P and β -H atoms surrounding the $\text{N}-\text{O}^\bullet$ function are highlighted and where the α' -C retains a (S) configuration. The main X-ray crystallographic features of **10b**, i.e., elongated $\text{C}_\alpha-\text{C}(\text{tBu})$, (α, α') -C–N and $\text{C}_\alpha-\text{P}$ bonds, and a large $\text{C}_\alpha-\text{N}-\text{C}_\alpha'$ bond angle (Table 2), result from the steric effect of the two bulky alkyl groups in α, α' positions. Similar geometries have been reported for the DEPN derivatives **20**^[16a] and **21**^[16b] (Figure 1, left panel) but unlike this latter nitroxide, the strongest stabilizing electrostatic interaction in crystallized **10b** is likely to establish between the partially

positively charged N-atom and the carboxylate anionic oxygen rather than the phosphonyl oxygen,^[16b] as shown by the $d(\text{N}\cdots\text{O})$ distance of 2.683 Å which is notably smaller than the NO van der Waals radii sum (~ 3.07 Å; see^[16b] and references therein). Indeed the preferred electronic structures indicated in the headings of Table 2 for **10b** and related compounds account for the fact that in the unprotonated (basic) form of the radical the electron-donating effect of the CO_2^- group will favor the resonance dipolar

form (b) of the nitroxide function while the electron-withdrawing effect of the CO_2H group in the protonated (acid) form will favor the non-polar form (a), as depicted in the right panel of Figure 1C.

To better understand the structural features responsible for the good stability in solution of acidic and basic forms of **10b** and its congeners **5a**, **5c** and **11b** (see below and Experimental Section), molecular dynamics (MD) simulations of the EPR

Table 1. ³¹P NMR pH calibration^[a] and relaxation^[b] characteristics of α -aminophosphonates esters in biologically relevant media^[c]

Compd	medium	pK _a	δ_a (ppm)	δ_b (ppm)	$\Delta \delta_{ab}$ (ppm)	T_1 (s)
P_i	KH	6.64	0.68	3.26	2.58	9.80–11.50
	CytM _h	6.78	0.68	3.22	2.54	1.30–3.80
3a	KH	3.63	22.31	32.69	10.38	3.60
	CytM _h	3.48	22.48	32.84	10.36	2.97
	CytM _l	3.52	22.48	32.71	10.23	2.41
3b	KH	3.35	22.29	32.68	10.39	2.90
	PB	3.24	21.81	32.21	10.40	3.00
3c	KH	2.60	22.15	32.52	10.37	2.40
	CytM _h	2.48	22.34	32.41	10.04	nd ^[d]
9a	KH	2.39	20.20	30.74	10.54	3.07
	CytM _l	2.47	20.81	30.62	9.81	0.30
	PB	2.54	20.63	30.09	9.46	1.91
9b	KH	2.99	19.73	29.91	10.18	3.20
	CytM _l	2.95	19.89	29.80	9.91	1.55
	PB	3.18	19.47	29.27	9.80	5.58
(2S,1'S)-16	PB	nd	27.76	27.74	0.02	6.83 ^[e]
						
(2S,1'R)-16	PB	nd	30.61	30.61	0	6.15 ^[e]
						

[a] δ_a and δ_b , limiting chemical shift of the protonated and unprotonated form, respectively; $\Delta \delta_{ab} = \delta_a - \delta_b$; determined at 22 °C and given as means from 3–10 repeated titrations with < 1% precision. [b] T_1 = longitudinal relaxation time measured at pH \approx pK_a. [c] P_i, inorganic phosphate; KH, Krebs-Henseleit medium; CytM_h, rat heart cytosolic homogenate; CytM_l, rat liver cytosolic homogenate; PB, phosphate buffer (i.e., 1 mM KH₂PO₄). [d] not determined. [e] pH 4.00.

spectra recorded at room temperature were performed to study the conformational changes of the solvated nitroxides (see Experimental Section for details). Because the chirality of the probes (at either C_α or $C_{\alpha'}$) can in principle affect the distribution of the conformations, (*R*)/(*S*) enantiomers were considered (four trajectories for each compound). Table 2 reports the results of MD simulations for the (*S*) enantiomer of the nitroxides and results for the (*R*) enantiomer led to the same conclusions below (Table S1, Supporting Information). Comparing the angle and bond lengths pointed out in the X-ray crystallographic structure of **10b** to computed values in solution revealed no or little variations suggesting that the β -H atom in all nitroxides remains sterically protected from disproportionation. Besides, none of the calculated $d(N\cdots O)$ distances in the unprotonated forms or $d(COOH\cdots O=P)$ and $d(COOH\cdots O-N)$ distances in the acidic forms (these being all

> 3.8 Å, data not shown) were compatible with intramolecular bonds, e.g., H-bonds. Therefore the strong interaction found above for **10b** in the solid state likely results from a crystal packing effect.

EPR characterization

To construct accurate EPR pH-titration curves for the novel ionizable nitroxides, high-resolution signals are needed in order to separate the contributions of different species and to calculate the best sets of hfs at a given pH value.

Table 2. Selected crystallographic data of **10b** ($R = \text{Me}$) and molecular dynamics simulation for the average geometry of the (*S*) enantiomer of **10b**, **11b** ($R = \text{H}$), **5a** ($R' = \text{Me}$) and **5c** ($R' = \text{Ph}$) in aqueous solution.^[a]

	X-ray	unprotonated	protonated	unprotonated	protonated
Compd	10b	10b	11b	10b	11b
5a	5c	5a	5c		
Bond lengths (Å)					
C(1)–P(53)	1.835(2)	1.84(3)	1.83(3)	1.83(3)	1.82(3)
C(1)–C(13)	1.569(3)	1.57(3)	1.56(3)	1.57(3)	1.56(3)
N(40)–C(1)	1.473(3)	1.50(3)	1.50(3)	1.50(3)	1.50(3)
N(40)–O(45)	1.288(2)	1.27(3)	1.27(3)	1.27(3)	1.27(3)
N(40)–C(3)	1.482(3)	1.50(3)	1.51(3)	1.51(3)	1.51(3)
Distances (Å)					
O(42)...N(40)	3.124	3.7(3)	3.6(4)	3.7(3)	3.6(3)
O(46)...N(40)	2.683	3.2(2)	3.2(2)	3.2(2)	3.2(2)
Bond angle (°)					
C(3)–N(40)–C(1)	126.77(19)	126(3)	126(3)	126(3)	126(3)
Dihedral angles (°)					
P(53)–C(1)–N(40)–O(45)	–76.47	–70(11)	–70(11)	–70(12)	–71(12)
H(2)–C(1)–N(40)–O(45)	170.90	–179(12)	177(12)	179(12)	175(12)

[a] Values in parentheses are the estimated SD. In all structures atom numbering is given similar to the deposited structure of **10b**. [b] Values obtained for alternative stable conformations.

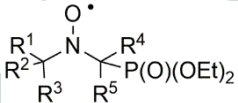
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This was achieved by recording the spectra in deoxygenated buffer using low modulation amplitude, as illustrated by Figure 3A for **10b** at room temperature in phosphate buffer, pH ~7. The 12-lines EPR signals given by all carboxylic nitroxides and their ester precursors in aqueous solution clearly show that, in addition to a_N and a_P coupling constants observed in this class of radicals, the $a_{H\beta}$ hfs were also resolved. Moreover, simulation of the highly resolved spectrum of **10b** gave the best fit ($r > 0.996$) assuming a single species with additional long-range hfs with both α -C-Me groups and the *t*Bu group, and a ^{13}C splitting pattern involving both α , α' - and the three β -carbons. Assuming the same long-range coupling model, the hfs and g -factors of the novel nitroxides at nearly neutral pH (except for **12**) were determined by simulation (Table 3) and used as starting sets in EPR titration curves of ionizable compounds (which are all unprotonated under these conditions, see below). In the case of the EPR spectrum of DEPN in phosphate buffer, where only six lines can be resolved, we performed simulations including also β - and γ -hydrogen hfs besides the nitrogen and phosphorous couplings, which improved dramatically the fit ($r > 0.996$) and yielded a realistic, yet too large $a_{H\beta}$ value ~1 G, not allowing to split the spectrum into a 12 lines pattern. According to the simplified Heller-McConnell relationship^[23] the mean EPR β -couplings, $\langle a_X \rangle$ ($X = \text{H}, \text{P}$), of our target nitroxides in solution follow a dihedral dependence

$$\langle a_X \rangle = B_X \times \langle \cos^2 \theta_X \rangle \quad (1)$$

where θ_X is the dihedral angle between the N-C-X plane and the direction of the $2p_z$ orbital at nitrogen atom of the N-O \cdot function (see Figure 2B in the case of **10b**). Accordingly, unlike DEPN, **20**, **21** and congeners which yield EPR sextets in organic solvents,^[16,17] all the nitroxides of our series should adopt a preferred conformation in aqueous medium with a θ_H value not close to 90° , i.e., the N-O and C α -H β (for **5a** and **5c**) or C α -H β (for **10b** and **11b**) bonds are not totally eclipsing. This geometry was observed in the X-ray structure of **10b** since packing constraints the H β -C α -N-O and P-C α -N-O torsion angles to about 171° and -76° , respectively (Table 2), corresponding to dihedral angles of $\theta_H \approx 99^\circ$ and $\theta_P \approx 13^\circ$ (Figure 2A). For both enantiomers of nitroxides **5a**, **5c**, **10b** and **11b**, the average values of the torsion angles obtained from the MD simulation studies in water over the last 99.5 ns of the trajectory are reported (Tables 2 and S1). Conformational changes due to the release of crystal packing and minimization of steric constraints always tended to increase H β -C α -N-O dihedral angles in both basic and acidic forms near to 180° (corresponding to $\theta_H = 90^\circ$) with consequences on the EPR couplings as discussed below.

Table 3. Simulated EPR parameters of β -diethoxyphosphoryl nitroxides at 22°C

											
Compd	Medium ^[a]	R ¹	R ²	R ³	R ⁴	R ⁵	a_N (G)	$a_{H\beta}$ (G)	a_P (G)	Long range couplings (G)	g
5a	PB (pH 6.62)	H	Me	CO ₂ ⁻	Me	Me	14.62	4.88	51.45	0.27(3H), 0.15(3H)	—
5c	PB (pH 6.25)	H	Ph	CO ₂ ⁻	Me	Me	14.50	4.48	49.47	0.35(3H)	—
10a	W (pH 7.12)	Me	Me	CO ₂ Et	H	<i>t</i> -Bu	14.38	1.29	41.75	0.45(3H), 0.26(3H)	2.00557
10b	W (pH 6.56)	Me	Me	CO ₂ ⁻	H	<i>t</i> -Bu	14.67	1.43	40.25	0.36(3H), 0.26(3H), 0.23(9H) 5.6(2 × ¹³ C), 7.4(3 × ¹³ C)	2.00547
11b	PB (pH 6.67)	Me	Me	CO ₂ ⁻	H	<i>i</i> -Pr	14.92	1.39	44.04	0.45(3H), 0.22(6H), 0.10(1H)	2.00636
12	W (pH 3.23)	Me	Me	CO ₂ Et	H	<i>i</i> -Pr	14.59	1.38	44.05	0.39(3H), 0.21(6H)	2.00543
(2 <i>S</i> ,1' <i>S</i>)- 17	W (pH 7.17)	Me	<i>i</i> -Pr	CO ₂ Et	H	<i>t</i> -Bu	13.69	1.17	38.46	0.30(1H)	2.00560
(2 <i>S</i> ,1' <i>R</i>)- 17	W (pH 7.08)	Me	<i>i</i> -Pr	CO ₂ Et	H	<i>t</i> -Bu	14.65	1.47	43.61	3.95(1H)	2.00554
DEPN	PB (pH 7.3)	Me	Me	Me	H	<i>t</i> -Bu	14.93	0.99	45.68	0.34(9H)	2.00542

[a] W, water; PB, 20 mM phosphate buffer.

