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# New Biocompatible $\beta$ -Phosphorylated Linear Nitrones Targeting Mitochondria: Protective Effect in Apoptotic Cells

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**Abstract:** Mitochondria, an essential organelle involved in cellular respiration, energy production, and cell death, is the main cellular source of reactive oxygen species (ROS), including superoxide. Mitochondrial diseases resulting from uncontrolled/excess ROS generation are an emerging public health concern and there is current interest for specific mitochondriotropic probes to get information on *in situ* ROS production. As such, nitrones vectorized by the triphenylphosphonium (TPP) cation have recently drawn attention despite reported cytotoxicity. Herein, we describe the synthesis of 13 low-toxic derivatives of *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN) alkyl chain-grafted to a pyridinium, triethylammonium or berberinium lipophilic cation. These nitrones showed *in vitro* superoxide quenching activity and EPR/spin-trapping efficiency towards biologically relevant free radicals, including superoxide and hydroxyl radicals. Their mitochondrial penetration was confirmed by <sup>31</sup>P NMR and anti-apoptotic properties assessed in hydrogen peroxide treated Schwann cells. Two pyridinium substituted PPNs were identified as potentially better alternatives to TPP nitrones conjugates for studies of mitochondrial oxidative damage.

## Introduction

Mitochondria are complex, metabolically active and dynamic organelles that regulate critical cellular processes from ATP-production to apoptosis. They are major oxygen-consumers with roughly 95% of the total amount of breathed O<sub>2</sub> undergoing, in desirable conditions, reduction to water by cytochrome *c* oxidase (complex IV) in four consecutive one-electron steps. Activation of O<sub>2</sub> by monovalent reduction leads to superoxide anion radical (O<sub>2</sub><sup>•-</sup>), a reactive oxygen species (ROS) participating in many key cellular pathways.<sup>[1]</sup> Since the 1970s, popular estimates of the steady state amount of mitochondrial O<sub>2</sub><sup>•-</sup> ranged 1–2% of inhaled oxygen (i.e., 215–430 mmol of O<sub>2</sub><sup>•-</sup> daily for an 80 kg human)<sup>[1a]</sup>, yet recent knowledge proposed it could actually be one order of magnitude less under non pathological conditions.<sup>[1b]</sup> Nevertheless, still the mitochondrial respiratory chain is regarded, together with cytosolic NADPH oxidases (Nox), as a major cellular source for O<sub>2</sub><sup>•-</sup>. In mitochondria electron transport chain, O<sub>2</sub><sup>•-</sup> formation involves complexes I (NADH ubiquinone oxidoreductase) and III (cytochrome *bc*<sub>1</sub> complex), releasing this free radical within the mitochondrial matrix and, mostly, into the intermembrane space, respectively.<sup>[1c]</sup>

About thirty years ago, the production and/or regulation of mitochondrial ROS have emerged as essential mechanisms to maintain cellular functions and signaling.<sup>[1d]</sup> Impairment of these processes results in overproduction of O<sub>2</sub><sup>•-</sup> and/or hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), the ROS specifically formed when O<sub>2</sub><sup>•-</sup> is neutralized by mitochondria-specific superoxide dismutases. The following excess of H<sub>2</sub>O<sub>2</sub>, if not cleared by detoxifying enzymes such as catalase (present in the mitochondria of some cell types),

glutathione peroxidases (GPx), and peroxiredoxins (Prdx) may then be converted to harmful hydroxyl radicals (HO•) in a general deleterious process termed as oxidative stress.<sup>[2]</sup> Mitochondrial dysfunction has been associated with a host of disease categories and metabolic conditions, including metabolic syndrome (diabetes/obesity), ischemia/reperfusion injury, inflammation, as well as chronic disorders such as cancer, neurodegeneration, Alzheimer's and Parkinson's diseases, and aging<sup>[3]</sup>, even if most of them have recognized polygenic and/or environmental origins.

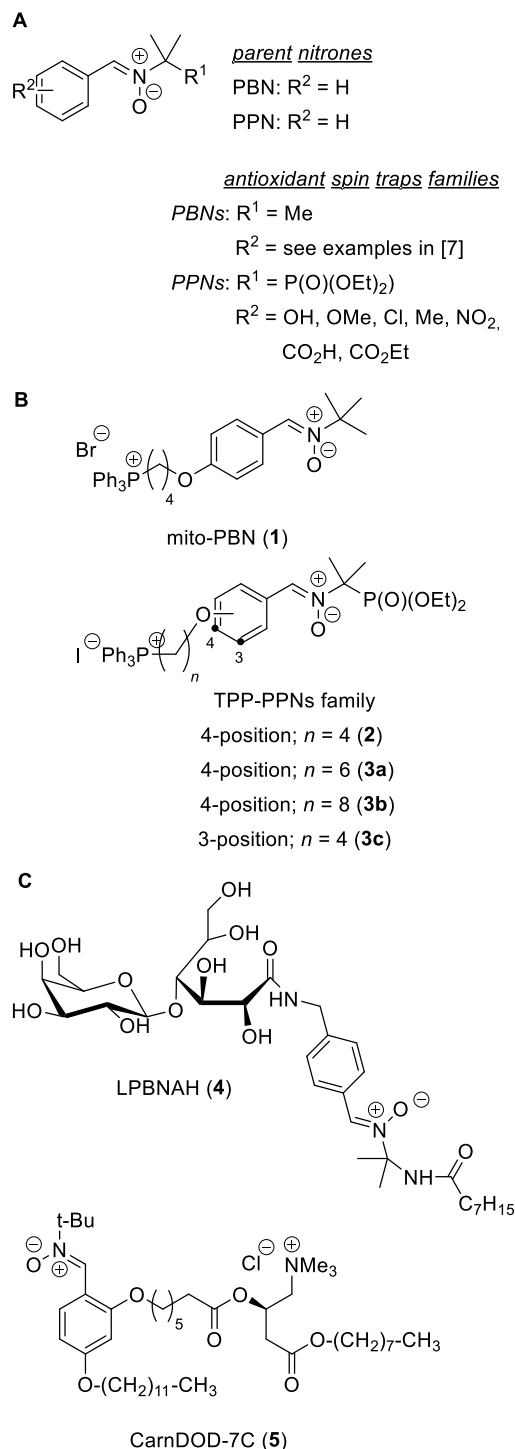
In this context the amount of space is expanding for chemically engineering mitochondria targeted antioxidants and free radical scavengers able to maintain the redox status of the cell and repair the dysfunctions linked to oxidative stress, or for other bioinspired mitochondriotropic probes for quantifying ROS variation, such as mito-peptides.<sup>[4]</sup> To this end synthetic strategies have favored hybrids made up of (i) a delocalized lipophilic cation vector for crossing the mitochondrial inner membrane by diffusion, most commonly a triphenylphosphonium cation (TPP), (ii) an aliphatic chain spacer, and (iii) a pharmacophore such as a chain-breaking antioxidant aryl moiety or an active free radical (FR) scavenging site. Because nitrones have been used for decades in electron paramagnetic resonance (EPR) spin-trapping for detection/identification purposes<sup>[5]</sup>, their chemical function C=N<sup>+</sup>-O<sup>-</sup> was considered suitable as a FR scavenging building block in designing these new hybrid compounds.<sup>[4c,6]</sup> Moreover, the  $\alpha$ -phenyl-*N*-tert-butyl nitronone (PBN; Figure 1A) scaffold has emerged as a privileged core for drug design in many pathologies related to FR formation. Thus, a wide range of PBN- or PBN-like derivatives has been developed in the last decades (jointly named PBNs family in Figure 1A) and many have demonstrated pharmacological action *in vivo*, assuming this may rely on the inhibition of ROS-dependent or -independent biological pathways considered as hallmarks of mitochondrial dysfunction.<sup>[7,8]</sup> Following the first reported PBN/TPP conjugate bromide, mito-PBN (**1**; Figure 1B)<sup>[9]</sup>, a number of TPP-based linear or cyclic nitrones have been synthesized, their mitochondrial vs. cytosolic uptake was found increased 100–1,000 times *in vitro*, and practically all these compounds exhibited pharmacological properties to reduce mitochondrial ROS content in various situations.<sup>[6]</sup> In particular, **1** has been found to inhibit cerium oxide nanoparticles toxicity in human fibroblasts by interacting with both mitochondrial ROS formation and membrane Nox2 and Nox4 activation.<sup>[10]</sup> It has been also reported that encapsulating **1** in liposomes-lipid nanoparticles decreased O<sub>2</sub><sup>•-</sup> levels in mitochondria of diabetic mice by targeting hepatocytes and Kupffer cells.<sup>[11]</sup>

In the PBNs structure the  $\alpha$ -*tert*-Bu group, introduced to increase the kinetic stabilization of the resulting nitroxide spin adduct and thus facilitate its EPR detection<sup>[5]</sup>, brings about increased lipophilicity of the nitronone but may enhance its toxicity (see<sup>[8]</sup> and references therein). In our prior approaches to discover improved spin-trapping probes of oxidative stress, we repeatedly found a lower cytotoxicity when one Me of the *tert*-Bu was replaced by a diethoxyphosphoryl group, (EtO)<sub>2</sub>P(O).<sup>[8,12]</sup> On this biocompatibility basis, we developed a family of  $\beta$ -phosphorylated, PBN-like hybrids, i.e., based on a *N*-benzylidene-1-diethoxyphosphoryl-1-methylethylamine *N*-oxide (PPN) scaffold, bearing, for a subset, an aryl substituent inspired by those found in natural phenolic acids (PPNs family; Figure 1A). Not only these PPNs demonstrated nitric oxide (NO)-donation properties, resulting in significant vasoprotection (at submillimolar doses) of rat aortic rings challenged by O<sub>2</sub><sup>•-</sup>, but those compounds bearing a methoxylated phenolic ligand showed strong antioxidant properties *in vitro*, while retaining a low toxicity in various cell lines. In contrast to many PBNs, most of these PPNs yielded EPR characterizable O<sub>2</sub><sup>•-</sup> and HO• spin adducts in aqueous media, taking advantage of the known stabilization effect provided by  $\alpha$ -P(O)(OEt)<sub>2</sub> substitution of linear nitronones.<sup>[8]</sup>

Bearing the above in mind, we recently synthesized a series of iodides having both a TPP-grafted aryloxy substituent as for **1**, and a PPNs-type nitronyl function (TPP-PPNs family; Figure 1B). <sup>31</sup>P NMR studies in rat livers confirmed that, for the lead, para-substituted compound **2**, the relative accumulation ratio in mitochondrial:cytosolic extracts was of 2:1, and its toxicity to murine fibroblasts was (i) decreased about 8 times as compared with its non-phosphorylated analogue **1**, but (ii) increased 1.6 times as compared with the 'untargeted' analogue where an uncharged diphenylphosphonyl group Ph<sub>2</sub>P(O) was present instead of TPP.<sup>[13]</sup> Altogether, these latter findings were considered of particular interest since they suggest the protective 'phosphonate effect'<sup>[12]</sup> expected in **2** may have compensated the TPP group cytotoxicity. Indeed, alkyl or aryl phosphonium salts are known to be highly toxic to human tumor mitochondria and, as such, can exhibit chemotherapeutic potency.<sup>[4c]</sup> Furthermore, high doses of a variety of TPP conjugates have been reported to cause mitochondria membrane depolarization of normal cells, depending on structural features such as the linker chain length and the pharmacophore (e.g., quinone, vitamin E, or a stable nitroxide).<sup>[6b,14]</sup>

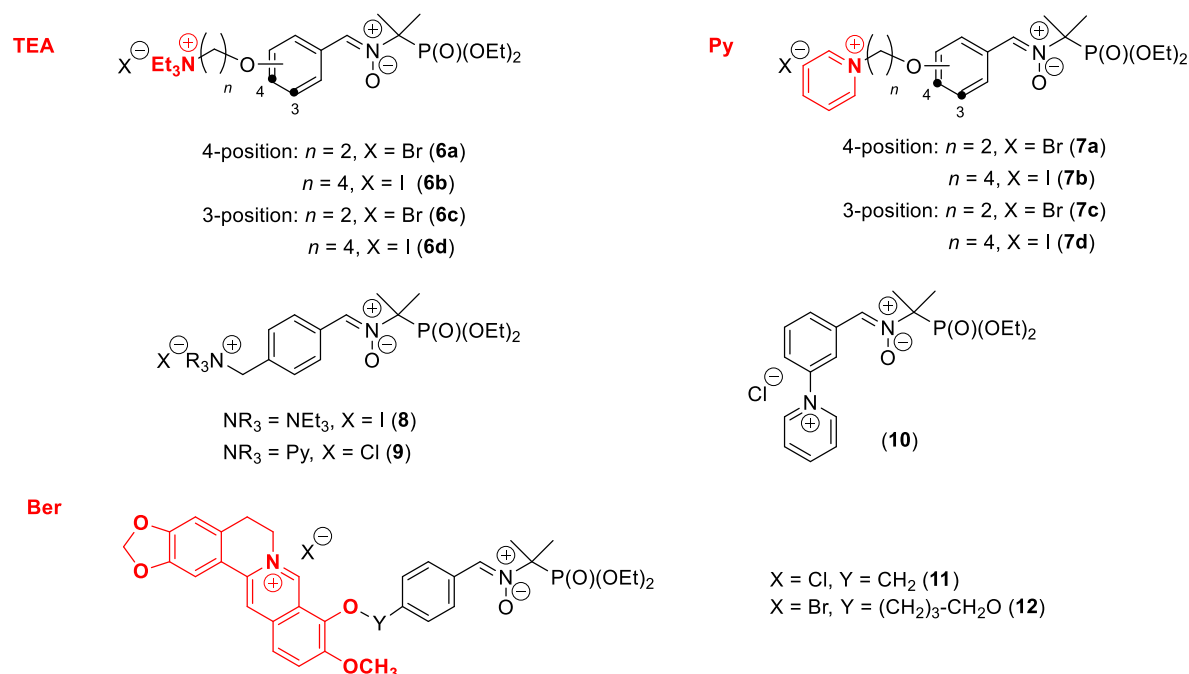
Given the relative toxicity issues of TPP, alternative mitochondria specific more biocompatible vectors to be coupled to nitronones have been proposed in the literature. Figure 1C shows typical compounds<sup>[15]</sup> such as the neuroprotective agent LPBNAH (**4**), an amide-substituted PBN vectorized by

a sugar moiety, and CarnDOD-7C (**5**), a PBNs/carnitine conjugate having demonstrated good FR scavenging properties (Figure 1C). Conceivably, other lipophilic derivatives of *N*-centered cations, as exemplified by the ammonium salt **5**, are potential mitochondriotropic candidates. Specifically considering nitrones, the preparation of PBNs pyridinium salts for spin trapping purposes has been reported decades ago<sup>[16]</sup> but only recently was a series of *N*-arylpyridinium PBNs designed as mitochondria targeted antioxidants, although their permeation property was not investigated.<sup>[17]</sup>



**Figure1.** General structures of noncyclic nitrones and related families exhibiting antioxidant and/or pharmacological activity (A) deriving from PBN and PPN<sup>[7,8]</sup>, or targeted to the mitochondrion (B) by a TPP cation<sup>[6a,8]</sup>, or (C) using alternative vectors.<sup>[15]</sup>

Accordingly, the focus of this work was to tailor the general structure of a new class of mitochondria targeted nitrones (jointly named mito-PPNs) to lower cytotoxicity while retaining good mitochondrial addressing, antioxidant power, and spin-trapping informative potential. For these purposes, the first mito-PPNs synthesized included eight compounds in which the aryloxy framework involved a triethylammonium (TEA; compounds 6a–d) or a pyridinium (Py; compounds 7a–d) lipophilic cation carrier instead of TPP. Three additional TEA or Py vectorized nitrones were prepared along an aryl framework (compounds 8–10). To expand structural diversity, we also prepared two mito-PPNs 11 and 12 where the aryl or aryloxy frameworks above were coupled with the berberinium iminium cation (Ber). Berberine, a plant alkaloid used for centuries in traditional medicine, has been recently employed as a core in drug synthesis.<sup>[18]</sup> Being highly delocalized and lipophilic, Ber has been shown to be an efficient mitochondria penetrating vector.<sup>[19]</sup> The chemical structures of the thirteen target mito-PPNs are shown in Figure 2, Cytotoxic activities were determined on normal human dermal fibroblasts (HDF) and newcomers combining optimal biocompatibility and O<sub>2</sub><sup>•-</sup> quenching properties were evaluated for their mitochondrial permeation in isolated perfused rat livers and protection in a cellular model of H<sub>2</sub>O<sub>2</sub> induced apoptosis. In addition, a series of spin-trapping experiments established a database of EPR coupling constants for O<sub>2</sub><sup>•-</sup>, HO<sup>•</sup>, and hydroxyalkyl radical adducts.



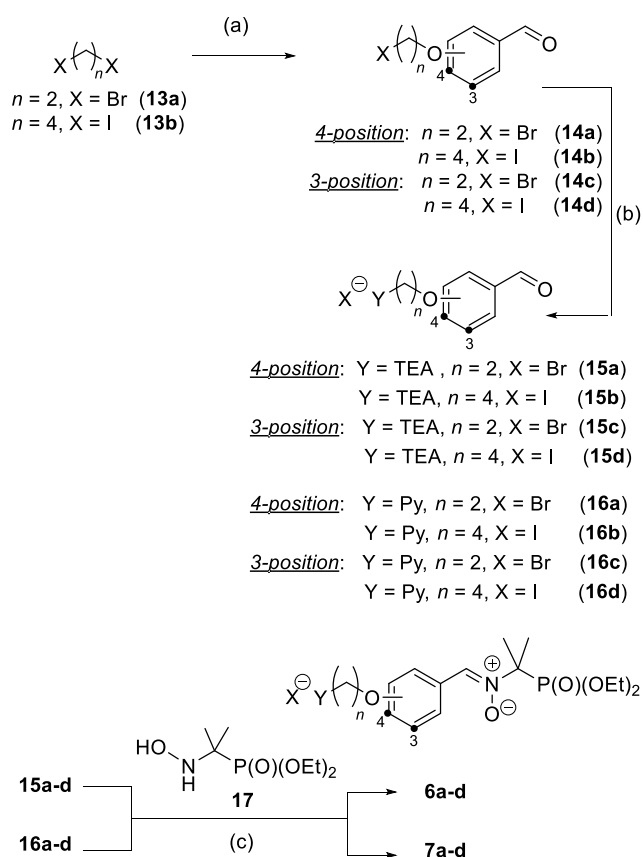
**Figure 2.** Structures of novel mito-PPNs 6–12 showing TEA, Py, and Ber vectors.

