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Design of New Derivatives of Nitrone DEPMPO Functionalized at C-4 for Further Specific Applications in Superoxide Radical Detection

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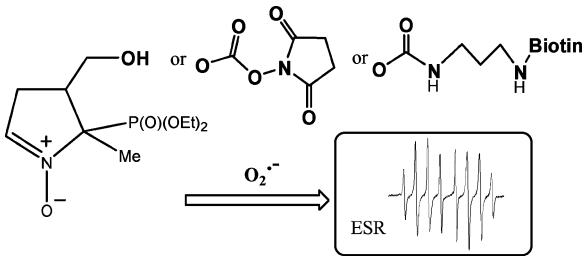

A general synthetic route to prepare derivatives of the DEPMPO nitrone (5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide) functionalized at C-4 was established via the synthesis of 4-HMDEPMPO nitrone (5-diethoxyphosphoryl-4-hydroxymethyl-5-methyl-1-pyrroline-*N*-oxide) that was obtained from reduction of the nitro compound **1**. (4*R**,5*S**)-4-HMDEPMPO was successfully separated from its minor diastereoisomer and could be used to generate various substituted analogues. Among them, 4-NHSDEPMPO, 5-diethoxyphosphoryl-4-succinimidyloxycarbonyloxymethyl-5-methyl-1-pyrroline-*N*-oxide, constitutes a NH_2-reactive precursor for further conjugation to relevant moieties such as targeting groups, labels, or drugs. From 4-NHSDEPMPO, a biotinylated nitrone was synthesized offering new perspectives for targeted delivery applications. A short study of the trapping behaviors of the (4*R**,5*S**)-isomer of these 4-HMDEPMPO analogues proved that they are as good as DEPMPO for detecting superoxide. For each isomer, only one diastereoisomer adduct was obtained, resulting from the addition of superoxide on the less hindered face of the nitrone, that is, *trans* to the phosphoryl group and the C-4 substituent. From spectra simulation and experiments in various solvents, we proved that ESR patterns of each adduct corresponded to the superimposed signals of two sets of conformers in a sufficiently slow chemical exchange to induce a widening and a dissymmetry of some of the signal lines. This phenomenon was drastically reduced when compared with that observed for DEPMPO superoxide and attributed to a similar chemical exchange, and it did not hamper spectrum assignment. Determination of the decay rate of the superoxide adduct of (4*R**,5*S**)-4-HMDEPMPO proved that it has a 25% longer half-life time than the superoxide adduct of DEPMPO.

Introduction

Reactive oxygen centered radicals (hereafter called RORs) accumulate after phagocyte activation, lipoxygenase activation, metal-ion release, or disruption of a mitochondrial electron transport chain. They contribute to the physiological disorders through tissue destruction and degeneration. Superoxide $O_2^{-\bullet}$ is often the primary upstream radical of the radical reaction chain of the oxidative stress, and the hydroxyl radical $HO^{\bullet}$ is the predominant species contributing to cellular damage.[1] Intensive

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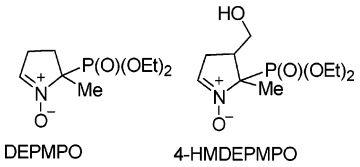
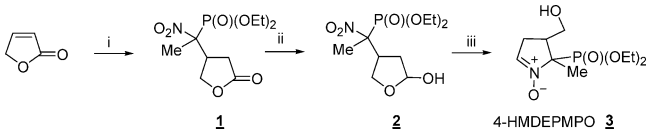
SCHEME 1. Chemical Structures of DEPMPO and 4-HMDEPMPO



investigation of the action of antioxidants in an in vivo setting has revealed that RORs stimulate inflammatory signaling and propagate growth stimulatory signals.[2] In the antioxidant therapeutic field, there is a need for novel drugs that function both as free radical scavengers and as nonsteroidal anti-inflammatory agents and that target the sites of radical generation. It is essential for rational drug design to determine what radicals are involved in a specific disease and in its progression, their exact role, their formation sites, and their molecular targets. Improvements have to be done in the field of radical detection. The informative as well as the therapeutic potential of nitrones such as PBN or DMPO,[3] which can trap various radicals and allow their characterization when used in association with electron spin resonance,[4] could be maximized by a better targeted biodistribution or by reduction of their trapping ability toward one specific radical species. New spin traps exhibiting targeting properties toward lectins,[5] mitochondria,[6] cell membranes,[7] and sulfhydryl-containing peptides[8] have recently been prepared. However, these nitrones exhibited, like their parent nitrone, the commonly used α-phenyl-*N*-*tert*-butyl spin trap (PBN), limited spin-trapping properties toward oxygen centered radicals. In our search for new specialized nitrones, we investigated some synthetic routes to obtain various derivatives of DEPMPO (Scheme 1), which is an efficient nitrone[9] known for the persistency of its adduct with superoxide.[10] For the series of DEPMPO analogues, the phosphorus coupling gives valuable information, affording greater reliability and confidence in the assignment of the adduct signals to the trapped radicals. Furthermore, DEPMPO crosses lipid bilayer membranes quite well[11] and is a good trapping agent in biological systems.[12] However, enhancing its biodistribution in subcellular compartments is a serious challenge. We focused also on the development of a series of efficient spin traps through the design of a single precursor bearing a reactive group for further conjugation to targeting groups, labels, or drugs. In a recent communication, we have described briefly the synthesis of a new family of DEPMPO-based spin traps and their use in preliminary spin-trapping experiments.[13] Hereafter, we detail the synthesis of the first one, the 4-HMDEPMPO nitrone (Scheme 1), and its change into another analogue, allowing further coupling at C-4, exemplified by the coupling of a biotinylated group. The ability of these new DEPMPO derivatives to trap superoxide radical and the assignment of the signals obtained are hereafter conclusively proved.