While establishing accurate sets of EPR hfs for all nitroxides we observed unusually intensive lines in the EPR signal of the ethyl ester (2*S*,1*R*)-**17** in degassed water (not shown). A nice fit of the spectrum was obtained ($r > 0.996$) assuming a very large γ -hydrogen splitting of ~ 4 G, almost 2.7 times exceeding $a_{\text{H}\beta}$ (Table 3). We propose such a large long-range coupling in (2*S*,1*R*)-**17**, but not in its diastereomer (2*S*,1*S*)-**17**, to result from a periplanar 'W-plan' arrangement, as reported earlier in *i*Pr-substituted cyclic nitroxides.^[24]

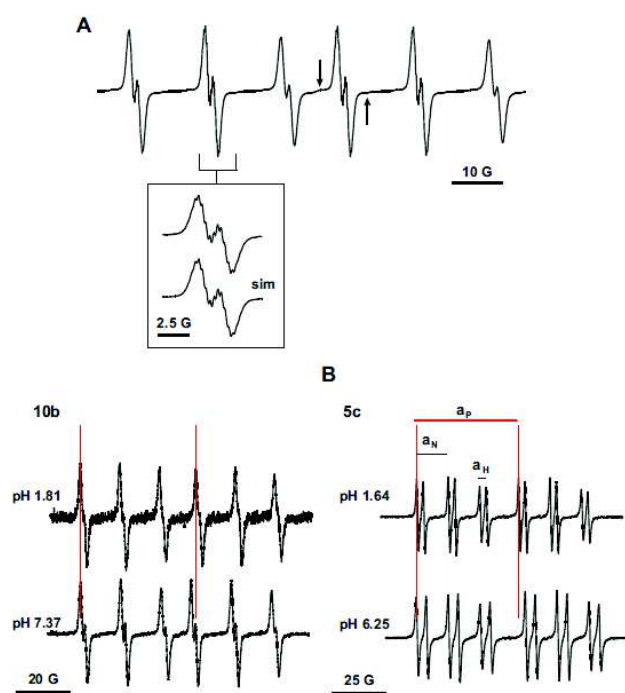


Figure 3. (A) EPR spectrum of **10b** (1 mM) in degassed phosphate buffer (20 mM; pH 7.21) showing long-range hydrogen couplings (expanded region with simulation) and two ^{13}C satellites (arrows). Settings: modulation amplitude, 0.03 G; time constant, 20.48 ms; gain, 3.2×10^4 ; scan rate, 0.57 G.s^{-1} ; accumulated scans, 10. (B) Limiting protonated and unprotonated EPR spectra of **10b** (0.36 mM) and **5c** (~ 0.1 mM) in 20 mM phosphate buffer. Settings (**10b**, **5c**): modulation amplitude, (0.06, 0.48) G; time constant, (40.96, 5.12) ms; gain, (1×10^5 , 4×10^4); scan rate, (1.13, 1.31) G.s^{-1} . Signals of **5c** were recorded in the reaction mixture 4 min (pH 6.25) and 40 min (pH 1.64) following oxidation of amine **4c** by mCPBA. The vertical lines allow a visual comparison of differences in a_{P} values.

EPR pH titration

In eq 1 the experimentally determined hyperconjugative parameters B_{H} and B_{P} in β -phosphorylated nitroxides are of 26 G and 58 G, respectively.^[14,25] We thus reasoned that in our novel ionizable stable nitroxides, even a minute pH-induced conformational change, leading to θ_{H} and/or θ_{P} variation, would translate to large Δa_{H} and/or Δa_{P} values, the latter being potentially larger due to the greater B_{P} constant. Indeed pH-dependent EPR spectra were obtained with the four carboxylic

nitroxides **5a**, **5c**, **10b** and **11b** (illustrated in Figure 3B for **10b** and **5c**) while non ionizable esters **10a**, **12** and **17** exhibited pH-insensitive EPR signals. With parameters of Table 3 as starting points of the simulations a set of three monophasic EPR pH titration curves involving a_{N} , a_{H} and a_{P} hfs were obtained for each carboxylic nitroxide in 20 mM phosphate buffer or cytosolic homogenates at 22 °C. Titration curves for **10b**, **5a** and **5c** are shown in Figures 4 and S3 (Supporting Information), respectively. Importantly, these plots were obtained by reversibly sweeping a 0.5–12 pH window for about 1.5 h without any noticeable loss of EPR signal.

Table 4 reports the limiting hfs and pK_{a} s obtained either by fitting the pH titration profiles (each spectrum was simulated individually) to the EPR variant of the Henderson-Hasselbalch eq 3 (Supporting Information) or by a two-dimensional (2-D) procedure^[26] involving simultaneous fitting of the whole set of parameters (hfs, g -value, linewidth and pK_{a}) for each titration. Regardless of the nucleus considered, limiting hfs and pK_{a} s of **5a**, **5c** and **10b** in synthetic buffer vs the various CytM prepared from rat organs were not substantially altered, varying by less than 0.1 G and 0.2 pH units, respectively. In particular a_{N} values remained almost constant although spin density at nitrogen in β -phosphorylated nitroxides is known to increase with solvent polarity.^[15a,17] For all nitroxides in a given milieu, acidic pK_{a} s extracted from the three a_{X} ($\text{X} = \text{N}, \text{H}, \text{P}$) vs pH titration curves ($n = 2-4$) showed little variation (≤ 0.06 pH units), except when a_{H} was used to probe pH using **11b**, probably because of the very small EPR sensitivity found in this case. As compared with these data, 2-D calculation yielded small differences in limiting hfs (< 0.2 G; not shown) but slightly different pK_{a} s which however followed the same trend among compounds (Table 4). In agreement with the known effect of electron-withdrawing substituents in α -position of the carboxylic acid function, the lowest pK_{a} in the nitroxide series found for **5c** vs **5a** follows the trends observed for **3c** vs **3a** in the ^{31}P NMR study (Table 1) and of the α -amino acids building blocks phenylglycine vs alanine, which show pK_{a} s of 1.83 vs 2.34, respectively. In another hand, protonation/deprotonation of the nitroxides generates a characteristic linewidth alternation responsible for slightly asymmetric EPR lines as seen in Figure 3. The proton exchange times calculated by 2-D simulation were 13.2, 13.8, 104 and 31 ns for **5a**, **5c**, **10b** and **11b**, respectively, reflecting a fast exchange on the EPR time scale.

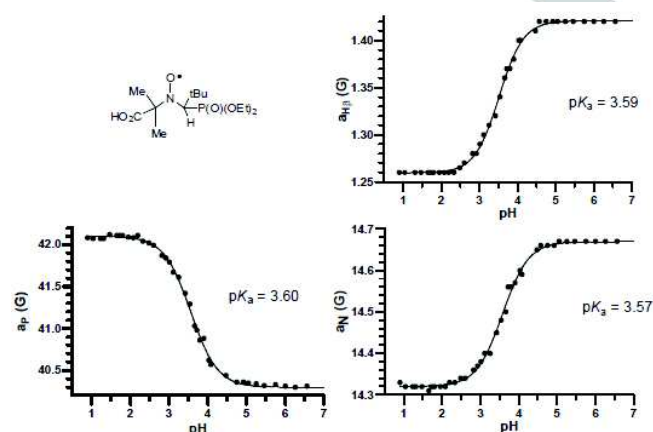
As anticipated, an important feature of novel nitroxides pH markers is their enhanced EPR sensitivity in the acidic region making them good candidates to probe the gastric pH in vivo. Consistent with the high potential of conformational-related variation of hfs, **5a** and **5c** offered the best properties, e.g., for **5a**: $\Delta a_{\text{H}} = 2.35$ G and $\Delta a_{\text{P}} \approx 3.7$ G while **10b** still showed appreciable interest, with $\Delta a_{\text{P}} \approx 1.9$ G (Table 4). It is worth underlining that unlike our lead compound **10b**, related nitroxides having demonstrated Δa_{P} values up to 3.5 G were not isolated.^[15]

Table 4. EPR pH-calibration parameters of β -phosphorylated carboxylic nitroxides at 22 °C^[a]

Compd	Medium ^[b]	limiting hyperfine splittings (G) ^[c] and pK _a values									
		from a _N titration curve			from a _H titration curve			from a _P titration curve			2-D ^[d]
		(a _N) _a	(a _N) _b	pK _a	(a _H) _a	(a _H) _b	pK _a	(a _P) _a	(a _P) _b	pK _a	
5a	PB	14.49 ± 0.02	14.67 ± 0.01	2.91 ± 0.06	2.53 ± 0.04	4.88 ± 0.01	2.97 ± 0.02	47.71 ± 0.04	51.45 ± 0.01	2.95 ± 0.01	2.57
	CytM _h	14.48 ± 0.04	14.67 ± 0.04	3.11 ± 0.04	2.47 ± 0.03	4.93 ± 0.09	3.06 ± 0.01	47.67 ± 0.03	51.56 ± 0.01	3.06 ± 0.01	
5c	PB	14.25 ± 0.01	14.50 ± 0.01	2.47 ± 0.06	2.69 ± 0.02	4.50 ± 0.01	2.48 ± 0.02	45.67 ± 0.06	49.55 ± 0.03	2.45 ± 0.02	2.49
	CytM _h	14.26 ± 0.01	14.50 ± 0.01	2.54 ± 0.07	2.74 ± 0.02	4.46 ± 0.01	2.50 ± 0.02	45.77 ± 0.09	49.45 ± 0.03	2.51 ± 0.02	
10b	PB	14.34 ± 0.01	14.67 ± 0.01	3.61 ± 0.03	1.28 ± 0.01	1.42 ± 0.01	3.59 ± 0.07	42.10 ± 0.02	40.23 ± 0.01	3.60 ± 0.03	3.76
	CytM _l	14.32 ± 0.01	14.66 ± 0.01	3.58 ± 0.02	1.27 ± 0.01	1.42 ± 0.01	3.44 ± 0.02	42.05 ± 0.02	40.21 ± 0.01	3.59 ± 0.02	
	CytM _s	14.32 ± 0.01	14.70 ± 0.01	3.56 ± 0.02	1.27 ± 0.01	1.42 ± 0.01	3.52 ± 0.01	42.05 ± 0.02	40.22 ± 0.01	3.58 ± 0.02	
11b	PB	14.56 ± 0.01	14.91 ± 0.01	3.41 ± 0.02	1.37 ± 0.01	1.39 ± 0.01	3.59 ± 0.29	44.36 ± 0.01	44.00 ± 0.01	3.35 ± 0.06	3.62

[a] Mean ± SD ($n = 2-4$ independent titrations). [b] PB, 20 mM phosphate buffer; CytM_h, rat heart cytosolic homogenate; CytM_l, rat liver cytosolic homogenate; CytM_s, rat stomach cytosolic medium. [c] (a_x)_a, limiting coupling constant in the protonated form; (a_x)_b, limiting coupling constant in the unprotonated form. [d] Two-dimensional EPR simulation.

In the Im-(1,2) and In-(1,2) families developed as EPR pH probes (Figure 1B) a through-bond mechanism operates by which protonation/deprotonation modifies spin density at nitrogen, and thereby pH sensing mainly relies on changes in a_N.^[6,11,12] In our designed nitroxides a deprotonation-induced rise in a_N occurred as the resonance form (b) becomes predominant (Figure 1C, left panel) with Δa_N values ranging 0.18–0.38 G (Table 4), yet below the sensitivities of In-1 nitroxides having acidic pK_as.^[12,13a]

**Figure 4.** EPR pH titration curves for **10b** in phosphate buffer at 22 °C.

In **10b** and **11b**, the finding that a_H increases (that is, θ_H decreases) while a_P decreases (that is, θ_P increases) upon increasing pH (Figure 4 and Table 4) is an obvious consequence

of eq 1 (schematized in Figure 2B) since in these nitroxides the coupling nuclei H and P are attached to the same C_α. In **5a** and **5c** where H and P are linked to C_α and C_{α'}, respectively, both a_H and a_P increased with pH (Figure S3 and Table 4). These opposite trends were confirmed by applying eq 1 with B_P = 58 G to θ_P angles derived from MD simulations of P–C_α–N–O dihedral angle in the protonated and unprotonated forms of the nitroxides (Tables 4 and S1). Assuming a (R)/(S) racemic mixture for **5a**, **5c**, **10b** and **11b** gave predicted absolute $\Delta\langle a_P \rangle$ values of 2.1, 4.1, –0.6 and –2.8 G, respectively, in relatively good agreement (especially the sign was well reproduced) with the corresponding experimental values of 3.74, 3.88, –1.87 and –0.36 G, respectively (Table 4). For **5a** and **5c** dihedral angle distributions revealed two symmetric positions in the protonated forms while the P(O)(OEt)₂ moiety adopts almost the same orientation in the unprotonated form (Figures S4 and S5, Supporting Information). For (**10b**, **11b**) no significant changes in dihedral distributions with pH were found (Figures S6 and S7, Supporting Information) suggesting that phosphoryl group rotation is almost frozen. Altogether these data could explain the differences (amplitude and sign) of $\Delta\langle a_P \rangle$ between the two couples of nitroxides. In accordance, MD simulation of H_β–C_α–N–O dihedral angle predicts smaller standard-deviation values for (**10b**, **11b**) vs **5a** and **5c** (Table 2) in agreement with larger experimental $\Delta a_{H\beta}$ values in these latter nitroxides (Table 4).

Bioreduction studies

In vivo, nitroxides are converted to diamagnetic hydroxylamines by antioxidants such as ascorbate and glutathione, or by enzymatic systems located, e.g., in liver microsomes. To assess the metabolic stability of **10b** near its pK_a it was incubated at 37 °C in the presence of CytM_l in phosphate-citrate buffer adjusted to

pH 3.5 and compared to non ionizable β -phosphorylated nitroxide 2-diethoxyphosphoryl-2,5,5-trimethylpyrrolidine 1-oxyl (TMPPO) and the widely studied 3-carboxy-2,2,5,5-tetramethylpyrrolidine 1-oxyl (3-CP) which demonstrates an acidic $pK_a = 4.0$ but a weak EPR pH reporting property ($\Delta a_N = 0.19$ G).^[27] At pH 3.5 the rates of reduction and half-lives, determined under pseudo first-order conditions from the relative decay curves of **10b**, 3-CP and TMPPO (Figure 5), were $k_{red} = 0.27 \pm 0.03$, 0.29 ± 0.02 and 0.07 ± 0.02 min⁻¹ and $t_{1/2} = 3.1$, 2.5 and 14.5 min, respectively, indicating that both carboxylic nitroxides are reduced at a significantly faster rate. When incubations were carried out in KH adjusted to pH 7.0, **10b** became the more resistant compound, with kinetic parameters ranking: $k_{red} = 0.10 \pm 0.01$, 0.16 ± 0.02 and 0.29 ± 0.04 min⁻¹ and $t_{1/2} = 9.5$, 4.3 and 2.4 min for **10b**, 3-CP and TMPPO, respectively. Such difference in stability with pH is not unexpected since the reduction of the carboxylate form, which is the dominant species at neutral pH for **10b** and 3-CP, is clearly unlikely.^[28] Abundant literature addressing structure-activity relationships for the stability toward reductants of cyclic nitroxides, including Im and In classes of EPR pH markers, has identified α, α' -tetraalkyl substitution of the ring as a key feature to sterically protect nitroxide function.^[11c,28,29] In this regard **10b** appears an exception to this rule and it may have some interest in probing acidic cell compartments.

