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Diastereoselective Synthesis and ESR Study of 4-PhenylDEPMPO Spin Traps

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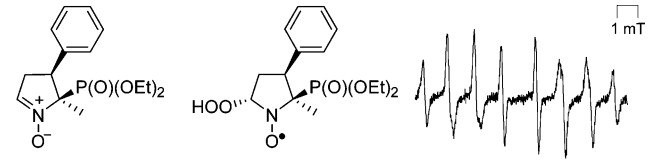

4-PhDEPMPOc 4-PhDEPMPOc-OOH and ESR spectrum

The cis and trans diastereoisomers of 5-(diethoxyphosphoryl)-5-methyl-4-phenylpyrroline *N*-oxide (4-PhDEPMPO*t* **8** and 4-PhDEPMPO*c* **9**) were prepared stereoselectively and used as spin traps for hydroxyl and superoxide radicals. The spin adduct formed by reaction of the cis stereoisomer **9** with superoxide radical anion exhibited an 8-line ESR spectrum, showing only a reduced alternating line width phenomenon. This spectrum is simpler than the 12-line spectrum of DEPMPO-OOH, which exhibits a strong alternating line width phenomenon. The half-life times of the 4-PhDEPMPO*c*-OOH and DEPMPO-OOH adducts were of the same order: 14.5 and 15.5 min, respectively.

Introduction

Reactive oxygen species ($HOO^{\bullet}$, $HO^{\bullet}$, H_2O_2) are considered to play an important role in many pathological disorders, such as ischemia-reperfusion, heart attack, stroke, Alzheimer's disease, aging, DNA damage, cancer,[1−5] and so on. To study the involvement of oxygen short-lived free radical species, the technique of spin trapping is one of the most valuable tools.[6−13] Among the nitrones used as spin traps, DMPO **1** has been for a long time one of the most popular to detect oxygen-centered radicals.[13−15] However, detection of the superoxide radical is limited by the instability of the DMPO-OOH adduct (half-life time < 1 min).[14] In the past 10 years, a new generation of spin traps related to DMPO has been developed.[16−18] Among them, DEPMPO **2** is becoming a widely used spin trap for biological studies because of the considerably better stability of the DEPMPO-OOH spin adduct (half-life time ∼18 min at physiological pH).[17,19] The related diisopropyl ester (DIPPMPO) **3** is even slightly better, with a half-life time of 21 min for the DIPPMPO-OOH adduct at physiological pH.[20] However, the dissymmetric

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nature of these nitrones, DEPMPO and DIPPMPO (Me-5 and phosphoryl-5 groups), leads to the formation of complex spectra because of the superimposition of the signals of two superoxide spin adducts. These signals are due to the formation of the cis and trans adducts **4** and **5,** respectively, in a ~1:9 ratio, which result from attack of the incipient free radical on both sides of the nitrone.[17,20] Moreover, the 12-line spectrum of the major signal, the trans spin adduct **5**, exhibits an alternating line width phenomenon because of the presence of two quickly exchanging conformers.[21,22] To limit these effects, introduction of a phenyl substituent on the pyrroline ring could be beneficial. Earlier studies showed that the presence of a phenyl group slows down the pseudorotation occurring within the ring of stable β-phosphorylated five-membered ring nitroxides.[23,24] Introduction of a phenyl group on the DMPO spin trap induced a rigidification of the ring conformation, beneficial for superoxide radical trapping.[25] In the DEPMPO series, synthesis of the *cis*-3-phenyl DEPMPO analogue **6** (DEPMPPO*c*) was recently reported.[26] The presence of a phenyl group led to an important steric effect on the course of addition of the superoxide radical. The DEPMPPO*c*-OOH adduct **7** showed an EPR spectrum with 12 separated lines with the same intensity, indicative of the absence of an alternating line width phenomenon. Unfortunately, the half-life time of this adduct was only around 2 min, compared to the 18 min of the DEPMPO-OOH adduct. Thus, the presence of a phenyl group on the C-3 carbon center has a largely positive effect on the pattern of the spectrum, but a negative one on the stability of the adducts. We considered that shifting the phenyl group from the C-3 center to the C-4 center should result in two beneficial effects: (a) the steric hindrance leading to only one free radical adduct should be maintained and (b) the absence of vicinal interactions destabilizing the spin adducts should be observed. In this paper, we report the stereoselective syntheses of the two diastereoisomers of 4-phenylDEPMPO, the trans isomer **8** (4-PhDEPMPO*t*) and the cis isomer **9** (4-PhDEPMPO*c*), and the study of their behavior toward oxygen free radicals (Figure 1).

Results and Discussion

DMPO-type nitrones can be prepared by three main routes (Scheme 1).[27,28] Two of them (a and b) require the synthesis of an intermediate pyrrolidine, eventually oxidized into the nitrone.[28] The third method (c) involves the reductive cyclization of a γ-nitroaldehyde in the last step.[29,30] In the case of ring-substituted DEPMPO derivatives, this reductive cyclization step leads to the formation of a pair of diastereoisomers.[26] However, diastereoselective synthesis should be possible by the route involving a pyrroline-type intermediate. Addition of a phosphite to 3-phenyl-2-methylpyrroline (**10**, R_1 = Me) should lead after oxidation to the *trans*-5-phosphoryl-4-phenyl nitrone derivative **8** [Scheme 2, R_1 = Me, R_2 = $P(O)(OEt)_2$], and addition of a methyl group to 3-phenyl-2-phosphorylpyrroline [**10**, R_1 = $P(O)(OEt)_2$] should lead after oxidation to the *cis*-5-phosphoryl-4-phenyl nitrone derivative **9** [Scheme 2, R_1 = $P(O)(OEt)_2$, R_2 = Me].

The trans nitrone **8**, 5-(diethoxyphosphoryl)-5-methyl-4-phenylpyrroline *N*-oxide, was prepared in six steps from cinnamonitrile by the sequence described in Scheme 3. 3-Phenyl-4-oxopentane-1-nitrile **11** was obtained by treatment of cinnamonitrile with Mg turnings in the presence of acetic anhydride/TMSCl in DMF according to the method of Ohno et al.[31] Protection of the carbonyl group as a dioxolane was performed by reaction of **11** with ethylene glycol in the presence of a catalytic amount of camphorsulfonic acid (CSA) in toluene under reflux, giving compound **12** in 91% yield. Reduction of **12** with

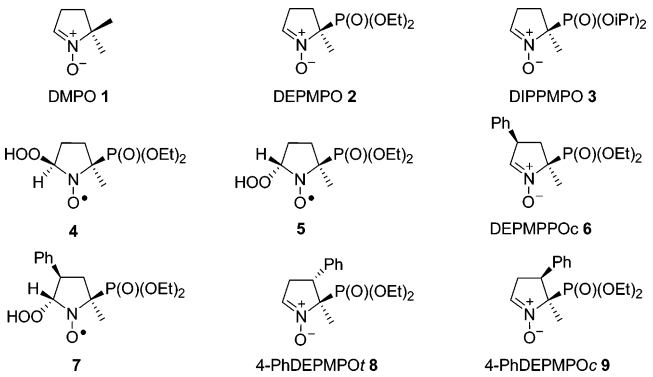
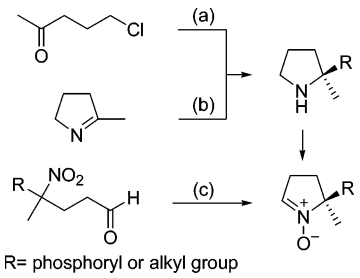
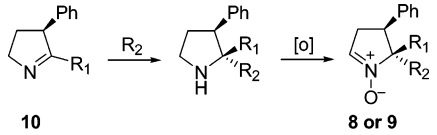


FIGURE 1. Chemical structures of DMPO, phosphorylated analogues, and some of their superoxide adducts.

