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Synthesis and Spin-Trapping Behavior of 5-ChEPMPO, a Cholesteryl Ester Analogue of the Spin Trap DEPMPO

Micaël Hardy,[†] Olivier Ouari,[†] Laurence Charles,[‡] Jean-Pierre Finet,*[,†] Gilles Iacazio,[§] Valérie Monnier,[‡] Antal Rockenbauer,[||] and Paul Tordo*[,†]

Laboratoire SREP, UMR 6517 CNRS et Universités d'Aix-Marseille 1, 2 et 3, Centre de Saint Jérôme, 13397 Marseille Cedex 20, France, Laboratoire TRACES, JE 2421, Universités d'Aix-Marseille 1 et 3, Centre de Saint Jérôme, 13397 Marseille Cedex 20, France, Laboratoire de Bioinorganique Structurale, UMR 6517 CNRS et Universités d'Aix-Marseille 1, 2 et 3, Centre de Saint Jérôme, 13397 Marseille Cedex 20, France, and Chemical Research Center, Institute for Structural Chemistry, H-1525 Budapest, PO Box 17, Hungary

tordo@srepir1.univ-mrs.fr; jean-pierre.finet@up.univ-mrs.fr

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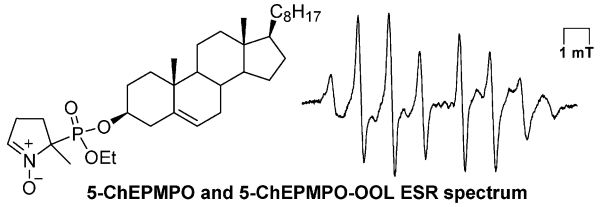

5-(Cholesteryloxyethoxyphosphoryl)-5-methylpyrroline *N*-oxide (5-ChEPMPO), a DEPMPO analogue bearing a cholesterol group on the phosphorus atom, has been prepared and used to trap peroxyl-, alkoxyl-, thiyl-, and carbon-centered radicals in organic solvent. The important steric hindrance in 5-ChEPMPO does not affect the properties of 5-ChEPMPO in comparison to DEPMPO for the spin trapping of an enantiopure linoleic acid hydroperoxide. The 5-ChEPMPO−OOL spin adduct was observed by ESR and confirmed by ESI-MS/MS experiments. The relaxation terms of the 5-ChEPMPO−lipid peroxyl spin adduct were compared with those of other peroxyl spin adducts, and it was shown that the cholesteryl group has only a weak influence on the exchange rate between adduct conformers.

Introduction

Free radicals, and especially reactive oxygen species (ROS), play an important role in living systems, and they are implicated in aging and in the pathology of a range of diseases including ischemic and post-ischemic reperfusion damage, inflammation processes, cancers, and neurodegenerative diseases.[1−3] In these pathologies, oxidative damage initiated by free radicals, such as lipid peroxidation process,[4] DNA cleavage,[5] and enzyme inactivation, is occurring. Thus, the development of a method allowing the detection of free radicals in biological systems is of great importance and is a major goal in free-radical research. Among the techniques available to study free radicals, ESR has emerged as a powerful tool for their direct detection and identification. However, in living systems, most free radicals are highly reactive species with very short lifetimes. To circumvent these limitations, the spin trapping technique, introduced in the late 1960s, is now a valuable tool for the study of free-radical processes.[6−13] In spin-trapping experiments, a diamagnetic probe, a nitrone or a nitroso compound,

* To whom correspondence should be addressed. Phone: 33 4 91 28 89 27. Fax: 33 4 91 28 87 58.
[†] Laboratoire SREP, UMR 6517 CNRS et Universités d'Aix-Marseille 1, 2 et 3.
[‡] Laboratoire TRACES, JE 2421.
[§] Laboratoire de Bioinorganique Structurale, UMR 6517 CNRS et Universités d'Aix-Marseille 1, 2 et 3.
[||] Chemical Research Center, Institute for Structural Chemistry.

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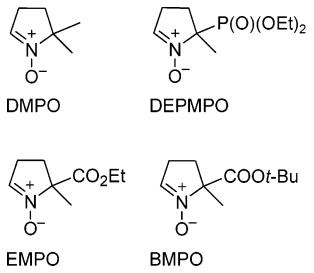
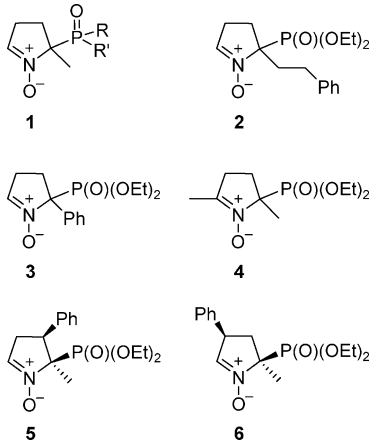
SCHEME 1. Chemical Structure of the Spin Traps



reacts with a free radical to produce a nitroxide spin adduct, showing a considerably higher persistence as compared to the parent free radical. The resulting ESR spectrum often exhibits a hyperfine splitting pattern characteristic of the trapped radical that allows its identification. In the last 15 years, the spin-trapping method coupled with ESR spectroscopy has been used successfully for in vitro,[14] ex vivo,[15] and in vivo[16,17] experiments. However, the spin-trapping technique still presents limitations when applied to biological systems, and the detection of free radicals is mainly limited by the short persistence of the spin adducts and by the difficulty in assigning ESR spectra of the spin adducts. To develop more efficient probes, different spin traps have been synthesized in the past decade. As a result, BMPO,[18,19] EMPO,[20] and DEPMPO[14,21] (Scheme 1) have been prepared, and they led to considerable improvements in the detection of the superoxide radical (adduct half-life time: 9, 9, and 17 min, respectively). Moreover, Karoui et al.[22] have recently shown that performing the trapping reaction in the presence of methylated β-cyclodextrin results in a 7-fold increase in the half-life time of the DEPMPO−OOH adduct ($t_{1/2}$ = 96 min) and that the hydroperoxyl adduct was protected partially from reduction by gluthatione peroxidase and by the ascorbate anion in in vitro experiments.