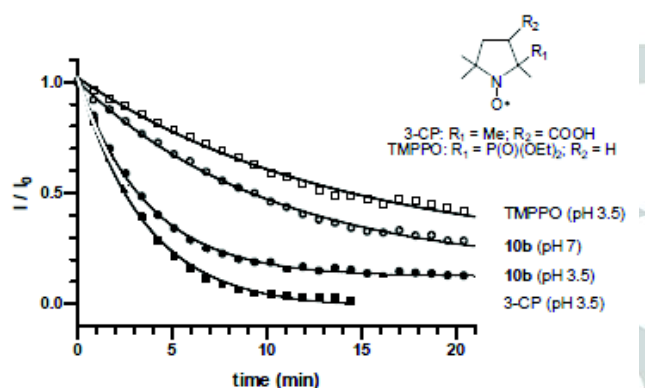


Figure 5. Reduction of nitroxides (0.1 mM) by cytosolic rat liver homogenate at 37 °C in phosphate-citrate buffer, pH 3.5 or Krebs-Henseleit medium, pH 7.0. I_0 is the initial intensity of the EPR signal recorded 1 min after mixing.

In vitro toxicity studies

Cytotoxicity assays were performed on **10b** and selected α -aminophosphonates esters to investigate their effects on cell viability and metabolic activity in vitro. IC_{50} values against human normal fibroblasts (NHLF) and lung cancer (A549) cell lines were determined by measuring the release of total intracellular lactate dehydrogenase (LDH) and by FMCA and MTT assays^[30] following a 48 h incubation at 37 °C with test compounds (0.1–50 mM). The results are summarized in Table 5 along with that of reference compounds and calculated lipophilicities. In general all novel compounds, TMPPO and DEPN demonstrated low cytotoxicity, with IC_{50} values in the millimolar range in the three assays, and in most cases A549 cells were found less sensitive than NHLF.

Nitroxides are being increasingly employed for their antioxidant properties, with the purpose of therapeutic use. Thus, we have reported on cardioprotective effects of low concentrations of TMPPO in ischemic rat hearts, with EPR signal of the nitroxide being still detected up to 30 min following reperfusion both in perfusate and tissue homogenate medium.^[31] In order to ensure that negligible nitroxide degradation into secondary species susceptible to interfere with the assay may have occurred in cell medium when incubation time was prolonged, A549 cells were exposed to 15 mM of **10b**, TMPPO or DEPN for 48 h at 37 °C and no significant loss of EPR signals was found (not shown).

Among the tested compounds the most hydrophilic diethyl(2-methylpyrrolidin-2-yl) phosphonate (DEPMPPH), a cyclic ³¹P NMR pH marker already applied ex vivo,^[10a] yielded the highest IC_{50} value. In contrast **3c** has increased cytotoxicity, a result that could account for its high lipophilicity and/or the presence of an aromatic substituent, as previously found in structural analogs such as those shown in Figure 1A.^[9b] Based on their toxicity data and in view of obtaining well-resolved signals with short acquisition times, the concentrations of 3 and 5 mM for **10b** and **3a**, respectively, were selected for the next ex vivo spectroscopic experiments.

In vivo pH measurement in the rat stomach

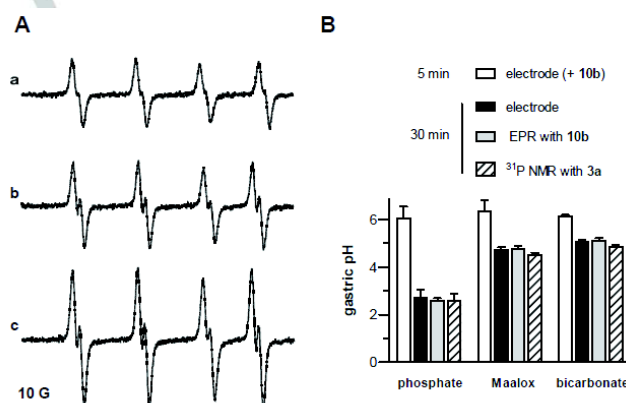


Figure 6. Low-field lines of EPR spectra recorded from the gastric fluid of a resting rat 30 min after receiving a solution of **10b** (3 mM) in (a) 5 mM phosphate buffer (pH 7.0), (b) Maalox:water (1:1, v/v; pH 8.7), or (c) 0.1 M sodium bicarbonate (pH 8.4) via a cannula inserted into the stomach. (B) Mean value \pm SEM ($n = 3$ –7/group) of gastric pH measured 30 min after drug administration using a pH electrode, or by EPR (from aE coupling) or ³¹P NMR using **10b** (3 mM) or **3a** (5 mM) as pH probes, respectively. Starting pH values at 5 min correspond to animals given **10b**.

According to the steepest part of their titration curves (Figures 4 and S2) and their $pK_a \approx 3.6$, **10b** and **3a** are appropriate to probe a ~ 2.1 – 5.1 pH window, which conforms to the admitted useful range of $pK_a \pm 1.5$ pH units for spectroscopic pH markers.^[6] When anesthetized rats had their stomach filled for 30 min with a solution of **10b** (3 mM) in either phosphate buffer (5 mM; starting pH 7.19), or in bicarbonate (0.1 M; starting pH 8.41) or Maalox taken as antacids (starting pH 8.72), pH-dependent EPR spectra were obtained in gastric fluid samples.

These signals, recorded at 22 °C five min after sampling, were strong enough to obtain good resolution (e.g., modulation amplitude < 0.1 G was applied) and a short sweep time (< 1.5 min), as can be seen in Figure 6A where only the four low-field doublets are shown to best visualize the changes in hfs. In normal gastric fluid, pH ~ 2.6, the EPR signal of **10b** exhibited a slower first-order decay ($k \approx 0.04 \text{ min}^{-1}$ and $t_{1/2} \approx 20 \text{ min}$) compared to the bioreduction process in CytM₁, with no change in hfs. In the rat stomach a better in vivo stability has been reported for two 1m-type EPR pH probes, which yet show a 2-times lower spectral sensitivity vs **10b**.^[13b]

Finally, a very good agreement was found between pH values determined spectroscopically and that obtained using a microelectrode (Figure 6B). We found differences ≤ 0.15 pH units among the three methods for a given condition when pH was obtained against the a_P EPR titration curve in CytM₁ but alternate use of a_N and a_H hfs resulted in identical precision. Comparing EPR to ³¹P NMR, it should be noted that while the amine **3a** was very stable in the stomach fluid (typical ³¹P NMR spectra are shown in Figure S8, Supporting Information), a longer acquisition time was necessary (at least 8 min) to achieve a similar accuracy in pH determination. A sampling time of 30 min was selected in all groups on the basis of reported almost full normalization of gastric pH level in rats given an EPR pH probe with $pK_a \sim 4.9$.^[13b] In this time frame, the pH of the stock solutions of **10b** and **3a** stored at 37 °C remained neutral or slightly basic, stabilizing at less than +0.15 pH units vs the corresponding starting value. Therefore Figure 6B demonstrates in particular that **10b** (i) did not chemically interact with the early (5 min) relaxation of stomach pH, and (ii) faithfully probed the expected slower pH decrease in the high-buffering capacity antacids vs phosphate buffer, in line with other EPR studies.^[13] Thus, 30 min after administration of **10b** in 5 mM phosphate buffer, a physiologically relevant value for gastric pH of 2.58 ± 0.11 ($n = 7$) could be determined.

Table 5. Cytotoxicity of α -phosphorylated nitroxides and amines pH markers against A549 cells and Normal Human Lung Fibroblasts (NHLF)^[a], and predicted lipophilicities.

Compd	IC ₅₀ (mM) ^[b]						AlogP ^[c]
	FMCA		MTT		LDH		
	A549	NHLF	A549	NHLF	A549	NHLF	
DEPN	4.4 ± 0.2	nd ^[d]	2.5 ± 0.1	nd	4.2 ± 0.3	nd	2.01
TMPPPO	12.1 ± 2.1	19.8 ± 2.4	10.5 ± 2.4	17.5 ± 0.9	> 45	40.8 ± 2.4	1.12
10b	15.8 ± 1.4	13.8 ± 0.4	13.2 ± 1.6	10.3 ± 0.9	13.5 ± 2.0	9.2 ± 1.3	1.71
3a	18.4 ± 1.6	10.8 ± 1.2	22.7 ± 1.2	13.8 ± 1.2	25.3 ± 1.9	12.5 ± 1.0	1.68
3b	12.7 ± 0.4	2.2 ± 0.5	6.7 ± 1.2	3.5 ± 0.3	12.4 ± 1.5	8.1 ± 0.2	2.27
3c	1.7 ± 0.4	1.8 ± 0.1	1.8 ± 0.2	1.0 ± 0.2	2.1 ± 0.2	1.1 ± 0.1	2.71
9a	3.9 ± 0.5	1.4 ± 0.3	4.9 ± 0.3	1.5 ± 0.1	5.1 ± 0.4	4.1 ± 0.1	2.21
9b	7.6 ± 0.1	3.4 ± 1.3	14.3 ± 0.2	4.2 ± 1.4	19.8 ± 0.2	3.8 ± 0.9	1.88
DEPMPH	70.2 ± 2.1	62.5 ± 0.8	72.5 ± 0.9	60.9 ± 1.1	126.3 ± 1.5	102.2 ± 1.2	1.04

[a] Cells seeded at 2.5×10^4 cells/well in DMEM (A549) or FBM (NHLF) until confluence were treated with test compounds at 0.01–100 mM for 48 h. Data are means ± SD of 3–6 independent experiments made in triplicate for at least four concentrations. [b] IC₅₀ defined as the concentration of compound resulting in 50% cell viability (FMCA and MTT; calculated from concentration-response curves) or 50% decrease of intracellular LDH content with respect to total LDH. [c] Obtained using the ALOGPS 2.1 software (www.vcclab.org/lab/alogps/). [d] not determined.

Conclusions

In our continuous efforts to discover a novel generation of spectroscopic pH markers, we have designed a series of α -hydrogen substituted, β -phosphorylated linear nitroxides based on an amino acid core structure. Notably, crystallized sodium carboxylate **10b** displayed triple sensing (using a_N , a_P or a_H couplings), pH dependent EPR spectra and its $pK_a = 3.6$ and good resistance to bioreduction render it particularly suited to probe acidic sub-cellular compartments or study normal and pathological conditions linked to low pH. Based on known rules for EPR conformation-dependent hfs of β -substituted nitroxides, introducing a non-titrating phosphorus moiety in **10b** improved its spectral sensitivity by ~2-fold, as compared to the more rigid cyclic probes described before. Interestingly, even better sensitivities were obtained for **5a** and **5c** that were not obtained as crystals under our experimental conditions.

On the EPR side of this study, further follow-up will include a MD-assisted structure-based rational nitroxide design, with the general structure shown in Figure 1C as a starting point. In the course of the syntheses, a series of novel α -aminophosphonates esters were produced and their ³¹P NMR constants will increment our existing library of about 50 analog pH probes with the purpose of optimizing structure-activity relationships.

Experimental Section

ROESY spectra of diastereomers of compound **16**, ³¹P NMR acid-base titration curves of compounds **3a**, **9a** and **9b**, additional EPR pH titration curves of compounds **5a** and **5c**, additional molecular dynamics data, protocols for FMCA and MTT assays and ³¹P NMR spectra from the gastric fluid of a rat given **3a** are provided in the Supporting Information.

Synthesis

Reagents and instruments

All solvents and chemicals were reagent grade from commercial suppliers and were used as such. Doubly distilled deionized water was used throughout and test solutions were filtered through a 0.2- μ m Millipore filter prior to use. Ultrapure DEPMPH^[32] and diethyl(2-propylaminoprop-2-yl)phosphonate (DPP)^[9a] were obtained as previously described. 3-CP was from Sigma-Aldrich Chimie (St. Quentin Fallavier, France) and TMPPPO was synthesized and purified as described.^[29a] DEPNI^[16a] was a kind gift of Dr. D. Gimes. Microwave reactions were conducted in an Anton Paar Monowave 300 reactor. Reactions were followed by TLC on Merck-Kieselgel 60 F254 precoated silica gel plates and the spots were visualized under UV light, or by staining with phosphomolybdic acid. Melting points were obtained using a Büchi Melting Point B-540 apparatus and are uncorrected. Analytical NMR spectra were recorded using a Bruker Avance III Nanobay spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) relative to internal Me₄Si (¹H and ¹³C) or 85% external H₃PO₄ (³¹P) and coupling constants (*J*) are given in hertz (Hz). Splitting patterns are reported as follows: s = singlet; d = doublet; dd = doublet of doublets; t = triplet; quint = quintuplet; hept = heptuplet; m = multiplet; br = broad peak. High-resolution mass spectrometry (HRMS) in electron spray ionization (ESI; Q-STAR Elite instrument, Applied Biosystems, USA) and combustion analyses (Thermo Finnigan EA 1112 series Flash elemental analyzer) were undertaken at the Spectropole (Analytical Laboratory) at Campus St. Jérôme (Marseille, France). Optical rotations were determined on an Anton Paar MCP200 polarimeter with a 0.2 cm length. The purity of all synthesized compounds was > 96% as ascertained by combustion analysis.