SCHEME 2. Synthesis of 4-HMDEPMPO[a]



[a] Reagents and conditions: (i) diethyl-(1-nitroethan-1-yl)phosphonate, PBu_3, cyclohexane/CH_2Cl_2 (10:1), rt, 80%; (ii) DIBAL-H, CH_2Cl_2, −78 °C, 50%; (iii) Zn, NH_4Cl, THF/H_2O (10:1), −5 °C, 64%.

Results and Discussion

Synthesis of Nitrones. (a) Synthesis of 4-HMDEPMPO. A DMPO derivative with a hydroxymethyl substituent at C-4 on the pyrroline ring has already been synthesized from dihydrofuranone.[14] Following a similar synthetic pathway, the phosphorylated analogue 4-HMDEPMPO **3** was easily prepared in a three-step synthesis in 26% yield (Scheme 2). Diethyl-(1-nitroethan-1-yl)phosphonate was deprotonated using tributylphosphane in catalytic quantities in an appropriate solvent mixture (cyclohexane/CH_2Cl_2 10:1). The so-formed anion[15] reacted with furanone, leading to 4-(1-diethylphosphoryl-1-nitroethyl)tetrahydrofuran-2-one **1**. Reduction of **1** with DIBAL-H into hemiacetal **2** required a chemical treatment at low temperature (−78 °C) to avoid diethylphosphite formation. Reduction of hemiacetal **2** to nitrone **3** was carried out using Zn−NH_4Cl in THF/H_2O in the absence of light and under argon atmosphere to avoid degradation of the intermediate hydroxylamine. The (4*R**,5*S**)-diastereoisomer of 4-HMDEPMPO, named hereafter 4-HMDEPMPO$_{cis}$, **3c**, where the phosphoryl group and the hydroxymethyl substituent are in *cis* position, was successfully separated (67%) by chromatography from the *trans* diastereoisomer (4-HMDEPMPO$_{trans}$, **3t**). Purification of nitrone **3** was much easier than that of DEPMPO.

(b) Synthesis of 4-NHSDEPMPO (Previously Named NHS-DEPMPO[13]). Modifying the hydroxyl function directly

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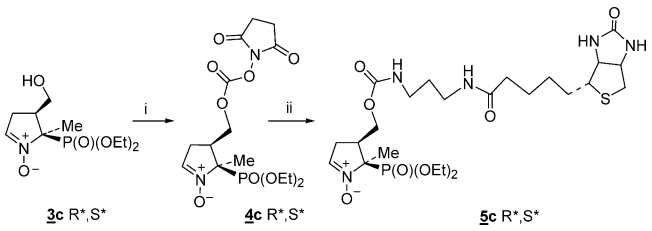
SCHEME 3. Synthesis of (4*R,5*S**)-4-BioS1DEPMPO[a]**



[a] Reactions and conditions: (i) disuccinimidyl carbonate, NEt_3, CH_3CN, rt, 95%; (ii) biotinylamidopropylammonium trifluoroacetate, NEt_3, DMSO rt, 39%.

on nitrone **3** to obtain various 4-HMDEPMPO derivatives functionalized at C-4 seemed unwise at first sight since the aminoxyl function is highly reactive. However, other approaches of DEPMPO analogues via modifications of the second or the third step of the synthetic route shown in Scheme 2 were unsuccessful.[16] Moreover, we succeeded to convert the alcoholic function of 4-HMDEPMPO **3** into an oxycarbonyloxyl group that is very sensitive to nucleophilic attack, therefore opening the route to introduce various specific moieties at C-4. Disuccinimidyloxycarbonate in the presence of imidazole in acetonitrile has already been used to transform the alcoholic function of an alkyl chain of a phosphonate into an oxycarbonyloxyl group.[17] Using these experimental conditions with **3t** afforded (4*R**,5*R**)-4-NHSDEPMPO **4t** in 20% yield after chromatography. However, imidazole reacted with the oxycarbonyloxyl group of **4t**, and a significant amount (16%) of the corresponding nitrone was also isolated. Therefore, triethylamine was used to synthesize from the diastereoisomer **3c** the (4*R**,5S*)-diastereoisomer **4c** (Scheme 3) that was isolated in 95% yield.

(c) Synthesis of a Biotinylated Nitrone, 4-BioS1DEPMPO. Synthesis of a biotinylated nitrone was attempted as the biotin moiety could offer targeted and/or monitored biodistribution by linkage of the new trap to an avidine complex including a specific binder and one or two probes.[18] It is worthy to note that Arya et al.[19] described the synthesis of a biotinylated DMPO; however, when they oxidized their pyrrolidinic precursor to generate the nitronyl function, the biotin sulfur group was converted into sulfoxide.

Our aim in building biotinylated DEPMPO was to keep the functions of the biotin moiety unchanged and to maintain its whole mobility for further applications through its complexation with avidine. Nitrone **4c** was converted into a biotinylated nitrone **5**, (4*R**,5*S**)-4-BioS1DEPMPO, by reaction with biotinylamidopropylammonium trifluoroacetate at room temperature in the presence of triethylamine (Scheme 3).

