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Amphiphilic NO-Donor Antioxidants

Konstantin Chegaev,[a] Loretta Lazzarato,[a] Barbara Rolando,[a] Elisabetta Marini,[a] Gloria V. Lopez,[b] Massimo Bertinaria,[a] Antonella Di Stilo,[a] Roberta Fruttero,[a] and Alberto Gasco*[a]

*Models of amphiphilic NO-donor antioxidants **24**–**26** were designed and synthesized. The products were obtained by linking a lipophilic tail (C_6, C_8, C_{10}) with a polar head constituted by the 2,6-dimethoxyphenol antioxidant joined to the NO-donor 3-furoxancarboxamide substructure through a bridge containing a quaternary ammonium group. Compound **23**, containing the shortest C_2-alkyl chain, was also studied as a reference. The antioxidant properties (TBARS and LDL oxidation assays) and the vasodilator properties of the compounds were studied in vitro. The ability of these products to interact with phospholipid vesicles was also investigated by NMR techniques. The results indicate that both activities are modulated by the ability of the compounds to accumulate on phospholipid layers.*

Introduction

The endothelium is a complex organ system involved in maintaining micro- and macrovascular homeostasis by releasing both vasodilator and vasoconstrictor factors.[1] The accumulation of low-density lipoproteins (LDL) and reactive oxygen species (ROS) in the sub-endothelial space induces a high degree of LDL oxidation. According to the oxidative hypothesis of atherosclerosis, this is an early event in a complex process that leads to the formation of foam cells that constitute a fatty streak, a forerunner to the development of mature atherosclerotic plaques.[2] An increasing number of studies seem to indicate that the administration of exogenous antioxidants might decrease the impact of atherosclerosis in animals and humans through the regulation and protection of several aspects of endothelial function.[1–4] Endothelium-dependent vascular relaxation is predominantly mediated by nitric oxide (NO). In vessels affected by atherosclerosis, there is enhanced inactivation of NO due to its reaction with the superoxide anion ($O_2^{-\bullet}$) leading to the formation of peroxynitrite ($ONOO^-$). As well as decreasing the bioavailability of protective NO, the peroxynitrite formed in this reaction is a precursor for the highly reactive and very toxic hydroxyl radical ($OH^\bullet$). In addition, peroxynitrite is recognized to be highly cytotoxic in its own right and can mediate lipid peroxidation reactions. Furthermore, a decrease in the production of NO by endothelial cells cannot be excluded, at least in advanced atherosclerotic disease.[1,5] On this basis, we recently proposed a new class of NO-donor hybrid drugs obtained by joining antioxidant phenol groups with appropriate NO-donor moieties as potential agents for the treatment of cardiovascular disease (CD) involving atherosclerotic vascular changes.[6] As a further development of this research, we designed new NO-donor antioxidants with an amphiphilic structure (compounds **24**–**26**). These products were obtained by linking a polar head with lipophilic tails. The polar head is constituted by an antioxidant substructure joined through a bridge containing a quaternary ammonium group to an NO-donor moiety. The antioxidant is represented by 2,6-dimethoxyphenol. The NO-donor moiety is the 3-carbamoylfuroxan residue present in the 4-hydroxymethylfuroxan-3-carboxyamide (CAS 1609), an orally active anti-hypertensive drug developed by Cassella–Hoechst.[7] The lipophilic tails are represented by the C_6, C_8, and C_{10} paraffin chains. These products should accumulate in membranes and, generally speaking, in lipophilic complex structures such as LDL, with their tails embedded in the lipophilic environment and their hydrophilic heads directed towards the aqueous medium. Compound **23**, which contains the shortest alkyl chain (C_2), was also studied as a reference compound. Herein we describe the synthesis and in vitro antioxidant and vasodilator properties of these products. Their ability to interact with liposomes, taken as a simple model of phospholipid vesicles, is also analyzed by NMR techniques.