Synthesis of methyl esters of amino acid based phosphonates 3a–c by the Kabachnik–Fields reaction: Dry ammonia was bubbled for 4 h into a suspension of the (S)-amino acid methyl ester hydrochloride **1a–c** in dry Et₂O (60 mL). After filtration, Et₂O was removed under reduced pressure and the resulting free amino acid methyl ester **2a–c** was processed without further purification as follows: a stirred mixture of diethylphosphite, **2a–c** and Na₂SO₄ (5.0 g, 35.2 mmol) was refluxed in acetone (30 mL) for 18 h. The mixture was filtered, concentrated in vacuum and the residue was purified to give the desired compound **3a–c** as detailed below.

(S)-Methyl 2-[[2-(diethoxyphosphoryl)propan-2-yl]-amino]propanoate (3a): Free alanine methyl ester **2a** was obtained as a colorless oil (4.6 g, 89%) from hydrochloride **1a** (7.0 g, 50.1 mmol). Compound **3a** was then obtained from **2a** (4.6 g, 44 mmol) and diethylphosphite (6.1 g, 44 mmol) according to the above procedure. Distillation of the residue under reduced pressure afforded **3a** as a light yellow oil (7.4 g, 59%): b.p. 80 °C (3.7 \times 10⁻² mmHg); ¹H NMR (300 MHz, CDCl₃) δ =4.15–4.12 (m, 4H, 2 \times CH₂), 3.78 (quint, 1H, *J* = 9.0 Hz, CHCO₂Me), 3.65 (s, 3H, CO₂CH₃), 1.89 (br s, 1H, NH), 1.29–1.18 (m, 15H, 5 \times CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ =177.5 (C=O), 62.5 (d, *J*_{CP} = 7.5 Hz, CH₂), 62.0 (d, *J*_{CP} = 7.5 Hz, CH₂), 53.1 (d, *J*_{CP} = 153.2 Hz, C), 51.8 (CO₂CH₃), 51.0 (d, *J*_{CP} = 4.5 Hz, CH), 23.9 (d, *J*_{CP} = 3.8 Hz, CCH₃), 22.6 (d, *J* = 3.8 Hz, CCH₃), 21.7 (CHCH₃), 16.5 (d, *J*_{CP} = 3.0 Hz, CH₂CH₃), 16.4 (d, *J*_{CP} = 3.0 Hz, CH₂CH₃); ³¹P NMR (121.5 MHz, CDCl₃) δ =28.9. Elemental analysis calcd (%) for C₁₁H₂₄NO₅P: C 46.97, H 8.60, N 4.98; found: C 46.63, H 8.60, N 4.77.

(S)-Methyl 2-[[2-(diethoxyphosphoryl)propan-2-yl]-amino]-3-methylbutanoate (3b): Free valine methyl ester **2b** was obtained as a colorless oil (4.6 g, 98%) from hydrochloride **1b** (8.9 g, 35.0 mmol). Compound **3b** was then obtained from **2b** (4.6 g, 37.3 mmol) and

diethylphosphite (5.2 g, 37.3 mmol) according to the above procedure. The residue was purified by silica gel column chromatography eluting with pentane/acetone/methanol (80/18/2) to afford **3b** as a yellow oil (5.9 g; 54%). ¹H (300 MHz, CDCl₃) δ =4.20–4.00 (m, 4H, 2 \times CH₂), 3.70 (s, 3H, CO₂CH₃), 3.37 (d, 1H, *J* = 6.0 Hz, CHCO₂Me), 1.94 (s, 1H, NH), 1.81 (m, 1H, CH(CH₃)₂), 1.33 (t, 3H, *J* = 7.1 Hz, OCH₂CH₃), 1.32 (t, 3H, *J* = 7.5 Hz, OCH₂CH₃), 1.25 (d, 3H, *J*_{HP} = 11.0 Hz, CCH₃), 1.23 (d, 3H, *J*_{HP} = 13.1 Hz, CCH₃), 0.91 (d, 3H, *J* = 6.8 Hz, CH(CH₃)₂), 0.89 (d, 3H, *J* = 6.8 Hz, CH(CH₃)₂); ¹³C NMR (75.5 MHz, CDCl₃) δ =176.6 (C=O), 62.3 (CH₂), 60.5 (NHCH), 53.3 (d, *J*_{HP} = 158.2 Hz, C), 51.6 (CO₂CH₃), 32.9 (CH(CH₃)₂), 23.4 (2 \times CCH₃), 19.4 (CH(CH₃)₂), 18.4 (CH(CH₃)₂), 16.7 (2 \times CH₂CH₃); ³¹P NMR (121.4 MHz, CDCl₃) δ =30.4. Elemental analysis calcd (%) for C₁₃H₂₈NO₅P: C 50.48, H 9.12, N 4.53; found: C 49.98, H 9.39, N 4.49.

(S)-Methyl 2-[[2-(diethoxyphosphoryl)propan-2-yl]-amino]-3-methylbutanoate (3c): Free phenylglycine methyl ester **2c** was obtained as a colorless oil (3.0 g, 92%) from hydrochloride **1c** (4.0 g, 19.8 mmol). Compound **3c** was then obtained from **2c** (3.0 g, 18.2 mmol) and diethylphosphite (5.0 g, 20.2 mmol) according to the above procedure. The residue was purified by silica gel column chromatography eluting with pentane/acetone (70/50) to afford **3c** as a light yellow oil (3.77 g; 60%). ¹H NMR (300 MHz, CDCl₃) δ =7.43–7.41 (m, 2H, *H*_{meta}), 7.32–7.24 (m, 3H, *H*_{ortho} and *H*_{para}), 4.99 (s, 1H, CH), 4.14–4.02 (m, 4H, 2 \times CH₂), 3.66 (s, 3H, CO₂CH₃), 2.51 (br s, 1H, NH), 1.34–1.18 (m, 12H, C(CH₃)₂ and 2 \times –OCH₂CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ =174.6 (C=O), 140.7 (Ar, Ph), 128.4 (Ar, Ph), 127.5 (Ar, Ph), 127.4 (Ar, Ph), 62.3 (d, *J*_{CP} = 7.5 Hz, CH₂), 62.1 (d, *J*_{CP} = 7.5 Hz, CH₂), 59.6 (d, *J*_{CP} = 3.8 Hz, CH), 54.4 (d, *J*_{CP} = 149.4 Hz, C), 52.3 (CO₂CH₃), 23.9 (d, *J*_{CP} = 3.0 Hz, CCH₃), 23.4 (d, *J*_{CP} = 3.8 Hz, CCH₃), 16.5 (d, *J*_{CP} = 2.3 Hz, CH₂CH₃), 16.4 (d, *J*_{CP} = 2.3 Hz, CH₂CH₃); ³¹P NMR (121.4 MHz, CDCl₃) δ =30.1. Elemental analysis calcd (%) for C₁₆H₂₆NO₅P: C 55.97, H 7.63, N 4.08; found: C 55.69, H 7.77, N 4.01.

Synthesis of amino acid based phosphonates 4a and 4c: The appropriate compound **3a** or **3c** (1.07 mmol) was stirred in aqueous NaOH (5 N, 3 mL) at RT until complete solubilization. The mixture was carefully acidified with H₂SO₄ to pH 1 and the aqueous phase was extracted with Et₂O (3 \times 5 mL). Combined organic layers were dried over MgSO₄, filtered and concentrated in vacuum to give the corresponding **4a** and **4c** which were used without further purification.

2-[[2-(Diethoxyphosphoryl)propan-2-yl]-amino]propanoic acid (4a): Saponification of **3a** (0.3 g, 1.07 mmol) afforded **4a** as a yellow oil (0.16 g, 56%). ¹H (300 MHz, CDCl₃) δ =5.36 (br s, 1H, NH), 4.17–4.06 (m, 4H, 2 \times CH₂), 3.71 (quint, 1H, *J* = 9 Hz, CHCO₂H), 1.34–1.24 (m, 15H, 5 \times CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ =176.7 (C=O), 62.9 (d, *J*_{CP} = 7.5 Hz, CH₂), 62.7 (d, *J*_{CP} = 7.5 Hz, CH₂), 53.2 (d, *J*_{CP} = 153.0 Hz, C), 51.4 (d, *J*_{CP} = 3.8 Hz, CH), 23.2 (d, *J*_{CP} = 1.5 Hz, CCH₃), 22.0 (d, *J*_{CP} = 2.3 Hz, CCH₃), 20.3 (CHCH₃), 16.4 (d, *J*_{CP} = 1.5 Hz, CH₂CH₃), 16.3 (d, *J*_{CP} = 1.5 Hz, CH₂CH₃); ³¹P NMR (121.4 MHz, CDCl₃) δ =29.72. Elemental analysis calcd (%) for C₁₁H₂₄NO₅P: C 46.97, H 8.60, N 4.98; found: C 46.75, H 8.70, N 4.99.

2-[[2-(Diethoxyphosphoryl)propan-2-yl]-amino]-2-phenyl-ethanoic acid (4c): Saponification of **3c** (0.2 g, 0.58 mmol) afforded **4c** as a yellow oil (0.12 g, 62%). ¹H (300 MHz, CDCl₃) δ =7.43–7.41 (m, 2H), 7.34–7.24 (m, 3H), 5.67 (br s, 2H, NH and CO₂H), 4.90 (s, 1H, CH), 4.17–4.04 (m, 4H, 2 \times CH₂), 1.36–1.18 (m, 12H, 4 \times CH₃); ¹³C NMR (75.47 MHz, CDCl₃) δ =174.4 (C=O), 139.9 (Ar, Ph), 128.6 (Ar, Ph), 127.9 (Ar, Ph), 127.4 (Ar, Ph), 62.8 (d, *J*_{CP} = 3.0 Hz, CH₂), 62.7 (d, *J*_{CP} = 2.3 Hz, CH₂), 59.9 (d, *J*_{CP} = 3.8 Hz, CH), 53.4 (d, *J*_{CP} = 150.9 Hz, C), 23.2 (d, *J*_{CP} = 3.8 Hz, CCH₃), 23.1 (d, *J*_{CP} = 3.0 Hz, CCH₃), 16.4 (d, *J*_{CP} = 1.5 Hz, CH₂CH₃), 16.3 (d, *J*_{CP} = 1.5 Hz, CH₂CH₃); ³¹P NMR (121.4

MHz, CDCl₃) δ =29.7. Elemental analysis calcd (%) for C₁₆H₂₆NO₅P: C 55.97, H 7.63, N 4.08; found: C 55.53, H 7.80, N 4.09.

In situ formation of carboxylic nitroxides 5a and 5c: Solutions of the appropriate amine **4a** or **4c** (100 mM) and a solution of *m*CPBA (10 mM) were first prepared in phosphate buffer (i.e., 20 mM KH₂PO₄, pH 7.4). An aliquot of NaOH (1 N) was added to the *m*CPBA solution (final concentration, 15%) to achieve complete solubilization of the peracid. Two hundred microliters of each solution of **4a** or **4c** were then diluted to 10 mM by adding phosphate buffer or CytM_n^[10a,31] (see preparation below), and an aliquot (30 μ L) of *m*CPBA solution was added to this mixture (final peracid concentration, 0.15 mM) to reach a final volume of 2 mL. The resulting working solutions of nitroxides (**5a** and **5c**) can be used in EPR titration experiments within 3–4 h following addition of the oxidant.

Synthesis of aminophosphonates esters 9a and 9b

Ethyl 2-amino-2-methylpropanoate hydrochloride (7): To a solution of 2-aminoisobutyric acid **6** (1.2 g, 11.72 mmol) in ethanol (50 mL) was added acetyl chloride (2.7 mL, 37.4 mmol, 3.2 equiv) at 0 °C under argon atmosphere. The resulting mixture was stirred at 85 °C for 24 h, cooled to RT and concentrated under reduced pressure to give **7** as a white powder (1.93 g, 11.5 mmol, quantitative yield); m.p. 155.7–155.9 °C (lit.^[33] m.p. 156–157 °C). ¹H NMR (300 MHz, D₂O) δ =4.18 (q, 2H, *J* = 7.2 Hz, CH₂), 1.49 (s, 6H, C(CH₃)₂), 1.19 (t, 3H, *J* = 7.2 Hz, CH₂CH₃); ¹³C NMR (75.5 MHz, CDCl₃) δ =172.5 (C=O), 63.7 (CO₂CH₂), 56.8 (C(CH₃)₂), 22.9 (C(CH₃)₂), 13.1 (CO₂CH₂CH₃).