ESR Study of the Trapping of Superoxide with 4-HMDEPMPO$_{cis}$, 4-NHSDEPMPO$_{cis}$, and 4-BioS1DEPMPO$_{cis}$. The ESR spectrum of the superoxide adduct with DEPMPO exhibits a major signal assignable mainly to *trans* diastereoisomer adduct (HOO− *trans* to $(EtO)_2P(O)-$), resulting from the addition of the radical on the less hindered face of the pyrroline ring.[9] This signal exhibits a dramatic alternating line

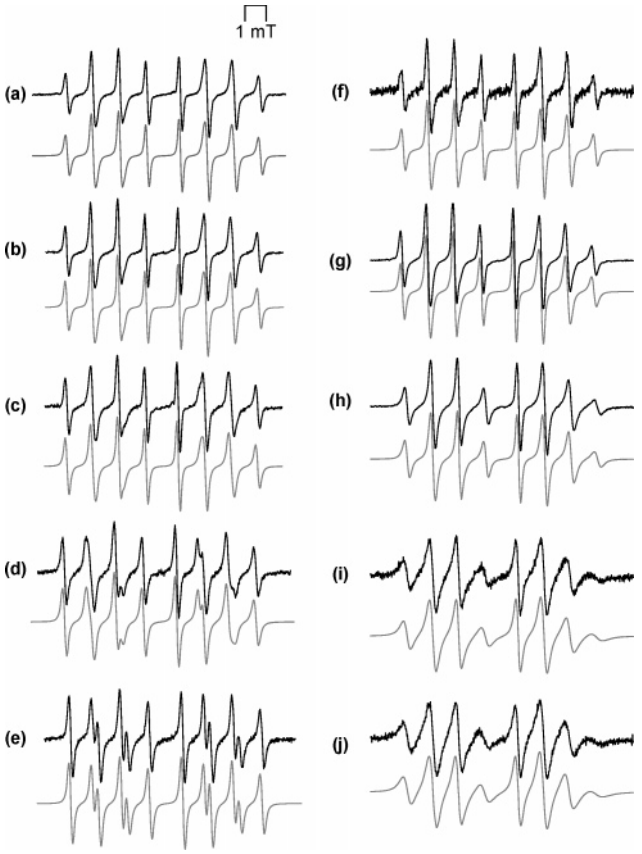


FIGURE 1. Spin trapping of superoxide by nitrones 4-HMDEPMPO$_{cis}$ and 4-NHSDEPMPO$_{cis}$ and 4-BioS1DEPMPO$_{cis}$. Spectra obtained (dark lines) and computer simulated (gray lines) after 1 min incubation of a mixture in phosphate buffer (0.1 M, pH 7.3) containing (a) [HX/O_2/XO] system and **3c** (50 mM); (b) [KO_2/crown ether] system and **3c** (50 mM); (c) [KO_2/crown ether] system and **4c** (50 mM); (d) [KO_2/crown ether] system and **5c** (20 mM); (e) as in (c) but after 10 min incubation; (f−j) [KO_2/crown ether] system and **3c** (30 mM) and glycerol (f) 10%; (g) 30%; (h) 50%; (i) 60%; (j) 70%. Spectrometer settings: microwave power 10 mW; modulation amplitude, 0.313 G (b), 0.702 (a, c−e), 0.500 (f−h); time constant, 0.128 s; gain 10^5; sweep time, 83.89 s; conversion time, 82 ms.

width, due to a sufficiently slow chemical exchange between two sets of conformers of this diastereoisomer. In the present work, the spin-trapping abilities toward superoxide of the *cis* diastereoisomers (4*R**,5*S**) of **3**, **4**, and **5** were tested in aqueous and organic phase. The *cis* diastereoisomers of **3**, **4**, and **5** were expected to afford exclusively *trans* addition of superoxide toward $(EtO)_2P(O)-$ and $(OH)H_2C-$ groups and to generate much simpler spectra than the corresponding *trans*-nitrone.

We checked by NMR studies that the nitrones did not decompose in an aqueous phase and that the C-4 substituents were inert toward water and were not lost. After 24 h incubation of the nitrones in chelexed water or in 0.1 M phosphate buffer (from chelexed water), the signals of nitroxides possibly formed from nucleophilic addition of water to the nitrones and subsequent oxidation of the ensuing hydroxylamines were not significant.

Superoxide was generated in 0.1 M phosphate buffer (pH 7.3) containing DETAPAC (diethylenetriaminepentaacetic acid) using a [KO_2/crown ether] system or a [hypoxanthine/oxygen/xanthine oxidase] (HX/O_2/XO) system. The spectra observed in these experiments are shown in Figure 1. A tentative assignment of the signals was done through their simulation

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TABLE 1. ESR Parameters of *trans* Superoxide Adduct with 4-HMDEPMPO$_{cis}$ or 4-NHSDEPMPO$_{cis}$ or 4-BioS1DEPMPO$_{cis}$

