Notes

Synthesis of Tetraalkyl (Pyrrolidine-2,2-diyl)bisphosphonates and 2,2-Bis(diethoxyphosphoryl)-3,4-dihydro-2*H*-pyrrole 1-Oxide; ESR Study of Derived Nitroxides

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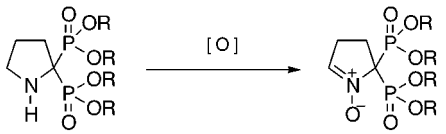
Introduction

Many investigations are currently undertaken to address the role played by oxygen-centered radicals in numerous pathological processes. We recently reported the synthesis of a new spin trap, the 2-diethoxyphosphoryl-2-methyl-3,4-dihydro-2*H*-pyrrole 1-oxide **1** (DEPMPO), which was shown to be particularly efficient in trapping oxygen-centered radicals in biological milieu.[1−4] DEPMPO is a phosphorylated analogue of the 2,2-dimethyl-3,4-dihydro-2*H*-pyrrole 1-oxide **2** (DMPO), which is the most popular spin trap in free radical biology.

The kinetics of $HO^{\bullet}$ and $O_2^{-\bullet}$ addition on **1** or **2** are almost identical. However, the DEPMPO superoxide spin adduct (DEPMPO−OOH) **3** has a significantly larger half-life (15 times at pH 7.4) than DMPO−OOH.[4]

To understand and, possibly, to optimize the influence of the phosphoryl group on the half-life of superoxide spin adducts[5,6] we prepared a series of new phosphorylated nitrones and investigated their spin trap properties. We showed that if a methylene spacer is inserted between the carbon adjacent to the nitrogen of the nitroxyl function and the phosphorylated group,[5] the spin trapping properties of the resulting nitrone and the persistence of the superoxide spin adduct are very similar to those of DMPO. This result clearly suggests that the electron-withdrawing effect exerted by the phosphoryl group takes part in the stabilization of DEPMPO−OOH. It was then interesting to investigate the influence of the introduction of a second phosphorylated group on the same carbon atom.

Scheme 1



5

Tetraalkyl (pyrrolidine-2,2-diyl)bisphosphonates **5** were selected as precursors[1,2,7−12] of the targeted *gem*-bisphosphorylated nitrones. We describe hereafter the synthesis of **5** and that of the bis(diethoxyphosphoryl)-3,4-dihydro-2*H*-pyrrole 1-oxide **6** (Scheme 1). We will also describe the spin trapping properties of **6** and the ESR features of various nitroxides derived from **5**.

Results and Discussion

Synthesis of Bisphosphonates 5. Aminomethylene *gem*-bisphosphonates exhibit a number of useful biological properties,[13−16] and different synthetic approaches for these compounds are now available. However, simple and efficient syntheses of the alicyclic analogues are still scarce.[14,17,18]

Yokomatsu[18] prepared the tetraethyl(piperidine-2,2-diyl)bisphosphonate **7** via a Beckman rearrangement. We first tried to extend his approach to the synthesis of **5** from cyclobutanone **8**. However, following his procedure, we obtained a crude reaction mixture containing at least seven products, with only 10% of the desired bisphosphonates. Then, we decided to prepare the bisphosphonates **5** through esterification of the corresponding (pyrrolidine-2,2-diyl)bisphosphonic acid **9**. The compound **9** was obtained from the pyrrolidin-2-one according to the general method of Plöger[14] modified by Zilch.[17] It is soluble in basic aqueous medium only, and all our

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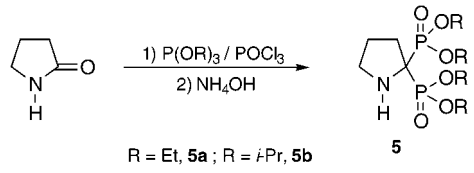
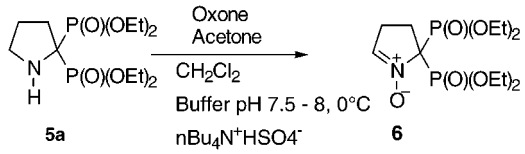
Table 1. Calculated ESR Parameters of 6−OOH and 6−SG Spin Adducts Obtained by Simulation with Chemical Exchange (Two-Site Model)