Methyl 2-[[1-(diethoxyphosphoryl)-2,2-dimethylpropan-1-yl]-amino]-2-methylpropanoate (9a): To a solution of hydrochloride **7** (4.86 g, 29 mmol) in dichloromethane (30 mL) under argon atmosphere was added dropwise triethylamine (4.2 mL, 30.5 mmol, 1.05 equiv) and the mixture was stirred at RT for 1 h. To this mixture was added MgSO₄ (3 spatula tips) and pyvaldehyde (3.8 mL, 34.8 mmol, 1.2 equiv) and the reaction mixture was stirred at 50 °C for 15 h. The mixture was cooled at RT, filtered and the filtrate was concentrated in vacuum. The residue was dissolved in dichloromethane (30 mL), the resulting solution was washed with water (2 \times 30 mL), dried over MgSO₄ and filtered. Evaporation of the solvents in vacuum afforded the imine **8a** (5.3 g, 26.6 mmol, 92%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ =7.38 (s, 1H, NCH), 4.08 (quint, 2H, *J* = 7.2 Hz, CH₂), 1.32 (s, 6H, C(CH₃)₂), 1.17 (t, 3H, *J* = 7.2 Hz, CH₂CH₃), 0.97 (s, 9H, C(CH₃)₃); ¹³C NMR (75.5 MHz, CDCl₃) δ =174.8 (C=O), 169.4 (C=N), 64.5 (C(CH₃)₂), 60.6 (CO₂CH₂), 36.2 (C(CH₃)₃), 26.7 (C(CH₃)₃), 26.1 (C(CH₃)₂), 14.1 (CO₂CH₂CH₃). HRMS-ESI: calcd for C₁₁H₂₂NO₅P⁺ [M+H]⁺ 200.1645; found 200.1642.

A mixture of imine **8a** (200 mg, 1 mmol) and diethylphosphite (200 μ L, 1.3 mmol, 1.3 equiv) was stirred inside a microwave reactor at 150 °C for 45 min. After cooling to RT, the solution was diluted with dichloromethane (20 mL), washed with water (2 \times 20 mL), dried over MgSO₄, filtered and evaporated in vacuum. The crude residue was purified by silica gel column chromatography eluting with pentane/acetone (5/1) to give **9a** as a colorless oil (250 mg; 74%). ¹H NMR (300 MHz, CDCl₃) δ =4.12–3.93 (m, 6H, 3 \times CH₂CH₃), 2.73 (d, 1H, *J* = 17.4 Hz, CH), 1.28–1.18 (m, 15H, 2 \times POCH₂CH₃, CO₂CH₂CH₃, 2 \times CH₃), 0.97 (s, 9H, C(CH₃)₃); ¹³C NMR (75.5 MHz, CDCl₃) δ =176.2 (C=O), 61.3 (d, *J*_{CP} = 7.2 Hz, POCH₂), 61.2 (d, *J*_{CP} = 7.2 Hz, POCH₂), 60.2 (CO₂CH₂), 58.5 (d, *J*_{CP} = 137.0 Hz, CH), 57.5 (C(CH₃)₂), 35.0 (d, *J*_{CP} = 9.4 Hz, C(CH₃)₃), 27.7 (d, *J*_{CP} = 6.1 Hz; C(CH₃)₃), 23.7 (C(CH₃)₂), 16.4 (d, *J*_{CP} = 6.6 Hz, POCH₂CH₃), 14.0 (CO₂CH₂CH₃); ³¹P NMR (121.4 MHz, CDCl₃) δ =28.01. Elemental analysis calcd (%) for C₁₅H₃₂NO₅P: C 53.40, H 9.56, N 4.15; found: C 53.00, H 9.90, N 4.15.

Methyl 2-[[1-(diethoxyphosphoryl)-2-methylpropan-1-yl]-amino]-2-methylpropanoate (9b): To a solution of hydrochloride **7** (2 g, 12 mmol) in dichloromethane (12 mL) under argon atmosphere was added dropwise triethylamine (1.8 mL, 13.2 mmol, 1.05 equiv) and the mixture was stirred at RT for 1 h. To this mixture was added MgSO₄ (3 spatula tips) and isobutyraldehyde (2.4 mL, 14.4 mmol, 1.2 equiv) and the reaction mixture was stirred at 50 °C for 6 h. The mixture was cooled to RT, filtered and the filtrate was concentrated in vacuum. The residue was dissolved in dichloromethane (30 mL), the resulting solution was washed with water (2 \times 30 mL), dried over MgSO₄ and filtered. Evaporation of the solvents in vacuum afforded the imine **8b** (2.1 g, 11.4 mmol, 95%) as a colorless oil. ¹H NMR (300 MHz, CDCl₃) δ =7.40 (d, *J* = 6.0 Hz, 1H, NCH), 4.12 (quint, 2H, *J* = 6.0 Hz, CH₂), 2.50–2.40 (m, 1H, CH(CH₃)₂), 1.37 (s, 6H, C(CH₃)₂), 1.21 (t, 3H, *J* = 6.0 Hz, CH₂CH₃), 1.02 (m, 6H, CH(CH₃)₂); ¹³C NMR (75.5 MHz, CDCl₃) δ =176.6 (C=O), 167.9 (C=N), 64.5 (C(CH₃)₂), 60.7 (CO₂CH₂), 34.6 (CH), 26.0 (C(CH₃)₂), 19.2 (CH(CH₃)₂), 14.1 (CO₂CH₂CH₃). HRMS-ESI: calcd for C₁₀H₂₀NO₂⁺ [M+H]⁺ 186.1489; found 186.1488.

A mixture of imine **8b** (400 mg, 2.16 mmol) and diethylphosphite (362 μ L, 2.81 mmol) was stirred inside a microwave reactor at 150 °C for 30 min. After cooling to RT, water (100 mL) was added and the mixture was treated with concentrated aqueous HCl until pH 2 and extracted with pentane (5 \times 50 mL). The combined organic layers were dried over MgSO₄, filtered and evaporated in vacuum. The crude residue was purified by silica gel column chromatography eluting with pentane/acetone (5/1) to give **9b** as light yellow oil (650 mg; 68%). ¹H NMR (300 MHz, CDCl₃) δ =4.15–4.08 (m, 6H, 3 \times CH₂CH₃), 3.02 (dd, 1H, *J* = 15.0 and 2.8 Hz, NHCH), 2.02–1.93 (m, 1H, CH(CH₃)₂), 1.32 (t, 3H, *J* = 8.0 Hz, POCH₂CH₃), 1.30 (t, 3H, *J* = 8.0 Hz, POCH₂CH₃), 1.29 (s, 6H, NHC(CH₃)₂), 1.27 (t, 3H, *J* = 8.0 Hz, CO₂CH₂CH₃), 1.00 (d, 6H, *J* = 8.0 Hz, CH(CH₃)₂); ¹³C NMR (75.5 MHz, CDCl₃) δ =176.8 (C=O), 62.2 (d, *J*_{CP} = 7.5 Hz, POCH₂), 61.4 (d, *J*_{CP} = 7.5 Hz, POCH₂), 60.6 (CO₂CH₂), 57.4 (d, *J*_{CP} = 8.3 Hz, NHC(CH₃)₂), 55.2 (d, *J*_{CP} = 148.6 Hz, CH), 30.1 (d, *J*_{CP} = 6.8 Hz, CH(CH₃)₂), 26.5 (NHCCCH₃), 25.5 (NHCCCH₃), 19.2 (d, *J*_{CP} = 10.6 Hz, CHCH₃), 18.7 (d, *J*_{CP} = 10.6 Hz, CHCH₃), 16.5 (d, *J*_{CP} = 6 Hz, POCH₂CH₃), 16.4 (d, *J*_{CP} = 6 Hz, POCH₂CH₃), 14.0 (CO₂CH₂CH₃); ³¹P NMR (121.4 MHz, CDCl₃) δ =27.6. Elemental analysis calcd (%) for C₁₄H₃₀NO₅P: C 52.00, H 9.35, N 4.33; found: C 52.36, H 9.71, N 4.19.

Synthesis of aminophosphonate ester 16

(S)-Methylvaline ethyl ester hydrochloride (14): This compound was prepared from (S)-methylvaline **13** (0.5 g, 3.82 mmol) and acetyl chloride (870 μ L, 12.2 mmol, 3.2 equiv) in ethanol (5 mL) according to the procedure used for preparing **7**, with a reaction time of 72 h. Compound **14** was obtained as a white powder (745 mg, 3.81 mmol, quantitative yield); m.p. 121.0–121.5 °C; [α]_D²⁰ = –13.5° (c=0.01 in water). ¹H NMR (300 MHz, D₂O) δ =4.21 (q, *J* = 7.0 Hz, 2H, CH₂), 2.14 (m, 1H, CH(CH₃)₂), 1.45 (s, 3H, CH₃), 1.21 (t, 3H, *J* = 7.0 Hz, CH₂CH₃), 0.91 (br s, 6H, CH(CH₃)₂); ¹³C NMR (75.5 MHz, CDCl₃) δ =172.0 (C=O), 66.0 (C_α), 63.7 (CO₂CH₂), 34.0 (CH(CH₃)₂), 18.9 (C_β(CH₃)), 16.2 (C(CH₃)₂), 15.4 (C(CH₃)₂), 13.1 (CO₂CH₂CH₃).

Methyl 2-[[1-(diethoxyphosphoryl)-2,2-dimethylpropan-1-yl]-amino]-2,3-dimethylbutanoate (16): To a solution of hydrochloride **14** (745 mg, 3.82 mmol) in dichloromethane (5 mL) under argon atmosphere was added dropwise triethylamine (558 μ L, 4.01 mmol, 1.05 equiv) and the mixture was stirred at RT for 1 h. To this mixture was added MgSO₄ (3 spatula tips) and pyvaldehyde (500 μ L, 4.6 mmol, 1.2 equiv) and the reaction mixture was stirred at RT for 72 h. The mixture was filtered, the filtrate concentrated in vacuum, then diluted in dichloromethane (10 mL). The solution was washed with water (2 \times 10

mL), dried on MgSO_4 and filtered. Evaporation of the solvents in vacuum afforded the imine **15** (563 mg, 2.48 mmol, 65%) as a colorless oil. ^1H NMR (300 MHz, CDCl_3) δ =7.37 (s, 1H, NCH), 4.08 (quint, 2H, J = 7.3 Hz, CH_2), 2.16 (hept, 1H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$), 1.17 (t, 3H, J = 7.3 Hz, $\text{CO}_2\text{CH}_2\text{CH}_3$), 1.14 (s, 3H, $\text{C}(\text{CH}_3)_3$), 0.97 (s, 9H, $\text{C}(\text{CH}_3)_3$), 0.85 (d, 3H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$), 0.77 (d, 3H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$); ^{13}C NMR (75.5 MHz, CDCl_3) δ =174.5 (C=O), 169.5 (C=N), 70.4 (C_α), 60.2 (CO_2CH_2), 36.5 ($\text{CH}(\text{CH}_3)_2$), 35.5 ($\text{C}(\text{CH}_3)_3$), 26.6 ($\text{C}(\text{CH}_3)_3$), 18.6 ($\text{C}_\alpha(\text{CH}_3)$), 17.3 and 16.9 ($\text{CH}(\text{CH}_3)_2$), 14.1 ($\text{CO}_2\text{CH}_2\text{CH}_3$); HRMS-ESI: calcd for $\text{C}_{13}\text{H}_{26}\text{NO}_2^+$ $[\text{M}+\text{H}]^+$ 228.1958; found: 228.1953.

A mixture of imine **15** (0.5 g, 2.20 mmol) and diethylphosphite (400 μL , 2.86 mmol, 1.3 equiv) was stirred for 15 days at 55 °C in a sealed vessel. After cooling down to rt, the mixture was diluted with dichloromethane (20 mL), washed with water (2 \times 20 mL), dried over MgSO_4 , filtered and evaporated in vacuum. The crude residue was purified by silica gel column chromatography eluting with pentane/acetone (5/1) to give compound **16** as a colorless oil (300 mg; 37%) containing a 1:1 ratio of diastereomers (determined by HPLC). Elemental analysis calcd (%) for $\text{C}_{17}\text{H}_{36}\text{NO}_5\text{P}$: C 55.87, H 9.93, N 3.83; found: C 55.97, H 9.99, N 3.75.

Separation of the two diastereomers of **16**

The two diastereomers were separated by chiral column chromatography under the following conditions: column, Chiralpak IC, 250 mm \times 10 mm; mobile phase, hexane/isopropanol/chloroform (8/1/1); flow rate, 5 mL \cdot min $^{-1}$; sample concentration, 300 mg/10 mL in hexane/isopropanol/chloroform (4/3/3); injected volume per run, 230 μL injected 45 times every 4.5 min; UV detection at 230 nm.