superoxide adduct	solvent	species R[c]	$k^a \times 10^{-7}$ (s^{-1})	g	a_P (mT)	a_N (mT)	$a_{H\beta}$ (mT)	$a_{H\gamma(Me)}$ (mT)	$a_{H\gamma(ring)}$ (mT)		
4-HMDEPMPO$_{cis}$-OOH ([HX/O$_2$/XO] experiments)	buffer[b]	I (62%)	1.73	2.00647	5.442	1.290	1.248	0.010	0.054	0.072	0.039
		II (38%) 0.996		2.00642	5.223	1.315	1.086	0.010	0.054	0.072	0.039
4-HMDEPMPO$_{cis}$-OOH ([KO$_2$/crown ether] experiments)	buffer[b]	I (57%)	3.28	2.00646	5.447	1.290	1.268	0.020	0.053	0.035	0.070
		II (43%) 0.994		2.00644	5.187	1.302	1.061	0.020	0.053	0.035	0.070
	buffer[b] with 10% glycerol	I (57%)	3.55	2.00648	5.459	1.289	1.285	0.010	0.054	0.072	0.027
		II (43%) 0.983		2.00642	5.174	1.290	1.067	0.010	0.054	0.072	0.027
	buffer[b] with 30% glycerol	I (59%)	2.83	2.00649	5.399	1.270	1.299	0	0.038	0.066	0.063
		II (41%) 0.992		2.00644	5.190	1.300	1.022	0	0.038	0.066	0.063
	buffer[b] with 50% glycerol	I (59%)	2.53	2.00654	5.385	1.258	1.327	0.018	0.054	0.074	0.026
		II (41%) 0.999		2.00647	5.151	1.294	0.987	0.018	0.054	0.074	0.026
	buffer[b] with 60% glycerol	I (59%)	1.50	2.00657	5.336	1.245	1.325	0.000	0.062	0	0
		II (41%) 0.994		2.00654	5.185	1.296	0.984	0.000	0.062	0	0
	buffer[b] with 70% glycerol	I (59%)	1.41	2.00660	5.305	1.245	1.336	0.024	0.029	0.060	0
		II (41%) 0.991		2.00658	5.161	1.287	0.930	0.024	0.029	0.060	0
4-NHSDEPMPO$_{cis}$-OOH ([KO$_2$/crown ether] experiments)	buffer[b]	I (59%)	1.05	2.00672	5.311	1.274	1.273	0.019	0.046	0.014	0.077
		II (41%) 0.995		2.00669	5.240	1.325	0.996	0.019	0.046	0.014	0.077
4-BioS1DEPMPO$_{cis}$-OOH ([KO$_2$/crown ether] experiments)	buffer[b]	I (61%)	0.48	2.00665	5.353	1.275	1.259	0.020	0.047	0.077	0
		II (52%) 0.995		2.00661	5.256	1.313	1.027	0.020	0.047	0.077	0

[a] Exchange rate. [b] 0.1 M phosphate buffer. [c] Correlation coefficient for the simulation given.

(gray lines in Figure 1) using the ESR software developed in the Central Research Institute of Chemistry, Hungary.[20] The simulated coupling constants and the associated g values are given in Table 1.

When superoxide was generated in an aqueous buffer in the presence of 4-HMDEPMPO$_{cis}$, **3c** (Figure 1a and 1b), or 4-NHSDEPMPO$_{cis}$, **4c** (Figure 1c), the ESR spectra observed in the first minutes exhibited intense and persistent signals with similar shapes. The signals were not observed if superoxide dismutase (SOD) was added to the generating superoxide mixture (HX/O$_2$/XO). These results support the assignment of the signals to the corresponding superoxide adducts. Each signal was composed of a doublet of quadruplets, showing a certain degree of dissymmetry together with a broadening of the third and sixth lines. Even under low amplitude modulation conditions, no $a_{H\gamma}$ hyperfine coupling constants could be detected. No satisfactory simulation of these signals could be obtained assuming that they correspond to a single species. The specific patterns of each signal could be explained assuming the superimposition of the signals of two different species with close phosphorus coupling constant values. The combination of the two diastereoisomers of the superoxide adduct was reasonably excluded since the phosphorus coupling constant values of these two diastereoisomers were expected to present a significant difference because of different geometries, as it is observed for the two diastereoisomers of the DEPMPO−superoxide adduct.[9b] It is likely that the trapping of superoxide is stereoselective, affording the *trans* diastereoisomer through addition on the less hindered face of the pyrroline ring. Therefore, the ESR signals observed could be explained assuming either the superimposition of the spectrum of the *trans* diastereoisomer and that of a second nitroxide or an exchange model between two "sets" of conformers of the *trans* diastereoisomer. The latter assumption was supported by the better fit observed between calculated and experimental spectra (regression parameters 0.993 and 0.997, respectively, for **3c** and **4c**).

When superoxide was trapped with 4-BioS1DEPMPO$_{cis}$, **5c**, the observed ESR signal (Figure 1d) resulted obviously, even in the first minute of the experiment, from the superimposition of the spectra of two different nitroxides, the superoxide adduct and a second minor nitroxide (a_N = 1.357 mT, a_P = 5.326 mT, $a_{H\beta}$ = 1.039 mT) that became rapidly predominant (Figure 1e). Comparison with an authentic sample generated by a Fenton reaction in the presence of **5c** clearly showed that it corresponded to the spin adduct of the hydroxyl radical, **5c**-OH (data not shown). The formation of **3c**-OH (a_N = 1.365 mT, a_P = 5.347 mT, $a_{H\beta}$ = 1.070 mT) and **4c**-OH (a_N = 1.359 mT, a_P = 5.257 mT, $a_{H\beta}$ = 1.046 mT) was also observed when superoxide was trapped by **3c** and **4c**, respectively; however, their formation was much slower than the formation of **5c**-OH.

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The formation of these three hydroxyl radical adducts was favored when the concentration of KO_2 was increased.

The relative simplicity of the figure patterns (8 lines) of the superoxide adducts compared to the signal of the DEPMPO-OOH adduct (12 lines)[9a] might suggest a much slower conformational exchange between the two conformer sets. However, the rate constants calculated for the exchange between the conformer sets of the adducts **3c**-OOH, **4c**-OOH, or **5c**-OOH ($0.5-3.3 \times 10^7$ s^{-1}, Table 1) were only slightly lower than that found for the exchange of DEPMPO-OOH rotamers (6.7×10^7 s^{-1},[9b] or 9×10^7 s^{-1} [21]). To confirm the existence of the conformational exchange, we tried to lower the rate of this exchange for **3c**-OOH by adding a viscous compound (glycerol) to the aqueous medium. The respective spectra obtained by adding from 10 to 70% glycerol (Figure 1f−j) showed an increased broadening of the lines and an increased dissymmetry between the internal lines or the external lines of the two quartets.

This phenomenon of line broadening without resolution of the long-range coupling patterns can be explained by the fact that the radical presented a faster relaxation rate as the rotational tumbling of the aminoxyl function was reduced with an increasing amount of glycerol. These spectra were simulated with some very close sets of coupling constants of two conformer nitroxides in a chemical exchange, with the exchange rate constant decreasing when the amount of glycerol increased.