Results and Discussion

Synthesis

The general synthetic pathway followed to prepare products **23**–**26** is reported in Scheme 1. 2-[4-(Benzyloxy)-3,5-dimethoxy-

[a] *Dr. K. Chegaev, Dr. L. Lazzarato, Dr. B. Rolando, Dr. E. Marini, Dr. M. Bertinaria, Prof. A. Di Stilo, Prof. R. Fruttero, Prof. A. Gasco*
Dipartimento di Scienza e Tecnologia del Farmaco
Università degli Studi di Torino
Via Pietro Giuria 9, 10125 Torino (Italy)
Fax: (+39) 011-670-7286
E-mail: alberto.gasco@unito.it

[b] *Dr. G. V. Lopez*
Departamento de Química Orgánica
Facultad de Química y Facultad de Ciencias
Universidad de la República
Iguá 4225, 11400 Montevideo (Uruguay)

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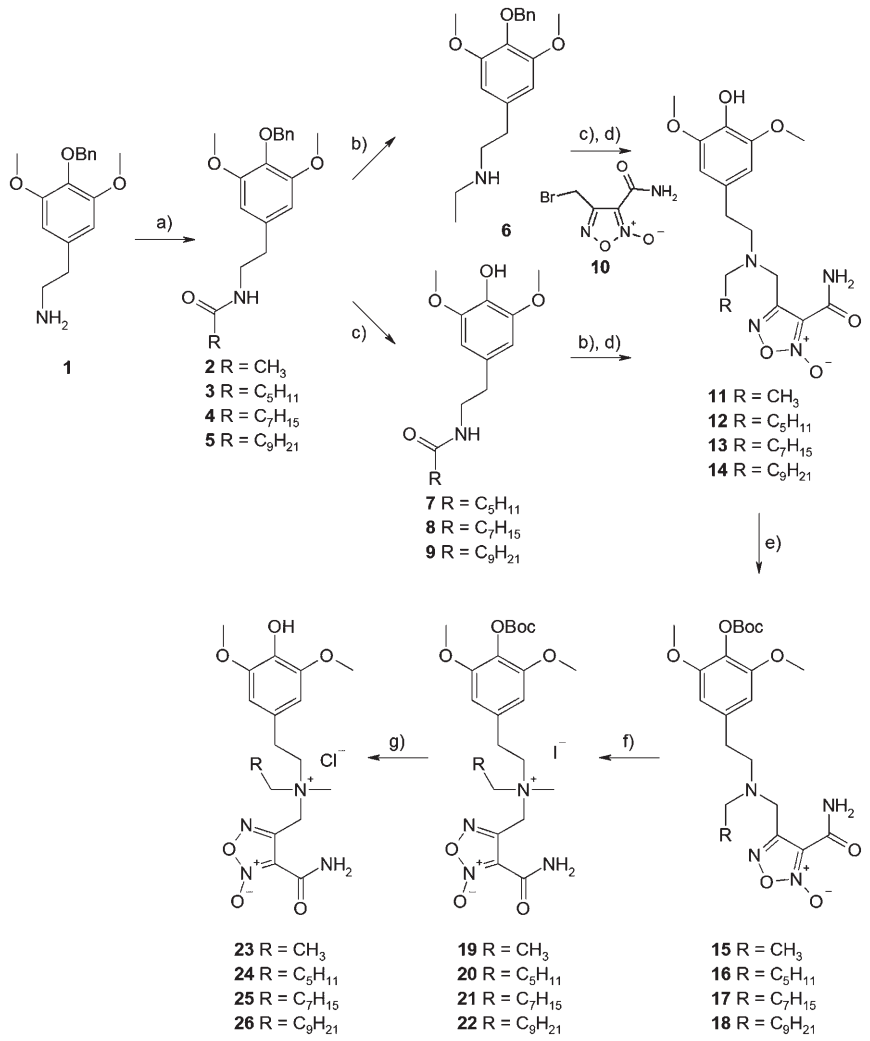