The design of spin traps giving more persistent spin adducts and a greater spectral resolution between the different spin adducts has been the major focus of recent research. Besides these improvements, the development of spin traps that can accumulate in relevant sites and cell compartments is an important issue.[23−25]

SCHEME 2. Chemical Structure of the DEPMPO Analogues



Recently, Anzai et al.[26] have shown that DMPO and DEPMPO diffuse quickly across lipid bilayer membranes by passive diffusion. Nevertheless, in line with the value of their partition coefficient K_p (0.06, 0.16, respectively), the distribution of DMPO and DEPMPO between an hydrophilic and an hydrophobic milieu is greatly in favor of the aqueous compartment.[14,27] Due to the implication of ROS in lipid peroxidation processes[28,29] and in the etiology of several diseases,[29,30] the development of spin traps that accumulate in lipophilic compartments is important. To improve the spin trapping properties of DEPMPO, a range of derivatives, **1**−**6**, have been prepared (Scheme 2). Modification of the alkoxy groups on the phosphorus atom did not alter significantly the spin-trapping properties [nitrone **1** (R, R′= alkoxy, alkyl, or aryl), Scheme 2].[31−34] Introduction of a substituent on the C-4 position led to a slightly modified spin-trapping behavior (nitrone **5**, Scheme 2).[35] In contrast, introduction of substituents on the pyrroline ring at C-2 or C-3 or modification at C-5 (such as replacement of the methyl group by longer chain alkyl or phenyl groups) led to an important decrease of the trapping properties (nitrones **2**−**4**, **6**, Scheme 2).[36−39]

To increase the lipophilic character of DEPMPO, we selected cholesterol as a carrier group because of its high

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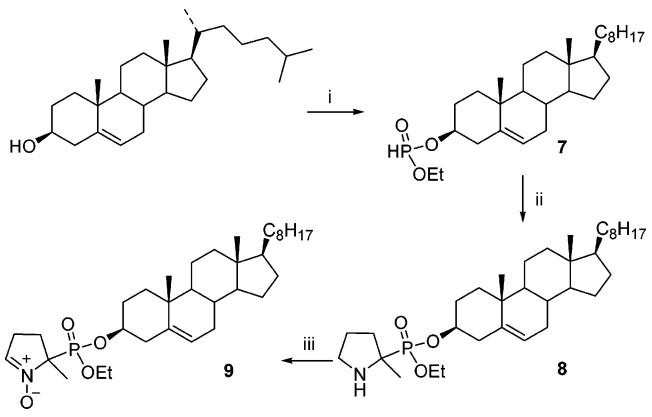
SCHEME 3[a]



[a] Reaction conditions: (i) PCl_3, EtOH, CH_2Cl_2, 10 °C, 1 h, 75%; (ii) 2-methyl-1-pyrroline, cat. $BF_3{\cdot}Et_2O$, rt, 5 d, 66%; (iii) H_2O_2, cat. Na_2WO_4, EtOH, H_2O, 0 °C, 2 d, 45%.

affinity for cell membranes and its relevance to numerous biological processes.[40] However, to avoid a dramatic slowing down of the spin adduct tumbling, monosubstitution of the phosphorus was preferred, and therefore, 5-ChEPMPO, a nitrone bearing a dissymmetric cholesteryl-ethyl phosphonate group, was synthesized, and its spin trapping properties were tested in organic solvents.

Results and Discussion

The nitrone **9**, 5-(cholesteryloxyethoxyphosphoryl)-5-methylpyrroline *N*-oxide (5-ChEPMPO), was synthesized in a three-step sequence from cholesterol and 2-methylpyrroline (Scheme 3) involving the preparation of the dissymmetric phosphite **7** and its addition onto 2-methylpyrroline followed by oxidation of the resulting aminophosphonate **8**.

The dissymmetric phosphite **7**[41] was obtained in 75% yield by adding PCl_3 to an equimolar solution of ethyl alcohol and cholesterol in CH_2Cl_2 at 10 °C. Reaction of phosphite **7** with 2-methyl-1-pyrroline in the presence of a catalytic amount of $BF_3{\cdot}OEt_2$ afforded 2-(cholesteryloxyethoxyphosphoryl)-2-methylpyrrolidine **8** in 66% yield. Compound **8** was obtained as a mixture of four diastereoisomers as indicated by ^{31}P NMR. Oxidation of the aminophosphonate **8** by hydrogen peroxide in the presence of a catalytic amount of sodium tungstate[42] in a mixture of EtOH−H_2O afforded the nitrone **9**, 5-ChEPMPO, in 45% yield. The structure of 5-ChEPMPO was determined by ^1H, ^{13}C, and ^{31}P NMR and confirmed by tandem mass spectrometry (MS/MS) of the protonated molecule $[M + H]^+$ produced by electrospray ionization (ESI).

ESR Studies. In the case of DEPMPO and its derivatives, introduction of a group on the pyrroline ring of DEPMPO has led in some cases to less efficient DEPMPO-based spin traps.[36,38] To determine if 5-ChEPMPO **9** could be a good candidate for the study of radical processes occurring in the lipophilic compartments present in biological systems, it was important to compare this nitrone, 5-ChEPMPO, with DEPMPO, a reference spin trap, by using the same conditions for the spin-trapping experiments. The reactions of the various oxygen-, sulfur-, and carbon-centered radicals with 5-ChEPMPO and DEPMPO were studied by ESR in organic media.