Our results indicate that substitution on the C-4 position inhibits neither the conformational flexibility of the ring nor the occurrence of the conformational equilibrium. However, the steric hindrance caused by both the phosphoryl group and each C-4 substituent on the same face of the pyrroline ring favors *trans* addition and not *cis* addition of superoxide. The line width alternation looked less significant for C-4-substituted DEPMPO, not because of the slower rate of conformational exchange, but owing to the stronger relaxation that made it impossible to resolve the long-range coupling patterns. Moreover, the preponderant set of the two sets of the conformers exhibited closer values for the hydrogen and nitrogen coupling constants (Table 1). The change in the size of the C-4 substituents does not modify the shape of the ESR signals of the superoxide adducts and the values of the coupling constants. Likely it does not modify the conformations of the resulting nitroxides; however, it modifies their exchange rate. Bigger substituent induced lower exchange rate.

Kinetic Decay of the Superoxide Adduct 4-HMDEPMPO$_{cis}$-OOH (3c-OOH). In spite of the formation of **3c**-OH observed with both superoxide generating systems, the half-life time of **3c**-OOH adduct was expected to reach at least the value of the half-life time of its parent analogue DEPMPO-OOH. This assumption was supported by the absence of vicinal interactions destabilizing the **3c**-OOH adduct and by the geometry of the preponderant conformer set of **3c**-OOH suggested by its phosphorus coupling constants that were higher than that of the preponderant conformer set of DEPMPO-OOH.[9b] A high phosphorus coupling constant could suggest a conformation with a more axial position of the carbon phosphorus bond that may favor anomeric or hyperconjugative effects,[22,23] increasing the half-life of the superoxide adduct.

As the signal of the **3c**-OH overlapped the signal of the **3c**-OOH, the decay of this latter signal could not be monitored following the decrease of one of its lines. The ROKI program[20] can be used to simulate the concomitant evolution of the different species composing an ESR spectrum. The decay rate of the **3c**-OOH adduct was computed by comparing the variation of the ESR amplitudes recorded in slow and fast sweeping conditions. The ESR amplitudes of the simulated fast recording spectrum were used in the simulation of the slow recording spectrum, modulated by a kinetic function describing either a first or second order or complex decay process. To compare the kinetics of the decay of the **3c**-OOH and DEPMPO-OOH adducts, the two trapping systems were tested in parallel under the same set of conditions using the (HX/XO) superoxide generating system. The computations showed that the half-life times were 21 min for the **3c**-OOH adduct and 15 min 30 s for the DEPMPO-OOH adduct, and that the decay of the **3c**-OOH adduct had a predominant first-order character. The higher persistency of **3c**-OOH can be explained by the more axial position of the C−P bond favoring hyperconjugative effects.

Conclusion

Investigation for a general synthetic route to prepare various specialized derivatives of DEPMPO led us to synthesize a new nitrone bearing a hydroxymethyl function at C-4 and named 4-HMDEPMPO. Its *cis* diastereoisomer (4*R**,5*S**) was converted into another nitrone, a NH_2-reactive precursor named 4-NHSDEPMPO, bearing an oxycarboxyoxyl group that allowed us to prepare a biotinylated DEPMPO. This biotinylated nitrone offers new perspectives for targeted delivery applications. For each nitrone, only one diastereoisomer adduct was obtained, resulting from the addition of superoxide on the less hindered face of the nitrone, that is, *trans* to the phosphoryl group and the C-4 substituent. From spectra simulation and experiments in various solvents, we proved that ESR patterns of each adduct corresponded to the superimposed signals of two sets of conformers in a sufficiently slow chemical exchange to induce a widening and a dissymmetry of some of the signal lines. This alternating line width phenomenon was drastically reduced when compared with that observed for DEPMPO superoxide and attributed to a similar chemical exchange, and it did not hamper spectrum assignment. Determination of the decay rate of the superoxide adduct of (4*R**,5*S**)-4-HMDEPMPO proved that it has a 25% longer half-life time than the superoxide adduct of DEPMPO, itself 22.5 times longer than those reported for the DMPO-OOH adducts.

Experimental Section

Synthesis. 4-(1-Diethoxyphosphoryl-1-nitroethyl)tetrahydrofuran-2-one (1). Under inert atmosphere and in darkness, 2.16 g of tributylphosphane (0.01 mol) was added dropwise to a mixture of diethyl-(1-nitroethan-1-yl)phosphonate[10] (12.58 g, 0.059 mol) and furanone (5 g, 0.059 mol) in cyclohexane (70 mL) and CH_2Cl_2 (7 mL). The mixture was stirred at room temperature for 66 h. Then the solvents were removed under reduced pressure, and the crude product was purified by flash chromatography on silica gel eluting with Et_2O/pentane (9:1). Nitrofuranone **1**, 14.01 g (0.047 mol, 80% yield) as a yellow oil was obtained as a mixture of two diastereoisomers in 55:45 ratio: ^{31}P NMR (121.49 MHz) δ 13.79 (45%), 13.70 (55%); ^1H NMR (300.13 MHz) δ 4.54−4.05 (6H,

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m), 3.78−3.56 (1H, m), 2.82−2.35 (2H, m), 1.77 (3H, d, J = 14.4 Hz), 1.75 (3H, d, J = 14.4 Hz), 1.38−1.25 (6H, m, J = 7.0 Hz); ^{13}C NMR (75.47 MHz) δ 174.5 (C^{IV}, s), 174.6 (C^{IV}, s), 90.1 (C^{IV}, d, J = 147.1 Hz), 90.4 (d, J = 148.2 Hz), 68.3 (d, J = 2.8 Hz), 68.1 (d, J = 9.3 Hz), 64.8 (d, J = 7.7 Hz), 65.1 (d, J = 7.2 Hz), 40.2 (s), 39.8 (s), 30.2 (d, J = 3.3 Hz), 29.9 (d, J = 7.7 Hz), 16.37 (d, J = 9.4 Hz), 16.36 (d, J = 7.7 Hz), 16.28 (d, J = 5.2 Hz), 16.25 (d, J = 5.2 Hz). $C_{10}H_{18}NO_7P$ requires: C, 40.68; H, 6.15; N, 4.74%. Found: C, 40.67; H, 6.07; N, 4.61%.