## Results and Discussion

### Chemical synthesis

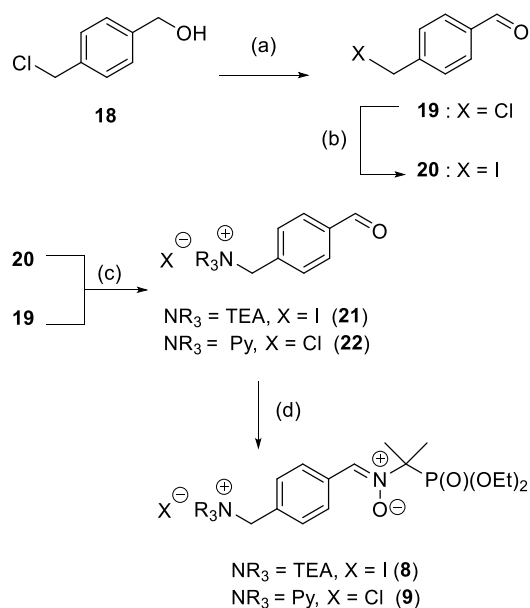
Our privileged synthetic route towards the novel mito-PPNs 6 and 7 bearing an aryloxy framework is outlined in Scheme 1 and started by grafting the TEA or Py vectors on a suitable hydroxy substituted benzaldehyde. The linkers 14a–d, prepared in moderate yields by reacting dihalogenoalkanes 13a and 13b with 3- or 4-hydroxybenzaldehyde, were converted in high yields into their TEA (15a–d) or Py (16a–d) salts under microwave irradiation. In the last step, introduction of the diethoxyphosphoryl moiety with subsequent formation of the nitronyl function involved the coupling of 15a–d and 16a–d with diethyl-2-(hydroxyamino)propan-2-yl-phosphonate) 17, a general method for preparing PPNs.<sup>[8]</sup> Use of microwave irradiation allowed to obtain the target mito-PPNs 6a–d and 7a–d with overall yields of 20–40%. Initial attempts to introduce the vectors onto the corresponding hydroxy substituted PPNs (see

Figure 1A with R<sup>2</sup> = 3- or 4-OH) only yielded traces of the desired compounds which degraded upon column purification.



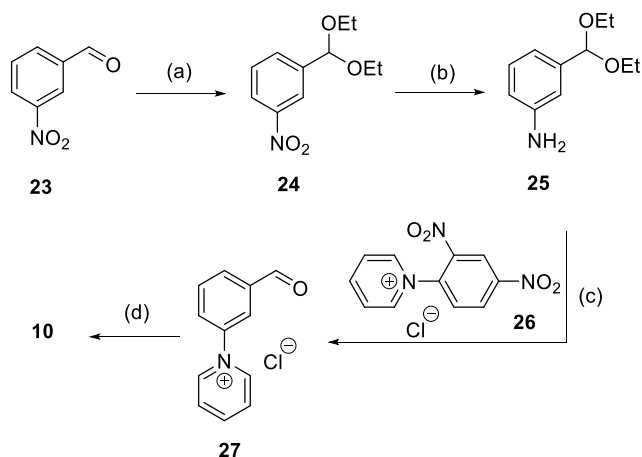
**Scheme 1.** Synthesis of triethylammonium (**6a–d**) and pyridinium (**7a–d**) vectorized mito-PPNs. (a) Hydroxybenzaldehyde, K<sub>2</sub>CO<sub>3</sub>, CH<sub>3</sub>CN, RT, 24 h; (b) NEt<sub>3</sub>, EtOH, 130 °C, 1 h, microwave or C<sub>5</sub>H<sub>5</sub>N, 100 °C, 20 min, microwave; (c) EtOH, 100 °C, 1 h, microwave.

The same grafting strategy was adopted to synthesize two mito-PPNs bearing a single methylene aliphatic linker (Scheme 2). Using PCC oxidation<sup>[20]</sup> 4-(chloromethyl)benzyl alcohol **18** was oxidized to 4-chloromethylbenzaldehyde **19**, which was subsequently converted to the corresponding iodide **20** in moderate yield by nucleophilic substitution. The final steps of the syntheses were carried out under microwave irradiation, starting by the conversion of **20** and **19** into their respective TEA (**21**) and Py (**22**) salts, followed by their treatment with **17** to afford the TEA (**8**) and Py (**9**) mito-PPNs in 10% and 69% overall yields, respectively. Preparation of **20** was necessary because chloride **19** was found poorly reactive towards TEA grafting.



**Scheme 2.** Synthesis of triethylammonium and pyridinium-vectorized mito-PPNs **8** and **9**. (a) PCC,  $\text{CH}_2\text{Cl}_2$ , RT, 3 h; (b) NaI,  $\text{CH}_3(\text{CO})\text{CH}_3$ , 55 °C, 24 h; (c)  $\text{NEt}_3$ , EtOH, 130 °C, 1 h, microwave or  $\text{C}_5\text{H}_5\text{N}$ , 100 °C, 20 min, microwave; (d) **17**, EtOH, 100 °C, 1 h, microwave.

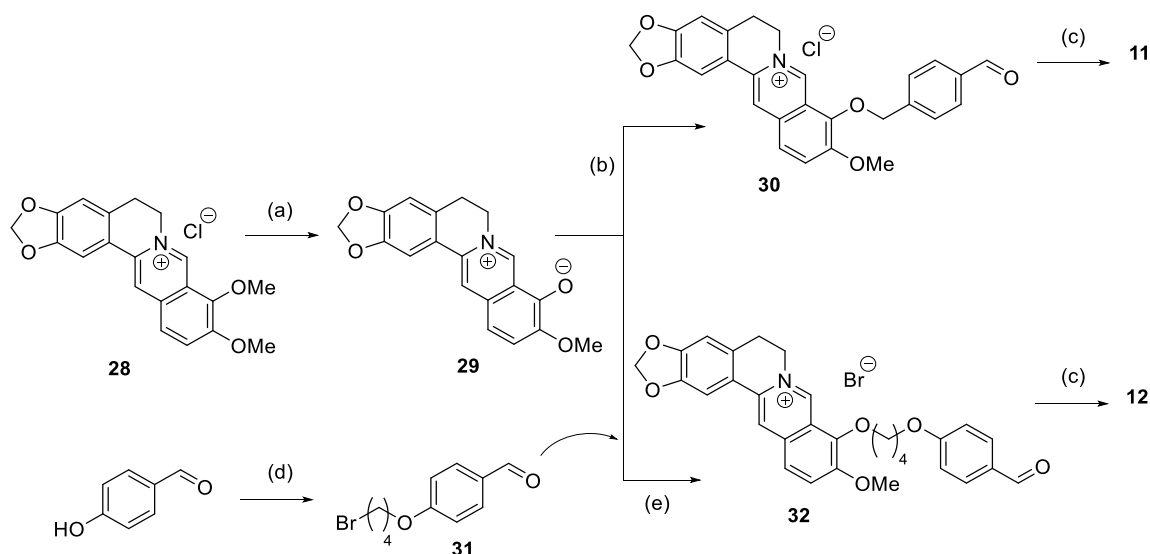
To prepare the TEA based nitrone **10** lacking the connecting aliphatic linker, 3-nitrobenzaldehyde **23** was protected into its diethoxyacetal **24** which was then hydrogenated to the amino derivative **25** in good yield with Adam's catalyst (Scheme 3). A Zincke reaction was then carried out between **25** and *N*-(2,4-dinitrophenyl)pyridinium chloride **26**<sup>[17]</sup> under microwave irradiation to give the fully deprotected pyridinium aldehyde **27** in quantitative yield. Treatment of **27** with **17** under microwave activation afforded the target nitrone **10** in 56% overall yield.



**Scheme 3.** Synthesis of nitrone **10**. (a)  $\text{HC}(\text{OEt})_3$ , EtOH, 80 °C, 24 h; (b)  $\text{PtO}_2$ ,  $\text{H}_2$ , EtOAc, RT, 72 h; (c) EtOH/ $\text{H}_2\text{O}$ , 130 °C, 3 h, microwave; (d) **17**, EtOH, 100 °C, 1 h, microwave.

Last, two Ber targeted mito-PPNs were prepared (Scheme 4) in three steps from commercially available berberine chloride **28** which was first demethylated into berberrubine **29** in quantitative yield according to a published protocol.<sup>[18]</sup> The phenolate form of zwitterionic **29** occurring in DMF reacted with chloroaldehyde **19** to give the Ber salt **30** which was subsequently condensed onto **17** under microwave activation to afford the target mito-PPNs **11** which features a single methylene aliphatic linker (75% overall yield). In a second synthesis, we undertook to prepare a Ber derived phosphorylated nitrone

featuring a longer etheroxy linker. Thus the bromoaldehyde **31** having a butoxy linker was first obtained in good yield by nucleophilic substitution of 1,4-dibromobutane by 4-hydroxybenzaldehyde. Nucleophilic substitution of **29** phenolate on **31** gave the aldehydic precursor **32** which was condensed onto **17** under microwave activation to afford the target compound **12** in 66% overall yield.



**Scheme 4.** Synthesis of berberinium vectorized mito-PPNs **11** and **12**. (a) DMF, 130 °C, 5 min, microwave then 180 °C, 15 min, microwave; (b) **19**, DMF, 80 °C, 15 h; (c) **17**, EtOH, 100 °C, 1 h, microwave; (d) 1,4-dibromobutane, K<sub>2</sub>CO<sub>3</sub>, CH<sub>3</sub>CN, RT, 16 h; (e) DMF, 85 °C, 24 h.

## Superoxide quenching

A first screen for mito-PPNs involved determining their O<sub>2</sub><sup>•-</sup> quenching capacity as an essential mitochondria relevant antioxidant property. Nitrones **1**, **2**, and **6–12** were evaluated at 37 °C in DMSO (0.1%)-supplemented glycine buffer (6.25 mM, pH 10.1) by an improved assay<sup>[8,21]</sup> using allopurinol (22.5 μM)/xanthine oxidase (3 mU/well) as the FR generator and lucigenin chemiluminescence as the detector. Table 1 shows that O<sub>2</sub><sup>•-</sup> quenching efficacy (determined as EC<sub>50</sub> values) was significantly better for all phosphorylated nitrones, as compared to PBN (for which EC<sub>50</sub> > 4 mM was reported<sup>[8]</sup>) and its TPP conjugate **1**. Such enhanced superoxide quenching property of β-phosphorylated derivatives was also observed for the non-vectorized PPN (EC<sub>50</sub> = 518.3 ± 32.5 μM). As discussed earlier<sup>[8]</sup>, this may partly originate from the electronegative effect of the β-diethoxyphosphoryl group that could accelerate the rate of O<sub>2</sub><sup>•-</sup> addition on the more electrophilic nitronyl carbon.

Besides this basic reaction which potentially yields nitroxides for EPR spin-trapping (see below), other mechanisms affecting the O<sub>2</sub><sup>•-</sup> quenching rate would likely superimpose. Thus, of the new mito-PPNs, the most active (ranked by decreasing scavenging activity) were the three *N*-arylpyridinium derivatives **10** and **7a,c**, followed by the TEA vectorized compound **8**. Moreover, only five other nitrones showed better EC<sub>50</sub> values than the phosphorylated, TPP vectorized **2**, and no clear trend was observed when comparing the 3- or 4-substitution position (Table 1). It is therefore possible that the inductive electron-attraction effect of the *N*-arylpyridinium group basically contributed to further increase the electrophilic character of the nitronyl carbon, as suggested in a recent study.<sup>[22]</sup> At this stage, this is consistent with the observed decrease of the superoxide scavenging effect as the chain length increased for compounds bearing the same electrophilic vector, e.g., [**6d** < **6c**], [**7b** < **7a**], and [**7d** < **7c**].



**Table 1.** Superoxide quenching property, cytotoxicity, predicted lipophilicity, and relative mitochondrial:cytosolic distribution in the rat liver

Cpd	O <sub>2</sub> <sup>•-</sup> quenching (EC <sub>50</sub> , μM) <sup>[a]</sup>	Cytotoxicity IC <sub>50</sub> (μM) <sup>[b]</sup>	AlogP <sup>[c]</sup>	Mitochondria:cytosol distribution <sup>[d]</sup>
<b>1</b>	> 1000	0.10 ± 0.01	6.05	<i>nm</i>
<b>2</b>	500.0 ± 20.0	0.53 ± 0.06	6.24	2:1
<b>6a</b>	533.3 ± 47.3	0.98 ± 0.05	0.49	<i>nm</i>
<b>6b</b>	505.0 ± 47.7	2.26 ± 0.09	0.65	6:1
<b>6c</b>	382.5 ± 38.6	1.04 ± 0.07	0.54	1:0
<b>6d</b>	672.4 ± 61.8	2.11 ± 0.19	0.63	<i>nm</i>
<b>7a</b>	293.8 ± 22.9	6.11 ± 0.34	-1.50	1:0
<b>7b</b>	> 700	1.55 ± 0.08	-1.32	2:1
<b>7c</b>	283.3 ± 25.2	3.80 ± 0.32	-1.47	1:0
<b>7d</b>	566.7 ± 62.9	1.75 ± 0.09	-1.33	<i>nm</i>
<b>8</b>	340.0 ± 31.2	3.04 ± 0.27	0.31	6:1
<b>9</b>	595.0 ± 27.8	2.90 ± 0.30	-1.65	<i>nm</i>
<b>10</b>	141.7 ± 7.6	0.86 ± 0.05	-1.00	1:0
<b>11</b>	> 500	> 0.025 <sup>[e]</sup>	0.73	<i>nm</i>
<b>12</b>	> 500	> 0.005 <sup>[e]</sup>	1.43	<i>nm</i>

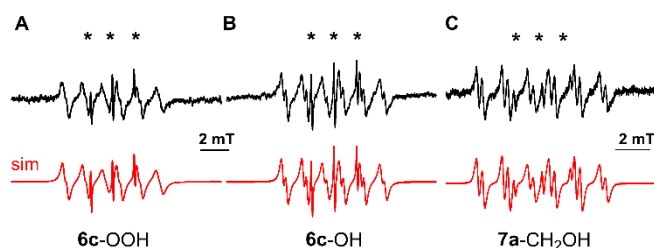
[a] Superoxide generator was the allopurinol/xanthine oxidase system and scavenging activity was determined by the lucigenin chemiluminescence assay; [b] IC<sub>50</sub> defined as the concentration of compound resulting in 50% cell viability after 72 h and calculated from concentration-response curves; [c] Obtained by using the ALOGPS 2.1 program<sup>[23]</sup>; [d] Determined from liver organic extracts by <sup>31</sup>P NMR; [e] Viability was 65–70% upon exposing the cells to either 0.025 μM of **11** or 0.05 μM of **12**. *nm*: not measured.

### ***In vitro* spin-trapping**

Note: From now onwards the expected mito-PPNs/R• nitroxide spin adducts will be termed as mito-PPNs-R.

In support of the quenching properties seen above we performed a series of EPR/spin-trapping experiments on O<sub>2</sub><sup>•-</sup>, HO•, hydroxymethyl (•CH<sub>2</sub>OH), and 1-hydroxyethyl (•CH(OH)CH<sub>3</sub>) radicals (taken as model carbon centered radicals) in buffers using enzymatic, Fenton (H<sub>2</sub>O<sub>2</sub> + FeSO<sub>4</sub>), or [Fenton + alcohol] radical generators, respectively. Additional trapping experiments involving methoxyl (MeO•) and ethoxyl (EtO•) radicals (taken as model alkoxy radicals) were carried out in organic milieu.

Unlike Ber-vectorized nitrones **11** and **12**, which repeatedly failed to afford the expected superoxide and hydroxyalkyl radicals adducts, almost all eleven Py- and TEA-based mito-PPNs gave EPR signals, yet often complex, upon trapping these six radicals. To the best of our knowledge only a few spin adducts of mitochondria targeted linear nitrones were reported earlier, namely 1-CH(OH)CH<sub>3</sub><sup>[9]</sup>, 5-CH<sub>2</sub>OH<sup>[15b]</sup>, and 1-octyloxyl adducts of **1**, **3b,c**.<sup>[13]</sup>



**Figure 3.** EPR spectra and simulations of typical **6c** (A,B) and **7a** (C) spin adducts at room temperature. Spectra were recorded in phosphate buffer (20 mM; pH 7.4) alone (C) or supplemented with 1 mM DTPA (A,B) using nitrones concentrations of 150 mM (A), 100 mM (B), and 20 mM (C). The composition of free radical generators and the main EPR parameters are given in the Experimental section. Other instrument settings for traces (A, B, C) were: modulation amplitude (0.041, 0.040, 0.063) mT; time constant (81.62, 81.92, 40.96) ms; receiver gain ( $3.2 \times 10^5$ ,  $5 \times 10^5$ ,  $1.25 \times 10^5$ ); sweep rate (0.18, 0.18, 0.14) mT/s. (\*) denotes a contaminant decomposition triplet (see Table 2 footnote).

In general, the EPR spectrum formed in the spin-trapping reaction onto a  $\beta$ -phosphorylated nitronone features 12 equivalent lines showing couplings with the nitrogen ( $a_N$ ),  $\beta$ -hydrogen ( $a_{H\beta}$ ), and phosphorus ( $a_P$ ) nuclei (Scheme S1 in Supporting Information). In most cases, the major EPR signals obtained with the new mito-PPNs could be assigned to the expected spin adducts (Figure 3) based on their sets of hyperfine coupling constants (hfcs) found in the range of known PPNs adducts analogs formed under the same conditions and free radical generators.<sup>[8]</sup>

Table 2 lists the simulated hfcs and  $g$ -values of the spectra shown in Figure 3. In the other experiments, EPR parameters for expected main and secondary species, if any, together with selected spectra are available in the Supporting Information (Tables S1–S6 and Figures S1–S5).