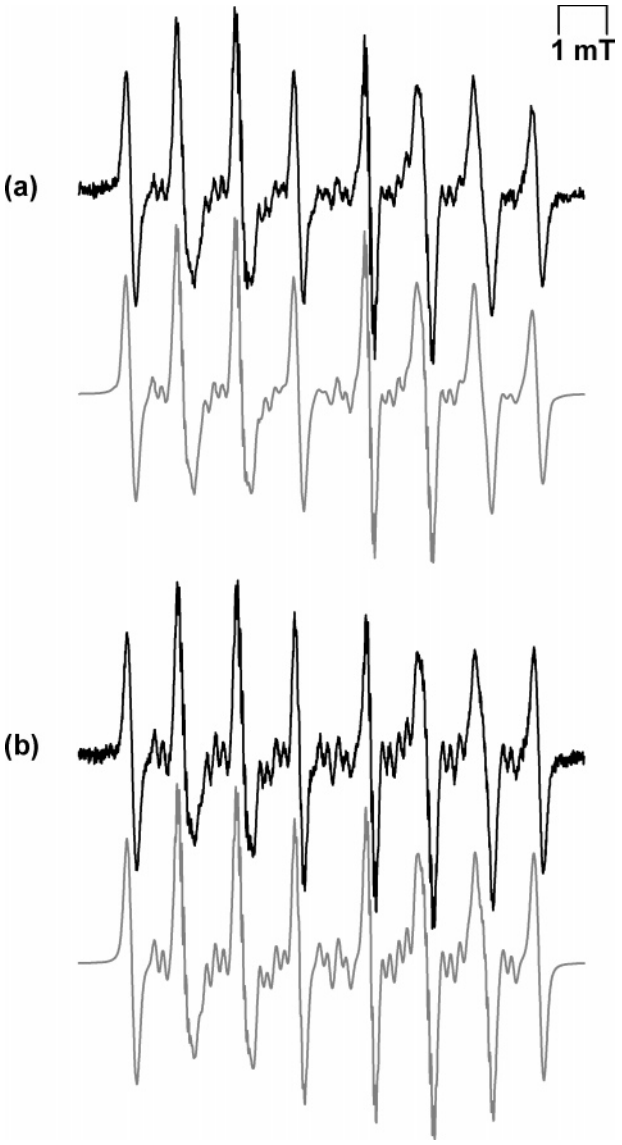


FIGURE 1. Nucleophilic addition of hydrogen peroxide on 5-ChEPMPO **9**. (a) Signal obtained after addition of H_2O_2 (0.16 M) to a solution of 5-ChEPMPO (20 mM) in deoxygenated pyridine. (b) Same conditions as in (a) with DEPMPO (20 mM). The gray lines represent the computer simulation of the spectra with parameters given in Table 1. Spectrometer settings: microwave power 10 mW (a, b); modulation amplitude, 0.0099 mT (a), 0.0313 mT (b); time constant, 1.28 ms (a, b); gain 5×10^4 (a), 10^5 (b); sweep time, 335 s (a, b); conversion time, 327 ms (a), 163 ms (b).

(a) Formation of 5-ChEPMPO Peroxyl, Alkoxyl, Thiyl and Carbon-Centered Radical Adducts by Photolysis or Nucleophilic Addition−Oxidation. The 5-ChEPMPO−OOH adduct was obtained by nucleophilic addition of H_2O_2 (160 mM) to 5-ChEPMPO (20 mM) in pyridine followed by in situ oxidation of the hydroxylamine.[43] The obtained spectrum (Figure 1a) showed the

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TABLE 1. Simulated Coupling Constants for Peroxyl Radical Adducts of 9 and DEPMPO

adducts	generating system	diastereoisomer	conformer	k^a (s^{-1})	a_P (mT)	a_N (mT)	$a_{H\beta}$ (mT)	$a_{H\gamma}{}^b$ (mT)
9−OOH	H_2O_2 (0.16 M)/pyridine	trans (89.8%)	T_1 (62.6%)	0.79×10^8	5.239	1.207	1.083	0.045 (3), 0.098, 0.044 (3)
			T_2 (37.4%)		4.468	1.246	0.979	
		cis (10.5%)			3.836	1.261	0.896	0.161
DEPMPO−OOH	H_2O_2 (0.16 M)/pyridine	trans (86.0%)	T_1 (59.2%)	1.69×10^8	5.190	1.212	1.165	0.098, 0.037 (3), 0.060, 0.051, 0.043
			T_2 (40.8%)		4.642	1.230	0.876	
		cis (14.0%)			3.814	1.262	0.903	0.157, 0.029 (3), 0.056, 0.047, 0.039
9−OO-*t*-Bu	*t*-BuOOH (1.5 M)	trans (91.9%)	T_1 (58.7%)	1.27×10^8	5.357	1.228	1.032	0.042 (5), 0.106, 0.120, 0.066
	hν, toluene		T_2 (41.3%)		4.505	1.207	0.896	
		cis (8.1%)			3.944	1.277	0.822	0.148, 0.041, 0.040 (3)
DEPMPO−OO-*t*-Bu[c]	*t*-BuOOH (1.5 M)	trans (93.5%)	T_1 (53%)	2.22×10^8	5.336	1.223	1.085	0.110, 0.040 (3), 0.066, 0.046, 0.045
	hν, toluene		T_2 (47%)		4.608	1.220	0.855	
		cis (6.5%)			3.903	1.278	0.913	0.157
9−OOL	LOOH (1.5 M)	trans (∼90%)	T_1 (62.2%)	0.18×10^8	5.129	1.200	1.073	0.032 (3), 0.110, 0.067, 0.055, 0.044
	hν, toluene		T_2 (37.8%)		4.574	1.237	0.762	
		cis (∼10%)			3.725	1.237	0.757	0.211
DEPMPO−OOL	LOOH (1.5 M)	trans (∼90%)	T_1 (63.0%)	0.18×10^8	5.086	1.198	1.059	0.048 (3), 0.111, 0.064, 0.030 (2)
	hν, toluene		T_2 (37.0%)		4.567	1.233	0.702	
		cis (∼10%)			3.775	1.231	0.794	0.219

[a] Exchange rate constants in s^{-1}, [b] Number of equivalent protons given in parentheses. [c] The better resolution allowed a more precise parameter determination compared to ref 31.

TABLE 2. Simulated Coupling Constants for Alkoxyl-, Thiyl-, and Carbon-Centered Radical Adducts of 9 and DEPMPO

adducts	generating system	diastereoisomer	a_P (mT)	a_N (mT)	$a_{H\beta}$ (mT)	$a_{H\gamma}{}^a$ (mT)
9−O-*t*-Bu	*t*-BuOO-*t*-Bu (0.5 M), *hν*, toluene	trans (100%)	4.723	1.283	0.812	0.032 (3), 0.132, 0.083, 0.053, 0.024
DEPMPO−O-*t*-Bu[30]	*t*-BuOO-*t*-Bu, (0.5 M) *hν*, toluene	trans (100%)	4.68	1.28	0.80	0.13
9−SEt	EtSSEt (1 M), *hν*, toluene	trans (100%)	4.742	1.313	1.072	0.12, 0.086, 0.047 (3), 0.024 (2)
DEPMPO−SEt	EtSSEt (1 M), *hν*, toluene	trans (100%)	4.721	1.313	1.073	0.110, 0.086, 0.046 (3), 0.023
9−Me	MeI (1 M), $C_{24}H_{54}Sn_2$	I (71.8%)	4.873	1.385	1.956	0.043 (3), 0.013 (2), 0.026, 0.056
	(0.03 M), *hν*, toluene	II (28.2%)	5.011	1.380	2.033	0.043 (3), 0.031, 0.102, 0.023, 0.056
DEPMPO−Me	MeI (1 M), $C_{24}H_{54}Sn_2$,	I (50.1%)	4.832	1.386	1.957	0.043 (3), 0.022, 0.057, 0.011, 0.034
	(0.03 M), *hν*, toluene	II (49.9%)	4.884	1.382	1.971	0.043 (3), 0.052, 0.057, 0.025, 0.034