SCHEME 1. General Routes for the Synthesis of DMPO Analogues



SCHEME 2. Synthesis of 4-PhDEPMPO Derivatives



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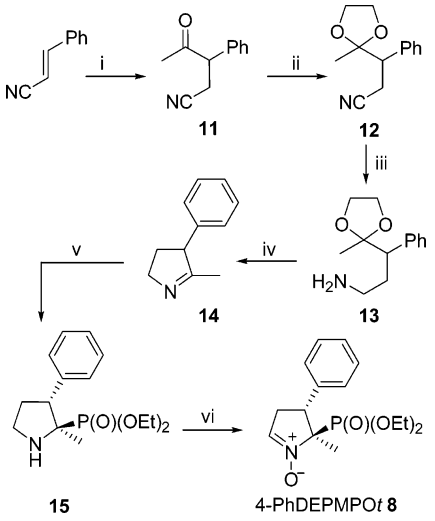
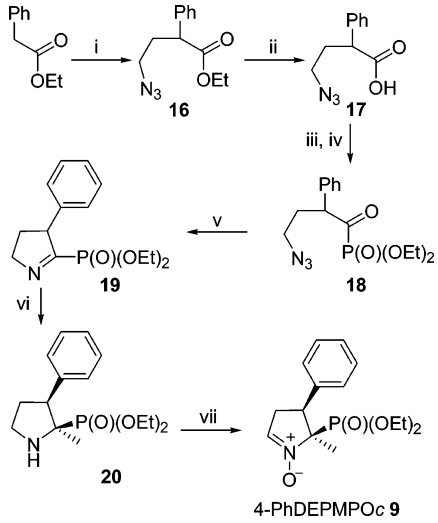
SCHEME 3. Synthesis of Nitrone 8[a]



[a] Reaction conditions: (i) Mg, Ac_2O-TMSCl, DMF, 70%; (ii) CSA, ethylene glycol, toluene, reflux, 8 h, 91%; (iii) $LiAlH_4$, THF, 0 °C, 85%; (iv) 3 N HCl, THF/H_2O, 4 h, then K_2CO_3, 1 h, 93%; (v) $HP(O)(OEt)_2$, cat. $BF_3{\cdot}OEt_2$, 7 d, 54%; (vi) H_2O_2, cat. Na_2WO_4, EtOH/H_2O, 0 °C, 72 h, 90%.

SCHEME 4 . Synthesis of Nitrone 9[a]



[a] Reaction conditions: (i) LDA, −78 °C, THF, then 1-azido-2-iodoethane, 12 h, rt, 59%; (ii) NaOH 1 M, EtOH, 3 h, rt, 100%; (iii) $(COCl)_2$, CH_2Cl_2; (iv) $P(OEt)_3$, CH_2Cl_2, 15 h, rt, 100% for the two steps; (v) PPh_3, Et_2O, 16 h, rt, 95%; (vi) cat. $BF_3{\cdot}OEt_2$, MeMgBr, 4 h, −78 °C, 76%; (vii) H_2O_2, cat. Na_2WO_4, EtOH/H_2O, 0 °C, 50 h, 64%

$LiAlH_4$ in THF at 0 °C led to **13** in 85% yield. The 3-phenyl-2-methylpyrroline[32] **14** was obtained in two steps from **13** by deprotection of the carbonyl by treatment with 3 N HCl followed by intramolecular cyclization of the amine in the presence of K_2CO_3 in a mixture of THF/H_2O. Reaction of **14** with diethyl phosphite in the presence of a catalytic amount of boron trifluoride diethyl etherate ($BF_3{\cdot}OEt_2$) at room temperature led to 3-PhDEPMP **15** in 54% yield. The addition of diethyl phosphite was stereoselective leading to the phosphorylated group in a trans position in relation with the phenyl group. Oxidation of **15** with H_2O_2 in the presence of a catalytic amount of sodium tungstate in a mixture of THF/H_2O led finally to 4-PhDEPMPO*t* **8** in 90% yield.

The cis nitrone **9**, 5-diethoxyphosphoryl-5-methyl-4-phenylpyrroline *N*-oxide, was prepared by the route described in Scheme 4. The azidoester **16** was obtained in 59% yield by alkylation of ethyl phenylacetate with 1-azido-2-iodoethane[33] by an adaptation of the procedure of Flintoft et al.[34] The azidoester **16** was easily saponified by treatment with dilute sodium hydroxide in methanol/water at room temperature leading to the acid **17** (100%). Synthesis of the iminophosphonate **19** was performed by the method of Vaultier et al.[35,36] Treatment of the acid **17** with oxalyl chloride in CH_2Cl_2 gave the crude acid chloride which was used without further purification. Arbuzov reaction of the azidoacid chloride with triethyl phosphite in CH_2Cl_2 led to the moisture-sensitive azido acylphosphonate **18** in a quantitative yield, after distillation under vacuum (10^{-3} Torr) of the volatile compounds. The crude acylphosphonate **18** was treated directly with 1 equiv of triphenylphosphane in anhydrous ether to give the 3-phenyl-2-diethoxyphosphorylpyrroline **19** in 95% yield. Addition of methylmagnesium bromide on the pyrroline **19** in the presence of $BF_3{\cdot}OEt_2$ at −78 °C in THF[37−39] afforded the 3-PhDEPMP*c* **20** in 76% yield. Attack of the nucleophile occurred anti to the phenyl group. Thus, the 3-phenyl group is now in the cis position in relation with the phosphoryl group. Oxidation of the pyrrolidine **20** by H_2O_2 in the presence of a catalytic amount of sodium tungstate in a mixture of THF/H_2O led to the nitrone **9**, 4-PhDEPMPO*c*, in 64% yield.

ESR Studies. In the present work, the spin-trapping capacities of the two nitrones **8** and **9** were tested only toward two oxygen-centered radical species, the superoxide and hydroxyl radicals, that are of the largest biological relevance.

(a) Spin Trapping of Superoxide Radical. Spin trapping of superoxide radical with 4-PhDEPMPO*c* **9** (20 mM) was performed in a buffered aqueous solution at pH 7.3, using two different superoxide generating systems. Similar signals were observed either by using hypoxanthine with xanthine oxidase (HX/XO system) in the presence of diethylenetriaminepentaacetic acid (DTPA) or by reaction with KO_2. When superoxide dismutase (SOD) was added to the HX/XO generating system, the ESR signal disappeared (Figure 2c) proving that spectrum (a) is due to the trapping of superoxide.