**Table 2.** EPR hyperfine coupling constants for **6c** and **7a** spin adducts.

Trapped radical	Spin adduct	hfcs (mT)			$g$ -factor	% in mixture <sup>[a]</sup>
		$a_N$	$a_{H\beta}$	$a_P$		
$O_2^{\bullet-}$	<b>6c</b> -OOH	1.431	0.249	3.864	<i>nm</i>	99
$HO^{\bullet}$	<b>6c</b> -OH	1.468	0.291	4.129	2.0054	99
$\bullet CH_2OH$	<b>7a</b> -CH <sub>2</sub> OH	1.463	0.325	4.098	2.0055	98

[a] A decomposition nitroxide exhibiting a triplet with  $a_N = 1.558$ – $1.628$  mT (\* in Figure 3) accounted for < 2% of total signal.  
*nm*: not measured.

Further confirmation of the assignment for **6c**-OH in Table 2 came upon running two alternative  $HO^{\bullet}$  spin adduct generators<sup>[8]</sup> which yielded EPR signals with very similar hfcs, i.e., (in mT), [ $a_N = 1.472$ ,  $a_{H\beta} = 0.297$ ,  $a_P = 4.136$ ] by photolyzing 3% m/m  $H_2O_2$  and [ $a_N = 1.469$ ,  $a_{H\beta} = 0.290$ ,  $a_P = 4.112$ ] using nucleophilic addition (NA) of water catalyzed by  $Fe^{3+}$ . Such consistency among the three aforementioned mito-PPNs-OH producing systems was almost general to all new nitrones, but no EPR signal was seen with **12** using the Fenton reagent, while the  $Na/Fe^{3+}$  method failed to afford signals with **6a** and **7a** (not shown). Due to the long known exothermic decomposition of  $H_2O_2$  in the presence of concentrated iodides<sup>[24]</sup> no photolytic  $HO^{\bullet}$  or  $HOO^{\bullet}$  generator containing  $H_2O_2 > 3\%$  m/m ( $\sim 1$  M) could be used with iodides **6b,d**, **7b,d**, and **8**, and a few attempts resulted in diamagnetic mixtures upon cooling.

Using an alternative photolytic hydroperoxyl radical generator (based on 30% m/m  $H_2O_2$ <sup>[8]</sup>) allowed detection of the expected  $HOO^{\bullet}$  adducts for a set of mito-PPNs-OOH adducts (Tables S1–S3), but not for **6c** (which gave a major signal with hfcs similar to that of **6c**-OH, see Tables 2 and S4), **10**, and all five iodides (for these latter the reason is discussed above). The assignment of **6c**-OOH vs. **6c**-OH given in Table 2 was strengthened by calculating the ranges of spectrum total width ( $STW = 2 \times a_N + a_{H\beta} + a_P$ ) which, for a given nitronone, was always found lower for  $HOO^{\bullet}$  vs.  $HO^{\bullet}$  adduct, regardless the FR generating system (Tables S1 and S2). Thus,  $STW = 6.975$  and  $7.356$  mT for **6c**-OOH and **6c**-OH, respectively to be compared to  $STW = 7.073$  and  $7.413$  mT for the known PPN-OOH and PPN-OH, respectively<sup>[8]</sup> (see also<sup>[25]</sup>).

Furthermore, almost all novel nitrones gave characteristic spin adducts with hydroxymethyl (as did **7c**; Figure 2), 1-hydroxyethyl, and both tested methoxyl and ethoxyl radicals (Figures S1–S3, Supporting Information), with hfcs in agreement with the literature<sup>[8]</sup> (Tables S1–S3). For example, **11**-OEt and **12**-OMe spin adducts devoid of any contaminant radical were obtained by adding ~1 mg of Pb(OAc)<sub>4</sub> to a solution of the parent nitron (35 mM) containing 25% EtOH or MeOH, respectively. The simulated hfcs of the strong detected signals were: **11**-OEt:  $a_N = 1.321$  mT,  $a_{H\beta} = 0.263$  mT,  $a_P = 3.884$  mT,  $g = 2.0059$ ; **12**-OMe:  $a_N = 1.325$  mT,  $a_{H\beta} = 0.264$  mT,  $a_P = 3.895$  mT,  $g = 2.0059$  (Figures S2 and S3).

Depending on the trapped ROS or carbon radicals tested above, and nitron chemical structure, a strong contribution (ideally 100%) of the expected spin adducts components to the total EPR signal, based on improved computer simulation<sup>[26]</sup>, can be considered a discriminant criterion for applicability in biological systems. Remarkably, the ratio of unexpected species was found highly variable, e.g., it was ~90% for [**9**/enzymatic superoxide], > 20% for [**6b**/Fenton] or > 70% for [**7d**/hydroxymethyl]. Obviously, secondary radicals could arise here from competing reactions on the linker (e.g., hydrogen abstractions, radical rearrangements) and/or by interactions with the vector. Although this latter possibility cannot be ruled out, it is noteworthy that no secondary adducts were reported in aqueous spin-trapping experiments involving photolytically generated HO• or carbon radicals and Py-substituted PBNs bearing no aliphatic chains.<sup>[16a]</sup>

Lastly, measuring spin-trapping rate constants (and spin adducts half-lives) for mito-PPNs superoxide and hydroxyl radical adducts, which requires appropriately stopping free radical formation, then running proper kinetic models for the build-up (and decay) of the EPR signals, was not among the goals of this study. Indeed, this would have been a tedious task in view of the complexity of formation and possible self-evolutionary pathways of spin-adducts *in vitro*. This is illustrated in Tables S4–S6 (Supporting Information) summarizing the sets of secondary species giving the best fits in the computer simulations.

Interestingly, EPR signals for mito-PPNs-OH and mito-PPNs-OOH in buffers, as shown in Figure 3, were found accumulating for at least 20 min, i.e., beyond the radical production peaks for HO• and O<sub>2</sub>•<sup>-</sup>, respectively (not shown). This remarkable apparent persistence confirms the general stabilizing property induced by β-P(O)(OEt)<sub>2</sub> substitution on those spin adducts, i.e., for PPNs vs. PBNs.<sup>[5a,8]</sup> Regarding EPR spin-trapping probing of O<sub>2</sub>•<sup>-</sup> in buffers, for which phenolic based PPNs were not very efficient<sup>[8]</sup>, the enhanced EPR detectability of the novel mito-PPNs is reminiscent of earlier data on TPP conjugates of β-dialkoxyposphorylated cyclic PPNs analogues<sup>[27]</sup> or Py-substituted PBNs.<sup>[5a]</sup>

To sum up the EPR screening, we empirically ranked the five best compounds according to their spin-trapping property towards the studied ROS and carbon centered radicals (i.e., expected adduct with low contaminants, apparent stability) as: [**6c**, **7c**, **10**] > [**7a**, **9**].

### Cytotoxicity studies and lipophilicity

The cytotoxicity of mitochondria targeted nitrones was assessed against HDF by running the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) cell viability assay. The enzymatic MTT reduction to a purple precipitate of MTT-formazan is catalyzed by mitochondrial succinate dehydrogenase of living cells. The absorbance of the precipitated MTT-formazan further dissolved in DMSO is read spectrophotometrically at 570 nm and is directly proportional to the amount of living cells. Cells were incubated in fibroblasts growth medium (FGM) for nitrones **6a–d**, **7a–d**, **8–10** or in FGM containing 0.1% DMSO for less soluble nitrones **1**, **2**, **11**, and **12**. Preliminary tests confirmed that adding 0.1% DMSO to FGM vs. FGM alone had no impact on HDF cells viability.

Table 1 shows cell viability (as IC<sub>50</sub> values) after incubating cells for 72 h with 0.001–100 μM of the nitrones. The cytotoxicity values for Ber vectorized compounds **11** and **12** could not be determined accurately because both nitrones precipitated at rather low concentrations, i.e., at 0.05 μM and 0.01 μM, respectively. Nevertheless, a 65–70% cell viability was estimated after exposing cells to **11** (0.025 μM) or **12** (0.005 μM). Compared to the TPP vectorized **1** and **2**, all novel mito-PPNs showed significantly lower cytotoxicity in the assay, with the most toxic **10** and the less toxic **7a** retaining EC<sub>50s</sub> about 1.7-times and 12.5-times higher than the β-phosphorylated **2**, respectively. We recently found the cytotoxicity of TPP-PPNs in the MTT assay to be modulated by the length of the alkyl chain linker<sup>[13]</sup> and some relevant mechanisms related to structural effects have been reported previously.<sup>[14,28]</sup> However, there was no consistent trend between cytotoxicity values of Table 1 and structural features of mito-PPNs as displayed in Figure 2, including TEA vs. Py vectors, 3- vs. 4-position, and *n* value. Hence, for a same alkyl chain length and a given vector, the 3- vs. 4-substitution pattern on the aromatic cycle had a marginal impact on IC<sub>50s</sub>, e.g., when comparing [**6a** vs. **6c**], [**6b** vs. **6d**], and [**7b** vs. **7d**]. Also, no clear tendency was found for Py vs. TEA vectors for nitrones sharing the same *n* value and substitution pattern, e.g., for [**6a** vs. **7a**], [**6b** vs. **7b**], [**6c** vs. **7c**], [**6d** vs. **7d**], and [**8** vs. **9**]. Interestingly, for a given

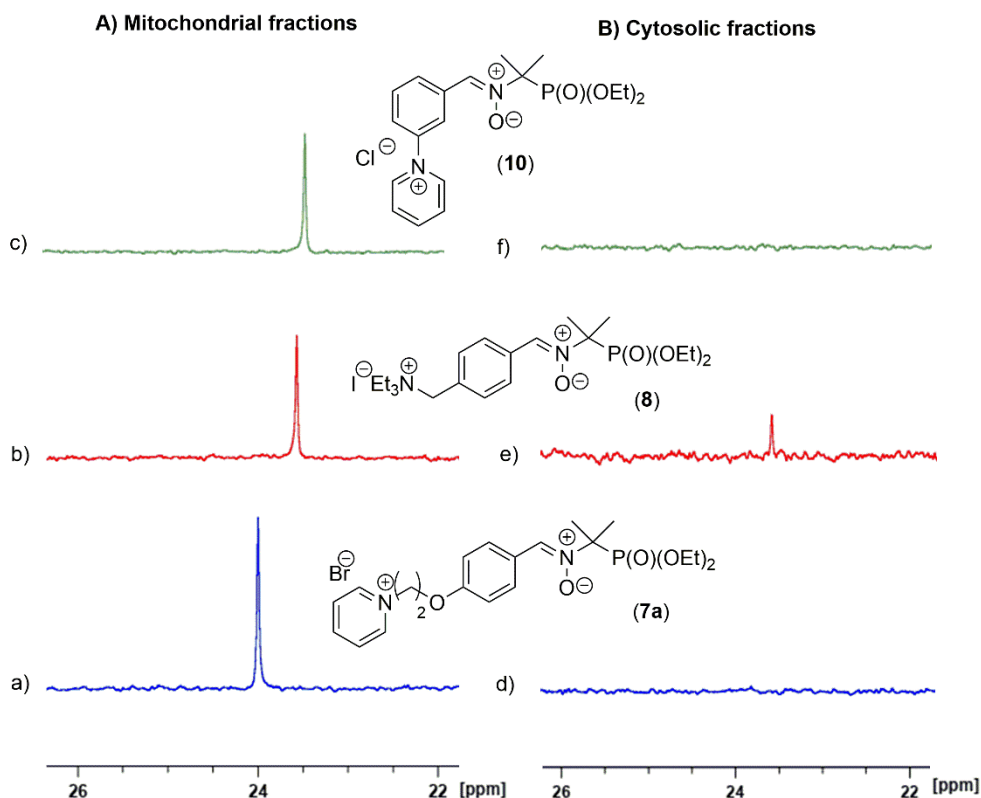
4- vs. 3-substitution pattern, cytotoxicity increased either (i) with decreasing  $n$  value in the Py series, as for [7a and 7c] vs. [7b and 7d] or (ii) with increasing  $n$  value in the TEA series, as for [6b and 6d] vs. [6a and 6c].

The lipophilic profile of nitrones  $AlogP$  (where  $P$  is the partition coefficient in water/octanol) was computed according to the ALOGPS 2.1 program and the values are shown in Table 1. Both 1 and 2 exhibited strong lipophilic values ( $AlogP > 6$ ) while narrow ranges of either positive (indicating a lipophilic character) or negative (indicating a hydrophilic character) values were found for TEA or Py grafted mito-PPNs, respectively. Not unexpected were the positive  $AlogP$  values calculated for 11 and 12, showing the above hydrophilic character of Py containing compounds is reversed upon fusing of the ring within the Ber aromatic network. We previously observed an inverse relationship between experimentally measured  $P$  values and cytotoxicity in a series of cyclic PPNS analogues. In view of these data<sup>[29]</sup>, we considered that, if they distribute adequately within the mitochondria, the less cytotoxic 7a,c could be valuable add-ons for the final screening.

### Mitochondrial permeation

On the basis of their biochemical and EPR properties studied above, we selected the six new nitrones 6b,c, 7a,c, 8, and 10 as potential mitochondriotropic hit compounds with anti-ROS action. In this latter regard, the poorly antioxidant 7b and the highly lipophilic 2 were included in the selection as controls.

Mitochondrial uptake of selected nitrones was investigated in the isolated recirculating rat liver, selected as a high metabolizing system. Livers were perfused for 1 h with a Krebs-Henseleit (KH) buffer containing a non-toxic nitrone concentration (1  $\mu$ M), then a 10-min washout with cold KH was carried out. Liver homogenates were then processed to prepare cytosol and mitochondrial fractions whose organic extracts were concentrated and quantified by <sup>31</sup>P NMR in DMSO-*d*<sub>6</sub>.<sup>[13]</sup> The mitochondrial vs. cytosolic distribution in the case of nitrones 7a, 8, and 10 is illustrated by Figure 4 showing the respective <sup>31</sup>P NMR peaks associated with the (EtO)<sub>2</sub>P(O) moiety at 23.39, 22.96 and 22.59 ppm, respectively. Despite their  $AlogP$  values indicate a low lipophilic character that would limit mitochondria uptake, compounds 7a and 10 were exclusively recovered in the mitochondrial extracts (Figure 4a,c), while compound 8, presumably more lipophilic, was present in both extracts, yet mostly in the mitochondria (Figure 4b,e). Although the studied system here represents a highly active metabolizing environment none of the other studied nitrones exhibited significant degradation yielding additional <sup>31</sup>P NMR peaks (not shown). The relative mitochondria:cytosol distributions, based on NMR peak integration are reported in Table 1, confirming the very efficient mitochondria targeting property of Py and TEA vectors in mito-PPNs. Unlike nitrones 7a,c, compound 7b, whose predicted hydrophilicity was similar did not exclusively reach the mitochondria. Although the infused mito-PPNs concentration was very low a good signal-to-noise ratio for <sup>31</sup>P NMR peaks was generally reached after only 90-s acquisition indicating a fast intracellular uptake.



**Figure 4.**  $^{31}\text{P}$  NMR signals in  $\text{DMSO-}d_6$  recorded in the (A) mitochondrial (traces a–c) and (B) cytosolic (traces d–f) extracts from the same rat liver that underwent a 60-min normothermic perfusion with Krebs–Henseleit buffer containing  $1\ \mu\text{M}$  of **7a** (traces a,d) or **8** (traces b,e), or **10** (traces c,f). Chemical shifts are given in ppm relative to 85%  $\text{H}_3\text{PO}_4$  in  $\text{D}_2\text{O}$

### Protective effect of mito-PPNs in $\text{H}_2\text{O}_2$ -stressed Schwann cells

In the last step of the study we investigated if the most efficient mitochondriotropic nitrones **7a** and **10** would provide a better cytoprotective effect vs. the non-exclusively targeted **7b** and **8** (Table 1) in a specific model of ROS induced cell apoptosis. Accordingly, primary cultures of Schwann cells<sup>[30]</sup> preincubated for 8 h with the four above compounds ( $0.1\ \mu\text{M}$ ) were submitted to  $\text{H}_2\text{O}_2$  ( $100\ \mu\text{M}$ )-induced oxidative stress for 16 h, a treatment known to induce apoptosis in these cells.<sup>[13,30]</sup> The nitrones PPN and its TPP conjugate **2** were also studied at the same concentration as untargeted and targeted/more cytotoxic references, respectively.

Indeed, exposure of the cells to  $\text{H}_2\text{O}_2$  resulted in a concomitant dramatic increase of lactate dehydrogenase (LDH) release (a marker of cell necrosis) and a strong loss of viability, being  $173 \pm 11\%$  and  $29 \pm 3\%$  of unexposed control cells, respectively. All tested nitrones significantly improved both indices above, with the following overall protection ranking:  $\text{PPN} < \mathbf{2} < \mathbf{7b} \approx \mathbf{8} < \mathbf{7a} \approx \mathbf{10}$  (Figure S6).

From studies on various cell lines<sup>[31,32]</sup>, there has been abundant biochemical and pharmacological evidence that oxidative stress following a prolonged exposure to  $\text{H}_2\text{O}_2$  damages the mitochondria leading to dysfunction, including membrane potential collapse, decrease of ATP level, membrane integrity impairment, and the release of calcium and cytochrome c which can in turn promote intra-mitochondrial superoxide formation. Owing to this complex pattern of detrimental events, the best anti-apoptotic properties found for the likely-safe mito-PPNs **7a** and **10** appear fully consistent with their unique affinity for mitochondria.