First eluted diastereomer (t_R = 4.87 min) (2*S*,1'*R*)-**16**: $[\alpha]_{\text{D}}^{25}$ = +28.8° (c =0.034 in chloroform); ^1H (300 MHz, CDCl_3) δ =4.24–4.02 (m, 6H, 3 \times CH_2CH_3), 2.73 (d, 1H, J = 16.3 Hz, CH), 1.82 (hept, 1H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$), 1.36–1.27 (m, 9H, 2 \times POCH_2CH_3 , $\text{CO}_2\text{CH}_2\text{CH}_3$), 1.11 (s, 3H, $\text{C}(\text{CH}_3)_3$), 1.09 (s, 9H, $\text{C}(\text{CH}_3)_3$), 0.98 (d, 3H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$), 0.83 (d, 3H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$); ^{13}C NMR (75.5 MHz, CDCl_3) δ =176.0 (C=O), 63.5 (CO_2CH_2), 61.5 (d, J_{CP} = 6.6 Hz, POCH_2), 60.6 (d, J_{CP} = 7.7 Hz, POCH_2), 60.0 ($\text{CH}(\text{CH}_3)_2$), 57.9 (d, J_{CP} = 129.9 Hz, CH), 38.8 (d, J_{CP} = 2.8 Hz, $\text{C}(\text{CH}_3)_3$), 35.7 (d, J_{CP} = 9.9 Hz; $\text{C}(\text{CH}_3)_3$), 28.2 ($\text{CH}(\text{CH}_3)_2$), 28.1 ($\text{CH}(\text{CH}_3)_2$), 23.5 ($\text{C}(\text{CH}_3)_3$), 20.2 ($\text{C}(\text{CH}_3)_3$), 17.6 (d, J_{CP} = 7.2 Hz, POCH_2CH_3), 16.9 (d, J_{CP} = 8.2 Hz, POCH_2CH_3), 14.0 ($\text{CO}_2\text{CH}_2\text{CH}_3$); ^{31}P NMR (121.4 MHz, CDCl_3) δ =28.08.

Second eluted diastereomer (t_R = 6.05 min) (2*S*,1'*S*)-**16**: $[\alpha]_{\text{D}}^{25}$ = -14.5° (c =0.014 in chloroform); ^1H (300 MHz, CDCl_3) δ =4.08–3.98 (m, 6H, 3 \times CH_2CH_3), 2.95 (d, 1H, J = 19.1 Hz, CH), 1.91 (hept, 1H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$), 1.26 (t, 3H, J = 6.9 Hz, POCH_2CH_3), 1.25 (t, 3H, J = 6.9 Hz, POCH_2CH_3), 1.21 (t, 3H, J = 7.2 Hz, $\text{CO}_2\text{CH}_2\text{CH}_3$), 1.11 (s, 3H, $\text{C}(\text{CH}_3)_3$), 0.95 (s, 9H, $\text{C}(\text{CH}_3)_3$), 0.91 (d, 3H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$), 0.76 (d, 3H, J = 6.8 Hz, $\text{CH}(\text{CH}_3)_2$); ^{13}C NMR (75.5 MHz, CDCl_3) δ =176.3 (C=O), 61.8 (d, J_{CP} = 6.2 Hz, POCH_2), 61.2 (d, J_{CP} = 7.7 Hz, POCH_2), 61.0 (CO_2CH_2), 60.5 ($\text{CH}(\text{CH}_3)_2$), 59.4 (d, J_{CP} = 132.1 Hz, CH), 37.4 (br s, $\text{C}(\text{CH}_3)_3$), 35.4 (d, J_{CP} = 10.5 Hz; $\text{C}(\text{CH}_3)_3$), 28.9 ($\text{CH}(\text{CH}_3)_2$), 28.8 ($\text{CH}(\text{CH}_3)_2$), 23.4 ($\text{C}(\text{CH}_3)_3$), 20.2 ($\text{C}(\text{CH}_3)_3$), 16.8 (d, J_{CP} = 6.2 Hz, POCH_2CH_3), 16.5 (d, J_{CP} = 5.5 Hz, POCH_2CH_3), 14.1 ($\text{CO}_2\text{CH}_2\text{CH}_3$); ^{31}P NMR (121.4 MHz, CDCl_3) δ =27.08.

General procedure for the synthesis of nitroxides β -esters **10a, **12** and **17**:** The corresponding aminoester (1 equiv) was dissolved in a volume of chloroform as to give a final concentration of 250 mM. This solution was cooled at 0 °C and a solution of *m*CPBA (2 equiv) in chloroform (same volume) was added dropwise in 2 h. The mixture was stirred for 2 h at 0 °C then the organic layer was washed with saturated

aqueous Na_2CO_3 (3 \times 4 mL) and brine (4 mL). The organic layer was dried over MgSO_4 , filtered and evaporated in vacuum to give the crude nitroxides β -esters.

N-(Ethyl-2-methylpropanoate)-(1-diethoxyphosphoryl)-2,2-

dimethylpropyl) nitroxide (10a): Title compound was prepared from solutions of **9a** (250 mg, 0.73 mmol) and *m*CPBA (360 mg, 1.5 mmol), both in chloroform (3 mL). Purification of the crude residue by silica gel column chromatography eluting with dichloromethane/acetone (5/1) afforded **10a** as an orange oil (197 mg, 80%). Elemental analysis calcd (%) for $\text{C}_{15}\text{H}_{32}\text{NO}_6\text{P}$: C 51.13, H 8.87, N 3.97; found: C 51.19, H 9.34, N 3.93.

N-(Ethyl-2-methylpropanoate)-(1-diethoxyphosphoryl)-2-

methylpropyl) nitroxide (12): Title compound was prepared from solutions of **9b** (30 mg, 0.09 mmol) and *m*CPBA (46 mg, 0.19 mmol), both in chloroform (1 mL). Compound **12** was obtained as pale orange oil (32 mg, quantitative yield). HRMS-ESI: calcd for $\text{C}_{14}\text{H}_{30}\text{NO}_6\text{P}$ $[\text{M}+\text{H}]^+$ 339.1805; found 339.1802. Elemental analysis calcd (%) for $\text{C}_{14}\text{H}_{29}\text{NO}_6\text{P}$: C 49.70, H 8.64, N 4.14; found: C 51.10, H 8.97, N 3.93.

N-(Ethyl-2,3-dimethylbutanoate)-(1-diethoxyphosphoryl)-2,2-

dimethylpropyl) nitroxide ((2*S*,1'*R*)-17**):** Title compound was prepared from solutions of (2*S*,1'*R*)-**16** (110 mg, 0.30 mmol) and *m*CPBA (149 mg, 0.60 mmol), both in chloroform (1.5 mL). Compound (2*S*,1'*R*)-**17** was obtained as a pale orange oil (67 mg, 60%). HRMS-ESI: calcd for $\text{C}_{17}\text{H}_{36}\text{NO}_6\text{P}$ $[\text{M}+\text{H}]^+$ 381.2275; found 381.2274. Elemental analysis calcd (%) for $\text{C}_{17}\text{H}_{35}\text{NO}_6\text{P}$: C 53.67, H 9.27, N 3.68; found: C 53.97, H 9.04, N 3.93.

N-(Ethyl-2,3-dimethylbutanoate)-(1-diethoxyphosphoryl)-2,2-

dimethylpropyl) nitroxide ((2*S*,1'*S*)-17**):** Title compound was prepared from solutions of (2*S*,1'*S*)-**16** (80 mg, 0.22 mmol) and *m*CPBA (108 mg, 0.44 mmol), both in chloroform (1 mL). Compound (2*S*,1'*S*)-**17** was obtained as a pale orange oil (54 mg, 65%). HRMS-ESI: calcd for $\text{C}_{17}\text{H}_{36}\text{NO}_6\text{P}$ $[\text{M}+\text{H}]^+$ 381.2275; found 381.2274. Elemental analysis calcd (%) for $\text{C}_{17}\text{H}_{35}\text{NO}_6\text{P}$: C 53.67, H 9.27, N 3.68; found: C 53.75, H 9.23, N 3.72.

N-(2-Methylpropanoate)-(1-diethoxyphosphoryl)-2,2-dimethylpropyl) nitroxide sodium salt (**10b**)

Nitroxide **10a** (197 mg, 0.6 mmol) was dissolved in a 5 M aqueous NaOH (1.2 mL, 10 equiv) and stirred vigorously for 15 h at RT. The mixture was extracted with dichloromethane (4 \times 5 mL), the organic layers were gathered, dried over MgSO_4 , filtered and concentrated in vacuum. The resulting crude residue was precipitated with pentane to give a precipitate which was filtered, affording **10b** as yellow crystals (81 mg, 42%); m.p. 111.6–112.0 °C. Elemental analysis calcd (%) for $\text{C}_{13}\text{H}_{26}\text{NO}_6\text{PNa}$: C 45.09, H 7.57, N 4.0; found: C 45.45, H 7.58, N 4.33.

In situ formation of carboxylic nitroxide **11b**

N-(2-Methylpropan)-(1-diethoxyphosphoryl)-2-methylpropyl)-oic

acid (11a): Ester **9b** (0.1 g, 310 μmol) was saponified according to the procedure used for preparing **10b**, affording carboxylic acid **11a** as a yellow oil (91 mg, quantitative yield). ^1H (300 MHz, CDCl_3) δ =6.43 (brs, 2H, CO_2H and NH), 4.18–4.08 (m, 4H, 2 \times CH_2CH_3), 3.04 (dd, 1H, J = 18.3 and 2.9 Hz, NHCH), 2.08 (m, 1H, $\text{CH}(\text{CH}_3)_2$), 1.37–1.29 (m, 12H, 2 \times POCH_2CH_3 and $\text{NHC}(\text{CH}_3)_2$), 1.03 (d, 3H, J = 6.0 Hz, $\text{CH}(\text{CH}_3)_2$), 1.01 (d, 3H, J = 6.0 Hz, $\text{CH}(\text{CH}_3)_2$); ^{13}C NMR (75.5 MHz, CDCl_3) δ =178.5 (C=O), 65.6 (CO_2CH_2), 62.2 (d, J_{CP} = 8.3 Hz, POCH_2), 62.1 (d, J_{CP} = 8.3 Hz, POCH_2), 58.4 (d, J_{CP} = 8.8 Hz, $\text{NHC}(\text{CH}_3)_2$), 55.1 (d, J_{CP}

= 151.3 Hz, CH), 29.8 (d, J_{CP} = 5.0 Hz, CH(CH₃)₂), 26.2 (NHCCH₃), 24.6 (NHCCH₃), 19.2 (d, J_{CP} = 11.0 Hz, CHCH₃), 18.7 (d, J_{CP} = 10.6 Hz, CHCH₃), 16.2 (d, J_{CP} = 1.6 Hz, POCH₂CH₃), 16.1 (d, J_{CP} = 1.6 Hz, POCH₂CH₃); ³¹P NMR (121.4 MHz, CDCl₃) δ = 27.1; HRMS-ESI: calcd for C₁₂H₂₇NO₅P⁺ [M+H]⁺ 296.1621; found 296.1623. Elemental analysis calcd (%) for C₁₂H₂₆NO₅P: C 48.81, H 8.87, N 4.74; found: C 49.13, H 8.98, N 4.45.

N-2-(2-Methylpropan)-(1-diethoxyphosphoryl-2-methylpropyl)-oic acid oxide (11b): Title compound was formed in situ from **11a** according to the procedure used for **5a** and **5c** and the resulting nitroxide working solution in 20 mM phosphate buffer can be used in EPR titration experiments within 3–4 h following addition of oxidant. Using a calibration curve established with 0.01–0.1 mM TMPPO, the initial concentration of the working solutions of nitroxides **5a**, **5c** and **11b** prepared in situ was found ranging 0.05–0.1 mM.

X-ray diffraction analysis: X-ray diffraction data were collected at 293 K on a Bruker-Nonius Kappa CCD diffractometer using graphite-monochromated Mo K α radiation (λ = 0.71073 Å).

Crystal data for **10b**: C₁₃H₂₆NNaO₆P M = 347.31 monoclinic space group C2/c, Hall group –C 2yc, a = 22.2170(5), b = 16.2403(5), c = 10.8482(3) Å, β = 91.788(2)°.

CCDC 793807 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from the Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data%5Frequest.cif.

³¹P NMR pH-calibration of α -aminophosphonate esters 3a–c, 9a, 9b and diastereomers of 16: Calibration experiments were carried out in the following media: phosphate buffer (1 mM KH₂PO₄), KH (containing: 118.5 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 25 mM NaHCO₃ and 1.2 mM KH₂PO₄) having a ionic strength of 0.154, or homogenates CytM_I or CytM_{II}.^[10a,31] Test compounds (5 mM) were dissolved in the selected medium and the pH was adjusted to 15–20 different values in the range 1.0–12.0 with 1 N solutions of HCl or NaOH. The pH was measured using a SevenCompact digital pH-meter equipped with an Inlab Micro Pro micro-electrode (Mettler Toledo, Switzerland).

The ³¹P NMR spectra of titration solutions were acquired (32 accumulated scans) at 22 °C on either Bruker AMX 400 (at 161.9 MHz) or Avance I 500 MHz (at 202.46 MHz) instruments equipped with 10- or 5-mm probes, respectively. The chemical shifts (referenced to external 85% H₃PO₄ at 0 ppm) were plotted against pH to fit the Henderson-Hasselbalch equation for NMR using a nonlinear regression:

$$\text{pH} = \text{p}K_a + \log \frac{\delta - \delta_a}{\delta_b - \delta} \quad (2)$$

where δ is the experimental ³¹P chemical shift and δ_a and δ_b correspond to the limiting chemical shift values of the protonated and unprotonated form, respectively. The T_1 values were determined at pH \approx pK_a using a standard inversion/recovery (180°– τ –90°)–TR pulse-sequence with a 180° composite pulse, with the repetition time TR being 6–7 times the T_1 value. The number of scans was 8 and 16 evolution decays (τ) were used. For both diastereomers of **16** T_1 was measured at pH 4.00. Data are means of n = 3–10 repeated titrations.