The two ESR spectra of the 4-PhDEPMPO*c*-OOH adducts consisted of a doublet of quadruplet showing a certain degree of dissymmetry together with a broadening of the lines (Figure 2a,b). The relative simplicity of the spectra can be attributed to the stereoselective

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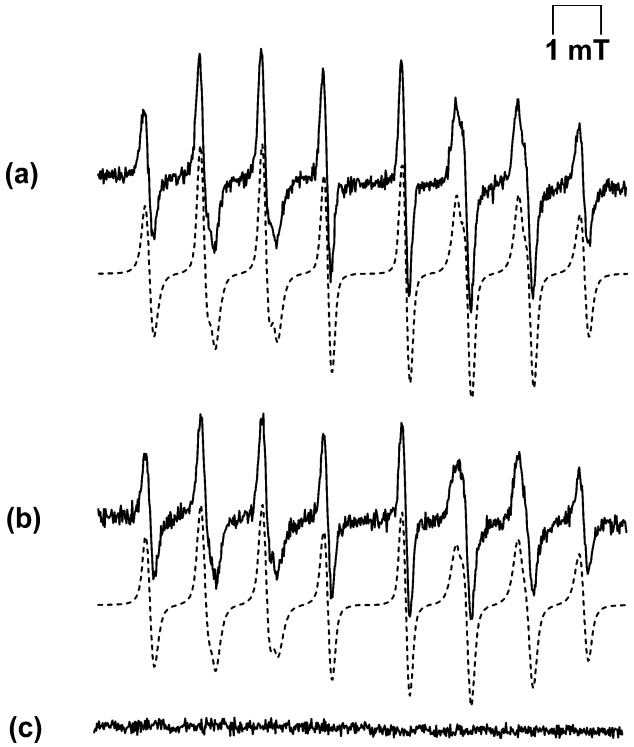

FIGURE 2. Spin trapping of superoxide radical by 4-PhDEPMPO*c* **9**: (a) Signal obtained after 5 min incubation of a mixture containing hypoxanthine (0.4 mM), xanthine oxidase (0.04 U mL^{-1}), DTPA (1 mM), and 4-PhDEPMPO*c* **9** (20 mM) in phosphate buffer (0.1 M, pH 7.3). (b) Signal obtained after 1 min incubation of a mixture containing KO_2 (10 mM) and 4-PhDEPMPO*c* **9** (20 mM) in phosphate buffer (0.1 M, pH 7.3). (c) As in (a) but in the presence of SOD (606 U mL^{-1}). The dotted lines in (a) and (b) represent the computer simulation of the spectra. Spectrometer settings: microwave power 10 mW; modulation amplitude, 0.702; time constant, 0.128 s; gain 10^5; sweep time, 83.89 s; conversion time, 0.819 s.

addition of the superoxide species on the less hindered face of 4-PhDEPMPO*c* giving only one adduct. The difference between the steric bulk of the methyl and the diethoxyphosphoryl groups present on the C-5 position combined with the presence of a phenyl group on the C-4 position forces the addition to take place on the face opposite to the phosphoryl group. The specific patterns of the two spectra (a) and (b) (Figure 2) could be explained either by an exchange model between two conformers in chemical equilibrium or by a superimposition model of two species. These species could be either two rigid conformers of the superoxide adduct or the combination of a hydroxyl adduct with one superoxide adduct conformer. Computer simulation of the spectra were made with the ROKI program[40] (dotted lines in Figure 2a,b). The best fit was obtained with the exchange model, with a regression parameter of 0.98739, the respective parameters being given in Table 1. Thus, the geometry of the 4-PhDEPMPO*c*-OOH adduct does not freeze the chemical exchange between two conformers of the superoxide spin adduct.

If we compare the ESR signals of the adducts of superoxide with 4-PhDEPMPO*c* and with DEPMPO, the spectrum of 4-PhDEPMPO*c*-OOH is simpler (8 lines) than the spectrum of DEPMPO-OOH showing 12 lines.[19] Moreover, the dissymmetry of the signal (compared to the baseline) and the alternate line width phenomenon are much less important in the case of the 4-PhDEPMPO*c*-OOH adduct than in the case of the corresponding DEPMPO-OOH adduct, even under the low amplitude modulation conditions that were used. Under these conditions, the γ_{AH} hyperfine coupling constants were not detected. The exchange rate constants between the respective pairs of conformers were not very different between 4-PhDEPMPO*c*-OOH and DEPMPO-OOH (0.34×10^8 s^{-1} for the system **9**/$O_2^{\bullet -}$ and 0.67×10^8 s^{-1} for the system **2**/$O_2^{\bullet -}$).[41] The values of the coupling constants of the 4-PhDEPMPO*c*-OOH and DEPMPO-OOH spin adducts are similar even if smaller differences between the coupling constants of the two conformers of 4-PhDEPMPO*c*-OOH compared to the corresponding values of the DEPMPO-OOH conformers can be noted (Table 1). These different facts can explain why, in the case of the 4-PhDEPMPO-OOH adduct, only a widening of the lines but no significant alternating line width phenomenon are observed.

(b) Spin Trapping of Hydroxyl Radical. The hydroxyl radical spin adduct, 4-PhDEPMPO*c*-OH, was obtained by incubation of 4-PhDEPMPO*c* **9** (20 mM) with a mixture of H_2O_2 (2 mM), $FeSO_4$ (2 mM), and DTPA (1 mM) in 0.1 M phosphate buffer at pH 7.3. The ESR spectrum of the 4-PhDEPMPO*c*-OH adduct consisted of 12 main lines (Figure 3), compared to the doublet of quadruplet detected for the corresponding DEPMPO-OH adduct.[19] This signal disappeared upon addition of catalase to the incubation mixture. Computer simulation showed that spectrum (a) results from the superimposition of the signals of the adducts of OH (80.4%), alkyl (14%) and one unidentified species. The parameters are given in Table 1.

(c) Kinetics of Decay of the Superoxide Adduct. The half-life time of the superoxide spin adducts depends on a variety of parameters, such as nitrone concentration, nature of the generating system, pH, etc.[42,43] In the classical method used for determination of the kinetics of decay of the DEPMPO-OOH adduct, the HX/XO system in the presence of DEPMPO in a phosphate buffer is used to generate the DEPMPO-OOH adduct.[41] Once the adduct concentration has reached an optimum value, the formation of superoxide is stopped by addition of a large amount of SOD. As superimposition of the ESR spectra of the DEPMPO-OOH and DEPMPO-OH adducts does not lead to overlap of the two signals, the decay of DEPMPO-OOH can be monitored by following the decrease of an appropriate line of the spectrum. In contrast, in the case of the 4-PhDEPMPOc nitrone, superimposition of the ESR signals of the two adducts (OOH and OH) leads to complete overlap of the two signals, and therefore, the decrease of one line cannot be used to determine the kinetics of decay of the 4-PhDEPMPOc-OOH adduct.