### Conclusion

In conclusion, the multifaceted approaches described in this study have validated the introduction of the pyridinium cation in the design of a series of improved mitochondria directed  $\beta$ -phosphorylated linear nitrones built on the PPN framework. Among the novel mito-PPNs described here, where the potential

of triethylammonium and berberinium substituted derivatives of PPN was also evaluated, the output of the screen identified the pyridinium vectorized nitrones **7a** and **10**. These rather hydrophilic and non-toxic hit nitrones showed good anti-superoxide activity which, combined to their fast intra-mitochondrial uptake and good EPR spin-trapping properties, could be promising probes for further *in vivo* studies of mitochondrial related pathological mechanisms involving ROS.

## Experimental Section

### Chemical synthesis

**Chemicals and instruments:** All chemical reagents were analytical grade from commercial sources (Sigma-Aldrich, Saint Quentin Fallavier, France; TCI, Zwijndrecht, Belgium; Acros Organics, Fisher Scientific, Illkirch, France) and were used without further purification. The hydroxylamine **17** was synthesized according to [8]. TPP vectorized nitrones **1**<sup>[10]</sup> and **2**<sup>[13]</sup> were prepared following improved methods. Compounds **26** and **29** were synthesized as previously reported, see the Supporting Information for full synthetic details. Microwave assisted reactions were carried out using a Monowave 400 reactor, equipped with a ruby-thermometer (Anton Paar, Les Ulis, France) and set at a microwave power of 400 W. Reactions were routinely monitored by TLC on Merck Kieselgel 60 F254 precoated silica gel plates, and the spots were visualized under UV light or by staining with phosphomolybdic acid. Melting points were obtained on a Buchi M-560 apparatus and are uncorrected. <sup>1</sup>H, <sup>13</sup>C and <sup>31</sup>P NMR spectra were recorded on Bruker spectrometers (Avance III nanobay – 300 or 400 MHz, Avance I – 500 MHz or Avance III – 600 MHz) in CDCl<sub>3</sub>, CD<sub>3</sub>OD or DMSO-*d*<sub>6</sub> solvents (Eurisotop, Saint-Aubin, France). Chemical shifts ( $\delta$ ) for <sup>1</sup>H and <sup>13</sup>C are given in ppm relative to tetramethylsilane (TMS) as internal standard, and to external H<sub>3</sub>PO<sub>4</sub> (<sup>31</sup>P); coupling constants (*J*) are listed in Hertz (Hz). Spin multiplicities are indicated as: s (singlet), d (doublet), dd (doublet of doublets), ddd (doublet of doublets), t (triplet), td (triplet of doublets), tt (triplet of triplets), q (quartet), quint (quintet), m (multiplet), br (broad). High-resolution mass spectrometry (HRMS) and mass spectrometry (MS) in electron spray ionization (ESI) were performed at the Spectropole (Analytical Laboratory) at Campus St. Jérôme (Marseille, France) using a Waters Synapt G2 HDMS spectrometer.

**General method for synthesis of the linkers (14a–d):** 3- or 4-Hydroxybenzaldehyde (1 eq.) and K<sub>2</sub>CO<sub>3</sub> (1.7 eq.) were stirred in acetonitrile (0.4 M of aldehyde) at 0 °C for 30 min. Then, the corresponding dihalogenoalkane **13a** or **13b** (1.5 eq.) was added dropwise and the mixture was stirred for 24 h at room temperature. After filtration and concentration *in vacuo*, the crude residue was purified by chromatography on SiO<sub>2</sub> to give the desired product.

**4-(2-Bromoethoxy)benzaldehyde (14a):** Prepared from 4-hydroxybenzaldehyde (4.00 g, 32.8 mmol), K<sub>2</sub>CO<sub>3</sub> (7.50 g, 55 mmol) and 1,2-dibromoethane (**13a**) (14.1 mL, 164 mmol) in acetonitrile (80 mL). Chromatography on SiO<sub>2</sub> (dichloromethane/pentane, 6/4, v/v) afforded **14a** as a yellow powder (3.98 g, 53%). m.p. 54.5 °C (Litt. 55–58°C<sup>[33]</sup>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$ =9.88 (s, 1H; CHO), 7.86 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; H-2), 7.14 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; H-3), 4.45 (t, <sup>3</sup>J<sub>H,H</sub>=5.3 Hz, 2H; 2'-CH<sub>2</sub>), 3.84 (t, <sup>3</sup>J<sub>H,H</sub>=5.3 Hz, 2H; 1'-CH<sub>2</sub>); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$ =191.8 (CHO), 163.3 (C-4), 132.3 (C-2), 130.5 (C-1), 115.6 (C-3), 68.6 (C-2'), 31.5 (C-1'); HRMS (ESI): *m/z* calcd for C<sub>9</sub>H<sub>10</sub>BrO<sub>2</sub><sup>+</sup>: 228.9859 [M+H]<sup>+</sup>; found 228.9859.

**4-(4-Iodobutoxy)benzaldehyde (14b):** Prepared from 4-hydroxybenzaldehyde (2.00 g, 16 mmol), K<sub>2</sub>CO<sub>3</sub> (3.76 g, 27.2 mmol) and 1,4-diiodobutane (**13b**) (3.4 mL, 24 mmol) in acetonitrile (40 mL). Chromatography on SiO<sub>2</sub> (dichloromethane, 100%) afforded **14b** as a yellow oil (2.29 g, 47%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$ =9.90 (s, 1H; CHO), 7.86 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; H-2), 7.01 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; H-3), 4.09 (t, <sup>3</sup>J<sub>H,H</sub>=5.9 Hz, 2H; 4'-CH<sub>2</sub>), 3.28 (t, <sup>3</sup>J<sub>H,H</sub>=6.6 Hz, 2H; 1'-CH<sub>2</sub>), 2.04 (m, 2H; 2'-CH<sub>2</sub> or 3'-CH<sub>2</sub>), 1.98 (m, 2H; 2'-CH<sub>2</sub> or 3'-CH<sub>2</sub>); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$ =191.8 (CHO), 163.9 (C-4), 132.0 (C-2), 130.1 (C-1), 114.8 (C-3), 67.1 (C-4'), 30.0 (C-2' or C-3'), 29.9 (C-2' or C-3'), 8.9 (C-1'); HRMS (ESI): *m/z* calcd for C<sub>11</sub>H<sub>14</sub>O<sub>2</sub>I<sup>+</sup>: 305.0033 [M+H]<sup>+</sup>, found 305.0032.

**3-(2-Bromoethoxy)benzaldehyde (14c):** Prepared from 3-hydroxybenzaldehyde (4.00 g, 32.8 mmol), K<sub>2</sub>CO<sub>3</sub> (7.50 g, 55 mmol) and **13a** (14.1 mL, 164 mmol) in acetonitrile (80 mL). Chromatography on SiO<sub>2</sub> (dichloromethane/pentane, 6/4, v/v) afforded **14c** as a yellow oil (3.83 g, 51%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$ =9.98 (s, 1H; CHO), 7.49 (s, 1H; H-2), 7.47 (t, <sup>3</sup>J<sub>H,H</sub>=7.3 Hz, 1H; H-5), 7.41 (d, <sup>3</sup>J<sub>H,H</sub>=2.6 Hz, 1H; H-4), 7.20 (dt, <sup>3</sup>J<sub>H,H</sub>=2.6 and 7.3 Hz, 1; H-6), 4.37 (t, <sup>3</sup>J<sub>H,H</sub>=6.1 Hz, 2H; 2'-CH<sub>2</sub>), 3.67 (t, <sup>3</sup>J<sub>H,H</sub>=6.1 Hz, 2H; 1'-CH<sub>2</sub>); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$ =191.8 (CHO), 158.7 (C-3), 137.9 (C-1), 130.3 (C-2), 124.1 (C-5), 122.1 (C-6), 113.0 (C-4), 68.1 (C-2'), 28.8 (C-1'); HRMS (ESI): *m/z* calcd for C<sub>9</sub>H<sub>10</sub>BrO<sub>2</sub><sup>+</sup>: 228.9859 [M+H]<sup>+</sup>, found 228.9859.

**3-(4-Iodobutoxy)benzaldehyde (14d):** Prepared from 3-hydroxybenzaldehyde (2.00 g, 16 mmol), K<sub>2</sub>CO<sub>3</sub> (3.76 g, 27.2 mmol) and **13b** (3.4 mL, 24 mmol) in acetonitrile (40 mL). Chromatography on SiO<sub>2</sub> (dichloromethane, 100%) afforded **14d** as a yellow oil (1.89 g, 39%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>):  $\delta$ =9.98 (s, 1H; CHO), 7.47 (s, 1H; H-2), 7.47 (m, 1H; H-5), 7.39 (m, 1H; H-6), 7.17 (dd, <sup>3</sup>J<sub>H,H</sub>=2.5 and 5.9 Hz, 1H; H-4), 4.06 (t, <sup>3</sup>J<sub>H,H</sub>=5.4 Hz, 2H; 4'-CH<sub>2</sub>), 3.28 (t, <sup>3</sup>J<sub>H,H</sub>=6.6 Hz, 2H; 1'-CH<sub>2</sub>), 2.06 (m, 2H; 2'-CH<sub>2</sub> or 3'-CH<sub>2</sub>), 1.95 (m, 2H; 2'-CH<sub>2</sub> or 3'-CH<sub>2</sub>); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>):  $\delta$ =192.0 (CHO), 159.5 (C-3), 137.9 (C-1), 130.1 (C-2), 123.6 (C-5), 121.9 (C-4), 112.7 (C-6), 67.0 (C-4'), 30.1 (C-2' or C-3'), 30.04 (sC-2' or C-3'), 6.02 (C-1'); HRMS (ESI): *m/z* calcd for C<sub>11</sub>H<sub>14</sub>O<sub>2</sub>I<sup>+</sup>: 305.0033 [M+H]<sup>+</sup>, found 305.0034.

**General method for synthesis of triethylammonium-based benzaldehydes (15a–d):** A mixture of benzaldehyde **14a–d** (1 eq.), absolute ethanol (volume to obtain 0.8 M) and triethylamine (4.5 eq.), in a closed vial, was heated for 1 h at 130 °C under microwave irradiation. Then, the reaction mixture was concentrated *in vacuo* to afford the desired product.

**N,N,N-Triethyl-2-(4-formylphenoxy)ethan-1-ammonium bromide (15a):** Prepared from **14a** (500 mg, 2.2 mmol), ethanol (2.8 mL) and triethylamine (1.4 mL), affording **15a** as a yellow oil (727 mg, quant. yield). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =9.80 (s, 1H; CHO), 7.89 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; H-2), 7.16 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; H-3), 4.51 (t, <sup>3</sup>J<sub>H,H</sub>=4.8 Hz, 2H; 2'-CH<sub>2</sub>), 3.70 (t, <sup>3</sup>J<sub>H,H</sub>=4.8 Hz, 2H; 1'-CH<sub>2</sub>), 3.36 (q,

$^3J_{\text{H,H}}=7.2$  Hz, 6H;  $3 \times \text{NCH}_2\text{CH}_3$ ), 1.23 (t,  $^3J_{\text{H,H}}=7.2$  Hz, 9H;  $3 \times \text{NCH}_2\text{CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta=192.0$  (CHO), 162.7 (C-4), 132.3 (C-2), 130.7 (C-1), 115.6 (C-3), 62.0 (C-2'), 55.6 (C-1'), 53.5 ( $3 \times \text{NCH}_2\text{CH}_3$ ), 7.73 ( $3 \times \text{NCH}_2\text{CH}_3$ ); HRMS (ESI):  $m/z$  calcd for  $\text{C}_{15}\text{H}_{24}\text{NO}_2^+$ : 250.1802 [M] $^+$ , found 250.1803.

**N,N,N-Triethyl-4-(4-formylphenoxy)butan-1-aminium iodide (15b)**: Prepared from **14b** (500 mg, 1.6 mmol), ethanol (2 mL) and triethylamine (1 mL), yielding **15b** as a yellow powder (670 mg, quant. yield). m.p. 156.8 °C;  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta=9.88$  (s, 1H; CHO), 7.87 (d,  $^3J_{\text{H,H}}=8.8$  Hz, 2H; H-2), 7.13 (d,  $^3J_{\text{H,H}}=8.8$  Hz, 2H; H-3), 4.16 (t,  $^3J_{\text{H,H}}=5.9$  Hz, 2H; 4'-CH $_2$ ), 3.30 (m, 2H; 1'-CH $_2$ ), 3.24 (q,  $^3J_{\text{H,H}}=7.2$  Hz, 6H;  $3 \times \text{NCH}_2\text{CH}_3$ ), 1.80 (m, 4H; 2'-CH $_2$  and 3'-CH $_2$ ), 1.18 (t,  $^3J_{\text{H,H}}=7.2$  Hz, 9H;  $3 \times \text{NCH}_2\text{CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta=191.8$  (CHO), 163.9 (C-4), 132.3 (C-2), 130.2 (C-1), 115.5 (C-3), 67.7 (C-4'), 55.7 (C-1'), 52.6 ( $3 \times \text{NCH}_2\text{CH}_3$ ), 25.5 (C-2'), 18.4 (C-3'), 7.63 ( $3 \times \text{NCH}_2\text{CH}_3$ ); HRMS (ESI):  $m/z$  calcd for  $\text{C}_{17}\text{H}_{28}\text{NO}_2^+$ : 278.2115 [M] $^+$ , found 278.2119.

**N,N,N-Triethyl-2-(3-formylphenoxy)ethan-1-aminium bromide (15c)**: Prepared from **14c** (500 mg, 2.2 mmol), ethanol (2.8 mL) and triethylamine (1.4 mL), yielding **15c** as a yellow powder (705 mg, 97%). m.p. 101.7 °C;  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta=9.99$  (s, 1H; CHO), 7.59 (s, 1H; H-2), 7.57 (dd,  $^3J_{\text{H,H}}=2.4$  and 3.3 Hz, 1H; H-5), 7.48 (d,  $^3J_{\text{H,H}}=2.4$  Hz, 1H; H-6), 7.33 (m, 1H; H-4), 4.47 (t,  $^3J_{\text{H,H}}=5.0$  Hz, 2H; 2'-CH $_2$ ), 3.69 (t,  $^3J_{\text{H,H}}=5.0$  Hz, 2H; 1'-CH $_2$ ), 3.36 (q,  $^3J_{\text{H,H}}=7.2$  Hz, 6H;  $3 \times \text{NCH}_2\text{CH}_3$ ), 1.24 (t,  $^3J_{\text{H,H}}=7.2$  Hz, 9H;  $3 \times \text{NCH}_2\text{CH}_3$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta=193.4$  (CHO), 158.4 (C-3), 138.1 (C-1), 131.0 (C-2), 124.1 (C-5), 122.0 (C-4), 113.9 (C-6), 61.8 (C-2'), 55.6 (C-1'), 53.51 ( $3 \times \text{NCH}_2\text{CH}_3$ ), 7.72 ( $3 \times \text{NCH}_2\text{CH}_3$ ); HRMS (ESI):  $m/z$  calcd  $\text{C}_{15}\text{H}_{24}\text{NO}_2^+$ : 250.1802 [M] $^+$ , found 250.1804.

**N,N,N-Triethyl-4-(3-formylphenoxy)butan-1-aminium iodide (15d)**: Prepared from **14d** (500 mg, 1.6 mmol), ethanol (2 mL) and triethylamine (1 mL), yielding **15d** as a yellow powder (670 mg, quant. yield). m.p. 108.7 °C;  $^1\text{H}$  NMR (400 MHz, DMSO- $d_6$ ):  $\delta=9.99$  (s, 1H; CHO), 7.53 (s, 1H; H-2), 7.51 (t,  $^3J_{\text{H,H}}=6.6$  Hz, 1H; H-5), 7.43 (m, 1H; H-4), 7.29 (dt,  $^3J_{\text{H,H}}=2.5$  and 6.6 Hz, 1H; H-6), 4.12 (t,  $^3J_{\text{H,H}}=5.6$  Hz, 2H; 4'-CH $_2$ ), 3.24 (m, 2H; 1'-CH $_2$ ), 3.24 (q,  $^3J_{\text{H,H}}=7.3$  Hz, 6H;  $3 \times \text{NCH}_2\text{CH}_3$ ), 1.80 (m, 4H; 2'-CH $_2$  and 3'-CH $_2$ ), 1.18 (t,  $^3J_{\text{H,H}}=7.3$  Hz, 9H;  $3 \times \text{NCH}_2\text{CH}_3$ );  $^{13}\text{C}$  NMR (100 MHz, DMSO- $d_6$ ):  $\delta=193.5$  (CHO), 159.4 (C-3), 138.1 (C-1), 130.9 (C-2), 123.3 (C-5), 121.9 (C-6), 113.9 (C-4), 67.4 (C-4'), 56.1 (C-1'), 52.5 ( $3 \times \text{NCH}_2\text{CH}_3$ ), 25.8 (C-2' or C-3'), 18.4 (C-2' or C-3'), 7.64 ( $3 \times \text{NCH}_2\text{CH}_3$ ); HRMS (ESI):  $m/z$  calcd for  $\text{C}_{17}\text{H}_{28}\text{NO}_2^+$ : 278.2115 [M] $^+$ , found 278.2115.

**General method for synthesis of the pyridinium-based benzaldehydes (16a–d)**: A solution of benzaldehyde **14a–d** (1 eq.) and pyridine (volume to obtain 0.8 M), in a closed vial, was heated for 20 min at 100 °C under microwave irradiation. Then, the reaction mixture was concentrated *in vacuo* to afford the desired product.

**1-(2-(4-Formylphenoxy)ethyl)pyridinium bromide (16a)**: Prepared from **14a** (500 mg, 2.2 mmol) in pyridine (2.8 mL), affording **16a** as a yellow oil (647 mg, 95%).  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta=9.87$  (s, 1H; CHO), 9.13 (d,  $^3J_{\text{H,H}}=5.9$  Hz, 2H; H-2"), 8.64 (t,  $^3J_{\text{H,H}}=7.5$  Hz, 1H; H-4"), 8.19 (dd,  $^3J_{\text{H,H}}=5.9$  and 7.5 Hz, 2H; H-3"), 7.85 (d,  $^3J_{\text{H,H}}=8.6$  Hz, 2H; H-2), 7.10 (d,  $^3J_{\text{H,H}}=8.6$  Hz, 2H; H-3), 5.08 (t,  $^3J_{\text{H,H}}=5.0$  Hz, 2H; 1'-CH $_2$ ), 4.65 (t,  $^3J_{\text{H,H}}=5.0$  Hz, 2H; 2'-CH $_2$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta=191.8$  (CHO), 162.7 (C-4), 146.6 (C-4"), 145.9 (C-2"), 132.3 (C-2), 130.8 (C-1), 128.4 (C-3"), 115.6 (C-3), 66.9 (C-2'), 60.3 (C-1'); HRMS (ESI):  $m/z$  calcd for  $\text{C}_{14}\text{H}_{14}\text{NO}_2^+$ : 228.1019 [M] $^+$ , found 228.1018.