[a] Number of equivalent protons given in parentheses.

characteristic pattern observed for DEPMPO−peroxyl type adducts, with a large apparent a_p characteristic of peroxyl adducts. Computation of this spectrum indicated the presence of a minor cis-adduct and a major trans-adduct (between the OOH and the phosphorus group). Moreover, the two conformers of the predominant trans-adduct undergo a slow exchange leading to an alternating line width phenomenon (Table 1).[14,44] To confirm the formation of the 5-ChEPMPO−OOH adduct, the same experiment was done using DEPMPO as the spin trap. A very closely related spectrum, characteristic of peroxyl adducts, was again observed (Figure 1b, Table 1).

Spin trapping of the *tert*-butylperoxyl radical by 5-ChEPMPO (20 mM) was performed in deoxygenated toluene by photolysis of *t*-BuOOH (1.5 M) in the presence of 5-ChEPMPO (20 mM) (Figure 2a).[45] The ESR spectrum showed the characteristic alternating line width phenomenon, and the hyperfine splitting constant's (hfsc's) parameters were close to those of the DEPMPO peroxyl adduct in toluene.

The 5-ChEPMPO−O-*t*-Bu adduct was obtained by photolysis of *t*-BuOO-*t*-Bu (0.5 M) in the presence of 5-ChEPMPO (20 mM) in deoxygenated toluene.[45] The observed ESR spectrum exhibited a symmetrical 12 lines signal (Figure 3a) showing the same pattern and similar hfsc's parameters as those observed for the corresponding DEPMPO−O-*t*-Bu adduct.

The 5-ChEPMPO−SEt spin adduct was obtained by photolysis of EtSSEt (1 M) in the presence of 5-ChEPMPO (20 mM) in deoxygenated toluene.[45] The observed ESR spectrum exhibited a symmetrical 12 lines signal (Figure 4a) showing the same ESR parameters as those observed with the corresponding DEPMPO−SEt obtained under the same conditions (Figure 4b).[45]

The 5-ChEPMPO−Me adduct was generated by photolysis of MeI (1 M) in the presence of bis(tributyltin) (0.03 M) and 5-ChEPMPO (20 mM) in deoxygenated toluene.[45] The observed ESR spectrum exhibited a 12 lines signal (Figure 5a) showing hfsc's parameters very close to those observed for the corresponding DEPMPO−Me obtained under the same conditions (Figure 5b).

In conclusion, 5-ChEPMPO has shown a spin-trapping behavior similar to that of DEPMPO in the case of radicals that are generated in organic solutions.[31,45]

(b) Formation of the Adducts of 13(*S*)-Hydroperoxy-9*Z*,11*E*-octadecadienoic Acid with 5-ChEPMPO and DEPMPO (5-ChEPMPO−OOL and DEPMPO−OOL). To study the ability of 5-ChEPMPO in trapping free radicals arising from lipid peroxidation processes, spin-trapping experiments were conducted with a linoleic acid hydroperoxide, used as a model of peroxidized PUFA (polyunsaturated fatty acids).

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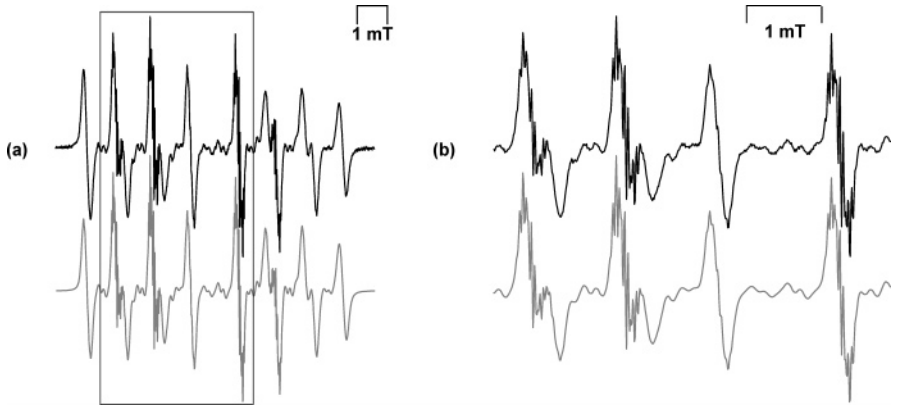

FIGURE 2. Spin trapping of *tert*-butylperoxyl radical by 5-ChEPMPO **9**. (a) Signal obtained by UV photolysis of a deoxygenated solution of *t*-BuOOH (1.5 M) and 5-ChEPMPO (20 mM) in toluene. (b) Enlargement of (a). The gray lines represent the computer simulation of the spectrum with parameters given in Table 1. Spectrometer settings: microwave power 10 mW; modulation amplitude, 0.0099 mT; time constant, 1.28 ms; gain 5×10^4; sweep time, 335 s; conversion time, 327 ms.

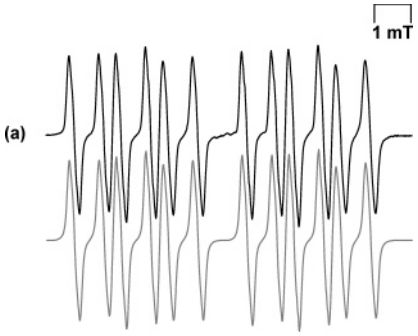

FIGURE 3. Spin trapping of *tert*-butoxyl radical by 5-ChEPMPO **9**. (a) Signal obtained by UV photolysis of a deoxygenated solution of *t*-BuOO-*t*-Bu (0.5 M) and 5-ChEPMPO (20 mM) in toluene. The gray line represents the computer simulation of the spectrum with parameters given in Table 2. Spectrometer settings: microwave power 10 mW; modulation amplitude, 0.0099 mT; time constant, 1.28 ms; gain 5×10^4; sweep time, 335 s; conversion time, 327 ms.