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TABLE 1. Simulated Coupling Constants for Superoxide and Hydroxyl Adducts of 4-PhDEPMPO*c* 9

nitrone/R•	generating system	diastereoisomer	conformer	A_p (mT)	A_N (mT)	$A_{H\beta}$ (mT)	$A_{H\gamma}$ (mT)
9/$O_2^{\bullet-}$	HX/XO system or KO_2/18-crown-6/DMSO in 0.1 M phosphate buffer	trans (100%)	T_1 (38%)[a]	5.458	1.284	1.316	0.074
			T_2 (62%)[a]	5.241	1.289	1.08	
9/HO•	Fenton reaction in 0.1 M phosphate buffer	trans (80.4%)		5.333	1.369	0.982	
		alkyl (14%)		5.594	1.410	1.660	

[a] Exchange rate constant between T_1 and T_2, $K = 0.34 \times 10^8$ s^{-1}.

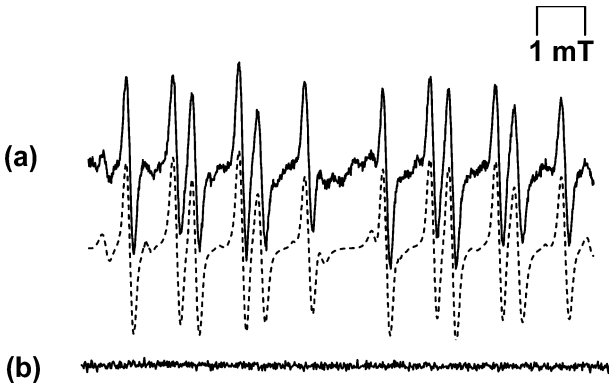


FIGURE 3. Spin trapping of hydroxyl radical by 4-PhDEPMPO*c* **9**. (a) Signal obtained after 1 min incubation of a mixture containing 4-PhDEPMPO*c* **9** (20 mM), H_2O_2 (2 mM), $FeSO_4$ (2 mM), DTPA (1 mM) in phosphate buffer (0.1 M, pH 7.3). (b) As in (a) but in the presence of catalase (600 U mL^{-1}). The dotted line in (a) represents the computer simulation of the spectrum. Spectrometer settings: microwave power 10 mW; modulation amplitude, 0.497; time constant, 0.128 s; gain 10^5; sweep time, 83.89 s; conversion time, 0.819 s.

A different method was used, taking advantage of the fact that the ROKI program is able to compute the concomitant evolution of different species. To compare the kinetics of decay of the 4-PhDEPMPO*c*-OOH and DEPMPO-OOH adducts, the two trapping systems were tested in parallel under the same set of conditions. A mixture containing hypoxanthine (0.4 mM), xanthine oxidase (0.04 U mL^{-1}), DTPA (1 mM), and the nitrone, either 4-PhDEPMPO*c* **9** or DEPMPO **2** (20 mM), in phosphate buffer (0.1 M, pH 7.3) was incubated at room temperature. Once the adduct concentration had reached a significant value (approximately 7 min), the formation of superoxide anion radicals was stopped by addition of a large amount of SOD and the spectra were recorded. Fast scan recording (30 s) gave the complete ESR spectrum. Slow scan recording (5368.71 s) was used to determine the decay rate of the superoxide spin adducts, which was computed by comparing the variation of ESR amplitudes recorded in the two different sweeping conditions (Figure 4). The ROKI program was first used to simulate the fast recording spectra in the traditional manner. Then the slow recording spectra were simulated, and the previous ESR amplitudes were modulated by a kinetic function describing either first or second order or complex decay process.

The computations showed that the half-life times were 14.5 min for the 4-PhDEPMPOc-OOH adduct and 15.5 min for the DEPMPO-OOH adduct and that the decay of the 4-PhDEPMPOc-OOH has a predominant first-order character. In contrast to the 3-PhDEPMPO*c*-OOH

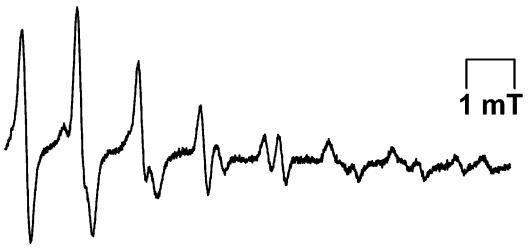
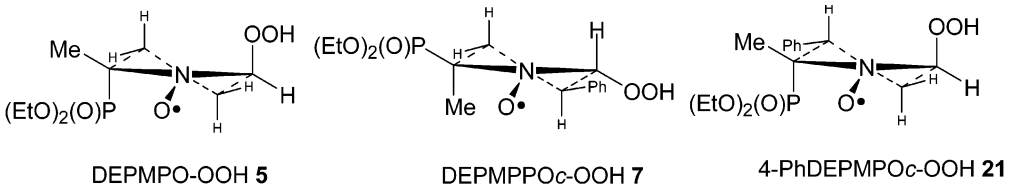


FIGURE 4. Kinetics of decay of the superoxide spin adducts. Signal obtained after incubation of a mixture containing hypoxanthine (0.4 mM), xanthine oxidase (0.04 U mL^{-1}), DTPA (1 mM), and 4-PhDEPMPO*c* **9** (20 mM) in phosphate buffer (0.1 M, pH 7.3). The formation of superoxide anion radicals is stopped by addition of a large amount of SOD (606 U mL^{-1}), and the spectrum was recorded by slow scan (5368.71 s). Spectrometer settings: microwave power 10 mW; modulation amplitude, 0.625; time constant, 0.128 s; gain 10^5; sweep time, 5368.71 s; conversion time, 2621.44 s.

(half-life time of 2 min),[30] the superoxide adducts obtained with the two nitrones **2** and **9** showed quite similar values. Thus, the steric hindrance induced by the presence of the 4-phenyl group does not lead to a destabilization of the superoxide spin adduct. In fact, addition of the superoxide radical on the face opposite to the phosphoryl group results in a conformation of the nitroxide in which the HOO and phosphoryl groups are in a pseudoaxial position, favorable for the stabilization by anomeric or hyperconjugative effects.[26,44] Moreover, the relative cis position between the 5-phosphoryl and 4-phenyl groups results, upon addition of the superoxide radical, in a conformation of the spin adduct **21**, in which the 4-phenyl group is in an equatorial position (Figure 5). A similar conformation is observed in spin adduct **5**, which is also stabilized. In contrast, the phosphoryl and OOH substituents occupy equatorial positions in spin adduct **7**, a fact which is not favorable for the stabilizing anomeric or hyperconjugative effects.[26,44]

(d) Addition on 4-PhDEPMPOt, 8. When addition of the two oxygen radical species, HOO• and HO•, was performed with the trans diastereoisomer (4-PhDEPMPO*t*, **8**), mixtures of diastereoisomeric spin adducts were observed, leading to complex ESR spectra. In the case of nitrone **8**, the steric bulk of the 5-phosphoryl and 4-phenyl groups is evenly distributed on the two faces of the pyrroline ring and stereoselectivity in the addition step cannot be observed. Therefore, the trans diastereoisomer **8** does not present the same interest as the cis nitrone **9** for spin-trapping experiments.

(44) Clément, J.-L.; Ferré, N.; Siri, D.; Karoui, H.; Rockenbauer, A.; Tordo, P. *J. Org. Chem.* **2005**, *70*, 1198−1203.