**1-(4-(4-Formylphenoxy)butyl)pyridinium iodide (16b)**: Prepared from **14b** (500 mg, 1.6 mmol) in pyridine (2 mL), affording **16b** as a yellow powder (610 mg, 99%). m.p. 84.4 °C;  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta=9.87$  (s, 1H; CHO), 9.11 (d,  $^3J_{\text{H,H}}=6.1$  Hz, 2H; H-2"), 8.61 (t,  $^3J_{\text{H,H}}=7.1$  Hz, 1H; H-4"), 8.17 (dd,  $^3J_{\text{H,H}}=6.1$  and 7.1 Hz, 2H; H-3"), 7.86 (d,  $^3J_{\text{H,H}}=8.6$  Hz, 2H; H-2), 7.10 (d,  $^3J_{\text{H,H}}=8.6$  Hz, 2H; H-3), 4.69 (t,  $^3J_{\text{H,H}}=7.3$  Hz, 2H; 1'-CH $_2$ ), 4.14 (t,  $^3J_{\text{H,H}}=6.1$  Hz, 2H; 4'-CH $_2$ ), 2.10 (m, 2H; 2'-CH $_2$ ), 1.78 (m, 2H; 3'-CH $_2$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta=191.8$  (CHO), 163.9 (C-4), 146.0 (C-4"), 145.3 (C-2"), 132.3 (C-2), 130.2 (C-1), 128.6 (C-3"), 115.4 (C-3), 67.8 (C-4'), 60.9 (C-1'), 28.1 (C-2'), 25.6 (C-3'); HRMS (ESI):  $m/z$  calcd for  $\text{C}_{16}\text{H}_{18}\text{NO}_2^+$ : 256.1332 [M] $^+$ , found 256.1330.

**1-(2-(3-Formylphenoxy)ethyl)pyridinium bromide (16c)**: Prepared from **14c** (585 mg, 2.55 mmol) in pyridine (3.2 mL), affording **16c** as a yellow powder (740 mg, 94%). m.p. 75.8 °C;  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta=9.97$  (s, 1H; CHO), 9.15 (d,  $^3J_{\text{H,H}}=5.5$  Hz, 2H; H-2"), 8.65 (t,  $^3J_{\text{H,H}}=7.9$  Hz, 1H; H-4"), 8.20 (dd,  $^3J_{\text{H,H}}=5.5$  and 7.9 Hz, 2H; H-3"), 7.56 (s, 1H; H-2), 7.54 (m, 1H; H-5), 7.41 (d,  $^3J_{\text{H,H}}=2.0$  Hz, 1H; H-6), 7.25 (m, 1H; H-4), 5.09 (t,  $^3J_{\text{H,H}}=4.8$  Hz, 2H; 1'-CH $_2$ ), 4.62 (t,  $^3J_{\text{H,H}}=4.8$  Hz, 2H; 2'-CH $_2$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta=193.3$  (CHO), 158.5 (C-3), 146.6 (C-4"), 145.9 (C-2"), 138.1 (C-1), 131.0 (C-2), 128.4 (C-3"), 124.1 (C-5), 121.8 (C-4), 114.0 (C-6), 66.9 (C-2'), 60.4 (C-1'); HRMS (ESI):  $m/z$  calcd for  $\text{C}_{14}\text{H}_{14}\text{NO}_2^+$ : 228.1019 [M] $^+$ , found 228.1019.

**1-(4-(3-Formylphenoxy)butyl)pyridinium iodide (16d)**: Prepared from **14d** (500 mg, 1.6 mmol) in pyridine (2 mL), affording **16d** as a yellow powder (600 mg, 95%). m.p. 131.7 °C;  $^1\text{H}$  NMR (300 MHz, DMSO- $d_6$ ):  $\delta=9.97$  (s, 1H; CHO), 9.10 (dd,  $^3J_{\text{H,H}}=1.3$  and 6.6 Hz, 2H; H-2"), 8.60 (t,  $^3J_{\text{H,H}}=1.3$  and 7.7 Hz, 1H; H-4"), 8.16 (dd,  $^3J_{\text{H,H}}=6.6$  and 7.7 Hz, 2H; H-3"), 7.53 (s, 1H; H-2), 7.51 (m, 1H; H-5), 7.39 (m, 1H; H-6), 7.25 (m, 1H; H-4), 4.68 (t,  $^3J_{\text{H,H}}=7.3$  Hz, 2H; 1'-CH $_2$ ), 4.09 (t,  $J=6.2$  Hz, 2H; 4'-CH $_2$ ), 2.11 (m, 2H; 2'-CH $_2$ ), 1.77 (m, 2H; 3'-CH $_2$ );  $^{13}\text{C}$  NMR (75 MHz, DMSO- $d_6$ ):  $\delta=193.4$  (CHO), 159.4 (C-3), 146.0 (C-4"), 145.3 (C-2"), 138.1 (C-1), 130.9 (C-2), 128.6 (C-3"), 123.3 (C-5), 121.6 (C-4), 113.8 (C-6), 67.6 (C-4'), 61.0 (C-1'), 28.1 (C-2'), 25.6 (C-3'); HRMS (ESI):  $m/z$  calcd for  $\text{C}_{16}\text{H}_{18}\text{NO}_2^+$ : 256.1332 [M] $^+$ , found 256.1332.

**4-(Chloromethyl)benzaldehyde (19)**: A mixture of 4-(chloromethyl)benzyl alcohol **18** (2.00 g, 12.8 mmol), pyridinium chlorochromate (4.00 g, 19.1 mmol), dichloromethane (20 mL), and a spatula of Celite was stirred for 3 h at room temperature. Then, the reaction mixture was filtrated through a Celite pad and concentrated *in vacuo* to give a brown oil. The crude residue was purified by chromatography on  $\text{SiO}_2$  (pentane/diethyl ether, 9/1, v/v) to give **19** as a white powder (1.64 g, 83%). m.p. 74.3 °C (Litt. 70–71 °C<sup>[20]</sup>);  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3$ ):  $\delta=10.04$  (s, 1H; CHO), 7.88 (d,  $^3J_{\text{H,H}}=7.9$  Hz, 2H; H-3), 7.59 (d,  $^3J_{\text{H,H}}=7.9$  Hz, 2H; H-2), 4.64 (s, 2H; -CH $_2$ );  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ ):  $\delta=191.5$  (CHO), 143.8 (C-4), 136.3 (C-1), 130.1 (C-3), 129.1 (C-2), 45.2 (CH $_2$ ); HRMS (ESI):  $m/z$  calcd for  $\text{C}_8\text{H}_7\text{OClAg}^+$ : 262.9221 [M+Ag] $^+$ , found 262.9221.

**4-(Iodomethyl)benzaldehyde (20):** A solution of **19** (1 g, 6.4 mmol) and NaI (970 mg, 6.4 mmol) in acetone (30 mL) was heated for 24 h at 55 °C. Then, the reaction mixture was filtrated to eliminate NaCl and concentrated *in vacuo*. The crude residue was dissolved in dichloromethane (25 mL) and washed with H<sub>2</sub>O (3 × 20 mL). The organic layer was dried over MgSO<sub>4</sub> and concentrated *in vacuo* to give **20** a rust colored powder (882 mg, 56%). m.p. 118.2 °C (Litt. 118 °C<sup>[34]</sup>). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ=10.00 (s, 1H; CHO), 7.83 (d, <sup>3</sup>J<sub>H,H</sub>=8.0 Hz, 2H; H-3), 7.55 (d, <sup>3</sup>J<sub>H,H</sub>=8.0 Hz, 2H; H-2), 4.49 (s, 2H; -CH<sub>2</sub>); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ=191.4 (CHO), 146.1 (C-4), 130.2 (C-3), 130.1 (C-1), 129.4 (C-2), 3.37 (CH<sub>2</sub>); HRMS (ESI): *m/z* calcd for C<sub>8</sub>H<sub>8</sub>IO<sub>2</sub>Na<sup>+</sup>: 268.9434 [M+Na]<sup>+</sup>, found 268,9434.

**N,N-Diethyl-N-(4-formylbenzyl)ethanaminium iodide (21):** A mixture of **20** (800 mg, 3.25 mmol), absolute ethanol (4.1 mL) and triethylamine (1.4 mL) was heated for 1 h at 130 °C under microwave irradiation. Then, the reaction mixture was concentrated *in vacuo* to afford **21** as a transparent oil (305 mg, 27%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ=10.10 (s, 1H; CHO), 8.02 (d, <sup>3</sup>J<sub>H,H</sub>=8.1 Hz, 2H; H-3), 7.75 (d, <sup>3</sup>J<sub>H,H</sub>=8.1 Hz, 2H; H-2), 4.58 (s, 2H; CH<sub>2</sub>), 3.19 (q, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 6H; 3 × NCH<sub>2</sub>CH<sub>3</sub>), 1.31 (t, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 9H; 3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ=195.4 (CHO), 137.6 (C-1), 134.4 (C-4), 133.9 (C-3), 130.2 (C-2), 59.5 (CH<sub>2</sub>), 52.9 (3 × NCH<sub>2</sub>CH<sub>3</sub>), 8.0 (3 × NCH<sub>2</sub>CH<sub>3</sub>); HRMS (ESI): *m/z* calcd for C<sub>14</sub>H<sub>22</sub>NO<sup>+</sup>: 220.1718 [M]<sup>+</sup>, found 220.1719.

**1-(4-Formylbenzyl)pyridinium chloride (22):** The title compound was prepared from **19** (200 mg, 1.6 mmol) in pyridine (2 mL) according to the above general procedure for the pyridinium-based benzaldehydes. The residue was triturated in diethyl ether to afford **22** as a yellow gum (316 mg, quant. yield). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ=10.03 (s, 1H; CHO), 9.27 (d, <sup>3</sup>J<sub>H,H</sub>=5.7 Hz, 2H; H-2'), 8.66 (t, <sup>3</sup>J<sub>H,H</sub>=7.7 Hz, 1H; H-4'), 8.21 (dd, <sup>3</sup>J<sub>H,H</sub>=5.7 and 7.7 Hz, 2H; H-3'), 7.96 (d, <sup>3</sup>J<sub>H,H</sub>=8.1 Hz, 2H; H-3), 7.71 (d, <sup>3</sup>J<sub>H,H</sub>=8.1 Hz, 2H; H-2), 6.03 (s, 2H; CH<sub>2</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ=193.2 (CHO), 146.7 (C-4'), 145.7 (C-2'), 140.9 (C-1), 137.0 (C-4), 130.6 (C-3), 129.8 (C-2), 129.1 (C-3'), 63.2 (CH<sub>2</sub>); HRMS (ESI): *m/z* calcd for C<sub>13</sub>H<sub>12</sub>NO<sup>+</sup>: 198.0913 [M]<sup>+</sup>, found 198.0913.

**1-(Diethoxymethyl)-3-nitrobenzene (24):** 3-Nitrobenzaldehyde **23** (4.00 g, 26.4 mmol) was dissolved in absolute ethanol (10 mL). Triethyl orthoformate (6.2 mL, 37.0 mmol) and a solution of HCl (5 M) in absolute ethanol (1 mL) were added dropwise and the mixture was heated for 24 h at 80 °C under argon atmosphere. After cooling at room temperature, the reaction mixture was diluted with dichloromethane and washed with a saturated K<sub>2</sub>CO<sub>3</sub> solution. The organic layer was dried over MgSO<sub>4</sub> and concentrated *in vacuo* to afford a yellow oil which was purified by chromatography on SiO<sub>2</sub> (pentane/diethyl ether, 9/1, v/v) to afford **24** as a yellow oil (5.25 g, 88%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ=8.35 (t, <sup>3</sup>J<sub>H,H</sub> 2.2 Hz, 1H; H-2), 8.19 (ddd, <sup>3</sup>J<sub>H,H</sub>=7.9, 2.2 and 1.1 Hz, 1H; H-4), 7.83 (dt, <sup>3</sup>J<sub>H,H</sub>=7.7 and 1.1 Hz, 1H; H-6), 7.54 (t, <sup>3</sup>J<sub>H,H</sub>=7.9 Hz, 1H; H-5), 5.58 (s, 1H; -CH(OEt)<sub>2</sub>), 3.61 (q, <sup>3</sup>J<sub>H,H</sub>=7.0 Hz, 4H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 1.27 (t, <sup>3</sup>J<sub>H,H</sub>=7.0 Hz, 6H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ=148.3 (C-1), 141.5 (C-3), 132.8 (C-6), 129.2 (C-5), 123.3 (C-4), 121.9 (C-2), 100.1 (CH(OEt)<sub>2</sub>), 61.3 (-OCH<sub>2</sub>CH<sub>3</sub>), 15.1 (-OCH<sub>2</sub>CH<sub>3</sub>); HRMS (ESI): *m/z* calcd for C<sub>11</sub>H<sub>15</sub>NO<sub>4</sub>Na<sup>+</sup>: 248.0893 [M+Na]<sup>+</sup>, found 248.0893.

**3-(Diethoxymethyl)aniline (25):** A stirred suspension of **24** (2.50 g, 11.1 mmol) and PtO<sub>2</sub> (50 mg) in ethyl acetate (20 mL) was first flushed with hydrogen, then allowed to react under a hydrogen atmosphere for 72 h at room temperature. The catalyst was removed by filtration through a celite pad and the solution was concentrated *in vacuo* to afford **25** as an orange oil (1.77 g, 83%). <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ=7.12 (t, <sup>3</sup>J<sub>H,H</sub>=7.9 Hz, 1H; H-5), 6.82 (td, <sup>3</sup>J<sub>H,H</sub>=7.5 and 0.9 Hz, 1H; H-4), 6.81 (t, <sup>3</sup>J<sub>H,H</sub>=2.4 Hz, 1H; H-2), 6.59 (ddd, <sup>3</sup>J<sub>H,H</sub>=0.9, 2.4 and 7.7 Hz, 1H; H-6), 5.40 (s, 1H; -CH(OEt)<sub>2</sub>), 3.69 (brs, 2H; -NH<sub>2</sub>), 3.55 (q, <sup>3</sup>J<sub>H,H</sub>=7.2 Hz, 4H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 1.22 (t, <sup>3</sup>J<sub>H,H</sub>=7.2 Hz, 6H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ=146.5 (C-3), 140.3 (C-1), 129.0 (C-5), 116.9 (C-4), 115.0 (C-6), 113.2 (C-2), 101.6 (CH(OEt)<sub>2</sub>), 61.0 (-OCH<sub>2</sub>CH<sub>3</sub>), 15.17 (-OCH<sub>2</sub>CH<sub>3</sub>); HRMS (ESI): *m/z* calcd for C<sub>11</sub>H<sub>18</sub>NO<sub>2</sub><sup>+</sup>: 196.1332 [M+H]<sup>+</sup>, found 196.1333.

**1-(3-Formylphenyl)pyridinium chloride (27):** A mixture of **26** (60 mg, 0.21 mmol) and 3-(diethoxymethyl) aniline **25** (125 mg, 0.64 mmol) in EtOH/H<sub>2</sub>O, 80/20, v/v (1 mL), in a closed vial, was heated for 3 h at 130 °C under microwave irradiation. After cooling at room temperature, the reaction mixture was diluted with H<sub>2</sub>O (20 mL) and washed with EtOAc (5 × 15 mL). The aqueous portion was concentrated *in vacuo* to afford **27** as a brown oil (49 mg, quant. yield). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ=10.17 (s, 1H; CHO), 9.40 (dd, <sup>3</sup>J<sub>H,H</sub>=1.6 and 6.8 Hz, 2H; H-2'), 8.82 (tt, <sup>3</sup>J<sub>H,H</sub>=1.6 and 7.7 Hz, 1H; H-4'), 8.42 (dd, <sup>3</sup>J<sub>H,H</sub>=1.8 and 2.4 Hz, 1H; H-2), 8.34 (dd, <sup>3</sup>J<sub>H,H</sub>=7.7, and 6.8 Hz, 2H; H-3'), 8.28 (td, <sup>3</sup>J<sub>H,H</sub>=1.8 and 7.7 Hz, 1H; H-4), 8.2 (ddd, <sup>3</sup>J<sub>H,H</sub>=8.1, 2.4 and 0.9 Hz, 1H; H-6), 7.99 (t, <sup>3</sup>J<sub>H,H</sub>=8.1 Hz, 1H; H-5); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ=192.4 (CHO), 147.5 (C-4'), 145.6 (C-2'), 143.8 (C-1), 137.9 (C-3), 133.0 (C-4), 131.6 (C-5), 131.0 (C-6), 128.6 (C-3'), 125.5 (C-2); HRMS (ESI): *m/z* calcd for C<sub>12</sub>H<sub>10</sub>NO<sup>+</sup>: 184.0757 [M]<sup>+</sup>, found 184.0757.