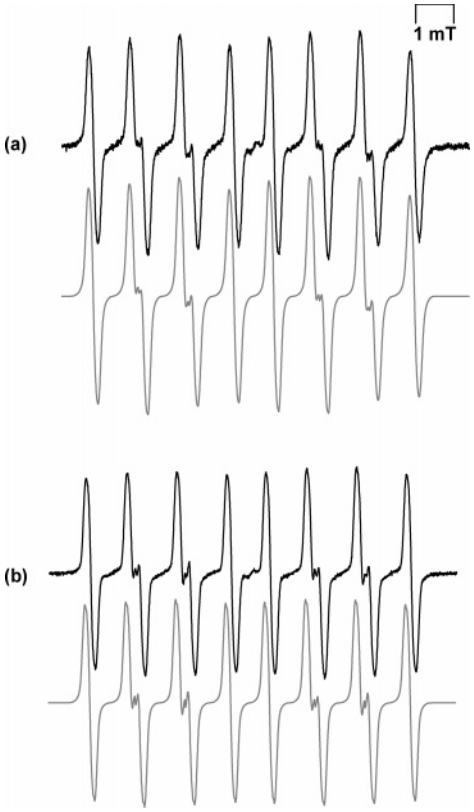

FIGURE 4. Spin trapping of ethylthiyl radical by 5-ChEPMPO **9** and by DEPMPO: (a) Signal obtained by UV photolysis of a deoxygenated solution of EtSSEt (1 M) and 5-ChEPMPO (20 mM) in toluene. (b) As in (a) with DEPMPO (20 mM). The gray lines represent the computer simulation of the spectra with parameters given in Table 2. Spectrometer settings: microwave power 10 mW (a, b); modulation amplitude, 0.00198 mT (a, b); time constant, 1.28 ms (a, b); gain 5×10^4 (a, b); sweep time, 335 s (a, b); conversion time, 163 ms (a, b).

In previous works, Davies et al.[46] and Dikalov et al.,[47] respectively, reported the detection of lipid oxygen- and carbon-centered radicals using DMPO in organic solvent and in aqueous media. In both cases, the observed ESR spectra were complicated by the overlapping of many signals with close hfsc's parameters. Stolze et al.[32] used DEPMPO as a spin trap to detect the radicals produced by mixing crude linoleic acid hydroperoxide and Fe(II) in a phosphate buffer. Different ESR spectra were observed depending on the use of oxygen-saturated or oxygen-free buffer solutions. The signals could not be fully assigned, but it has been assumed that $LOO^{\bullet}$ or possibly $LO^{\bullet}$ spin adducts as well as $HO^{\bullet}$ and carbon-centered spin adducts were observed. More recently and using DEPMPO as spin trap, Kambayashi et al.[48] have reported the formation of superoxide radicals during the ferrous ion-induced decomposition of linoleic acid hydroperoxide under aerobic conditions in water.

In these studies, the linoleic acid hydroperoxide was prepared either by a nonspecific process[49] of oxidation of linoleic acid by molecular oxygen during 72 h[32,46] or by mixing linoleic acid, soybean lipoxygenase, and the spin trap.[47] However, the limited ability of DMPO to detect peroxyl radicals[50] or the use of a mixture of crude

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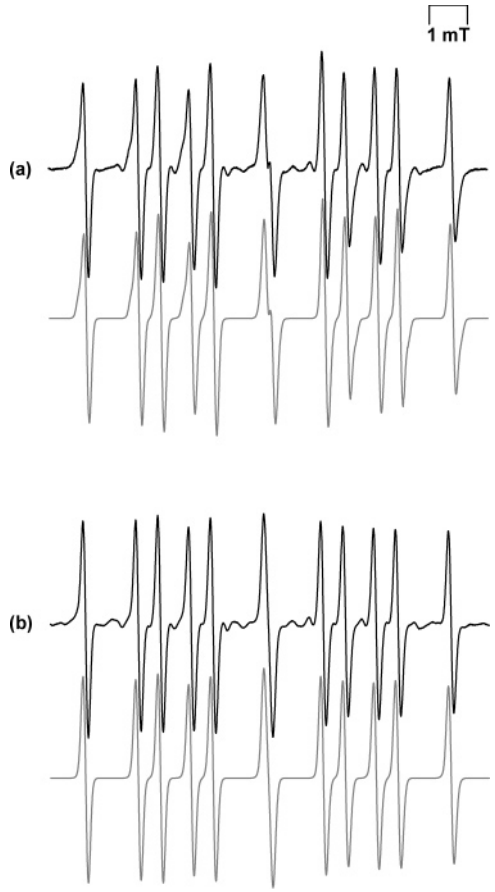

FIGURE 5. Spin trapping of methyl radical by 5-ChEPMPO **9** and by DEPMPO: (a) Signal obtained by UV photolysis of a deoxygenated solution of CH_3I (1 M), bis(tributyltin) (0.03 M), and 5-ChEPMPO (20 mM) in deoxygenated toluene. (b) As in (a) with DEPMPO (20 mM). The gray lines represent the computer simulation of the spectra with parameters given in Table 2. Spectrometer settings: microwave power 10 mW (a, b); modulation amplitude, 0.0099 mT (a, b); time constant, 1.28 ms (a, b); gain 5×10^4 (a, b); sweep time, 335 s (a, b); conversion time, 327 ms (a, b).

peroxidized linoleic acid, containing 9- and 13-hydroperoxide isomers and overoxidation products, explains the observation of complex spectra and the difficult assignment of the trapped radicals. Therefore, to study unambiguously the behavior of **9** toward a PUFA-peroxidized species, we decided to prepare a pure linoleic acid hydroperoxide sample.