4-(1-Diethoxyphosphoryl-1-nitroethyl)-2-hydroxytetrahydrofurane (2). A solution (11.45 mL) of 1 M DIBAL-H in hexane was added dropwise to a solution of **1** (1.3 g, 4.4 mmol) in CH_2Cl_2 (35 mL) at −76 °C under inert atmosphere. The reaction mixture was stirred at −78 °C for 3 h, and 95% ethanol (8 mL) was then added at the same temperature. The solution was filtered on silica gel and dried on Na_2SO_4. After removing the solvent, the expected hemiacetal **2** was isolated by flash chromatography on silica gel eluting with CH_2Cl_2/Et_2O (50:50) as a pale yellow oil containing four diastereoisomers in 50% yield (650 mg, 2.19 mmol): ^{31}P NMR (121.49 MHz) δ 15.15 (50%), 15.28 (25%), 15.48 (25%); ^{1}H NMR (300.13 MHz) δ 5.51 (1H, t, J = 5.3 Hz), 4.31−3.97 (6H, m), 3.94−3.56 (m, 1H), 2.43−1.86 (2H, m), 1.83 (3H, d, J = 14.4 Hz), 1.79 (3H, d, J = 14.8 Hz), 1.73 (3H, d, J = 14.8 Hz), 1.69 (3H, d, J = 14.5 Hz), 1.38−1.27 (6H, m); ^{13}C NMR (75.47 MHz) δ 98.5 (s), 98.3 (s), 97.7 (s), 97.6 (s), 91.4 (C^{IV}, d, J = 149.3 Hz), 91.2 (C^{IV}, d, J = 149.7 Hz), 90.9 (C^{IV}, d, J = 150.3 Hz), 90.5 (C^{IV}, d, J = 148.2 Hz), 67.0 (d, J = 10.4 Hz), 66.9 (d, J = 2.0 Hz), 65.8 (d, J = 10.4 Hz), 66.6 (s), 64.7 (d, J = 7.2 Hz), 64.5 (d, J = 7.2 Hz), 64.4 (d, J = 6.6 Hz), 64.3 (d, J = 7.6 Hz), 43.1 (s), 42.9 (s), 42.3 (s), 42.0 (s), 35.6, (d, J = 4.4 Hz), 35.5 (d, J = 4.9 Hz), 34.9 (d, J = 9.3 Hz), 34.8 (d, J = 9.9 Hz), 16.5 (d, J = 1.1 Hz), 15.8 (d, J = 1.1 Hz), 15.2 (s), 15.1 (s), 16.2 (d, J_{CP} = 5.5 Hz), 16.1 (d, J = 5.5 Hz). $C_{10}H_{20}NO_7P$ requires: C, 40.41; H, 6.78; N, 4.71%. Found: C, 40.42; H, 6.65; N, 4.49%.

5-Diethoxyphosphoryl-5-methyl-4-hydroxymethyl-1-pyrroline-*N*-oxide (3). To a solution of hemiacetal **2** (3.8 g, 0.0128 mol) in a mixture of THF/H_2O (10:1) were added at −5 °C first NH_4Cl (1.71 g, 0.032 mol) then powder zinc (2.09 g, 0.032 mol) in 2 h. The reaction mixture was stirred for 6 h at room temperature in the dark. The $ZnCl_2$ precipitate was filtered and washed with CH_2Cl_2 (3 × 40 mL) and MeOH. The mixed filtrates were concentrated under reduced pressure, and the residue was then dissolved in CH_2Cl_2 (40 mL) and washed with brine (10 mL). The organic layer was dried over Na_2SO_4, filtered, and the solvent removed under reduce pressure. The oil residue was purified by flash chromatography on silica gel eluting with CH_2Cl_2/EtOH (85:15), giving rise to three fractions of nitrone **3** (2.17 g) with 64% yield. One of these three fractions was a white hygroscopic solid (1.22 g, 0.006 mol, 38% yield; mp = 48 °C with decomposition) corresponding to diastereoisomer **3c** (4*S**,5*R**). The second fraction was a pale yellow oil (0.63 g, 0.0024 mol, 19% yield) and corresponded to the other diastereoisomer **3t**. The last fraction (0.32 g) contained the mixture. The stereochemistry of both nitrones was confirmed by NMR NOESY. **3c**: ^{31}PNMR (121.49 MHz) δ 20.85; ^{1}H NMR (300.13 MHz) δ 6.90 (1H, t, J = 2.7, 2.7 Hz), 4.39−4.14 (4H, m), 3.90 (2H, d, J = 6.1 Hz), 2.78−2.39 (4H, m), 1.76 (3H, d, J = 14.5 Hz), 1.38 (3H, t, J = 7.0 Hz), 1.34 (3H, t, J = 7.0 Hz); ^{13}C NMR (75.47 MHz) δ 134.5 (d, J = 8.8 Hz), 76.9 (C^{IV}, d, J = 149.8 Hz), 63.9 (d, J = 7.1 Hz), 63.8 (d, J = 7.7 Hz), 62.2 (d, J = 5.0 Hz), 49.3 (d, J = 2.8 Hz), 29.2 (s), 21.3 (d, J = 1.6 Hz), 16.4 (d, J = 5.5 Hz), 16.3 (d, J = 6.0 Hz). $C_{10}H_{18}NO_6P$, 1/2 H_2O requires: C, 43.80; H, 7.72; N, 5.11%. Found: C, 43.96; H, 7.61; N, 5.39%. **3t**: ^{31}PNMR (121.49 MHz) δ 22.0; ^{1}H NMR (300.13 MHz) δ 6.86 (1H, t, J = 2.5, 2.5 Hz), 4.40−4.11 (4H, m), 3.8 and 3.7 (2H, ABd, J = 11.0, 7.0, 5.9 Hz), 3.25−3.05 (1H, m), 2.87 (1H, ABdt, J = 18.3, 6.6, 2.5, 2.5 Hz), 2.48 (2H, m), 1.67 (3H, d, J = 16.0 Hz), 1.34 (3H, t, J = 6.9 Hz), 1.33 (3H, t, J = 6.9 Hz); ^{13}C NMR (75.47 MHz) δ 134.9 (d, J = 8.8 Hz), 76.7 (d, J = 158.1 Hz), 64.1 (d, J = 6.6 Hz), 63.3 (d, J = 7.3 Hz), 61.3 (d, J = 6.6 Hz), 40.9 (s), 30.1 (d, J = 4.8 Hz), 16.2 (d, J = 5.9 Hz), 14.0 (1C, s). $C_{10}H_{18}NO_6P$, 1/2 H_2O requires: C, 43.80; H, 7.72; N, 5.11%. Found: C, 44.06; H, 7.60; N, 5.33%. ESI-MS/MS (20 eV) *m*/*z* (%): 266.3 (79) $[M + H]^+$, 248 (12), 234 (24), 220 (5), 190 (3), 162 (6), 155 (9), 127 (75), 112 (24), 94 (100).