**4-(Berrubinemethyl)benzaldehyde chloride (30):** A solution of **29** (417 mg, 1.17 mmol) and **19** (198 mg, 1.28 mmol) in DMF (15 mL) was heated for 15 h at 80 °C under stirring. Then, the reaction mixture was concentrated *in vacuo* and the residue was dissolved in methanol (3 mL) and precipitated with diethyl ether (100 mL). The precipitate was filtrated and dried under vacuum to afford **30** as a black powder (551 mg, 99%). m.p. decomp. > 150 °C; <sup>1</sup>H NMR (600 MHz, DMSO-*d*<sub>6</sub>): δ=10.04 (s, 1H; CHO), 9.84 (s, 1H; 8-CH), 8.96 (s, 1H; 13-CH), 8.22 (d, <sup>3</sup>J<sub>H,H</sub>=9.3 Hz, 1H; 11-CH), 8.02 (d, <sup>3</sup>J<sub>H,H</sub>=9.3 Hz, 1H; 12-CH), 7.96 (d, <sup>3</sup>J<sub>H,H</sub>=8.5 Hz, 2H; 2 × H-3'), 7.83 (d, <sup>3</sup>J<sub>H,H</sub>=8.5 Hz, 2H; 2 × H-2'), 7.80 (s, 1H; 1-CH), 7.09 (s, 1H; 4-CH), 6.17 (s, 2H; -OCH<sub>2</sub>O-), 5.46 (s, 2H; -OCH<sub>2</sub>-Ph), 4.94 (t, <sup>3</sup>J<sub>H,H</sub>=5.5 Hz, 2H; 6-CH<sub>2</sub>), 4.07 (s, 3H; -OCH<sub>3</sub>), 3.20 (t, <sup>3</sup>J<sub>H,H</sub>=5.5 Hz, 2H; 5-CH<sub>2</sub>); <sup>13</sup>C NMR (150.9 MHz, DMSO-*d*<sub>6</sub>): δ=192.9 (CHO), 150.6 (C-10), 149.9 (C-3), 147.8 (C-2), 145.3 (C-8), 143.3 (C-4'), 141.9 (C-9), 137.6 (C-13a), 135.9 (C-1'), 133.0 (C-12a), 130.8 (C-4a), 129.6 (C-3'), 128.8 (C-2'), 126.6 (C-11), 124.0 (C-12), 121.7 (C-8a), 120.5 (C-13b), 120.3 (C-13), 108.5 (C-4), 105.5 (C-1), 102.2 (-OCH<sub>2</sub>O-), 74.6 (-OCH<sub>2</sub>-Ph), 57.1 (OCH<sub>3</sub>), 55.4 (C-6), 26.4 (C-5); HRMS (ESI): *m/z* calcd for C<sub>27</sub>H<sub>22</sub>NO<sub>5</sub><sup>+</sup>: 440.1492 [M]<sup>+</sup>, found 440.1489.

**4-(4-Bromobutoxy)benzaldehyde (31):** To a solution of 4-hydroxybenzaldehyde (1.00 g, 8.19 mmol) in acetonitrile (25 mL) was added 1,4-dibromobutane (4.9 mL, 41.0 mmol) and potassium carbonate (2.26 g, 16.38 mmol), and the mixture was stirred overnight at room temperature. After filtration the reaction mixture was concentrated *in vacuo* and the crude residue was purified on SiO<sub>2</sub> chromatography (pentane/diethyl ether, 8/2, v/v) to afford **31** as a white solid (1.91 g, 91%). m.p. 40 °C (Litt. 43 °C<sup>[35]</sup>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>): δ=9.87 (s, 1H; CHO), 7.82 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; 2 × H-2'), 6.98 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; 2 × H-3'), 4.08 (t, <sup>3</sup>J<sub>H,H</sub>=6.1 Hz, 2H; -CH<sub>2</sub>-CH<sub>2</sub>O-Ph), 3.48 (t, <sup>3</sup>J<sub>H,H</sub>=6.4 Hz, 2H; BrCH<sub>2</sub>-), 2.13-1.92 (m, 4H; -CH<sub>2</sub>-CH<sub>2</sub>-); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>): δ=190.8 (CHO), 164.0 (C-4), 132.1 (C-2), 130.2 (C-1), 114.9 (C-3), 67.4 (-CH<sub>2</sub>-CH<sub>2</sub>O-), 33.2 (BrCH<sub>2</sub>-), 29.4 (BrCH<sub>2</sub>-CH<sub>2</sub>-), 27.8 (-CH<sub>2</sub>-CH<sub>2</sub>O-); HRMS (ESI): *m/z* calcd for C<sub>11</sub>H<sub>14</sub>BrO<sub>2</sub>: 257.0172 [M-H]<sup>+</sup>, found 257.0169.



**4-(4-Berrubinebutoxy)benzaldehyde bromide (32):** A solution of **29** (380 mg, 1.06 mmol) and **31** (410 mg, 1.60 mmol) in DMF (10 mL) was stirred for 24 h at 85 °C under argon atmosphere. Then, the reaction mixture was concentrated *in vacuo* and the residue dissolved in methanol (2 mL) and precipitated with diethyl ether (50 mL). The precipitate was filtrated and dried under vacuum to afford **32** as a brown powder (575 mg, quant.). m.p. decomp. > 150 °C; <sup>1</sup>H NMR (300 MHz, CD<sub>3</sub>OD): δ=9.81 (s, 1H; CHO), 9.68 (s, 1H; 8-CH), 8.70 (s, 1H; 13-CH), 8.12 (d, <sup>3</sup>J<sub>H,H</sub>=9.2 Hz, 1H; 11-CH), 8.00 (d, <sup>3</sup>J<sub>H,H</sub>=9.2 Hz, 1H; 12-CH), 7.84 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 1H; 2 × H-2'), 7.67 (s, 1H; 1-CH), 7.07 (d, <sup>3</sup>J<sub>H,H</sub>=8.8 Hz, 2H; 2 × H-3'), 6.96 (s, 1H; 4-CH), 6.11 (s, 2H; -OCH<sub>2</sub>O-), 4.91 (t, <sup>3</sup>J<sub>H,H</sub>=6.2 Hz, 2H; 6-CH<sub>2</sub>), 4.52 (t, <sup>3</sup>J<sub>H,H</sub>=5.9 Hz, 2H; BBR-CH<sub>2</sub>-CH<sub>2</sub>-), 4.25 (t, <sup>3</sup>J<sub>H,H</sub>=5.9 Hz, 2H; -CH<sub>2</sub>-CH<sub>2</sub>O-Ph), 4.09 (s, 3H; -OCH<sub>3</sub>), 3.24 (t, <sup>3</sup>J<sub>H,H</sub>=6.2 Hz, 2H; 5-CH<sub>2</sub>), 2.17–2.08 (m, 4H; -CH<sub>2</sub>-CH<sub>2</sub>-); <sup>13</sup>C NMR (100.6 MHz, DMSO-*d*<sub>6</sub>): δ=191.2 (s, CHO), 163.6 (C-4'), 150.3 (C-10), 149.8 (C-3), 147.7 (C-2), 145.2 (C-8), 142.8 (C-9), 137.4 (C-13a), 133.0 (C-12a), 131.8 (C-2'), 130.6 (C-4a), 129.5 (C-1'), 126.6 (C-11), 123.3 (C-12), 121.6 (C-8a), 120.5 (C-13b), 120.2 (C-13), 114.9 (C-3'), 108.4 (C-4), 105.4 (C-1), 102.0 (-OCH<sub>2</sub>O-), 73.9 (BBR-CH<sub>2</sub>-CH<sub>2</sub>-), 67.9 (-CH<sub>2</sub>-CH<sub>2</sub>O-Ph), 57.1 (OCH<sub>3</sub>), 55.3 (C-6), 26.3 (C-5), 26.2 (BBR-CH<sub>2</sub>-CH<sub>2</sub>-), 25.0 (-CH<sub>2</sub>-CH<sub>2</sub>O-Ph); HRMS (ESI): *m/z* calcd for C<sub>30</sub>H<sub>28</sub>NO<sub>6</sub><sup>+</sup>: 498.1911 [M]<sup>+</sup>, found 498.1914.

**General microwave assisted procedure for synthesis of nitrones (6–12):** A mixture of the appropriate vectorized benzaldehyde (1 eq.) and **17** (1.2 eq) in a given volume of absolute ethanol, in a closed vial, was heated for 1 h at 100 °C under microwave irradiation. After cooling at room temperature, the reaction mixture was concentrated *in vacuo* and, unless otherwise indicated, the residue was diluted with one portion of water, then washed with five portions of ethyl acetate, and concentrated *in vacuo* to afford the desired product which was further used as obtained.

**2-(Diethoxyphosphoryl)-N-(4-(2-triethylammonioethoxy)benzylidene)propan-2-amine oxide bromide (6a):** Prepared from **15a** (546 mg, 1.7 mmol) and **17** (420 mg, 2.0 mmol) in ethanol (10 mL). Treatment: single portion volume, 10 mL. Obtained compound: **6a** as a yellow gum (423 mg, 49%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ=8.37 (d, <sup>3</sup>J<sub>H,H</sub>=9.1 Hz, 2H; H-2), 7.86 (d, <sup>3</sup>J<sub>H,H</sub>=2.5 Hz, 1H; HC=NO), 7.08 (d, <sup>3</sup>J<sub>H,H</sub>=9.1 Hz, 2H; H-3), 4.46 (t, <sup>3</sup>J<sub>H,H</sub>=4.7 Hz, 2H; 2'-CH<sub>2</sub>), 4.07 (q, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 4H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 3.70 (t, <sup>3</sup>J<sub>H,H</sub>=4.7 Hz, 2H; 1'-CH<sub>2</sub>), 3.38 (q, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 6H; 3 × NCH<sub>2</sub>CH<sub>3</sub>), 1.73 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.69 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.24 (t, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 6H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 1.23 (t, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 9H; 3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ=158.9 (C-4), 131.5 (d, <sup>3</sup>J<sub>C,P</sub>=6.6 Hz; HC=NO), 131.0 (C-2), 125.2 (C-1), 114.8 (C-3), 72.1 (d, <sup>1</sup>J<sub>C,P</sub>=156.0 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 63.0 (d, <sup>2</sup>J<sub>C,P</sub>=6.6 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 61.6 (C-2'), 55.6 (C-1'), 53.5 (3 × NCH<sub>2</sub>CH<sub>3</sub>), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 16.7 (d, <sup>3</sup>J<sub>C,P</sub>=5.9 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 7.8 (3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>31</sup>P NMR (162 MHz, DMSO-*d*<sub>6</sub>): δ=23.39; HRMS (ESI): *m/z* calcd for C<sub>22</sub>H<sub>40</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 443.2669 [M]<sup>+</sup>, found 443.2667.

**2-(Diethoxyphosphoryl)-N-(4-(4-triethylammonio)butoxy)benzylidene)propan-2-amine oxide iodide (6b):** Prepared from **15b** (610 mg, 1.5 mmol) and **17** (380 mg, 1.8 mmol) in ethanol (12 mL). Treatment: single portion volume, 12 mL. Obtained compound: **6b** as a yellow gum (670 mg, 75%). <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ=8.35 (d, <sup>3</sup>J<sub>H,H</sub>=8.9 Hz, 2H; H-3), 7.82 (d, <sup>3</sup>J<sub>H,H</sub>=2.5 Hz, 1H; HC=NO), 7.04 (d, <sup>3</sup>J<sub>H,H</sub>=8.9 Hz, 2H; H-2), 4.08 (q, <sup>3</sup>J<sub>H,H</sub>=7.0 Hz, 4H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 4.07 (m, 2H; 4'-CH<sub>2</sub>), 3.24 (q, <sup>3</sup>J<sub>H,H</sub>=7.3 Hz, 6H; 3 × NCH<sub>2</sub>CH<sub>3</sub>), 3.23 (m, 2H; 1'-CH<sub>2</sub>), 1.80 (m, 4H; 2'-CH<sub>2</sub> and 3'-CH<sub>2</sub>), 1.73 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.68 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.23 (t, <sup>3</sup>J<sub>H,H</sub>=7.0 Hz, 6H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 1.19 (t, <sup>3</sup>J<sub>H,H</sub>=7.3 Hz, 9H; 3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ=160.0 (C-4), 131.6 (d, <sup>3</sup>J<sub>C,P</sub>=6.6 Hz; HC=NO), 131.1 (C-3), 124.5 (C-1), 114.6 (C-2), 72.0 (d, <sup>1</sup>J<sub>C,P</sub>=156.0 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 67.2 (C-4'), 63.0 (d, <sup>2</sup>J<sub>C,P</sub>=5.9 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 56.1 (C-1'), 52.2 (3 × NCH<sub>2</sub>CH<sub>3</sub>), 25.8 (C-2'), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 18.4 (C-3'), 16.8 (d, <sup>3</sup>J<sub>C,P</sub>=5.9 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 7.7 (3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>31</sup>P NMR (121.5 MHz, DMSO-*d*<sub>6</sub>): δ=23.43; HRMS (ESI): *m/z* calcd for C<sub>24</sub>H<sub>44</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 471.2982 [M]<sup>+</sup>, found 471.2983.

**2-(Diethoxyphosphoryl)-N-(3-(2-triethylammonioethoxy)benzylidene)propan-2-amine oxide bromide (6c):** Prepared from **15c** (546 mg, 1.7 mmol) and **17** (420 mg, 2.0 mmol) in ethanol (11 mL). Treatment: single portion volume, 11 mL. Obtained compound: **6c** as a brown gum (425 mg, 48%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ=8.18 (s, 1H; H-2), 7.92 (d, <sup>3</sup>J<sub>H,H</sub>=2.5 Hz, 1H; HC=NO), 7.82 (d, <sup>3</sup>J<sub>H,H</sub>=8.1 Hz, 1H; H-6), 7.40 (t, <sup>3</sup>J<sub>H,H</sub>=8.1 Hz, 1H; H-5), 7.07 (dd, <sup>3</sup>J<sub>H,H</sub>=2.5 and 7.8 Hz, 1H; H-4), 4.41 (t, <sup>3</sup>J<sub>H,H</sub>=4.4 Hz, 2H; 2'-CH<sub>2</sub>), 4.07 (q, <sup>3</sup>J<sub>H,H</sub>=7.3 Hz, 4H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 3.69 (t, <sup>3</sup>J<sub>H,H</sub>=4.4 Hz, 2H; 1'-CH<sub>2</sub>), 3.37 (q, <sup>3</sup>J<sub>H,H</sub>=6.9 Hz, 6H; 3 × NCH<sub>2</sub>CH<sub>3</sub>), 1.73 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.69 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.23 (t, <sup>3</sup>J<sub>H,H</sub>=7.3 Hz, 6H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 1.23 (t, <sup>3</sup>J<sub>H,H</sub>=6.9 Hz, 9H; 3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ=157.6 (C-3), 133.7 (d, <sup>3</sup>J<sub>C,P</sub>=5.9 Hz; HC=NO), 132.8 (d, <sup>4</sup>J<sub>C,P</sub>=1.5 Hz; C-1), 129.9 (C-5), 122.8 (C-6), 117.0 (C-4), 114.5 (C-2), 72.7 (d, <sup>1</sup>J<sub>C,P</sub>=155.3 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 63.1 (d, <sup>2</sup>J<sub>C,P</sub>=6.6 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 61.6 (C-2'), 55.8 (C-1'), 53.5 (3 × NCH<sub>2</sub>CH<sub>3</sub>), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 16.7 (d, <sup>3</sup>J<sub>C,P</sub>=5.1 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 7.8 (3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>31</sup>P NMR (162 MHz, DMSO-*d*<sub>6</sub>): δ=23.08; HRMS (ESI): *m/z* calcd for C<sub>22</sub>H<sub>40</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 443.2669 [M]<sup>+</sup>, found 443.2668.

**2-(Diethoxyphosphoryl)-N-(3-(4-triethylammonio)butoxy)benzylidene)propan-2-amine oxide iodide (6d):** Prepared from **15d** (500 mg, 1.2 mmol) and **17** (304 mg, 1.4 mmol) in ethanol (10 mL). Treatment: single portion volume, 10 mL. Obtained compound: **6d** as a yellow gum (473 mg, 66%). <sup>1</sup>H NMR (400 MHz, DMSO-*d*<sub>6</sub>): δ=8.24 (s, 1H; H-2), 7.91 (d, <sup>3</sup>J<sub>H,H</sub>=2.8 Hz, 1H; HC=NO), 7.71 (d, <sup>3</sup>J<sub>H,H</sub>=7.9 Hz, 1H; H-6), 7.37 (t, <sup>3</sup>J<sub>H,H</sub>=7.9 Hz, 1H; H-5), 7.03 (ddd, <sup>3</sup>J<sub>H,H</sub>=0.5, 2.2 and 8.1 Hz, 1H; H-4), 4.07 (m, 2H; 4'-CH<sub>2</sub>), 4.07 (q, <sup>3</sup>J<sub>H,H</sub>=7.2 Hz, 4H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 3.25 (m, 2H; 1'-CH<sub>2</sub>), 3.25 (q, <sup>3</sup>J<sub>H,H</sub>=7.2 Hz, 6H; 3 × NCH<sub>2</sub>CH<sub>3</sub>), 1.80 (m, 4H; 2'-CH<sub>2</sub> and 3'-CH<sub>2</sub>), 1.75 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.70 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.24 (t, <sup>3</sup>J<sub>H,H</sub>=7.2 Hz, 6H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 1.19 (t, <sup>3</sup>J<sub>H,H</sub>=7.2 Hz, 9H; 3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (100 MHz, DMSO-*d*<sub>6</sub>): δ=158.6 (C-3), 132.7 (d, <sup>4</sup>J<sub>C,P</sub>=1.7 Hz; C-1), 132.2 (d, <sup>3</sup>J<sub>C,P</sub>=6.1 Hz; HC=NO), 129.8 (C-5), 122.3 (C-6), 117.1 (C-4), 114.3 (C-2), 72.4 (d, <sup>1</sup>J<sub>C,P</sub>=155.4 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 67.1 (C-4'), 63.1 (d, <sup>2</sup>J<sub>C,P</sub>=6.6 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 56.2 (C-1'), 52.6 (3 × NCH<sub>2</sub>CH<sub>3</sub>), 26.0 (C-3'), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 18.5 (C-2'), 16.72 (d, <sup>3</sup>J<sub>C,P</sub>=5.5 Hz; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 7.63 (3 × NCH<sub>2</sub>CH<sub>3</sub>); <sup>31</sup>P NMR (162 MHz, DMSO-*d*<sub>6</sub>): δ=23.07; HRMS (ESI): *m/z* calcd for C<sub>24</sub>H<sub>44</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 471.2982 [M]<sup>+</sup>, found 471.2982.