13(*S*)-Hydroperoxy-9*Z*,11*E*-octadecadienoic acid was obtained as a pure enantiomer by reaction of linoleic acid with soybean lipoxygenase under oxygen pressure according to the procedure of Iacazio et al.[51,52] The structure of the linoleic acid hydroperoxide was confirmed by the ESI-MS/MS fragmentation pattern. Photolysis for a few seconds of a mixture containing this linoleic acid hydroperoxide (1.5 M) and 5-ChEPMPO (20 mM) or DEPMPO (20 mM), respectively, in deoxygenated toluene afforded the spectra shown in Figure 6. The ESR spectra obtained with 5-ChEPMPO and DEPMPO used as spin traps were very similar and exhibited a main signal presenting the characteristic pattern and hfsc's parameters of peroxyl DEPMPO spin adducts in toluene (parameters given in Table 1). When the irradiation was maintained for a few minutes, an additional weak signal appeared, with hfsc's parameters characteristic of a carbon-centered spin adduct ($a_P = 4.90$ mT, $a_N = 1.35$ mT, $a_H = 1.90$ mT).

Analysis of the relaxation data calculated by the automatic parameter fitting program[53] has great importance (Table 3), since the bulky substituents on the phosphoryl side (cholesteryl) and on the opposite side (OOL) can exert a predominant role on the type and rate of molecular rotation, which have a characteristic effect on the relaxation terms. The relaxation formula used[53] was

$$\text{width}\,(M_N, M_P) = \alpha + \beta_1 M_N + \gamma_1 (M_N \times M_N) + \beta_2 M_P + \gamma_2 (M_N \times M_P)$$

where M_N stands for the magnetic quantum number of nitrogen (−1, 0, +1) and M_P stands for the magnetic quantum number of phosphorus (−1/2, +1/2). The α parameter is very complex, as it depends on the *g*-tensor anisotropy, the rotation rates, the spin−spin encounters (bimolecular and radical-oxygen interaction), and thus, this parameter is less suitable to derive information on the molecular dynamics. The β and γ terms depend on the rate and the direction of molecular rotation. In the case of fast rotation, these terms are small: this condition holds for DEPMPO−OOH where all these parameters are smaller than 3 μT. As a consequence of the presence of bulky substituents, the rotation is slowed and the spectrum can become anisotropic. The bulky cholesteryl group increases the β and γ relaxation terms, while the linoleyl group has a more marked and more specific impact, primarily the β_2 and γ_2 terms have large values. The β_1 and γ_1 relaxation terms are less sensitive to the size of the substituent, since the principal axis of the A_N hyperfine tensor is nearly perpendicular to the C(2)−C(5) axis. Due to the fast rotation around this C(2)−C(5) axis, it can easily average out the hyperfine anisotropy. On the other hand, the β_2 and γ_2 terms become large, since the C(2)−C(5) axis forms a small angle with the principal direction of the phosphorus hf tensor, which makes the averaging process less effective. The strongest relaxation can be observed when both C(2) and C(5) have large substituents, for example, in **9**−OOL (Table 3). The bulky linoleyl group has also a significant impact on the rate of chemical exchange caused by the rotation around the O−O bond: the rate of exchange is reduced by 1 order of magnitude (see Tables 1 and 2). In the case of the OOL adducts, the exchange rates were the same for DEPMPO and for **9**, but in the case of the smaller OOH and OO-*t*-Bu adducts, the cholesteryl substitution induced a slower exchange by a factor of 2. Although a faster exchange is expected for OOH than for OO-*t*-Bu, this effect is compensated by the fact that different solvents were used for the trapping of OOH and OO-*t*-Bu, respectively, pyridine and toluene.

To confirm the structure of the observed adducts, the sample containing the assumed 5-ChEPMPO−OOL ad-

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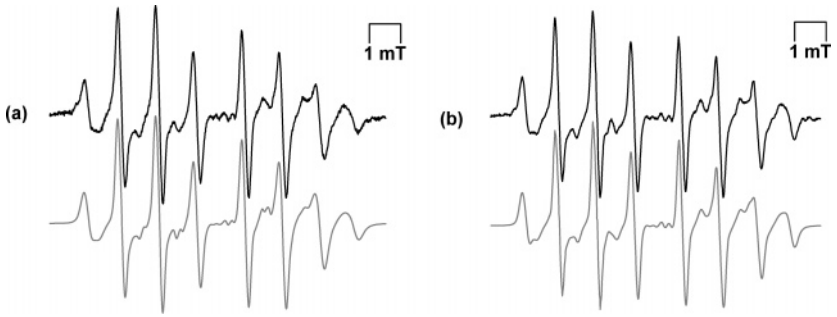
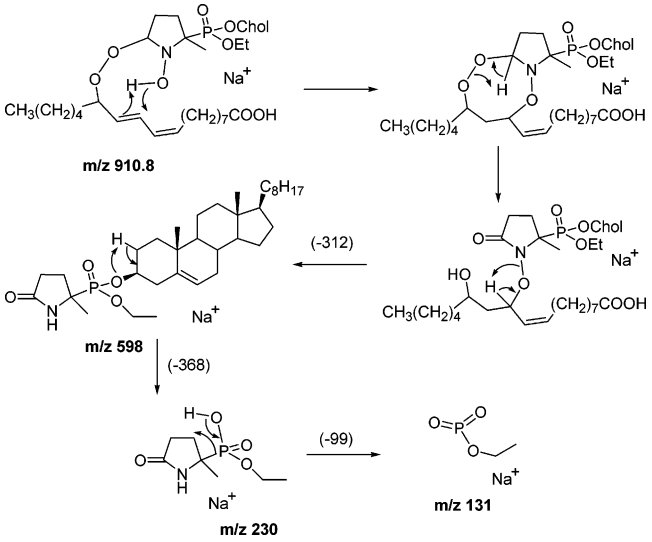


FIGURE 6. Spin trapping of LOO• radical by 5-ChEPMPO **9** and DEPMPO. (a) Signal obtained after photolysis of a deoxygenated solution of LOOH (1.5 M) and 5-ChEPMPO (20 mM) in toluene. (b) As in (a) with DEPMPO (20 mM). The gray lines represent the computer simulation of the spectra with parameters given in Table 1. Spectrometer settings: microwave power 10 mW (a, b); modulation amplitude, 0.0395 (a, b); time constant, 1.28 ms (a, b); gain 5×10^4 (a, b); sweep time, 84 s (a, b), 0.167 s (a, b); conversion time, 82 ms (a, b).