5-Diethoxyphosphoryl-5-methyl-4-(succinimidyloxycarbonyloxymethyl)-1-pyrroline-*N*-oxide (4). Imidazole (0.03 g, 0.43 mmol) and nitrone **3t** (0.115 g, 0.43 mmol) or triethylamine (0.75 mL, 0.55 mmol) and nitrone **3c** (0.115 g, 0.43 mmol) were added at room temperature under inert atmosphere to a solution of disuccinimide carbonate (0.122 g, 0.48 mmol) in anhydrous acetonitrile (5 mL). The reaction mixture was stirred for 36 h, then concentrated under reduced pressure. The residue dissolved in CH_2Cl_2 (10 mL) was washed by a solution saturated with $NaHCO_3$ (5 mL) and next by brine (5 mL). The mixed organic phases were dried on Na_2SO_4, and the solvent was removed. The purification of the yellow crude product by flash chromatography on silica gel eluting with CH_2Cl_2/EtOH (85:15 for **4t** and 90:10 for **4c**) gave rise to the pure expected nitrone 4-SOCOMDEPMPO with 20% yield for the oily diastereoisomer **4t** (0.036 g, 0.088 mmol) or 95% yield for the white powder of **4c** (0.166 g, 0.352 mmol). **4c**: Mp 176 °C; ^{31}P NMR (81.01 MHz) δ 18.0; ^{1}H NMR (200.13 MHz) δ 6.97 (1H, q, J = 2.9, 2.9 Hz), 4.76 (1H, A in ABd, J = 10.8, 5.9 Hz), 4.57 (1H, B in ABd, J = 10.8, 7.8 Hz), 4.36−4.08 (4H, m), 2.83 (4H, s), 2.8−2.57 (3H, m), 1.71 (3H, d, J = 13.9 Hz), 1.31 (3H, t, J = 7.0 Hz), 1.33 (3H, t, J = 7.0 Hz); ^{13}C NMR (50.32 MHz) δ 168.4 (s), 151.1 (s), 133.7 (d, J = 7.7 Hz), 75.8 (d, J = 148.7 Hz), 70.0 (d, J = 3.0 Hz), 64.5 (d, J = 6.5 Hz), 62.8 (d, J = 7.2 Hz), 45.4 (d, J = 2.3 Hz), 29.7 (d, J = 0.8 Hz), 25.3 (s), 20.1 (s), 16.1 (d, J = 5.7 Hz). HRMS calcd for $C_{15}H_{23}N_2O_9P$ $[C_{15}H_{23}N_2O_9P + Na]^+$: 429.1039. Found: 429.1062. **4t**: ^{31}P NMR (81.01 MHz) δ 22.67; ^{1}H NMR (200.13 MHz) δ 6.86 (1H, q, J = 2.6, 2.6 Hz), 4.55 (1H, A in ABd, J = 10.8, 4.9 Hz), 4.40−4.07 (m, 5H), 3.5−3.25 (m, 1H, C*H*CH_2O), 3.07 (1H, A in ABdt, J = 18.4, 8.7, 3.0 Hz), 2.54 (1H, B in ABdt, J = 18.4, 2.8, 3.0 Hz), 2.83 (4H s), 1.62 (3H, d, J = 15.6 Hz), 1.35 (6H, t, J = 7.1 Hz); ^{13}C NMR (50.32 MHz) δ 168.3 (s), 151.3 (s), 132.5 (d, J = 8.4 Hz), 76.3 (d, J = 160.3 Hz), 69.8 (d, J = 7.0 Hz), 64.4 (d, J = 6.2 Hz), 63.0 (d, J = 7.3 Hz), 38.0 (s), 30.1 (d, J = 4.7 Hz), 25.44 (s, 2C, *C*H_2CO), 16.31 (d, J = 5.8 Hz), 14.66 (s). High-resolution mass spectrum for $[C_{15}H_{23}N_2O_9P + Na]^+$: 429.1039; found 429.1060. In synthesis of **4t**, (4*R**,5*R**)-5-diethoxyphosphoryl-4-(1-imidazolylcarbonyloxymethyl)-5-methyl-1-pyrroline-*N*-oxide was also obtained in 16% yield (27 mg, 0.075 mmol): ^{31}P NMR (81.01 MHz) δ 22.85; ^{1}H NMR (200.13 MHz) δ 8.10 (1H, s), 7.38 (1H, t, J = 1.5 Hz), 7.08 (1H, dd, J_{HH} = 1.5, 0.76 Hz), 6.88 (1H, dd, J_{HH} = 2.8, 2.6 Hz), 4.62 (1H, A in Abd, J = 11.1, 5.5 Hz), 4.44 (1H, B in Abd, J = 11.1, 7.7 Hz), 4.36−4.09 (m, 4H), 3.61−3.32 (m, 1H), 3.06 (1H, A in ABdt, J = 18.4, 2.8, 8.8, 3.0 Hz), 2.52 (1H, B in ABdt, J = 18.4, 2.7, 6.1, 2.8 Hz), 3.36 (3H, d, J = 15.7 Hz), 1.35 (3H, t, J = 7.0 Hz), 1.34 (3H, t, J = 7.0 Hz); ^{13}C NMR (50.32 MHz) δ 148.2 (s), 137.0 (s), 132.0 (d, J = 8.8 Hz), 131.1 (s), 116.9 (s), 76.43 (d, J = 159.9 Hz), 67.1 (d, J_{CP} = 6.6 Hz), 64.6 (d, J = 6.2 Hz), 63.0 (d, J = 7.3 Hz), 37.9 (s), 30.4 (d, J = 4.8 Hz), 16.3 (d, J = 5.8 Hz), 14.79 (s). HRMS calcd for $C_{14}H_{22}N_3O_6P$ $[C_{14}H_{22}N_3O_6P + H]^+$: 360.1324. Found: 360.1304.