adduct	source	a_N, G	$a_{H\beta}$, G	a_{P1}, G	a_{P2}, G	$a_{H\gamma}$, G	%	exchng time, ns
6−OOH	H_2O_2 30%	12.8	10.3	42.0	39.3	0.2(4)	52	47.9
		12.9	11.1	47.7	39.1	0.2(4)	48	
6−OOH	HX/XOD/buffer, pH 5.8	12.9	8.2	39.2	39.4	0.75(4)	41	12.6
		12.7	11.8	46.7	38.6	0.75(4)	59	
6−SG	GSSG/ UV/buffer, pH 5.8	13.7	17.2	43.5	41.5	0.4	90	30.8
		11.8	16.1	54.7	27.7	0.4	10	

Scheme 2



Scheme 3



attempts to get the corresponding esters failed, even with the use of ethylorthoformate.[17]

Finally, we succeeded in preparing **5** in reasonable yield by modifying the Zilch synthesis (Scheme 2). $POCl_3$ (2 mol) was added to a mixture of pyrrolidin-2-one (1 mol) and trialkyl phosphite (2 mol) at −5 °C under nitrogen. The reaction mixture was stirred for 5 h at room temperature and then poured over a cold aqueous saturated NH_4OH solution. After workup, the aminobisphosphonates **5a** or **5b** were obtained in about 50% yield.

Resulting from the coupling with two phosphorus nuclei which exhibit the same chemical shift, the α and β carbons of **5** (C_β-C_α-O-P(O)-O-$C_{\alpha'}$-$C_{\beta'}$) were expected to appear as second-order quintets (AA′X systems) in the ^{13}C NMR spectra. However, the two outer lines of the quintet were not observed, thus indicating that $^2J_{PP'} \geq 15$ Hz.[19] If we assume that $^5J_{CP}$ is negligible, then the magnitude of the different coupling constants can be measured directly on the observed virtual triplets: $^2J_{C\alpha P}$ ($+ {}^4J_{C\alpha P'}$) = 5.3 Hz, $^2J_{C\alpha' P}$ ($+ {}^4J_{C\alpha' P'}$) = 5.8 Hz, $^3J_{C\beta P}$ = 5.5 Hz, $^3J_{C\beta' P}$ = 7.2 Hz for **5a** and $^2J_{C\alpha P}$ ($+ {}^4J_{C\alpha P'}$) = 6.2 Hz, $^2J_{C\alpha' P}$ ($+ {}^4J_{C\alpha' P'}$) = 6.7 Hz, $^3J_{C\beta 1P}$ = 6.2 Hz, $^3J_{C\beta 2P}$ = 6.7 Hz for **5b**. No coupling was observed for Cβ′1 and Cβ′2.

The oxidation of **5a** at 0 °C with dimethyldioxirane (DMD), prepared in situ from oxone and acetone in biphasic conditions (Brik procedure),[20] led to the *gem*-bisphosphorylated nitrone **6** in 11% yield after purification (Scheme 3). The use of DMD in an acetone solution[21] instead of the in situ procedure did not lead to a substantial increase of the yield.

ESR Studies. Spin Trapping. Spin trapping with **6** using a Fenton system in aqueous phosphate buffer (pH 5.8) led to the formation of the **6**−OH spin adduct (a_N = 13.5 G, $a_{H\beta}$ = 13.3 G, a_{P1} = 44.1 G, a_{P2} = 43.4 G, $a_{H\gamma}$ (4H) = 0.5 G, determined from computer simulation[22]).