**2-(Diethoxyphosphoryl)-N-(4-(2-N-pyridinylethoxy)benzylidene)propan-2-amine oxide iodide (7a):** Prepared from **16a** (620 mg, 2.0 mmol) and **17** (507 mg, 2.7 mmol) in ethanol (12 mL). Treatment: single portion volume, 12 mL. Obtained compound: **7a** as a yellow powder (682 mg, 68%). m.p. 57.7 °C; <sup>1</sup>H NMR (300 MHz, DMSO-*d*<sub>6</sub>): δ=9.13 (d, <sup>3</sup>J<sub>H,H</sub>=5.6 Hz, 2H; H-2'), 8.64 (t, <sup>3</sup>J<sub>H,H</sub>=7.8 Hz, 1H; H-4'), 8.30 (d, <sup>3</sup>J<sub>H,H</sub>=9.1 Hz, 2H; H-2), 8.19 (dd, <sup>3</sup>J<sub>H,H</sub>=5.6 and 7.8 Hz, 2H; H-3'), 7.81 (d, <sup>3</sup>J<sub>H,H</sub>=2.5 Hz, 1H; HC=NO), 6.99 (d, <sup>3</sup>J<sub>H,H</sub>=9.1 Hz, 2H; H-3), 5.06 (t, <sup>3</sup>J<sub>H,H</sub>=5.1 Hz, 2H; 1'-CH<sub>2</sub>), 4.57 (t, <sup>3</sup>J<sub>H,H</sub>=5.1 Hz, 2H; 2'-CH<sub>2</sub>), 4.05 (q, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 4H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>), 1.69 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.66 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.20 (t, <sup>3</sup>J<sub>H,H</sub>=7.1 Hz, 6H; 2 × -OCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (75 MHz, DMSO-*d*<sub>6</sub>): δ=158.9 (C-4), 146.6 (C-4'), 145.9 (C-2'), 131.5 (d, <sup>3</sup>J<sub>C,P</sub>=6.6 Hz; HC=NO), 131.0 (C-2), 128.4 (C-3'), 125.3 (C-1), 114.8 (C-3), 72.1 (d, <sup>1</sup>J<sub>C,P</sub>=156.1 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 66.6 (C-2'), 63.1

(d,  $^2J_{C,P}$ =6.6 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 60.4 (C-1'), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 16.8 (d,  $^3J_{C,P}$ =5.1 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{31}P$  NMR (121.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =23.39; HRMS (ESI): *m/z* calcd for C<sub>21</sub>H<sub>30</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 421.1887 [M]<sup>+</sup>, found 421.1888.

**2-(Diethoxyphosphoryl)-N-(4-(4-N-pyridinyl)butoxy)benzylidene)propan-2-amine oxide iodide (7b):** Prepared from **16b** (500 mg, 1.3 mmol) and **17** (329 mg, 1.6 mmol) in ethanol (10 mL). Treatment: single portion volume, 10 mL. Obtained compound: **7b** as a yellow gum (540 mg, 72%).  $^1H$  NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =9.11 (d,  $^3J_{H,H}$ =6.1 Hz, 2H; H-2'), 8.61 (t,  $^3J_{H,H}$ =7.6 Hz, 1H; H-4'), 8.31 (d,  $^3J_{H,H}$ =8.8 Hz, 2H; H-2), 8.17 (dd,  $^3J_{H,H}$ =6.1 and 7.6 Hz, 2H; H-3'), 7.81 (d,  $^3J_{H,H}$ =2.0 Hz, 1H; HC=NO), 6.98 (d,  $^3J_{H,H}$ =8.8 Hz, 2H; H-3), 4.68 (t,  $^3J_{H,H}$ =7.3 Hz, 2H; 1'-CH<sub>2</sub>), 4.07 (m, 2H; 4'-CH<sub>2</sub>), 4.07 (q,  $^3J_{H,H}$ =7.1 Hz, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 2.12 (m, 2H; 2'-CH<sub>2</sub>), 1.75 (m, 2H; 3'-CH<sub>2</sub>), 1.71 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.68 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.22 (t,  $^3J_{H,H}$ =7.1 Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{13}C$  NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =160.0 (C-1), 146.0 (C-4'), 145.3 (C-2'), 131.6 (d,  $^3J_{C,P}$ =7.3 Hz; HC=NO), 131.1 (C-2), 128.6 (C-3'), 124.5 (C-4), 114.6 (C-3), 72.0 (d,  $^1J_{C,P}$ =156.2 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 67.4 (C-4'), 63.0 (d,  $^2J_{C,P}$ =7.3 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 61.0 (C-1'), 28.1 (C-2'), 25.7 (C-3'), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 16.7 (d,  $^3J_{C,P}$ =5.1 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{31}P$  NMR (162 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =23.47; HRMS (ESI): *m/z* calcd for C<sub>23</sub>H<sub>34</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 449.2200 [M]<sup>+</sup>, found 449.2200.

**2-(Diethoxyphosphoryl)-N-(3-(2-N-pyridinylethoxy)benzylidene)propan-2-amine oxide bromide (7c):** Prepared from **16c** (700 mg, 2.3 mmol) and **17** (583 mg, 2.8 mmol) in ethanol (14 mL). Treatment: single portion volume, 14 mL. Obtained compound: **7c** as a yellow gum (969 mg, 84%).  $^1H$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =9.14 (d,  $^3J_{H,H}$ =5.4 Hz, 2H; H-2'), 8.64 (t,  $^3J_{H,H}$ =7.8 Hz, 1H; H-4'), 8.19 (s, 1H; H-2), 8.19 (dd,  $^3J_{H,H}$ =5.4 and 7.8 Hz, 2H; H-3'), 7.88 (d,  $^3J_{H,H}$ =2.5 Hz, 1H; HC=NO), 7.71 (d,  $^3J_{H,H}$ =7.8 Hz, 1H; H-6), 7.35 (t,  $^3J_{H,H}$ =8.1 Hz, 1H; H-5), 6.98 (dd,  $^3J_{H,H}$ =2.5 and 8.1 Hz, 1H; H-4), 5.06 (t,  $^3J_{H,H}$ =4.9 Hz, 2H; 1'-CH<sub>2</sub>), 4.53 (t,  $^3J_{H,H}$ =4.9 Hz, 2H; 2'-CH<sub>2</sub>), 4.06 (q,  $^3J_{H,H}$ =7.1 Hz, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 1.71 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.68 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.22 (t,  $^3J_{H,H}$ =7.1 Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{13}C$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =157.6 (C-3), 146.6 (C-4'), 145.9 (C-2'), 132.8 (d,  $^4J_{C,P}$ =1.7 Hz; C-1), 132.0 (d,  $^3J_{C,P}$ =6.6 Hz; HC=NO), 129.9 (C-5), 128.4 (C-3'), 123.0 (C-6), 117.0 (C-4), 114.3 (C-2), 72.5 (d,  $^1J_{C,P}$ =155.2 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 66.7 (C-2'), 63.1 (d,  $^2J_{C,P}$ =6.6 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 60.5 (C-1'), 23.4 (s, C(CH<sub>3</sub>)<sub>2</sub>), 16.72 (d,  $^3J_{C,P}$ =5.5 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{31}P$  NMR (121.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =23.06; HRMS (ESI): *m/z* calcd for C<sub>21</sub>H<sub>30</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 421.1887 [M]<sup>+</sup>, found 421.1882.

**2-(Diethoxyphosphoryl)-N-(3-(4-N-pyridinyl)butoxy)benzylidene)propan-2-amine oxide iodide (7d):** Prepared from **16d** (500 mg, 1.3 mmol) and **17** (329 mg, 1.6 mmol) in ethanol (10 mL). Treatment: single portion volume, 10 mL. Obtained compound: **7d** as a yellow gum (370 mg, 53%).  $^1H$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =9.11 (d,  $^3J_{H,H}$ =6.6 Hz, 2H; H-2'), 8.61 (t,  $^3J_{H,H}$ =7.7 Hz, 1H; H-4'), 8.17 (dd,  $^3J_{H,H}$ =6.6 and 7.7 Hz, 2H; H-3'), 8.17 (s, 1H; H-2), 7.88 (d,  $^3J_{H,H}$ =2.6 Hz, 1H; HC=NO), 7.69 (d,  $^3J_{H,H}$ =7.7 Hz, 1H; H-6), 7.34 (t,  $^3J_{H,H}$ =8.1 Hz, 1H; H-5), 6.98 (dd,  $^3J_{H,H}$ =2.6 and 8.1 Hz, 1H; H-4), 4.69 (t,  $^3J_{H,H}$ =7.3 Hz, 2H; 1'-CH<sub>2</sub>), 4.02 (m, 2H; 4'-CH<sub>2</sub>), 4.01 (q,  $^3J_{H,H}$ =7.2 Hz, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 2.11 (m, 2H; 2'-CH<sub>2</sub>), 1.76 (m, 2H; 3'-CH<sub>2</sub>), 1.73 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.68 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.23 (t,  $^3J_{H,H}$ =7.2 Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{13}C$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =158.4 (C-3), 146.0 (C-4'), 145.3 (C-2'), 132.7 (d,  $^4J_{C,P}$ =1.7 Hz; C-1), 132.3 (d,  $^3J_{C,P}$ =6.1 Hz; HC=NO), 129.8 (C-5), 128.6 (C-3'), 122.2 (C-6), 117.08 (C-4), 114.3 (C-2), 72.4 (d,  $^1J_{C,P}$ =155.4 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 67.3 (C-4'), 63.1 (d,  $^2J_{C,P}$ =6.6 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 61.0 (C-1'), 28.2 (C-2'), 25.8 (C-3'), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 16.8 (d,  $^3J_{C,P}$ =5.5 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{31}P$  NMR (121.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =23.07; HRMS (ESI): *m/z* calcd for C<sub>23</sub>H<sub>34</sub>N<sub>2</sub>O<sub>5</sub>P<sup>+</sup>: 449.2200 [M]<sup>+</sup>, found 449.2200.

**2-(diethoxyphosphoryl)-N-(4-(triethylammoniumethyl)benzylidene)propan-2-amine oxide iodide (8):** Prepared from **21** (304 mg, 0.88 mmol) and **17** (222 mg, 1.05 mmol) in ethanol (6 mL). Treatment: single portion volume, 6 mL. Obtained compound: **8** as a yellow powder (371 mg, 78%). m.p. 43.3 °C;  $^1H$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =8.46 (d,  $^3J_{H,H}$ =8.1 Hz, 2H; H-3), 8.03 (d,  $^3J_{H,H}$ =2.2 Hz, 1H; CH=NO), 7.58 (d,  $^3J_{H,H}$ =8.1 Hz, 2H; H-2), 4.49 (s, 2H; CH<sub>2</sub>), 4.09 (q,  $^3J_{H,H}$ =7.1 Hz, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 3.16 (q,  $^3J_{H,H}$ =7.1 Hz, 6H; 3 x NCH<sub>2</sub>CH<sub>3</sub>), 1.75 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.71 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.30 (t,  $^3J_{H,H}$ =7.1 Hz, 9H; 3 x NCH<sub>2</sub>CH<sub>3</sub>), 1.24 (t,  $^3J_{H,H}$ =7.1 Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{13}C$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =133.0 (C-2), 131.7 (C-4), 131.6 (d,  $^3J_{C,P}$ =2.2 Hz; HC=NO), 129.7 (C-1), 129.2 (C-3), 73.0 (d,  $^1J_{C,P}$ =155.0 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 63.2 (d,  $^2J_{C,P}$ =6.6 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 60.2 (CH<sub>2</sub>), 52.6 (3 x NCH<sub>2</sub>CH<sub>3</sub>), 23.3 (C(CH<sub>3</sub>)<sub>2</sub>), 16.7 (d,  $^3J_{C,P}$ =5.1 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 8.0 (3 x NCH<sub>2</sub>CH<sub>3</sub>);  $^{31}P$  NMR (121.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =22.96; HRMS (ESI): *m/z* calcd for C<sub>21</sub>H<sub>38</sub>N<sub>2</sub>O<sub>4</sub>P<sup>+</sup>: 413.2564 [M]<sup>+</sup>, found 413.2565.

**2-(diethoxyphosphoryl)-N-(4-(N-pyridinemethyl)benzylidene)propan-2-amine oxide chloride (9):** Prepared from **22** (222 mg, 0.95 mmol) and **17** (241 mg, 1.14 mmol) in ethanol (4 mL). Treatment: single portion volume, 5 mL. Obtained compound: **9** as a yellow powder (370 mg, 93%). m.p. 63.2 °C;  $^1H$  NMR (300 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =9.22 (d,  $^3J_{H,H}$ =5.7 Hz, 2H; H-2'), 8.63 (t,  $^3J_{H,H}$ =7.7 Hz, 1H; H-4'), 8.37 (d,  $^3J_{H,H}$ =8.4 Hz, 2H; H-2), 8.17 (dd,  $^3J_{H,H}$ =5.7 and 7.7 Hz, 2H; H-3'), 7.95 (d,  $^3J_{H,H}$ =2.6 Hz, 1H; HC=NO), 7.57 (d,  $^3J_{H,H}$ =8.4 Hz, 2H; H-3), 5.92 (s, 2H; CH<sub>2</sub>), 4.06 (q,  $^3J_{H,H}$ =7.2 Hz, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 1.72 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.68 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.21 (t,  $^3J_{H,H}$ =7.2 Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{13}C$  NMR (75 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =146.5 (C-4'), 145.4 (C-2'), 136.1 (C-4), 132.3 (d,  $^4J_{C,P}$ =1.7 Hz; C-1), 131.7 (d,  $^3J_{C,P}$ =6.6 Hz; HC=NO), 129.6 (C-2), 129.2 (C-3), 129.0 (C-3'), 72.6 (d,  $^1J_{C,P}$ =155.1 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 63.4 (CH<sub>2</sub>), 63.1 (d,  $^2J_{C,P}$ =7.2 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 23.4 (C(CH<sub>3</sub>)<sub>2</sub>), 16.7 (d,  $^3J_{C,P}$ =5.5 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{31}P$  NMR (121.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =22.92; HRMS (ESI): *m/z* calcd for C<sub>20</sub>H<sub>28</sub>N<sub>2</sub>O<sub>4</sub>P<sup>+</sup>: 391.1781 [M]<sup>+</sup>, found 391.1784.

**2-(Diethoxyphosphoryl)-N-(3-(N-pyridinyl)benzylidene)propan-2-amine oxide chloride (10):** Prepared from **27** (209 mg, 0.95 mmol) and **17** (241 mg, 1.14 mmol) in ethanol (4 mL). Treatment: single portion volume, 5 mL. Obtained compound: **10** as a brown powder (70 mg, 78%). m.p. 76.7 °C;  $^1H$  NMR (400 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =9.34 (dd,  $^3J_{H,H}$ =1.1 and 6.8 Hz, 2H; H-2'), 9.07 (s, 1H; H-2), 8.79 (tt,  $^3J_{H,H}$ =1.1 and 7.9 Hz, 1H; H-4'), 8.41 (d,  $^3J_{H,H}$ =7.9 Hz, 1H; H-6), 8.31 (dd,  $^3J_{H,H}$ =6.8 and 7.9 Hz, 2H; H-3'), 8.18 (d,  $^3J_{H,H}$ =2.6 Hz, 1H; HC=NO), 7.91 (ddd,  $^3J_{H,H}$ =0.5, 1.8 and 7.7 Hz, 1H; H-4), 7.81 (t,  $^3J_{H,H}$ =8.0 Hz, 1H; H-5), 4.06 (q,  $^3J_{H,H}$ =7.0 Hz, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 1.77 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.72 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.23 (t,  $^3J_{H,H}$ =7.0 Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{13}C$  NMR (100 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =147.3 (C-4'), 146.5 (C-3), 145.5 (C-2'), 143.3 (C-1), 131.7 (C-6), 131.3 (d,  $^3J_{C,P}$ =5.9 Hz; HC=NO), 130.7 (C-5), 128.7 (C-3'), 126.7 (C-4), 124.0 (C-2), 73.1 (d,  $^1J_{C,P}$ =155.0 Hz; C(CH<sub>3</sub>)<sub>2</sub>), 63.3 (d,  $^2J_{C,P}$ =6.6 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 23.3 (C(CH<sub>3</sub>)<sub>2</sub>), 16.81 (d,  $^3J_{C,P}$ =5.1 Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>);  $^{31}P$  NMR (121.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =22.59; HRMS (ESI): *m/z* calcd for C<sub>19</sub>H<sub>26</sub>N<sub>2</sub>O<sub>4</sub>P<sup>+</sup>: 377.1625 [M]<sup>+</sup>, found 377.1627.