Molecular dynamics simulations: All calculations were performed using GROMACS 5.0.5 package.^[34] The best conformer of the

protonated and unprotonated forms of each titrating nitroxide was fully optimized at the PBE0/6-31+G(d,p) level using the ab initio program Gaussian 09 revision D.01 package.^[35] Each nitroxide was solvated in a quasi-cubic box containing about 800 water molecules. In the case of unprotonated species the net charge of the system was neutralized with one sodium ion. The force fields employed in the simulations were TIP3P^[36] (description of the water molecules) and AMBER (ff99SB)^[37] including additional parameters for nitroxide moieties.^[38] The atomic charges were computed at the HF/6-31G(d) level of theory with the RESP scheme.^[39] To optimize the simulation box size a NPT calculation was performed at 300 K and 1 bar during 200 ps with a time step of 0.5 fs. After this first stage, a NVT trajectory was performed at 300 K during 100 ns with a time step of 0.5 fs. The last 99.5 ns of the trajectory were kept in the simulation and the data analysis calculations were performed on 99500 structures with system coordinates saved every 1 ps.

In vitro EPR experiments

All EPR measurements were performed at 22 °C unless otherwise noted, using an X-band (9.79 GHz) Bruker ESP 300 spectrometer (Karlsruhe, Germany) with a TM₁₁₀ microwave cavity, using a modulation frequency of 100 KHz and a microwave power of 10 mW. The magnetic field strength and microwave frequency were measured with a Bruker ER 035M proton probe gaussmeter and a Hewlett-Packard 5350B frequency meter, respectively. The temperature inside the resonant cavity was controlled by a Bruker ER 4111VT variable temperature unit. Coupling constants and g -factors were determined by computer simulation of spectra having 4k resolution using the program of Rockenbauer and Korecz.^[40] In order to resolve long-range EPR couplings, samples were deoxygenated by argon bubbling before their transfer into 50 μ L glass capillary tubes sealed at both ends with Critoseal. To optimize signal resolution of each tested nitroxide, other instrument settings were adjusted within the following ranges: modulation amplitude, 0.02–0.48 G; time constant, 5.12–81.92 ms; receiver gain, 2.5×10^4 – 2×10^5 ; scan rate, 0.25–1.51 G.s^{–1}; sweep width, 83–110 G (see also Figure legends).

EPR pH-calibration: Working solutions of the nitroxides **5a**, **5c** and **11b** prepared in situ as described above or aqueous stock solutions of isolated nitroxides prepared by weighing and diluted in appropriate solvent to yield a final concentration of 0.05 mM (**10b**), 1 mM (**10a**, **12**) or 3 mM (diastereomers of **17**) were used in calibration experiments. The pH was serially adjusted to 10–15 different values in the range 0.5–12.0 with 1 N HCl or NaOH, and the EPR scan was initiated at RT 90 s following stabilization of the pH.

The plot of hfs as a function of pH as determined from spectral simulations of experimental EPR spectra yielded the pK_a value and the EPR sensitivity Δa_X for **5a**, **5c**, **10b** and **11b** using the Henderson-Hasselbalch equation for EPR

$$\text{pH} = \text{p}K_a + \log \frac{a_X - (a_X)_a}{(a_X)_b - a_X} \quad (3)$$

where a_X is the experimental coupling constant of the nucleus X, and $(a_X)_a$ and $(a_X)_b$ are the corresponding limiting coupling constants in the protonated and unprotonated form of the nitroxide, respectively. Data are means of n = 3 repeated titrations.

Bioreduction of 10b, TMPPO and 3-CP: Twenty microliters of an aqueous stock solution of tested compound were diluted with an aliquot (80 μ L) of CytM_I in either KH buffer, pH 7.0 or a phosphate-citrate buffer (0.25 M; pH 3.5) to reach a final nitroxide concentration of 0.1 mM (see

preparation below). The mixture was vortexed for 10 s, transferred into a glass capillary and the decay of EPR double integrated simulations was monitored over time at 37 °C. Spectral acquisition was started 1 min after mixing using the settings: modulation amplitude, 0.31–0.48 G; time constant, 20.48 ms; receiver gain, $1-4 \times 10^5$; scan rate, 2.4–4.8 G.s⁻¹; sweep width, 50 G (for 3-CP) or 100 G (for **10b** and TMPO). For each nitroxide data \pm SD were computed from at least $n = 5$ independent determinations in each medium.

Biology

General

Doubly distilled deionized water was used throughout. Dulbecco's modified Eagle's medium (DMEM), phosphate buffered saline (PBS), fetal calf serum (FCS), antibiotics and antimycotic agents were from Gibco Life Technologies (Thermo Fisher Scientific, Saint Aubin, France). Stock solutions were freshly diluted in the appropriate culture medium prior to use and filtered through a 0.2- μ m Millipore filter.

Cell Culture and Exposure: Cell culture was performed according to the conditions described earlier.^[30] Briefly human alveolar epithelial A549 cells (CCL-185; ATCC, LGC Standards, Molsheim, France) were grown in DMEM containing 10% FCS, 100 U.mL⁻¹ penicillin, 100 μ g.mL⁻¹ streptomycin and 2 mM GlutaMax (Gibco) at 37 °C in a 5% CO₂ humidified atmosphere. NHLF (Lonza, Amboise, France) were seeded in culture dishes and incubated in fibroblast basal medium (FBM; Lonza) supplemented by growth factors (FGM; Clonetics FGM-2 Bullet Kit; Lonza). The exponential growth phase of the cells was performed at 37 °C in 5% CO₂ humidified atmosphere and the culture medium was renewed every two days. Confluent cells (in 25 cm² flasks) were trypsinized, seeded onto 96-well plates (at a density of 2.5×10^4 cells/well) and incubated up to confluence in appropriate medium. The medium was renewed and cells were exposed to varying concentrations of test compounds for 48 h in wells containing the appropriate medium.

Evaluation of Cytotoxicity: Following exposure 10 μ L of medium was removed from each well to determine LDH activity released into the incubation medium according to the instructions of the Biolabo LDH kit (Maizy, France). The total cellular LDH content was obtained by treating control wells with Triton X-100 (1% final) to induce 100% loss of viability and total LDH release. After removal of the remaining incubation medium cells were washed two times with PBS 1X (+/-) and cytotoxicity was determined using the fluorometric microculture cytotoxicity assay (FMCA)^[30] and the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay (Supporting Information).

Animal procedures and ethics: Seventeen Sprague-Dawley male rats weighing ~450 g (CERJ, Le Genest St Isle, France) were used in the study. Rats were maintained in the local animal house under conventional conditions including an enrichment of the structural and social environment while promoting physical and cognitive activity, in a room with controlled temperature (22 ± 3 °C) and a reverse 12 h light/dark cycle with food (standard Teklad 2016 diet, Harlan Laboratories, Gannat, France) and water available ad libitum. All research involving animals were performed in strict compliance with the guidelines of the [Directive 2010/63/EU of the European Parliament](#). The CNRS and Aix Marseille Université have currently valid license for animal housing and experimentation (agreement C13-055-06) delivered by the French Government and the study was under the supervision of a DVM at CNRS (agreement N°13-122). The protocol was approved by the National Research Committee for the project.

Preparation of rat organ homogenates: Heart, liver or stomach extracts were prepared from freshly excised organs as previously described.^[10a] Briefly, 8 g of tissue were minced in 12 mL of 125 mM KCl at 2 °C, homogenized in a blender for 10 min and centrifuged at $10,000 \times g$ for 20 min at 2–3 °C. The clear supernatant solution taken as a cytosolic-like medium was used for ³¹P NMR titrations. For EPR bio-reduction assays rat liver homogenates were prepared by mixing in a blender 2 g of freshly excised tissue with 6 mL of either KH buffer (pH 7.0) or phosphate-citrate buffer (0.25 M, pH 3.5).

pH measurement in the rat stomach fluid: Rats fasted for 10 h were anesthetized for 1 h with 1.8% sevoflurane using previously described procedures^[41] and instrumented with a thin teflon cannula inserted into the stomach. This allowed administration of 3.5 mL aliquots of **3a** (5 mM) or **10b** (3 mM) dissolved in 5 mM phosphate buffer (pH 7.01; to keep physiological conditions), 100 mM bicarbonate buffer (pH 8.27), or a 1:1 dilution of Maalox in water (pH 8.67). Maalox, a solid formulation of 34.9 mg.mL⁻¹ of Al(OH)₃ and 39.9 mg.mL⁻¹ of Mg(OH)₂, was from Theraplix (France).

Five min after drug administration the gastric medium (0.5 mL) was drawn and the pH was measured using a pH microelectrode. After 30 min equilibration samples of the stomach fluid containing **3a** (~2 mL) or **10b** (~0.5 mL) were drawn, scanned by ³¹P NMR or EPR, respectively (see below), and the pH calculated from the corresponding titration curves. In parallel, the pH of each gastric fluid was measured with a pH electrode. ³¹P NMR spectra were acquired from 2 mL samples of stomach fluid on a Bruker AVL 400 (at 162 MHz) instrument equipped with a 10-mm probe and using sequences of at least 64 accumulated scans. EPR analysis was carried out 3 min after the end of sampling using the following settings: modulation amplitude, 0.088 G; time constant, 20.48 ms; receiver gain, 8×10^4 ; scan rate, 1.13 G.s⁻¹; sweep width, 95 G.

During anesthesia heart function was monitored by the tail-cuff method using a Letica Scientific LE 5000/5500 instrument (Barcelona, Spain) and a programmed Electro Sphygmomanometer (LE 5160-R; Panlab, Barcelona, Spain). At the end of experiments animals were kept alive and no mortality or cognitive troubles associated with anesthesia and compound toxicity were observed in the long term (up to two weeks). Data represent means \pm SEM with $n = 3-7$ (electrode) or $n = 7$ (spectroscopic measurements) / experimental condition.

Data calculations and statistics

Titration curves were obtained using Prism 5.0 software (GraphPad, San Diego, CA). Data are expressed as mean \pm SD or SEM for the indicated number of independent experiments. 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 $P < 0.05$.

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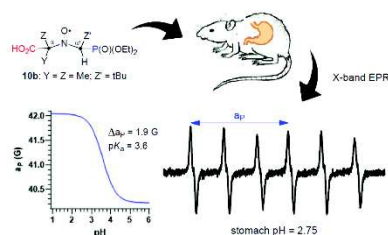
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McConnell's equation that helps EPR pH

probing. β -Phosphorylated nitroxides with a β' -amino acid core provide enhanced pH sensing in the acidic pH region. To illustrate, a stable, biocompatible, crystallized model carboxylate resisting to bioreduction was applied to assess rat stomach acidity.



Sophie Th  tiot-Laurent, Ga  lle Gosset, Jean-Louis Cl  ment, Mathieu Cassien, Anne Mercier, Didier Siri, Anouk Gaudel-Siri, Antal Rockenbauer, Marcel Culcasi,* and Sylvia Pietri

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Novel Amino-Acid Based β -Phosphorylated Nitroxides for Probing Acidic pH in Biological Systems by EPR Spectroscopy

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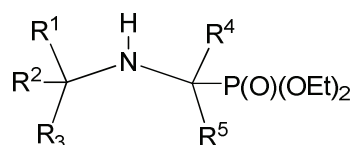
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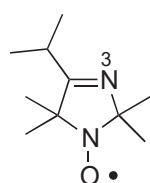
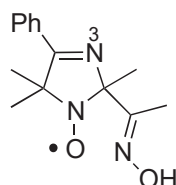
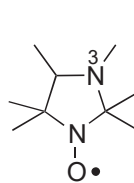
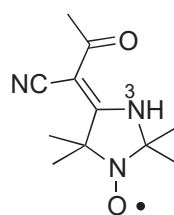


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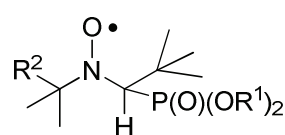
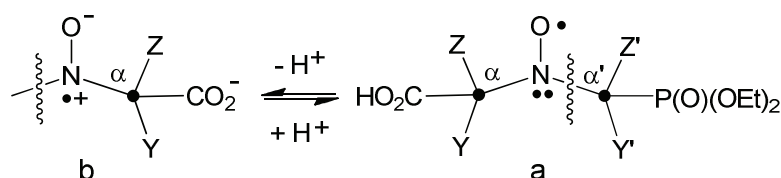
electron donating groups
at α -C and α' -C

electron withdrawing groups
at α -C and/or α' -C

	pK_a		pK_a
$R^1 = R^2 = H; R^3 = Et$ (DPP) $R^4 = R^5 = Me$	6.99	$R^1 = R^2 = Me; R^3 = CH_2OH$ $R^4 = H; R^5 = Me$	5.66
$R^1 = R^2 = H; R^3 = nPr$ $R^4 = H; R^5 = Me$	6.92	$R^1 = R^2 = H; R^3 = Et$ $R^4 = Me; R^5 = 4-NO_2-Ph$	5.03
$R^1 = R^2 = R^3 = Me$ $R^4 = H; R^5 = Me$	7.02	$R^1 = R^2 = R^3 = Me$ $R^4 = H; R^5 = P(O)(OEt)_2$	3.22
$R^1 = R^2 = R^3 = Me$ $R^4 = H; R^5 = iPr$	6.61	$R^1 = R^2 = H; R^3 = Ph$ $R^4 = Me; R^5 = CH_2Ph$	4.76