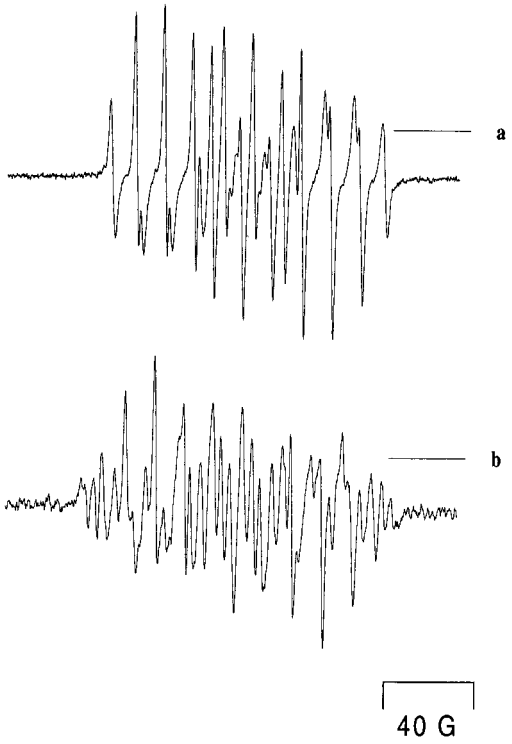


Figure 1. (a) ESR spectra of the **6**−OOH adduct (H_2O_2, 30%). (b) Complex adduct obtained with **6** (50 mM) with a DTPA (4.5 mM)/light/riboflavin system (0.1 M) in phosphate buffer pH 6.0.

The **6**−CH_3 and **6**−CO_2^- adducts were also generated using a Fenton system, in the presence of DMSO or sodium formate, respectively. (**6**−CH_3:a_N = 14.6 G, $a_{H\beta}$ = 21.8 G, a_{P1} = 45.5 G, a_{P2} = 44.3 G, $a_{H\gamma}$ (4H) = 0.5 G; **6**−CO_2^-: a_N = 14.3 G, $a_{H\beta}$ = 20.3 G, a_{P1} = 43.0 G, a_{P2} = 42.3 G, $a_{H\gamma}$ (4H) = 0.4 G) Moderate line width distortions were observed in the ESR spectra of these spin adducts, and very good calculated spectra were obtained considering slightly different couplings for the two diastereotopic phosphorus nuclei.

The **6**−OOH adduct was formed with three different generating systems (Table 1). It was well characterized from the UV photolysis of 30% H_2O_2 in the presence of **6** (Figure 1a). The use of the hypoxanthine/xanthine oxidase (HX/XOD) system at pH 6.0 also led to **6**−OOH together with **6**−OH, the later probably being formed from the decomposition of the superoxide adduct. Working in the presence of superoxide dismutase (SOD) (85 units mL^{-1}) inhibited the formation of the signal attributed to **6**−OOH; when glutathione (100 mM)/glutathione peroxidase (10 units mL^{-1}) was added to **6** in the HX/XOD system, **6**−OOH was reduced to **6**−OH. When superoxide was produced from a riboflavin/light/DTPA system in phosphate buffer at pH 5.8, a complex spectrum was obtained resulting from the superposition of **6**−OOH with other spin adducts (**6**−OH and carbon-

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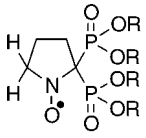
Scheme 4



10a : R = Et
10b : R = *i*-Pr

centered radical adducts) and no "pure" ESR line of the superoxide adduct could be found (Figure 1b) to measure its kinetic of decay. At pH 7.0, **6**−OOH was not identified. The kinetic of decay of the superoxide adduct, generated in pyridine by the lumiflavin/light/DTPA system, was estimated. With this superoxide generating system, the persistence of **6**−OOH was found to be similar to that of DMPO−OOH but 4.8 times lower than that of DEPMPO−OOH. UV photolysis of glutathione disulfide in the presence of **6** in phosphate buffer pH 5.8 led to **6**−SG. For **6**−OOH (Figure 1a) and **6**−SG, the line width alternation was rather important and satisfactory simulations could only be obtained taking into account a chemical exchange between two forms of these adducts (two-site model). The calculated ESR parameters are listed in Table 1.

The trapping of the superoxide with the bisphosphorylated nitrone **6** presented the same limitations as those observed with DMPO. The persistence of **6**−OOH is low (not observed in phosphate buffer at pH 7.0), and the decay of **6**−OOH in pyridine is closer to that of DMPO−OOH rather than to that of DEPMPO−OOH. On the other hand, a fast decomposition of the superoxide adduct into the hydroxyl adduct was observed. Work in progress in our laboratory[23] indicates that, in addition to its strong electron-withdrawing effect,[24] steric crowding of the $(EtO)_2P(O)$ plays a role in the stabilization of the DEPMPO−OOH spin adduct. However, for **6**, the important increase of intramolecular steric strain related to the introduction of a second phosphorylated group can induce the opposite, a destabilization of the spin adducts. Furthermore, we have shown that when only one $(EtO)_2P(O)$ is present, low-energy conformations with the phosphorus group in a pseudoaxial position are adopted by the molecule. When a second $(EtO)_2P(O)$ group is introduced in a *gem*-position, one group is forced to occupy a high-energy pseudoequatorial position.