DEPMPO-OOH **5** DEPMPPO*c*-OOH **7** 4-PhDEPMPO*c*-OOH **21**

FIGURE 5. Conformations of the superoxide adducts **5**, **7**, and **21**.

Conclusion

From the two diastereoisomeric C(4)-phenyl analogues of DEPMPO **8** and **9**, only the cis isomer, 4-PhDEPMPO*c* **9**, trapped hydroxyl and superoxide radicals in a stereoselective manner. The ESR spectrum of the 4-PhDEPMPO*c*-OOH spin adduct was more easily assignable than that of DEPMPO-OOH since the alternating line width phenomenon due to a chemical exchange has considerably been reduced. Moreover the 4-PhDEPMPO*c*-superoxide spin adduct **21** has a persistency similar to the one of DEPMPO-OOH (∼15 min) but a greater persistency than the DEPMPPO-OOH adduct **7** ($T_{1/2\ \mathrm{DEPMPPO}} < 2$ min). The conformational effect exerted by the presence of a phenyl group on the pyrroline ring is strongly dependent upon its position. When the phenyl is on the C-3 position, a strong destabilization takes place.[26] In contrast, when the phenyl is on the C-4 position, a modest stabilization of the superoxide adduct is observed. Thus, the presence of a phenyl group on the pyrroline ring in the most suitable position as in the 4-PhDEPMPO-c **9** does not freeze enough the conformational flexibility of the ring to forbid the occurrence of the particular conformation of the superoxide spin adduct that is prone to dismutation of the nitroxide, leading to decrease of the ESR signal.

Experimental Section

General Methods. The solvents were distilled under dry argon atmosphere: THF, Et_2O, and toluene in the presence of sodium and benzophenone and CH_2Cl_2 in the presence of P_2O_5. All chemicals and organic solvents were commercially available and were used as supplied. The reactions were monitored by CCM using silica gel and by ^{31}P NMR. Crude materials were purified by flash chromatography on silica gel 60 (0.040−0.063 mm). ^{31}P NMR, ^1H NMR, and ^{13}C NMR spectra were recorded at 121.49, 300.13, and 75.54 MHz, respectively, or at 81.01, 200.13, and 50.32 MHz, respectively. ^{31}P NMR was taken in $CDCl_3$ using 85% H_3PO_4 as an internal standard with broadband ^1H decoupling. ^1H NMR and ^{13}C NMR were taken in $CDCl_3$ using TMS or $CDCl_3$ as internal reference, respectively. Chemical shifts (δ) are reported in ppm and J values in hertz. The assignments of ^1H NMR signals of the compounds **20** and **9** were facilitated by use of the HMQC ($^1H-^{13}C$) sequence. Melting points are uncorrected. Elemental analyses and mass spectra were determined at the University of Aix-Marseille III. HRMS was determined at the Service Central d'Analyse du CNRS at Vernaison, France.

Synthesis of the Trans Nitrone (4-PhDEPMPOt) (8). 3-(2-Methyl-1,3-dioxolan-2-yl)-3-phenylpropionitrile (12). In a Dean−Stark apparatus a mixture of 3-phenyl-4-oxopentanenitrile[31] (2.5 g, 14 mmol), ethylene glycol (6.5 mL, 0.11 mol), and camphorsulfonic acid (0.5 g, 2.1 mmol) in anhydrous toluene (40 mL) was stirred under reflux for 8 h. The solvent was distilled under reduced pressure. The oily residue was purified by flash chromatography over silica gel (pentane/Et_2O 3:1) to afford **12** as a white solid (2.82 g, 91%): mp 65.9 °C; ^1H NMR (200.13 MHz) δ 7.35−7.25 (5H, m), 4.05−3.90 (4H, m), 3.26 (1H, dd, J = 5.1, 9.8), 2.92 (1H, dd, J = 16.8, 5.1), 2.72 (1H, dd, J = 16.8, 9.8), 1.13 (3H, s); ^{13}C NMR (50.32 MHz) δ 137.8 ($1C^{IV}$, s), 128.8 (2C, d), 128.5 (2C, s), 127.8 (1C, s), 119.1 (1C, s), 109.8 ($1C^{IV}$, s), 65.3 (1C, s), 64.4 (1C, s), 50.7 (1C,s), 22.6 (1C, s), 18.8 (1C, s). Anal. Calcd for $C_{13}H_{15}NO_2$: 217.26; C, 71.87; H, 6.96; N, 6.45. Found: C, 72.06; H, 6.85; N, 6.41.

3-(2-Methyl-1,3-dioxolan-2-yl)-3-phenylpropylamine (13). Lithium aluminum hydride (6 mL of a 1 M solution in THF) was added dropwise to a solution of **12** (430 mg, 1.98 mmol) in 10 mL of THF at 0 °C under argon. The mixture was stirred for 2 h at 0 °C then 7 h at room temperature. Potassium hydroxide (1 mL of a 10% aqueous solution) was added dropwise at 0 °C. The solution was filtered and diluted with CH_2Cl_2 (50 mL). The organic layer was washed with water (10 mL) and brine (10 mL). The organic layer was dried over Na_2SO_4, filtered, and distilled under reduced pressure to yield **13** as a pale yellow oil (370 mg, 85%): ^1H NMR (200.13 MHz) δ 7.40−7.10 (5H, m), 4.25−3.82 (4H, m), 2.86 (1H, dd, J = 10.9, 4.1), 2.44 (2H, td, J = 8.5, 6.2), 2.10−1.73 (2H, m), 1.27 (2H, s), 1.13 (3H, s); ^{13}C NMR (75.47 MHz) δ 140.4 ($1C^{IV}$, s), 129.2 (2C, d), 127.9 (2C, s), 126.5 (1C, s), 111.2 ($1C^{IV}$, s), 65.0 (1C, s), 64.5 (1C, s), 52.1 (1C, s), 40.3 (1C, s), 33.2 (1C, s) 22.9 (1C, s).

2-Methyl-3-phenylpyrroline (14). A solution of **13** (3.7 g, 16.8 mmol) in a mixture of THF (75 mL) and aqueous HCl (50 mL of a 3 M solution) was stirred for 4 h. The mixture was colored in red in a few moments. Solid K_2CO_3 was added to the solution up to pH 9, and the mixture was stirred for 1 h. A fluorescent orange color appeared. The aqueous layer was then saturated with NaCl and extracted with CH_2Cl_2 (3 × 60 mL) and ethyl acetate (50 mL). The combined organic layers were dried over Na_2SO_4 and distilled under reduced pressure to afford **14** (2.5 g, 93%) as a yellow oil: lit.[32] bp 58−60 °C/0.25 mm); ^1H NMR (300.13 MHz) δ 7.38−7.20 (3H, m), 7.15−7.10 (2H, m), 3.85−3.75 (2H, m), 2.55−2.39 (1H, m), 1.98−1.88 (2H, m), 1.85 (3H, s); ^{13}C NMR (75.47 MHz) δ 176.3 ($1C^{IV}$, s), 141.7 ($1C^{IV}$, s), 128.7 (2C, s), 127.5 (2C, s), 126.7 (1C, s), 59.5 (1C, s), 57.6 (1C, s), 33.5 (1C, s), 18.0 (1C, s).