(4*S,5*R**)-4-BioS1DEPMPO (5c).** A solution of biotinylamidopropylammonium trifluoroacetate (0.0665 g, 0.22 mmol) and of triethylamine (0.034 mL, 0.24 mmol) in 3 mL of DMSO was added to a solution of nitrone **4c** (0.090 g, 0.22 mmol) in DMSO (2 mL) at room temperature under inert atmosphere. The reaction mixture was stirred for 24 h at room temperature, and brine (5 mL) was added. The organic layer was separated, and the expected nitrone was extracted again twice from the aqueous phase with CH_2Cl_2 (10 mL). The mixed organic phases were dried with Na_2SO_4, and the solvent was removed under reduced pressure. The crude product, which was composed mainly of the nitrone expected from NMR (^{1}H and ^{31}P) analysis, was purified by flash chromatography on silica gel with a gradient of ethanol (15 up 100%) in CH_2Cl_2.

4-BioS1DEPMPOc **5c** was obtained as a white powder (51 mg, 0.087 mmol) with 39% yield: mp 176 °C (decomposition); ^{31}P NMR (81.01 MHz, CD_3OD, Me_4Si) δ 21.33; ^{1}H NMR (200.13 MHz, CD_3OD, Me_4Si) δ 7.28 (1H, q, J = 3 Hz), 4.56−4.38 (2H, m), 4.37−4.13 (6H, m), 3.27−3.10 (5H, m), 2.95 (1H, d, J = 12.9 Hz), 2.70 (1H, dd, J = 12.90, 4.93 Hz), 2.87−2.61 (3H, m), 2.22 (2H, t), 1.74 (3H, d, J = 14.4 Hz), 1.76−1.42 (8H, m), 1.37 (2t, 6H, J = 7.0, 7.2 Hz); ^{13}C NMR (50.32 MHz, CD_3OD, Me_4Si) δ 176.2 (s), 166.0 (s), 158.4 (s), 140.7 (d, J = 5.7 Hz), 77.5 (d, J = 151.4 Hz), 66.0 (d, J = 6.6 Hz), 64.9 (d, J = 7.7 Hz), 64.8 (s), 63.4 (s), 61.6 (s), 57.0 (s), 47.3 (s), 41.0 (s), 39.2 (s), 37.6 (s), 36.8 (s), 31.5 (s), 30.6 (s), 29.7 (s), 29.5 (s), 26.9 (s), 20.7 (s),16.73 (s), 16.67 (2d, J = 5.7 Hz). HRMS calcd for $C_{24}H_{42}N_5O_8PS$ [$C_{24}H_{42}N_5O_8PS$ + H]$^+$: 592.2570. Found: 592.2529. ESI/MS/MS (20 eV) *m*/*z* (%): 592.4 (100) [M + H] $^+$, 574 (5), 436 (4), 327 (17), 301 (45), 284 (5), 266 (5), 248 (10), 230 (8), 218 (14), 138 (5).

Spin-Trapping Studies. (a) Superoxide Trapping with KO_2/18-Crown-6 Generating System. ESR was signal observed upon incubating the reaction mixture obtained after adding 10% of 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, pH 7.3) containing nitrone (from 20 to 50 mM final concentration) and if necessary from 10 to 70% glycerol.

(b) Superoxide Trapping with Hypoxanthine-Xanthine Oxidase Generating System. XO (0.04 U mL^{-1}) was added to a solution of nitrone (50−60 mM), DTPA (1 mM), and HX (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 for an inhibition test of superoxide trapping, it was added before XO addition.

(c) 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. Once the adduct concentration had reached a significant value (approximately 7 min), 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). 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.

Supporting Information Available: Supplementary synthesis and experimental sections concerning unexpected results; NRM spectra copies; decay curves of the superoxide adducts of **3c** and DEPMPO. This material is available free of charge via the Internet at http://pubs.acs.org.

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