ESR Features of Bisphosphorylated Nitroxides Derived from Aminobisphosphonates 5a and 5b and Their Corresponding Acid. The oxidation of **5a** and **5b** with *m*-CPBA led to nitroxides **10a** and **10b** (Scheme 4) that exhibited 27-line ESR spectra (a_N(1N), a_H(2H), a_P(2P)). The ESR spectrum of **10a** was recorded in different solvents. No significant changes were observed in aprotic solvents (a_N = 13.9−14.0 G, a_P = 42.3−42.8 G, a_H = 17.8−18.1 G, g = 2.0060) and even in water changes are not very large (a_N = 14.5 G, a_P = 44.3 G, a_H = 19.3 G, g = 2.0058). This result could be accounted for by either the existence of a largely predominant conformer or the presence of an equilibrium between equivalent conformers. We have recently shown[25] that at low temperature (below 250 K) the ESR spectrum of

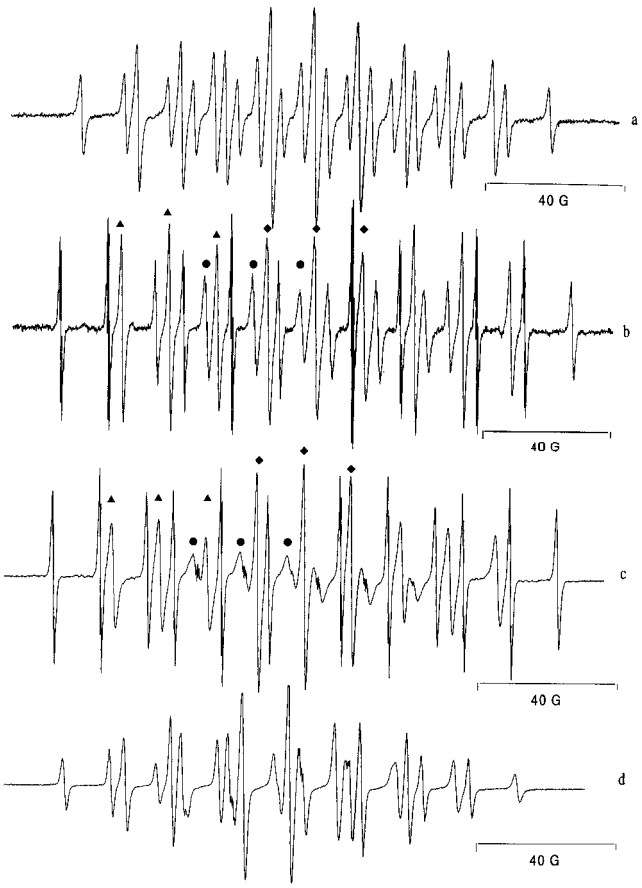


Figure 2. ESR spectra at 25 °C: (a) radical **10a** in methylene chloride, (b) radical **10b** in methylene chloride,[a] (c) **10b** in benzene,[a] (d) **11** in aqueous solution of NaOH (pH = 13). [a](▲, lines for which $m_I^{H_1} + m_I^{H_2} = 0$; ●, $m_I^{P_1} + m_I^{P_2} = 0$; ♦, $m_I^{H_1} + m_I^{H_2} = 0$ and $m_I^{P_1} + m_I^{P_2} = 0$).

10a could be explained assuming a complex chemical exchange between four sites. However, at 298 K, the interconversion rate between the different sites is sufficiently high to average the ESR parameters, and no significant line broadening was observed (Figure 2a).

The ESR spectrum of radical **10b** was recorded in either methylene chloride or benzene. The lines corresponding either to $m_I^{H_1} + m_I^{H_2} = 0$ or $m_I^{P_1} + m_I^{P_2} = 0$ are broadened, the broadening being much stronger in benzene (Figure 2c) than in methylene chloride (Figure 2b). The existence of a π complex between benzene and a nitroxide has been previously reported[26] and could explain such a difference.The calculated ESR parameters obtained by using a two-site chemical exchange model are listed in Table 2. The complete analysis of the chemical exchange observed for **10b** will be published in a forthcoming paper.