TABLE 3. Relaxation Data for DEPMPO−R and 5-ChEPMPO−R

adduct	generating system	α (μT)	β_1 (μT)	γ_1 (μT)	β_2 (μT)	γ_2 (μT)
DEPMPO−OOH	H_2O_2 (0.16 M)/pyridine	27.1	−2.4	2.4	0.6	−0.5
9−OOH	H_2O_2 (0.16 M)/pyridine	10.5	−9.1	8.5	18.9	10.2
DEPMPO−OOL	LOOH (1.5 M), *hν*, toluene	14.4	−10.0	3.0	46.8	−21.7
9−OOL	LOOH (1.5 M), *hν*, toluene	14.0	−16.5	7.1	95.5	−53.5
9−OO-*t*-Bu	*t*-BuOOH (1.5 M), *hν*, toluene	6.0	−12.7	6.6	4.8	−2.1
9−O-*t*-Bu	*t*-BuOO-*t*-Bu (0.5 M), *hν*, toluene	34.0	−1.9	8.1	3.4	1.2

SCHEME 4. MS/MS Fragmentation Pathway of the [5-ChEPMPOH−OOL + Na]$^+$ at *m/z* 910.8



duct was analyzed by mass spectrometry. Two major peaks, attributed to $[M + Na]^+$ and $[M + K]^+$ adducts at *m/z* 910.8 and *m/z* 926.8, respectively, were observed in the ESI-MS spectrum. MS/MS spectrum of *m/z* 910.8 ion gave fragment ions at *m/z* 598, 230, and 131 arising from the respective cleavage of the peroxide bond, followed by cleavage of the C−O bond of the cholesteryl ester and finally by dephosphorylation of the pyrrolidinone ring (Scheme 4). The fragmentation pattern confirmed unambiguously that the peak observed at *m/z* 910.8 corresponds to the 5-ChEPMPOH−OOL sodium adduct.

Conclusion

Despite the presence of the bulky cholesteryl substituent, 5-ChEPMPO **9** is as efficient as DEPMPO to trap peroxyl-, alkoxyl-, thiyl-, and carbon-centered radicals in organic solvent. The 5-ChEPMPO−OOL adduct derived from the trapping of an enantiopure linoleyl peroxide gave a signal that did not contain additional species as observed in the case of the trapping of an air oxygenation-generated linoleyl peroxide with DMPO.[46] In addition, the structure of the 5-ChEPMPO−OOL adduct was unambiguously confirmed by ESI-MS/MS studies. Therefore, 5-ChEPMPO can constitute an interesting lipophilic spin trap to study oxidative processes occurring in lipophilic compartments. Due to the easy inclusion of cholesteryl derivatives in β-cyclodextrin,[54−56] inclusion complexes between 5-ChEPMPO and β-cyclodextrin could also be useful for the study of oxidative processes in aqueous compartments.

Experimental Section

General Methods. CH_2Cl_2 was distilled under dry argon atmosphere in the presence of P_2O_5. All reagents were used as received without further purification. The reactions were monitored by TLC on 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 75.47 MHz, respectively. ^{31}P NMR was taken in $CDCl_3$ using 85% H_3PO_4 as an external standard with broad-band ^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 coupling constant *J* values in Hertz. The assignments of NMR signals were facilitated by use of the DEPT 135 sequence for all products.

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13(*S*)-Hydroperoxy-9*Z*,11*E*-octadecadienoic acid was prepared by reaction of linoleic acid with Soybean Lipoxygenase under oxygen pressure according to the procedure described by Iacazio et al.[52]

Synthesis of the Nitrone 5-ChEPMPO 9. Ethyl Cholesteryl Phosphite (7). Distilled PCl_3 (5 g, 36.5 mmol) was added dropwise to a cooled mixture of cholesterol (54.75 mmol, 21.2 g) and EtOH (2.5 g, 54.75 mmol) in CH_2Cl_2 (25 mL). The mixture was stirred at 10 °C for 1 h. The resulting solution was poured into ice (30 mL), and KOH pellets were added until pH > 8. The mixture was extracted with CH_2Cl_2 (80 mL), and the organic layer was washed with water (20 mL) and dried over Na_2SO_4. The solvent was distilled under reduced pressure and the residue was purified by column chromatography (pentane/acetone 15:2) to afford the ethyl cholesteryl phosphite **7** as a yellow oil (12.8 g, 75%): ^{31}P NMR (121.49 MHz) δ 7.52; ^{1}H NMR (300.13 MHz) δ 6.85 (1H, d, J = 690.1), 5.40−5.35 (1H, m), 4.40−4.15 (3H, m), and 2.44−0.80 (46H, m); ^{13}C NMR (75.47 MHz) δ 140.0, 123.0, 76.8, 62.0, 60.3, 56.6, 56.1, 50.0, 43.4, 42.3, 39.8, 39.6, 39.5, 37.3, 36.4, 36.1, 33.3, 31.7, 28.2, 28.0, 24.0, 23.8, 22.8, 22.5, 21.0, 20.9, 19.3 (d, J = 4.0), 18.7, and 11.8.

5-(Cholesteryloxyethoxyphosphoryl)-5-methylpyrrolidine (8). A mixture of ethyl cholesteryl phosphite (12.7 g, 26.5 mmol), 2-methyl-1-pyrroline (2.00 g, 24.1 mmol), and BF_3·Et_2O (0.2 mL, 1.4 mmol) was stirred at room temperature for 5 days. The resulting solution was poured into water (10 mL), and the mixture was extracted with CH_2Cl_2 (3 × 50 mL). The organic extracts were combined and dried over Na_2SO_4. The solvent was distilled under reduced pressure. Purification by column chromatography (gradient of pentane/acetone from 9/1 to 6/4) afforded a yellow oil (9.7 g, 66%), corresponding to a mixture of four diastereoisomers of the pyrrolidine **8**: ^{31}P NMR (121.49 MHz) 30.41 (25%), 30.37 (25%), 30.33 (28%), and 30.30 (22%); ^{1}H NMR (300.13 MHz) δ 5.39−5.31 (1H, m), 4.38−4.21 (1H, m), 4.20−4.04 (2H, m), 3.10−2.91 (2H, m), 2.45−2.35 (2H, m), 2.25−1.02 (37H, m), 0.99 (3H, s), 0.87 (9H, m, J = 6.4), and 0.65 (3H, s); ^{13}C NMR (75.47 MHz) δ 139.71, 139.69, 122.71, 122.69, 76.5 (d, J = 7.1), 76.4 (d, J = 7.7), 62.2 (d, J = 7.7), 62.1 (d, J = 8.2), 61.95 (d, J = 7.1), 61.92 (d, J = 7.1), 59.4 (d, J = 164.7), 56.6, 56.1, 50.0, 47.1 (d, J = 7.1), 42.3, 40.6 (d, J = 2.7), 40.2 (d, J = 5.5), 39.7, 39.5, 37.0, 36.4, 36.2, 35.8, 34.6 (d, J = 2.2), 34.5 (d, J = 2.7), 31.9, 31.8, 28.2, 28.0, 25.8 (d, J = 4.9), 25.7 (d, J = 4.9), 24.31 (d, J = 5.0), 24.29, 24.25, 23.8, 22.8, 22.5, 21.0, 19.3, 18.7, 16.6 (d, J = 4.4), 16.5 (d, J = 3.8), and 11.8; ESI-MS/MS (20 eV) *m/z* 562.4 (M^+ + H, 54.3), 452.3 (0.6), 369.3 (4.2), 194.1 (20.1), and 84.2 (100).