2-(Diethoxyphosphoryl)-2-methyl-3-phenylpyrrolidine (15). A mixture of **14** (0.9 g, 5.65 mmol) and boron trifluoride ethyl etherate (71 μL, 0.565 mmol) was stirred for 20 min. Diethyl phosphite (86.8 μL, 6.78 mmol) was then added, and the solution was stirred at room temperature for 7 days. After dilution with water (2 mL), aqueous HCl (5%) was added to the mixture to reach pH 2, and the mixture was washed with CH_2Cl_2. The aqueous layer was treated with a saturated $NaHCO_3$ solution to reach pH 9. After extraction with CH_2Cl_2 (3 × 10 mL), the organic layer was dried over Na_2SO_4 and distilled under reduced pressure to yield the pyrrolidine **15** (900 mg, 54%) as a brown oil: ^{31}P NMR (81.01 MHz) δ 29.30; ^1H NMR (200.13 MHz) δ 7.81−7.32 (5H, m), 4.59−4.18 (4H, m), 3.94 (1H, m), 3.54−3.24 (2H, m), 2.74−2.23 (3H, m), 1.52 (6H, t, J = 7.1), 1.14 (3H, d, J = 16.3); ^{13}C NMR (50.32 MHz) δ 139.9 ($1C^{IV}$, d, J = 4.6), 129.0 (2C, s), 127.6 (2C, s), 126.4 (1C, s), 62.5 (1C, d, J = 164.1), 62.3 (1C, d, J = 7.4), 62.1 (1C, d, J = 8.0), 48.8 (1C, d, J = 4.0), 45.6 (1C, d, J = 6.3), 32.8 (1C, d, J = 5.7), 20.5 (1C, d, J = 5.7), 16.3 (2C, d, J = 5.7).

***trans*-5-(Diethoxyphosphoryl)-5-methyl-4-phenylpyrroline *N*-Oxide (4-PhDEPMPOt) (8).** A solution of the pyrrolidine **15** (360 mg, 1.2 mmol) in ethyl alcohol (3 mL) was added to a solution of sodium tungstate (40 mg, 0.12 mmol) in demineralized water (6 mL). The mixture was cooled at −5 °C, and hydrogen peroxide (0.25 mL of a 30% aqueous solution, 2.54 mmol) was then added dropwise over a period of 1 h. The mixture was then stirred at 3−4 °C for 48 h. The aqueous layer was saturated with sodium chloride and extracted with CH_2Cl_2 (3 × 10 mL). The organic layer was dried over Na_2SO_4 and distilled under reduced pressure. The residual oil was purified by flash chromatography on silica gel (CH_2Cl_2/EtOH 9:1) to afford **8** (300 mg, 80%): ^{31}P NMR (121.49 MHz) δ 21.81; ^{1}H NMR (300.13 MHz) δ 7.35−7.26 (3H, m), 7.18−7.09 (3H, m), 4.37−4.15 (4H, m), 3.42−3.28 (1H, m), 2.86−2.74 (1H, m), 2.65−2.10 (1H, m), 1.37 (3H, t, J = 7.0), 1.35 (3H, t, J = 7.2), 1.18 (3H, d, J = 15.8); ^{13}C NMR (75.47 MHz) δ 139.1 (1C^{IV}, d, J = 9.7), 134.2 (1C, d, J = 9.2), 128.6 (2C, d), 128.6 (2C, s), 127.8 (1C, s), 78.4 (1C, d, J = 173.8), 64.2 (1C, d, J = 6.3), 62.8 (1C, d, J = 8.0), 44.9 (1C, d, J = 1.8), 34.1 (1C, s), 16.4 (2C, d, J = 6.3), 16.3 (1C, s); HRMS calcd for $C_{15}H_{22}NO_4P$ $[C_{15}H_{22}NO_4P]^+ + Na^+$ 334.1184, found 334.1172.

Synthesis of the Cis Nitrone (4-PhDEPMPOc) (9). 4-Azido-2-phenylbutyric Acid Ethyl Ester (16). Under argon, a stirred solution of lithium diisopropylamide (36.5 mL of a 2 M solution in hexanes) in THF (20 mL) was cooled to −78 °C, and a solution of ethyl phenylacetate (8 g, 0.049 mol) in THF (40 mL) was added dropwise. The mixture was stirred at −78 °C for 1 h. A solution of 1-azido-2-iodoethane[33] (12.42 g, 0.0633 mol) in THF (20 mL) was added over a period of 10 min. The solution was gradually warmed to room temperature and stirred for 12 h. A saturated aqueous NH_4Cl solution was added to the mixture up to pH 8, and the aqueous layer was extracted with CH_2Cl_2 (3 × 80 mL). The organic layers were combined, washed with brine, and dried over Na_2SO_4. The solvent was distilled under reduced pressure, and the residual oil was purified by flash chromatography on silica gel (gradient of pentane/CH_2Cl_2 4:1 to pure CH_2Cl_2) to afford **16** as a pale yellow oil (6.73 g, 59%): ^{1}H NMR (300.13 MHz) δ 7.39−7.20 (5H, m), 4.24−4.01 (2H, m), 3.68 (1H, t, J = 7.7), 3.29 (1H, ddd, J = 12.3, 6.3, 6.6), 3.18 (1H, ddd, J = 12.3, 6.3, 7.2), 2.33 (1H, m, J = 14.1, 7.7, 7.2, 6.5), 2.01 (1H, m, J = 14.1, 7.7, 6.3, 6.3), 1.20 (3H, t, J = 7.1). ^{13}C NMR (75.47 MHz) δ 173.1 (1C^{IV}, s), 138.0 (1C^{IV}, s), 128.8 (2C, s), 127.9 (2C, s), 127.5 (1C, s), 60.9 (1C, s), 49.2 (1C, s), 48.5 (1C, s), 32.5 (1C, s), 14.0 (1C, s). Anal. Calcd for $C_{12}H_{15}N_3O_2$: 233.27; C, 61.79; H, 6.48; N, 18.01. Found: C, 61.91; H, 6.50; N, 18.01.

4-Azido-2-phenylbutyric Acid (17). A minimum of methanol was added to a mixture of the azidoester **16** (6.62 g, 0.028 mol) and aqueous solution of NaOH (1 N, 0.035 mol) to make the reaction mixture homogeneous. After the mixture was stirred for 3 h at room temperature, the methanol was distilled under reduced pressure. The aqueous solution was acidified with HCl (10% aqueous solution) to reach pH 0. The aqueous phase was extracted with Et_2O (3 × 70 mL), and the organic phase was dried over Na_2SO_4. The solvent was distilled under reduced pressure to afford **17** as a white solid (5.8 g, 100%): mp 58−59 °C; ^{1}H NMR (300.13 MHz) δ 10.11−9.19 (1H, s, OH), 7.30−7.13 (5H, m), 3.64 (1H, t, J = 7.8), 3.22 (1H, ddt, J = 12.7, 6.4, 6.4), 3.09 (1H, ddd, J = 12.7, 6.4, 7.4), 2.25 (1H, m, J = 7.4, 6.4, 14.2, 7.8), 1.94 (1H, m, J = 14.2, 7.8, 6.4, 6.4); ^{13}C NMR (75.47 MHz) δ 179.2 (1C^{IV}, s), 137.1 (1C^{IV}, s), 128.9 (2C, s), 128.0 (2C, s), 127.8 (1C, s), 48.9 (1C, s), 48.3 (1C, s), 31.9 (1C, s). Anal. Calcd for $C_{10}H_{11}N_3O_2$: 205.21; C, 58.53; H, 5.40; N, 20.48. Found: C, 58.77; H, 5.49; N, 20.30.