Oxidation of **9** with *m*-CPBA in an aqueous solution of NaOH (pH 13) led to the ESR signal shown in Figure 2d, which was assigned to nitroxide **11** (Scheme 5). The ESR parameters of **11** were obtained by computer simulation: a_N = 16.1 G, a_H = 21.1 G, a_P = 40.6 G, g = 2.0054. Assuming that the pK_{a4} of **9** is lower than 11,[15,27] the nitroxide **11** is expected to be formed as a tetrasodium salt.

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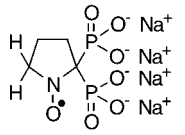
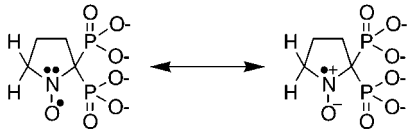
Table 2. Calculated ESR Parameters of Nitroxide 10b in Benzene and Methylene Chloride at 25 °C Obtained by Simulation with Chemical Exchange[22,25] (Two-Site Model)

	methylene chloride	benzene
a_N, G	13.9, 14.3	13.8, 13.8
a_{P1}, G	44.4, 40.0	43.6, 38.8
a_{P2}, G	41.2, 45.3	41.1, 44.9
a_{H1}, G	18.8, 16.3	18.5, 15.6
a_{H2}, G	17.2, 19.9	17.1, 19.9
other couplings, G	0.3(2)	0.4(2)
	0.4(2)	0.4(2)
g	2.0060	2.0060
population, %	68, 32	66, 34
exchng time, ns	11.1	38.5

Scheme 5



11



11_a **11_b**

In water, nitroxides **10a** and **11** exhibit significantly different nitrogen couplings and g factors ($a_N = 16.1$ G, $g = 2.0054$ for the former and $a_N = 14.5$ G, $g = 2.0058$ for the latter). These changes result from the strong donor effect exerted by the phosphonato groups, which favors the mesomeric form **11b** (Scheme 5). Although this increase in a_N should be accompanied by an increase of the β-phosphorus coupling constant a_P, actually we observed a decrease in a_P for nitroxide **11** as compared to **10a** (**11**, $a_P = 40.6$ G; **10a**, $a_P = 44.3$ G). This could be reasonably attributed to a decrease in the hyperconjugative proportionality constant value, B, which appears in the McConnell relationship ($a_P = B_P \cos^2 \theta$; $B_P = B\rho_N$; ρ_N is the spin density on nitrogen), rather than to conformational changes.

Although the dismutation was reported to be the main disappearance process for β-hydrogen-bearing nitroxides, we observed that nitroxides **10a**, **10b**, and **11** were persistent for days regardless of the presence of two β-hydrogens.

In addition to the previously reported 2,2-bis(diethoxyphosphoryl)-5,5-dimethylpyrrolidinoxyl,[25] nitroxides **10a**, **10b**, and **11** are, to our knowledge, the first reported examples of *gem*-β-bisphosphorylated nitroxides. We will now study the extension of this synthetic route to other cyclic aminobisphosphonates by varying the nature of both the phosphite and the lactam.

Further ESR studies of bisphosphonic acid-bearing nitroxides are planned, including metal-complexation properties and pH-correlated variations of the coupling constants. The good persistence of nitroxide **11** opens interesting perspectives for the use of this kind of nitroxide as MRI contrast enhancing agents.

Experimental Section

^{1}H and ^{13}C NMR spectra were recorded on Bruker AC 100, 200, and 400 spectrometers, and the chemical shifts (δ) in ppm were referenced to internal TMS or HOD (4.81 ppm) for D_2O solutions. Proton-decoupled ^{31}P NMR spectra were recorded on a Bruker AC 100 at 40.54 MHz, and the chemical shifts (δ) in ppm were referenced to external 85% H_3PO_4. All J values are given in hertz. IR spectra were recorded on a MATTSON 1000 series FTIR. ESR measurements were performed on a Brucker ESP 300 spectrometer equipped with an X-band resonator (9.41 GHz). All ESR spectra were recorded at 100 kHz magnetic field modulation. Solvents were purchased from SDS. Pyrrolidin-2-one, *m*-CPBA (*m*-chloroperbenzoic acid) (57−86%), phosphorus oxychloride, and triethyl phosphite were Aldrich reagents and were used as purchased, as was phosphorous acid purchased from Acros Chimica. Ammonia solution (ca. 32%) was a Prolabo reagent and was used as purchased.