5-(Cholesteryloxyethoxyphosphoryl)-5-methylpyrroline *N*-Oxide (9). Sodium tungstate (0.044 g, 0.13 mmol) was dissolved in demineralized water (6 mL) at room temperature and added to a solution of the pyrrolidine **8** (1 g, 1.77 mmol) in a mixture of EtOH/pentane (71/29). After the solution was cooled at 0 °C, a solution of hydrogen peroxide 30% (0.35 mL, 3.55 mmol) was then added dropwise over 1 h. The mixture was then stirred at 3−4 °C for 72 h. The aqueous layer was saturated with sodium chloride and extracted by CH_2Cl_2 (3 × 30 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 95:5) to afford **9** (0.46 g, 45%): ^{31}P NMR (121.49 MHz) 21.90 (35%), 21.86 (37%), 21.78 (16%), and 21.76 (12%); ^{1}H NMR (300.13 MHz) δ 6.93 (1H, dd, J = 6.93), 5.41−5.39 (1H, m), 4.49−4.15 (3H, m), 2.93−2.72 (2H, m), 2.66−2.36 (3H, m), 2.18−0.97 (36H, m), 0.94−0.87 (9H, m, J = 6.42, 6.61), and 0.69 (3H, s); ^{13}C NMR (75.47 MHz) δ 139.4, 139.3, 135.1 (d, J = 8.0), 134.8 (d, J = 6.9), 123.1, 123.0, 77.2, 75.0 (d, J = 156.6), 63.9 (d, J = 5.7), 62.9 (d, J = 6.9), 56.6, 56.1, 49.9, 42.3, 40.3 (d, J = 3.4), 40.1 (d, J = 4.6), 39.6, 39.5, 36.9, 36.7, 36.4, 36.1, 31.84, 31.79, 31.0, 30.9, 30.0 (d, J = 2.9), 29.8 (d, J = 4.0), 28.2, 28.0, 25.8, 24.2, 23.8, 22.8, 22.5, 21.0, 20.8, 18.7, 16.4 (d, J = 5.7), and 11.8; ESI-MS/MS (10 eV) *m/z* 576.1 $[M + H]^+$ (100), 369.3 (4.3), 208 (43.1), and 162.1 (0.7). Anal. Calcd for $C_{34}H_{58}NO_4P$·$1.5H_2O$: C, 67.74; H, 10.20; N, 2.32. Found: C, 67.48; H, 9.81; N, 2.31.

Spin-Trapping Studies. (a) ESR Measurements. ESR spectra were recorded at room temperature at 9.5 GHz (X-band) employing 100 kHz field modulation. Reaction mixtures were prepared in anhydrous toluene or in anhydrous pyridine. All solutions containing organic solvents were deoxygenated by freeze/thaw cycles before photolysis.

(b) Superoxide Adduct: H_2O_2/Pyridine System. H_2O_2 (160 mM) was added to a deoxygenated solution of 5-ChEPMPO **9** or DEPMPO (20 mM) in pyridine.

(c) *tert*-Butylperoxyl Trapping: *t*-BuOOH/*hν* System. The *tert*-butylperoxyl radical adduct was generated by photolysis of *t*-BuOOH (1.5 M) in the presence of 5-ChEPMPO **9** (20 mM) in deoxygenated toluene.

(d) Linoleyl Peroxyl Adduct: LOOH/Toluene System. 13(*S*)-Hydroperoxy-9*Z*,11*E*-octadecadienoic acid (1.5 M) was added to a deoxygenated solution of 5-ChEPMPO **9** or DEPMPO (20 mM) in toluene and photolyzed during 30 s.

(e) *tert*-Butoxyl Trapping: *t*-BuOO-*t*-Bu/*hν* System. The *tert*-butoxyl radical adduct was generated by photolysis of *t*-BuOO-*t*-Bu (0.5 M) in the presence of 5-ChEPMPO **9** (20 mM) in deoxygenated toluene.

(f) Ethylthiyl Trapping. The ethylthiyl radical adduct was generated by photolysis of EtSSEt (1 M) in the presence of 5-ChEPMPO **9** or DEPMPO (20 mM) in deoxygenated toluene.

(g) Methyl Trapping. The methyl radical adduct was generated by photolysis of MeI (1 M) in the presence of bis-(tributyltin) (0.03 M) and 5-ChEPMPO **9** or DEPMPO (20 mM) in deoxygenated toluene.

ES-Mass Spectrometry of 5-ChEPMPO−OOL. Positive-ion ES mass spectra and tandem mass spectra were acquired in a triple quadrupole mass spectrometer equipped with a pneumatically assisted ES source (nebulizing gas, air at a 0.8 L/min flow rate). The sample was prepared by diluting a milliliter amount of the sample with 500 μL of toluene and then diluted 1/10 with a methanol solution containing 0.5% of formic acid. The sample was introduced into the mass spectrometer at a 5 μL/min flow rate, setting the ES voltage at 5000 V with cone voltage at 50 V. Tandem mass spectra (MS/MS) of the molecule sodium adduct were obtained by collision-induced dissociation (CID), using argon as the collision gas and a collision energy at 40 eV: ESI-MS/MS (40 eV) *m/z* 910.8 $[M + Na]^+$ (0.4); 598.0 (100); 230.1 (31.7); 131.2 (1.3).

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Supporting Information Available: Structures and NMR spectra for compounds **7**−**9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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