**2-(Diethoxyphosphoryl)-N-(4-(berberubinemethyl)benzylidene)propan-2-amine oxide chloride (11):** Prepared from **30** (300 mg, 632  $\mu$ mol) and **17** (200 mg, 947  $\mu$ mol) in ethanol (3 mL). At the end of the procedure, the reaction mixture was concentrated *in vacuo* and the residue dissolved in methanol (2 mL) and precipitated with diethyl ether (75 mL). The precipitate was filtrated and dried under vacuum to afford **11** as a brown powder (320 mg, 76%). m.p. decomp. > 150 °C;  $^1H$  NMR (600 MHz, DMSO-*d*<sub>6</sub>):  $\delta$ =9.77 (s, 1H; 8-CH), 8.93 (s, 1H;

13-CH), 8.36 (d,  $^3J_{H,H}=8.1$  Hz, 2H; 2 x H-2'), 8.20 (d,  $^3J_{H,H}=8.9$  Hz, 1H; 11-CH), 8.00 (d,  $^3J_{H,H}=8.9$  Hz, 1H; 12-CH), 7.93 (d,  $^3J_{H,H}=2.4$  Hz, 1H; HC=NO), 7.78 (s, 1H; 1-CH), 7.65 (d,  $^3J_{H,H}=8.1$  Hz, 2H; 2 x H-3'), 7.08 (s, 1H; 4-CH), 6.17 (s, 2H; -OCH<sub>2</sub>O-), 5.40 (s, 2H; -OCH<sub>2</sub>-Ph), 4.92 (t,  $^3J_{H,H}=6.2$  Hz, 2H; 6-CH<sub>2</sub>), 4.08–4.05 (m, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 4.09 (s, 3H; -OCH<sub>3</sub>), 3.19 (t,  $^3J_{H,H}=6.2$  Hz, 2H; 5-CH<sub>2</sub>), 1.73 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.70 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.22 (t,  $^3J_{H,H}=7.3$  Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (150.9 MHz, DMSO-*d*<sub>6</sub>):  $\delta=150.6$  (C-10), 149.9 (C-3), 147.7 (C-2), 145.3 (C-8), 141.8 (C-9), 138.3 (C-4'), 137.4 (C-13a), 132.9 (C-12a), 131.4 (d,  $^3J_{C,P}=6.3$  Hz; HC=NO), 130.9 (C-1'), 130.4 (C-4a), 128.5 (C-2'), 128.4 (C-3'), 126.6 (C-11), 123.8 (C-12), 121.8 (C-8a), 120.4 (C-13b), 120.3 (C-13), 108.4 (C-4), 105.4 (C-1), 102.1 (-OCH<sub>2</sub>O-), 74.9 (-OCH<sub>2</sub>-Ph), 73.0 (d,  $^1J_{C,P}=156.6$  Hz; C(CH<sub>3</sub>)<sub>2</sub>), 62.6 (d,  $^2J_{C,P}=6.3$  Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 57.1 (OCH<sub>3</sub>), 55.3 (C-6), 26.4 (C-5), 22.9 (C(CH<sub>3</sub>)<sub>2</sub>), 16.2 (d,  $^3J_{C,P}=7.8$  Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>); <sup>31</sup>P NMR (121.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta=23.05$ ; HRMS (ESI): *m/z* calcd for C<sub>19</sub>H<sub>16</sub>NO<sub>4</sub><sup>+</sup>: 633.2360 [M]<sup>+</sup>, found 633.2361.

**2-(Diethoxyphosphoryl)-N-(4-(4-berberrubinebutoxy)benzylidene)propan-2-amine oxide bromide (12)**: Prepared from **32** (328 mg, 614  $\mu$ mol) and **17** (390 mg, 1.848 mmol) in ethanol (5 mL). At the end of the procedure, the reaction mixture was concentrated *in vacuo* and the residue dissolved in methanol (2 mL) and precipitated with diethyl ether (75 mL). The precipitate was filtrated and dried under vacuum to afford **12** as a brown powder (327 mg, 73%). m.p. decomp. > 142 °C; <sup>1</sup>H NMR (500 MHz, DMSO-*d*<sub>6</sub>):  $\delta=9.77$  (s, 1H; 8-CH), 8.94 (s, 1H; 13-CH), 8.32 (d,  $^3J_{H,H}=8.7$  Hz, 2H; 2 x H-2'), 8.19 (d,  $^3J_{H,H}=9.1$  Hz, 1H; 11-CH), 8.00 (d,  $^3J_{H,H}=9.1$  Hz, 1H; 12-CH), 7.81 (d,  $^3J_{H,H}=2.2$  Hz, 1H; HC=NO), 7.79 (s, 1H; 1-CH), 7.08 (s, 1H; 4-CH), 7.00 (d,  $^3J_{H,H}=8.7$  Hz, 2H; 2 x H-3'), 6.17 (s, 2H; -OCH<sub>2</sub>O-), 4.94 (t,  $^3J_{H,H}=5.7$  Hz, 2H; 6-CH<sub>2</sub>), 4.36 (t,  $^3J_{H,H}=5.9$  Hz, 2H; BBR-CH<sub>2</sub>-CH<sub>2</sub>-), 4.16 (t,  $J=5.9$  Hz, 2H; -CH<sub>2</sub>-CH<sub>2</sub>O-Ph), 4.11–4.00 (m, 4H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 4.04 (s, 3H; -OCH<sub>3</sub>), 3.20 (t,  $^3J_{H,H}=5.7$  Hz, 2H; 5-CH<sub>2</sub>), 2.09–2.01 (m, 2H; BBR-CH<sub>2</sub>-CH<sub>2</sub>-), 2.02–1.95 (m, 2H; -CH<sub>2</sub>-CH<sub>2</sub>O-Ph), 1.71 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.68 (s, 3H; C(CH<sub>3</sub>)<sub>2</sub>), 1.22 (t,  $^3J_{H,H}=7.3$  Hz, 6H; 2 x -OCH<sub>2</sub>CH<sub>3</sub>); <sup>13</sup>C NMR (125.8 MHz, DMSO-*d*<sub>6</sub>):  $\delta=159.8$  (C-4'), 150.4 (C-10), 149.8 (C-3), 147.7 (C-2), 145.2 (C-8), 142.8 (C-9), 137.5 (C-13a), 133.5 (C-12a), 131.2 (d,  $^3J_{C,P}=5.7$  Hz; HC=NO), 130.7 (C-4a), 130.6 (C-2'), 126.7 (C-11), 123.9 (C-1'), 123.4 (C-12), 121.6 (C-8a), 120.5 (C-13b), 120.2 (C-13), 114.1 (C-3'), 108.4 (C-4), 105.5 (C-1), 102.1 (-OCH<sub>2</sub>O-), 73.9 (BBR-CH<sub>2</sub>-CH<sub>2</sub>-), 72.3 (d,  $^1J_{C,P}=156.4$  Hz; C(CH<sub>3</sub>)<sub>2</sub>), 67.4 (-CH<sub>2</sub>-CH<sub>2</sub>O-Ph), 62.5 (d,  $^2J_{C,P}=6.5$  Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>), 57.1 (OCH<sub>3</sub>), 55.3 (C-6), 26.3 (C-5), 26.3 (BBR-CH<sub>2</sub>-CH<sub>2</sub>-), 25.2 (-CH<sub>2</sub>-CH<sub>2</sub>O-Ph), 22.9 (C(CH<sub>3</sub>)<sub>2</sub>), 16.3 (d,  $^3J_{C,P}=5.5$  Hz; 2 x -OCH<sub>2</sub>CH<sub>3</sub>); <sup>31</sup>P NMR (202.5 MHz, DMSO-*d*<sub>6</sub>):  $\delta=23.5$ ; HRMS (ESI): *m/z* calcd for C<sub>37</sub>H<sub>44</sub>N<sub>2</sub>O<sub>9</sub>P<sup>+</sup>: 691.2779 [M]<sup>+</sup>, found 691.2778.

## Superoxide quenching

Allopurinol was from Acros and glycine buffer, xanthine oxidase (XO; from buttermilk), lucigenin (*N,N*-dimethyl-9,9-biacridinium dinitrate), PBN, were from Sigma-Aldrich. PPN were prepared and purified as described.<sup>[8,36]</sup> White non-treated Nunc 96-well plates were from Thermo Fisher scientific (Waltham, MA, USA).

Superoxide quenching property of nitrones was evaluated at 37 °C in DMSO supplemented glycine buffer (GB, 6.25 mM, pH 10.1) on 96-well plates by measuring the concentration dependent inhibition of lucigenin derived chemiluminescence produced by an allopurinol/XO O<sub>2</sub><sup>-</sup> generating system.<sup>[8,21]</sup> Stock solutions of nitrones (0.1 M) in DMSO were diluted in GB to obtain test solutions (1–1,000  $\mu$ M) containing up to 0.1% DMSO. Briefly, 185  $\mu$ L of test solution (or GB alone for blanks) were first introduced in each well, followed by sequential addition of 15  $\mu$ L of GB solutions of allopurinol (working concentration, 375  $\mu$ M), 20  $\mu$ L of XO (working concentration, 150 mU/mL), and 30  $\mu$ L of lucigenin (working concentration, 500  $\mu$ M). Luminescence of the sample was monitored for 10 min following lucigenin addition using an Infinite M200 microplate reader (TECAN, Switzerland). Superoxide quenching activity, calculated by subtracting areas-under-curve against blank, was expressed as IC<sub>50</sub> values (in  $\mu$ M) from 3 independent experiments made in triplicate.

## Spin adduct formation and EPR studies

**Chemical and samples preparation**: Diethylenetriaminepentaacetic acid (DTPA), H<sub>2</sub>O<sub>2</sub>, KH<sub>2</sub>PO<sub>4</sub>, hypoxanthine, XO from bovine liver, and all other reagents and solvents were of the highest purity or grade from Sigma-Aldrich. Aqueous solutions were prepared using doubly distilled deionized water. The phosphate buffer (10–40 mM, pH 7.4), containing 0.01–1 mM DTPA for removal of adventitious metal catalysts, was passed through a 0.2-mm Millipore filter before use.

Mito-PPNs spin adducts were produced in aqueous medium by reacting near saturated solutions of the nitrones (20–150 mM) with established HO•, O<sub>2</sub><sup>-</sup>, or carbon centered radical generators.<sup>[6]</sup> These included a FeSO<sub>4</sub> (1 mM) / H<sub>2</sub>O<sub>2</sub> (1–3 mM) Fenton system in DTPA (1 mM) added phosphate buffer (20 mM; pH 7.0–7.4) for HO•, a hypoxanthine (0.4 mM)/xanthine oxidase (0.08 units/mL) generator in DTPA (1 mM) added phosphate buffer (pH 7.4) for O<sub>2</sub><sup>-</sup>, and the above Fenton system run in the presence of 40% MeOH or EtOH for CH<sub>2</sub>OH and •CH(OH)CH<sub>3</sub>, respectively. For details on spin-trapping experiments using photolytic HOO• and HO• generating systems, see the Supporting Information.

**CAUTION**: Solutions of iodides **6b,d**, **7b,d**, and **8** undergo a spontaneous exothermic reaction with H<sub>2</sub>O<sub>2</sub> at room temperature, with formation of a foam, and therefore their handling with H<sub>2</sub>O<sub>2</sub> solutions > 1% m/m should be avoided (see main Text).

After addition of the last component of the free radical generator (e.g., iron salt or enzyme), the mixture was vortexed for 5 s and the solution was loaded into a calibrated 50  $\mu$ L glass capillary, sealed at lower end by a proper wax, positioned within a TM<sub>110</sub> cavity and EPR spectra were monitored 40–75 s later.

**EPR spectroscopy**: EPR measurements were carried out on a Bruker ESP 300 spectrometer (Karlsruhe, Germany) operating at X-band (~ 9.8 GHz) with a 100 kHz modulation frequency to provide usual first derivative signals. For determining *g*-factors, a Hewlett-Packard 5350B frequency counter and a Bruker ER 035M NMR gaussmeter were operated. Unless otherwise stated, all spectra resulted from 10 accumulated scans at a microwave power of 10 mW and a field resolution of 2018 points. Other settings are indicated under Figure 3 legend. Throughout, the hyperfine splitting constants of spin adducts were optimized by simulation with an automatic fitting program.<sup>[26]</sup>

## Cell culture and cytotoxicity measurements

HDF and FGM were from Sigma-Aldrich. Trypsin-EDTA (1X) and fetal calf serum (FCV) were from Gibco Technologies (Thermo Fisher Scientific). T75 flasks were purchased from Sarstedt (Nümbrecht, Germany) and Nunc 96-well plates were from Thermo Fisher Scientific. Phosphate-buffered saline (PBS 1X (+/+)) was from Life Technology Corp. (St Aubin, France).

Stock solutions of nitrones **6–10** were prepared in FGM (50 mM) and stored at -20 °C. Nitrones **1, 2, 11** and **12** were dissolved in DMSO (500 mM) and diluted with FGM to obtain 500 µM stock solutions containing up to 0.1 % DMSO and stored at -20 °C. Nitrone solutions were then freshly diluted (0.001–100 µM) in culture medium prior to use and filtered through a 0.2-µm Millipore filter.

**Cell culture:** HDF cells were frozen in a medium containing FGM supplemented with 10% fetal calf serum and 10% DMSO. A cell aliquot ( $0.5 \times 10^6$  cells) was rapidly thawed, and cells were transferred into cold medium (15 mL) in a T75 flask, cultured at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> for 24 h. After 24 h, the medium was replaced by new FGM (15 mL). Cell adhesion and growth were observed under microscope until confluency by renewing the medium once or twice a week. Flasks containing confluent cells were trypsinized and cells were seeded in 96-well plates at a density of  $3 \times 10^3$  cells per well in FGM (100 µL).

**Cytotoxicity measurements (MTT assay):** Twenty-four hours after seeding, the medium is replaced by the diluted nitrone solutions (100 µL per well) and plate was incubated at 37 °C under controlled atmosphere for 72 h. Wells were then rinsed with PBS. An aliquot of 100 µL of FGM containing 0.5 mg/mL MTT (Acros) in PBS 1X (+/+) was added to the wells and incubation was extended for 2 h at 37 °C protected from the light. The medium was replaced by 100 µL/well DMSO and incubation was prolonged for 15 min at room temperature under stirring. The conversion of MTT to a purple formazan precipitate was monitored at 600 nm (UVmc2 spectrophotometer, SAFAS, Monaco). The inhibition of cell viability was calculated as IC<sub>50</sub> values using regression calculations from at least 3 different concentrations for each sample.

## Mitochondrial permeation

**Chemicals:** Sucrose, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), CH<sub>2</sub>Cl<sub>2</sub>, and DMSO were from Sigma Aldrich. KH buffer consisted of (in mM): KH<sub>2</sub>PO<sub>4</sub> (1.2), NaCl (119), KCl (4.8), MgSO<sub>4</sub> (1.2), NaHCO<sub>3</sub> (25), and CaCl<sub>2</sub> (1.3).

**Animals:** All animal care, protocols and procedures were completed under the guidelines of the Directive 2010/63/EU of the European Parliament. The study was under the supervision of a DVM at CNRS (agreement No.13-122), The study was approved by the Ethics Committee of Provence (CEEA 14) at Aix Marseille University and the French Ministry of National Education, Higher Education and Research (protocol code APAFIS#2396-2015112910476544, date of approval: 03/01/2016). All participating institutions have currently allowance for animal housing and experimentation (agreement C13-055-06) delivered by the French Government. Male Sprague-Dawley rats (~ 120 g; CERJ, Le Genest St Isle, France) were used for the experiments. Housing and feeding conditions have been described previously.<sup>[8,13]</sup>

**Liver perfusion and preparation of mitochondrial and cytosolic fractions:** Perfusion fluids and procedures were previously described.<sup>[11,12b,13,37]</sup> Animals were fasted for 24 h and subsequently anesthetized with 100 mg/kg intraperitoneal sodium pentobarbital (Ceva Santé Animale, Libourne, France) before surgery.

Livers were perfused via cannulation of the portal vein with KH buffer (pH 7.35, 37 °C) bubbled with a 95% O<sub>2</sub> and 5% CO<sub>2</sub> gas mixture in a nonrecirculating perfusion mode. The starting perfusion rate was set at 20 mL/min, assuming the liver to be about 4% of the body weight. After weighing of the excised liver, the flow rate was set at 3 mL/min/g for a 30-min equilibration period; afterwards an aliquot of a stock solution of the tested nitrone in DMSO (1 mM) was infused as to expose the liver to 1 µM nitrone for 1 h. Then, test compound was eliminated from the extracellular space by switching the perfusion medium to an ice cold plain KH buffer for 10 min.

The liver was removed from the cannula, cut into small pieces and homogenized in five volumes of 0.25 M sucrose and 10 mM HEPES. After filtration, the homogenate was centrifuged at 750 × g for 10 min at 2 °C. The pellet was used for preparation of the mitochondria and the supernatant was used for preparation of the cytosol by differential centrifugations as described.<sup>[12b,13,37]</sup> The mitochondrial and cytosolic fractions were finally resuspended in 1 mL KH buffer. These samples were extracted at 20 °C with CH<sub>2</sub>Cl<sub>2</sub> (2 × 5 mL), the organic layers combined and concentrated *in vacuo* to give the corresponding dry residues which were pooled (3–4 livers/test compound), dissolved in 20 µL DMSO and frozen until <sup>31</sup>P NMR analysis.

**<sup>31</sup>P NMR determination of mitochondria:cytosol distribution:** After thawing, each residue solution was diluted with DMSO-*d*<sub>6</sub> (0.5 mL, Eurisotop, Saclay, France) and <sup>31</sup>P NMR spectra were acquired at 242.94 MHz in 5-mm tubes on a Bruker Avance III – 600 MHz spectrometer equipped with a BBFO+ probe, under DMSO-*d*<sub>6</sub> lock using a ZIGIG pulse program with a 30° (17.07 ms) pulse width, acquisition time of 0.84 s, repetition delay of 2.00 s, and a 32 scans acquisition. Relative mitochondria:cytosol distribution was then determined from the ratio of peak integrals.

## Protective effect of mito-PPNs in H<sub>2</sub>O<sub>2</sub>-stressed Schwann cells

Schwann cells from bilateral sciatic nerves of Sprague Dawley male rats (provided by the Institute of Neuroscience, Hôpital de la Timone, Marseille) were cultured in Dubelcco's Modified Eagle Medium (DMEM) / Ham's F-12 nutrient (Gibco Technologies) as reported.<sup>[13,30,31]</sup> Cells were pretreated for 8 h at 37 °C with aliquots of test nitrones or the reference compounds PPN and **2** dissolved in culture medium containing 1% DMSO to reach a final concentration of 0.1 µM. Apoptosis was then triggered by adding 100 µM H<sub>2</sub>O<sub>2</sub> and incubation was prolonged for 16 h. The extent of cell damage was assessed by the MTT assay and by measuring LDH release using a commercial kit (Biolabo, Maizy, France) as reported.<sup>[10,13]</sup>

## Statistics

Data are expressed as mean  $\pm$  SD for the indicated number of independent experiments. Statistical significance was assessed by one-way analysis of variance (ANOVA) followed by Duncan test. (Prism 5.0 software, GraphPad, San Diego, CA). Differences were considered significant when  $p < 0.05$ .

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**Keywords:** cellular viability • EPR spin-trapping • mitochondria-targeted nitrones •  $^{31}\text{P}$  NMR • superoxide quenching

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