Preparation of Tetraalkyl (Pyrrolidine-2,2-diyl)bisphosphonates 5a and 5b). Phosphorus oxychloride (40 mL, 0.44 mol) was added over 1.25 h at −5 °C to a mixture of pyrrolidin-2-one (18.49 g, 0.22 mol) and trialkyl phosphite (0.42 mol). The reaction mixture was stirred for 5 h at room temperature and then poured over a mixture of ice (300 g) and ammonia 32% (300 mL). The aqueous layer was extracted with methylene chloride (4 × 100 mL). The organic layer was concentrated to obtain a yellow oil. This oil was dissolved in 100 mL of methylene chloride. Water was added (200 mL), and then concentrated hydrochloric acid (37%) was added until pH 1. The aqueous layer was separated and washed with methylene chloride (4 × 50 mL). Sodium hydroxide and sodium carbonate were added until pH 10 to the aqueous layer, which was extracted with methylene chloride (4 × 50 mL). The organic layer was dried over sodium sulfate and filtered. Removal of the solvent afforded compound **5a** or **5b**.

5a (33.4 g, 47%): IR (neat) 3480 (NH), 1243 (P═O), 1164 (P−O−C_2H_5) cm^{-1}; ^{1}H NMR (C_6D_6, 400 MHz) δ 4.17 (8H, m), 2.88 (2H, t, $J = 6.5$), 2.42 (2H, n, $J_{PH} = 17.7$, $J_{HaHb} = 7.2$), 1.69 (2H, q, $J = 6.8$, $J_{HH} = 7.2$), 1.11 (6H, t, $J = 7.1$), 1.10 (6H, t, $J = 7.1$); ^{31}P NMR ($CDCl_3$) δ 22.5; ^{13}C NMR (C_6D_6, 100.61 MHz) δ 63.4 (t, $J_{CP} = 5.3$), 62.7 (t, $J_{CP} = 3.6$), 62.8 (t, $J_{CP} = 151.8$), 47.7 (t, $J_{CP} = 4.0$), 31.2 (t, $J_{CP} = 3.0$), 26.5 (t, $J_{CP} = 3.1$), 16.6 (t, $J_{CP} = 5.5$), 16.5 (t, $J_{CP} = 7.2$); p$K_a = 3.5$; bp 140 °C at 6×10^{-2} mmHg. **5a** gave a picrate: mp 128−130 °C; ^{1}H NMR ($CDCl_3$, 100 MHz) δ 8.86 (2H, s), 7.99 (2H, s), 4.24 (8H, m), 3.64 (2H, t, $J = 6.9$), 2.67−2.15 (4H, m), 1.36 (6H, t, $J = 7.1$), 1.33 (6H, t, $J = 7.1$); ^{31}P NMR ($CDCl_3$) δ 16.23. Anal. Calcd for $C_{18}H_{30}N_4O_{13}P_2$: C, 37.75; H, 5.28; N, 9.79. Found: C, 37.24; H, 5.34; N, 9.28.

5b (37.2 g, 45%): IR (neat) 3471 (NH), 1246 (P═O), 989 (P−O−CH−$(CH_3)_2$) cm^{-1}; ^{1}H NMR (C_6D_6, 400 MHz) δ 5.00 (1H, m), 4.87 (1H, m), 2.95 (2H, t, $J = 6.5$), 2.37 (2H, tt, $J_{PH} = 17.7$, $J_{HH} = 7.3$), 1.75 (2H, q, $J = 6.8$, $J_{HH} = 6.9$), 1.31 (6H, d, $J_{HH} = 6.2$), 1.28 (6H, d, $J_{HH} = 6.3$), 1.27 (6H, d, $J_{HH} = 6.4$), 1.22 (6H, d, $J_{HH} = 6.1$); ^{31}P NMR ($CDCl_3$) δ 21.2; ^{13}C NMR (C_6D_6, 100.61 MHz) δ 71.7 (t, $^2J_{CP} = 6.2$), 70.8 (t, $^2J_{CP} = 6.7$), 63.1 (t, $^1J_{CP} = 151.1$), 47.7 (t, $^3J_{CP} = 4.6$), 31.0 (t, $^2J_{CP} = 3.4$), 26.4 (t, $^3J_{CP} = 3.3$), 24.7 (s), 24.4 (s), 24.0 (t, $^3J_{CP} = 6.4$), 23.8 (t, $^3J_{CP} = 6.7$). Anal. Calcd for $C_{16}H_{35}NO_6P_2$: C, 48.12; H, 8.83; N, 3.51. Found: C, 48.21; H, 8.80; N, 3.51. mp = −27 °C.