4-Azido-2-phenylbutyrylphosphonic Acid Diethyl Ester (18). Under argon, oxalyl chloride (2.31 mL, 0.027 mol) was added via a syringe to a solution of the acid **17** (3.68 g, 0.018 mol) in CH_2Cl_2 (11 mL) at 0 °C. The solution was stirred at room temperature for 1 h and refluxed for 2 h. After the solution was cooled to room temperature, distillation of the volatiles under reduced pressure (10^{-3} Torr, 2 h) afforded a crude oil that was dissolved in CH_2Cl_2 (20 mL). Triethyl phosphite (3.38 mL, 0.019 mol) was then added dropwise over a period of 2 h at 0 °C. The mixture was kept at room temperature for 15 h. Distillation under reduced pressure (10^{-3} Torr) afforded **18** as a pale yellow oil (5.8 g, 100%): ^{31}P NMR (121.49 MHz) δ −2.69; ^{1}H NMR (300.13 MHz) δ 7.41−7.18 (5H, m), 4.45 (1H, t, J = 7.6), 4.18−3.94 (2H, m), 3.87−3.68 (2H, m), 3.27 (1H, ddt, J = 12.5, 6.5, 6.6), 3.11 (1H, ddd, J = 12.5, 6.5, 8.0), 2.31 (1H, m, J = 8.0, 6.6, 14.2, 7.6), 1.98 (1H, m, J = 14.2, 7.6, 6.5, 6.5), 1.27 (3H, t, J = 7.1), 1.10 (3H, t, J = 7.2); ^{13}C NMR (75.47 MHz) δ 209.3 (1C^{IV}, d, J = 166.3), 134.2 (1C^{IV}, s), 129.3 (2C, s), 129.0 (2C, s), 128.0 (1C, s), 63.7 (1C, d, J = 7.1), 63.2 (1C, d, J = 7.1), 55.2 (1C, d, J = 53.3), 48.7 (1C, s), 30.5 (1C, d, J = 3.9), 16.1 (1C, d, J = 5.5), 15.9 (1C, d, J = 6.0). Anal. Calcd for $C_{14}H_{20}N_3O_4P$: 325.30; C, 51.69; H, 6.20. Found: C, 51.89; H, 6.17.

2-Diethoxyphosphoryl-3-phenylpyrroline (19). Under argon, a solution of triphenylphosphane (4.3 g, 0.018 mol) in Et_2O (30 mL) was added dropwise to a solution of the acylphosphonate **18** (5.8 g, 0.018 mol) in Et_2O (20 mL) at 10 °C. The mixture was stirred at room temperature for 16 h. The solution was filtered, and the solid was washed with anhydrous Et_2O. After distillation of the solvent under reduced pressure, a mixture of anhydrous Et_2O/petroleum ether (1/1) was added. The precipitate was filtered and washed with anhydrous Et_2O at 0 °C. After distillation of the solvent under reduced pressure, the procedure was repeated until complete removal of triphenylphosphine oxide. The iminophosphonate **19** was obtained as a pale yellow oil (4.8 g, 95%): bp 140 °C/10^{-3} Torr; ^{31}P NMR (121.49 MHz) δ 7.94; ^{1}H NMR (300.13 MHz) δ 7.36−7.27 (3H, m), 7.17−7.13 (2H, m), 4.32−4.70 (6H, m), 2.56−2.39 (1H, m, J = 13.2, 7.0), 2.03−1.88 (1H, m, J = 18.7, 13.2), 1.38−1.24 (1H, m, J = 18.7, 7.0), 1.21 (3H, t, J = 6.9), 1.07 (3H, t, J = 6.8); ^{13}C NMR (75.47 MHz) δ 173.8 (1C^{IV}, d, J = 207.9), 140.4 (1C^{IV}, s), 128.5 (2C, s), 127.7 (2C, s), 126.9 (1C, s), 63.4 (1C, d, J = 34.4), 62.8 (1C, d, J = 6.2), 62.2 (1C, d, J = 6.2), 57.7 (1C, d, J = 30.4), 32.6 (1C, d, J = 5.1), 15.9 (1C, d, J = 6.6), 15.8 (1C, d, J = 6.6).

2-(Diethoxyphosphoryl)-2-methyl-3-phenylpyrrolidine (20). Under argon, boron trifluoride diethyl etherate (1.97 mL, 7.46 mmol) was added dropwise to a solution of the iminophosphonate **19** (1 g, 3.55 mmol) in THF (20 mL) at −78 °C. The mixture was stirred for 2 h at this temperature. Methylmagnesium bromide (2.5 mL of a 3 M solution in THF) was added dropwise over a period of 1 h at −78 °C. The solution was stirred at this temperature for 4 h, and then a saturated $NaHCO_3$ aqueous solution was added to the mixture kept at −78 °C. The aqueous layer was washed with CH_2Cl_2 (3 × 50 mL). The aqueous phase was treated with an aqueous HCl (10%) solution to reach pH 1. The aqueous layer was washed with CH_2Cl_2 and treated with a saturated $NaHCO_3$ solution to reach pH 9. After extraction with CH_2Cl_2, the organic layer was dried over Na_2SO_4 and the solvent distilled under reduced pressure to give the pyrrolidine **20** as a pale yellow oil (0.8 g, 76%): ^{31}P NMR (121.49 MHz) δ 26.50; ^{1}H NMR (300.13 MHz) δ 7.46−7.43 (2H, d, J = 7.37, H_{ar}), 7.33−7.18 (3H, m, H_{ar}), 4.04−3.92 (2H, m, CH_2OP), 3.74−3.62 (1H, m, J = 6.7, CHOP), 3.47−3.30 (2H, m, J = 6.7, CHOP, Hd, dtd, J_{HdHe} = 16.4, J_{HdHb} = 8.7, J_{HdHc} = 2.4), 3.17 (He, ddd, J_{HeHd} = 16.4, J_{HeHb} = 9.1, J_{HeP} = 1.1), 3.02 (Ha, ddd, J_{HaP} = 30.9, J_{HaHb} = 12.1, J_{HaHc} = 7.4), 2.81 (Hb, m, J_{HbHc} = 17.6, J_{HbHa} = 12.1, J_{HbHe} = 9.1), 2.18−2.05 (Hc, m, J_{HcHb} = 17.6, J_{HcHa} = 7.4, J_{HcHd} = 2.4), 1.91 (1H, s, NH), 1.47 (3H, d, J_{HP} = 13.6, CH_3C^{IV}), 1.16 (3H, t, J = 7.2, CH_3CH_2OP), 0.88 (3H, t, J = 7.2, CH_3-CH_2OP); ^{13}C NMR (75.47 MHz) δ 138.0 (1C^{IV}, s, C_{ar}), 128.9 (2C, s, C_{ar}), 127.4 (2C, s, C_{ar}), 126.4 (1C, s, C_{ar}), 64.7 (1C, d, J_{CP} = 158.6, $C^{IV}NH$), 61.9 (1C, d, J_{CP} = 7.7, CH_2OP), 60.6 (1C, d, J_{CP} = 7.7, CH_2OP), 55.5 (1C, d, J_{CP} = 5.5, *CH*Ph), 44.1 (1C, d, J_{CP} = 2.7, CH_2NH), 30.6 (1C, s, CH_2CH), 24.0 (1C, d, J_{CP} = 6.0, CH_3C^{IV}), 16.2 (1C, d, J_{CP} = 5.5, CH_3CH_2OP), 15.6 (1C, d, J_{CP} = 6.0, CH_3CH_2OP).