B**Imidazoline****Im-1****Im-2****Imidazolidine****In-1****In-2**

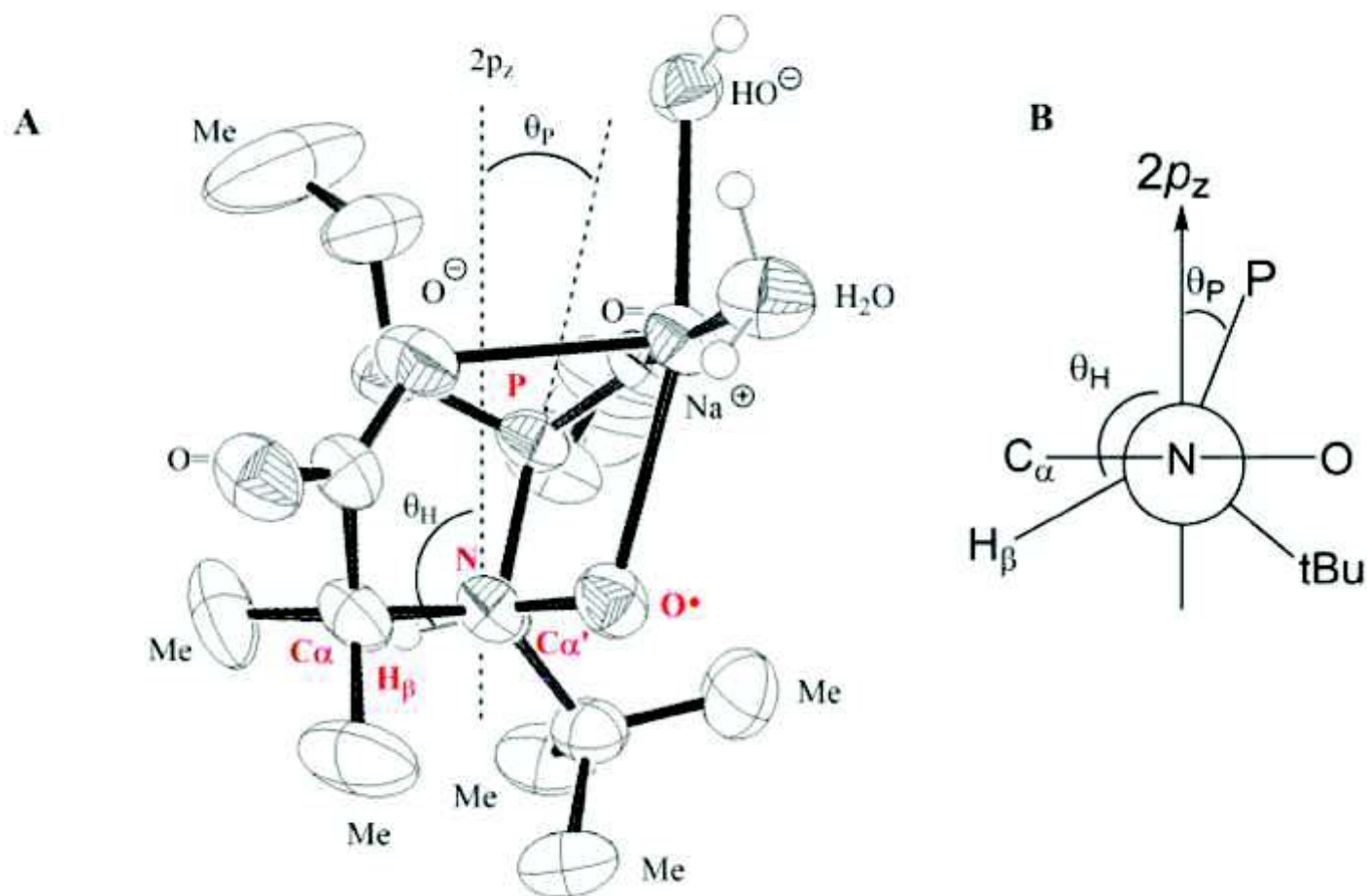
pK_a	0.8	1.0 (and 11.5)	4.7	9.7
Δa_N (G)	0.85	0.72 (and 0.23)	1.27	0.71

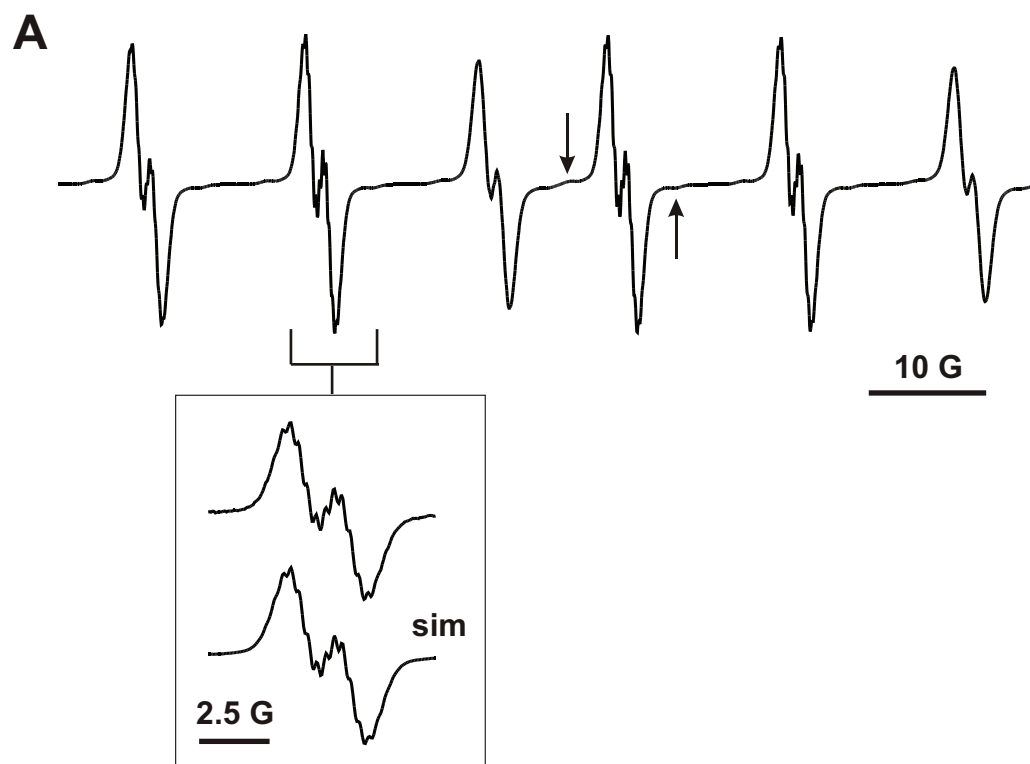
C**DEPN** ($R^1 = Et; R^2 = Me$)**20** ($R^1 = Bz; R^2 = Me$)**21** ($R^1 = Et; R^2 = CH_2OH$)**Target**Y and Y' = Me, *i*Pr, *t*Bu, Ph

Z or Z' = H

Figure 2

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B

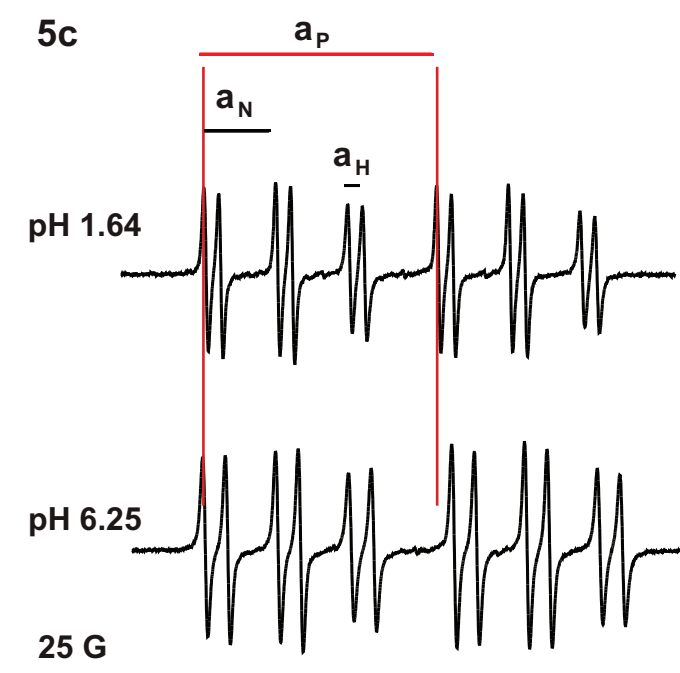
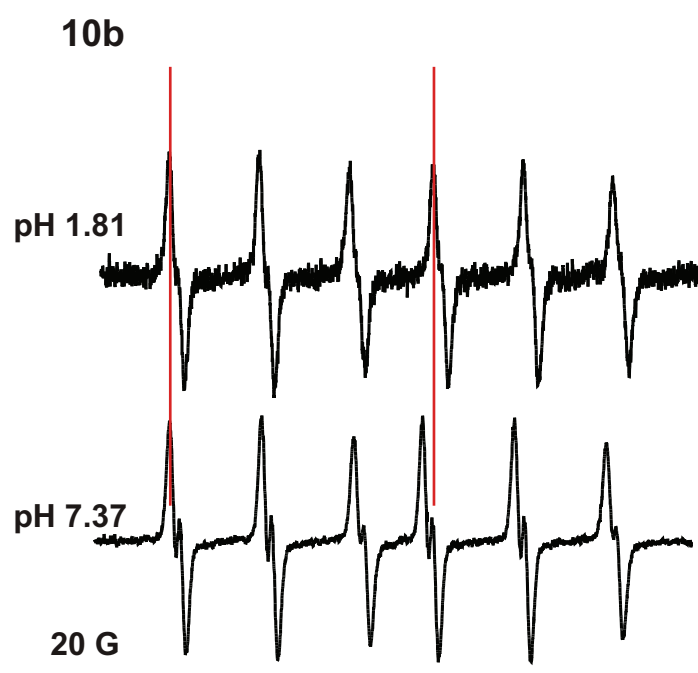


Figure 4

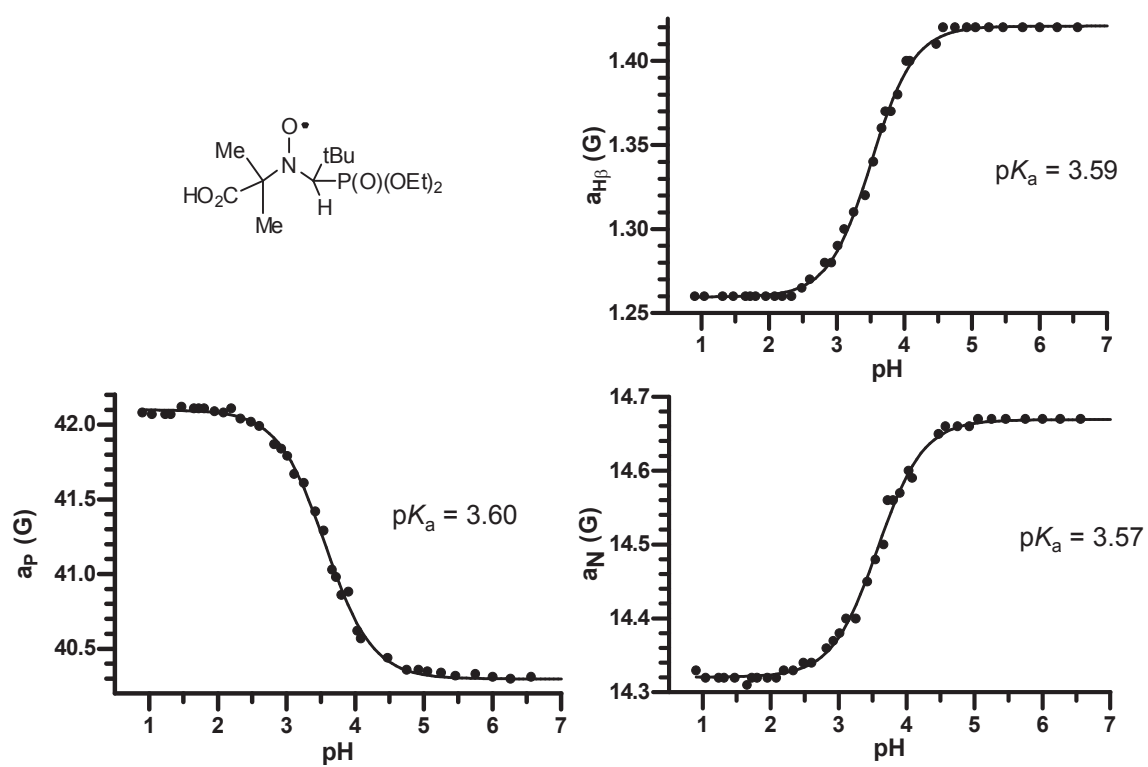
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Figure 5

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