Preparation of Cyclobutanone Oxime 8.[28,29] An aqueous solution (10 mL of water) of sodium carbonate (4.80 g, 45.3 mmol) was added under stirring to an aqueous solution (11 mL of water) of hydroxylamine hydrochloride (6.22 g, 89.5 mmol) and cyclobutanone (5.15 g, 73.5 mmol), and the temperature was kept below 45 °C. The reaction mixture was stirred for 1 h. The aqueous layer was extracted with diethyl ether (4 × 25 mL), and the solvent was removed to dryness to give a white powder. The powder was then dissolved in acetone (10 mL), dried over sodium sulfate, filtered, and evaporated to dryness to give compound **8** (4.7 g, 75%) as white crystals: ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.88 (1H, s, −OH), 2.92 (2H, dt, $J_1 = 8.3$, $J_2 = 1.0$), 2.87 (2H, dt, $J_1 = 8.2$, $J_2 = 1.0$), 1.97 (2H, q, $J = 8.1$); ^{13}C NMR ($CDCl_3$) δ 159.86, 31.51, 30.61, 14.54; mp 87 °C (lit. 84−85 °C).[28]

Preparation of (Pyrrolidine-2,2-diyl)bisphosphonic acid 9.[17] The pyrrolidin-2-one (17.36 g, 0.2 mol) and phosphorous acid (32.9 g, 0.4 mol) were heated under nitrogen at 80 °C until

(28) Iffland, D. C.; Criner, G. X.; Koral, M.; Lotspeich, F. J.; Papanastassiou, Z. B.; White, S. M., Jr. *J. Am. Chem. Soc.* **1953**, *75*, 4044−4046.

(29) Hawkes, G. E.; Herwig, K.; Roberts, J. D. *J. Org. Chem.* **1974**, *39*, 1017−1028.

the acid was dissolved. The phosphorus oxychloride (39 mL, 0.4 mol) was carefully added over 1 h. The reaction mixture was stirred for 8 h at 100 °C to give a yellow solid. Water (1 L) was added and the mixture was heated for 1 h at 100 °C and then filtered on a Büchner. Crystallization of the filtrate afforded compound **6** (8.0 g, 18%): IR (KBr) 2979 (ν_{CHsp3}), 2778 (ν_{NH}+), 2707 (P−OH), 2462 (P−OH), 1614 (δ_{NH}+), 1435 (δ_{CHsp3}), 1241 (P═O), 1119, 986 (P−O−H) cm^{-1}; ^1H NMR (D_2O/NaOD, 200 MHz) δ 3.42 (2H, t, $J = 7.02$), 2.38 (2H, h, $J_{HcHb} = 7.13$, $J_{PHc} = 14.46$), 2.06 (2H, q, $J = 7.04$); ^{31}P NMR (D_2O/NaOD) δ 15.01; ^{13}C NMR (D_2O/NaOD) δ 65.77 (t, $J_{CP} = 118$), 48.61, 31.31, 25.36; mp 281 °C (dec). Anal. Calcd for $C_4H_{11}NO_6P_2$: C, 20.78; H, 4.80; N, 6.06; P, 26.82. Found C, 20.65; H, 4.90; N, 5.64; P, 26.37.