The pyrrolidine **20** was characterized as the picrate, after recrystallization in ethyl alcohol. Anal. Calcd for $C_{21}H_{27}N_4O_{10}P$: 526.43; C, 47.91; H, 5.17; N, 10.64. Found: C, 47.93; H, 5.31; N, 10.46.

***cis*-5-(Diethoxyphosphoryl)-5-methyl-4-phenylpyrroline *N*-Oxide (4-PhDEPMPOc) (9).** A solution of the pyrrolidine **20** (300 mg, 1 mmol) in ethyl alcohol (3 mL) was added to a solution of sodium tungstate (33 mg, 0.1 mmol) in demineralized water (6 mL). The mixture was cooled at −5 °C, and hydrogen peroxide (0.21 mL of a 30% aqueous solution, 2.22 mmol) was then added dropwise over a period of 1 h. The mixture was then stirred at 3−4 °C for 50 h. The aqueous layer was saturated with sodium chloride and extracted with CH_2Cl_2 (3×10 mL). The organic layer was dried over Na_2SO_4 and distilled under reduced pressure. The crude oil was purified by flash chromatography on silica gel (CH_2Cl_2/EtOH 95:5) to afford **9** (200 mg, 64%): ^{31}P NMR (121.49 MHz) δ 19.93; ^{1}H NMR (300.13 MHz) δ 7.55−7.25 (5H, m, H_{ar}), 7.19 (1H, q, J = 2.5, CHN), 4.24−4.04 (2H, m, J = 7.0, CH_2OP), 3.75−3.58 (1H, m, J = 15.5, CHOP), 3.67 (Ha, ddd, J_{HaP} = 20.5, J_{HaHc} = 11.7, J_{HaHb} = 9.7), 3.51 (Hb, dtd, J_{HbHc} = 20.2, J_{HbHa} = 9.7, J_{HbP} = 2.4), 3.35 (1H, m, J = 15.5, CHOP), 2.69 (Hc, dtd, J_{HcHb} = 20.2, J_{HcHa} = 9.7, J_{HcP} = 2.5), 1.77 (3H, d, J_{HP} = 13.5, CH_3C^{IV}), 1.25 (3H, t, J = 7.1, CH_3CH_2OP), 0.75 (3H, t, J = 7.1, CH_3CH_2OP); ^{13}C NMR (75.47 MHz) δ 135.7 (1C, d, J_{CP} = 6.0, HCN), 135.3 ($1C^{IV}$, d, J_{CP} = 3.3, C_{ar}), 129.16 (2C, s, C_{ar}), 128.1 (2C, s, C_{ar}), 127.7 (1C, s, C_{ar}), 78.5 (1C, d, J_{CP} = 149.8, $C^{IV}NO$), 64.1 (1C, d, J_{CP} = 6.0, CH_2OP), 61.0 (1C, d, J_{CP} = 7.7, CH_2OP), 52.9 (1C, d, J_{CP} = 2.2, *CH*Ph), 30.7 (1C, d, J_{CP} = 1.7, CH_2CH), 19.6 (1C, s, CH_3C^{IV}), 16.3 (1C, d, J_{CP} = 6.0, CH_3CH_2OP), 15.4 (1C, d, J_{CP} = 7.1, CH_3CH_2OP); ESI/MS/MS (20 eV) *m/z* 312.1 (M^+ + H), 294.3 (51.3), 266.1 (19.5), 238.0 (64.6), 219.9 (16.8), 174.1 (74.3).

Spin-Trapping Studies. (a) General Information. Xanthine oxidase (XO), bovine erythrocyte superoxide dismutase (SOD), catalase, diethylenetriaminepentaacetic acid (DTPA), and other chemicals were purchased from commercial suppliers.

(b) ESR Measurements. ESR spectra were recorded at room temperature using a spectrometer at 9.5 GHz (X-band) employing 100 kHz field modulation. Reaction mixtures were prepared in a chelex-treated phosphate buffer (0.1 M, pH 7.3). Standard ESR spectra were simulated using the ESR software developed by A. Rockenbauer from the Central Research Institute of Chemistry, Hungary.[40]

(c) Superoxide Trapping: Hypoxanthine-Xanthine Oxidase System. Xanthine oxidase (0.04 U mL^{-1}) was added to a solution of 4-PhDEPMPO (20 mM), DTPA (1 mM), and hypoxanthine (0.4 U mL^{-1}) in phosphate buffer (0.1 M, pH 7.3).

When SOD (606 U mL^{-1}) was added to the HX/XO generating system, the ESR signal was not observed.

(d) Superoxide Trapping: KO_2/18-Crown-6 System. The ESR signal was observed upon incubating the reaction mixture obtained after adding a DMSO solution of KO_2 (10 mM final concentration) and 18-crown-6 ether (10 mM final concentration) to a phosphate buffer solution (0.1 M) containing 4-PhDEPMPOc (20 mM final concentration).

(e) Hydroxyl Trapping. The hydroxyl radical was generated by addition of $FeSO_4$ (2 mM) to a solution of 4-PhDEPMPO (20 mM), DTPA (1 mM), and H_2O_2 (2 mM) in phosphate buffer (0.1 M, pH 7.3).

No ESR signal was observed when catalase (600 U mL^{-1}) was added to the incubation mixture.

(f) Kinetics of Decay of the Superoxide Spin Adducts. The previously described HX/XO system was used to generate superoxide in phosphate buffer (0.1 M, pH 7.3). The spin-trap concentration was 20 mM for DEPMPO and 4-PhDEPMPOc. Once the adduct concentration had reached a significant value (approximately 7 min), the formation of superoxide anion radicals was stopped by addition of a large amount of SOD (606 U mL^{-1}), and the spectrum was recorded by slow scan (5368.71 s). Computer simulations were performed using the ROKI[40] program developed by A. Rockenbauer at the Central Research Institute for Chemistry in Budapest. Spectrometer settings: microwave power 10 mW; modulation amplitude, 0.625; time constant, 0.128 s; gain 10^5; sweep time, 5368.71 s; conversion time, 2621.44 s.

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