2,2-Bis(diethoxyphosphoryl)-3,4-dihydro-2*H*-pyrrole 1-Oxide 6. A mixture of **5** (1.6 g, 4.6 mmol), acetone (80 mL), methylene chloride (60 mL), tetrabutylammoniumhydrogenosulfate (81.4 mg, 0.24 mmol), 0.1 M Na_2HPO_4 buffer (60 mL), and 2 M aqueous sodium hydroxide (2 mL) was cooled to 0 °C. A solution of oxone (9.5 g; 15.5 mmol) in water (90 mL) was added over 1 h. Then, the organic layer was separated, and the aqueous layer was extracted with methylene chloride (2 × 100 mL). The combined organic fractions were reconcentrated under vacuum. Acetone (1 mL) was added to the residual material. Preparative TLC on Silicagel (CH_2Cl_2/anhydrous EtOH, 10/1 v/v; extraction with MeOH) afforded nitrone **6** in 11% yield (0.154 g): ^1H NMR (C_6D_6, 400 MHz) δ 1.09 (6H, t, $J = 7.1$), 1.11 (6H, t, $J = 7.1$), 2.11 (2H, m, $J_{HH} = 2.7$, $J_{HP} = 2.9$, $J_{HH} = 7.3$), 2.69 (2H, m, $J_{HH} = 7.2$, $J_{HP} = 17.2$), 4.20 (4H, m, $J = 10.3$), 4.37 (4H, m, $J = 10.3$), 6.42 (1H, q, $J_{HH} = 2.8$, $J_{HP} = 3.1$); ^{13}C NMR ($CDCl_3$, 100 MHz) 16.4 (s), 27.7 (s), 27.8 (s), 63.9 (s), 64.5 (s), 136.5 (t); ^{31}P NMR ($CDCl_3$, 40 MHz) 15.5; HRMS for $C_{12}H_{25}NO_7P_2$ calcd 357.1106, found 357.1127; MS (*m*/*z*) 220, 203, 138, 94, 67, 28. R_f (methylene chloride/ethanol 19/1) 0.26.

ESR Study of Nitroxides 10a, 10b, and 11. Compounds **5a**, **5b**, or **9** (0.03 mmol) were dissolved in 100 μL of solvent. *m*-CPBA (7.2 mg, 0.03 mmol) was added, and the spectrum was immediately recorded after nitrogen bubbling.

Spin Trapping Experiments. Xanthine oxidase (XOD) and bovine erythrocyte superoxide dismutase (SOD) were purchased from Boerhringer Mannheim Biochemica Co. Glutathione peroxidase, diethylenetriaminepentaacetic acid (DTPA) and chelating iminodiacetic resin Chelex 100 were purchased from Sigma Chemical Co. Phosphate buffers (0.1 M) were stirred in the presence of Chelex (4 h, 4 g/100 mL) to remove traces of metal impurities. UV photolysis was performed with an Oriel Xe−Hg (1000 W) lamp. Illumination of flavins/DTPA solutions was achieved with a tungsten filament 100 W lamp. The concentrations indicated below correspond to initial conditions after mixing of all reactants.

Spin Trapping of the Radical Hydroxyl (HO•). A classical Fenton system was used. A solution of $FeSO_4$ (2 mM)/DTPA (1 mM) in phosphate buffer pH 5.8 was added to a solution of nitrone **6** (100 mM) and H_2O_2 (2 mM). The spectrum was recorded 40 s after the addition of $FeSO_4$. The **6**−CH_3 and **6**−CO_2^- adducts were obtained in the same manner but with the addition of dimethyl sulfoxide (DMSO) (100 mM) or sodium formiate (50 mM) prior to the addition of ferrous sulfate.

Production of the Superoxide Adduct; Photolysis of Hydrogen Peroxide 30%. A solution of **6** (50 mM) in hydrogen peroxide (30%) was illuminated by UV light for 15 s in the ESR cavity, and the spectrum was recorded just after photolysis.

Hypoxanthine/Xanthine Oxidase System. A solution of xanthine oxidase (0.4 U mL^{-1}) was added to an air-bubbled solution of **10** (100 mM), DTPA (1 mM), and hypoxanthine (0.4 mM) in phosphate buffer pH 6.0. Air was bubbled into the reaction mixture for 30 s, and the ESR spectra were recorded 40 s after the addition of xanthine oxidase.

Flavin/Light/DTPA Systems. A solution of **6** (50 mM), DTPA (4.5 mM), and riboflavin (0.1 M) in phosphate buffer pH 6.0 was prepared. Air was bubbled into the reaction mixture for 30 s. The superoxide production was initiated by irradiation with the tungsten lamp. In pyridine, the riboflavin was replaced by lumiflavin.

GS• Trapping. GS• was produced by UV photolysis of a solution containing glutathione disulfide (50 mM) and nitrone **6** (50 mM) in phosphate buffer pH 5.8. The ESR spectrum was recorded after 15 s of UV photolysis.

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Supporting Information Available: ^1H NMR and ^{13}C NMR, with attributions, of compounds **5a**, **5b**, **6**, **8**, and **9** (2 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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