Scheme 1. Reagents and conditions: a) $(CH_3CO)_2O$, $(C_2H_5)_3N$, CH_2Cl_2 for **2**; CDI, RCOOH, CH_2Cl_2 for **3**–**5**; b) $LiAlH_4$, dry THF, reflux; c) H_2, $Pd(OH)_2/C$, MeOH; d) **10**, $KHCO_3$, acetone; e) Boc_2O, DMAP, CH_2Cl_2; f) CH_3I, reflux; g) HCl (concd). CDI = carbonyldiimidazole, THF = tetrahydrofuran, Boc = *tert*-butoxycarbonyl, DMAP = 4-dimethylaminopyridine.

phenyl]ethanamine (**1**) was coupled with the appropriate carboxylic acid using *N*,*N*′-carbonyldiimidazole (CDI) or acetic anhydride in the presence of triethylamine (in the case of compound **2**) in dichloromethane solution. The benzyl group present in amides **3**–**5** so obtained was removed by hydrogenolysis on $Pd(OH)_2/C$ in ethanol to give intermediates **7**–**9.** Further reduction with $LiAlH_4$ in THF afforded the corresponding amines that were used without purification. For the case in which R = methyl, these two steps were reversed for synthetic reasons. Nucleophilic displacement of bromine in 4-bromomethylfuroxan-3-carboxamide (**10**) by these products gave rise to the furoxan derivatives **11**–**14**. Subsequent protection of the phenol OH group (in **15**–**18**) with di-*tert*-butyldicarbonate (Boc_2O) in the presence of 4-dimethylaminopyridine (DMAP) followed by treatment with methyl iodide afforded the Boc-protected iodide quaternary salts **19**–**22**. Cleavage of the protection at OH groups with concentrated hydrochloric acid and subsequent anion exchange with AgCl yielded the final expected products.

NMR studies

The partitioning of amphiphilic drug molecules into complex lipid vesicles or membranes can be studied by using simple phospholipid liposomes. A number of methods have been developed to quantify this kind of interaction.[8] One of these relies on the evaluation, in ^{1}H NMR spectra, of the change in proton spin-lattice (T_1^{-1}) and spin–spin (T_2^{-1}) relaxation rates of observable signals of the drug molecule (solute) in the presence of liposomes. Changes in proton relaxation rates are related to a change (decrease) in the motional freedom of the solute in the presence of macromolecules, and therefore can be used to quantify the degree of interaction of the molecule with liposomes.[9,10] A direct measurable relaxation rate parameter is the line width at half peak height ($\Delta\nu_{1/2}$) of a given proton signal, which is proportional to T_2^{-1} according to Equation (1). For the theoretical background of this method, see references [9, 10].

$$\Delta\nu_{1/2} = \frac{1}{T_2\pi} \qquad (1)$$

This parameter is determined at different phospholipid (PPL) concentrations at a constant solute concentration, and it is linearly dependent on the lipid concentration within the range studied. The slope of the linear regression ($\Delta\nu_{1/2}$ slope), obtained by plotting the observed $\Delta\nu_{1/2}$ versus the PPL/ligand ratio, can be used to quantify the degree of interaction of the molecule with liposomes, provided that no other factors produce signal broadening. In fact, the amphiphilic nature of the compounds could afford molecular self association, such as micelle formation, if the concentration used exceeds the critical micellar concentration (CMC). Controls were performed to exclude this possibility (see Experimental Section). Plots reported in Figure 1 represent the change in $\Delta\nu_{1/2}$ of selected signals as a function of PPL/ligand ratio, and the corresponding $\Delta\nu_{1/2}$ slope is reported in Table 1. It can be observed that within the series of compounds **24**–**26**, a given signal becomes broader with increasing length of the hydrophobic alkyl chain. Compound **26**, which has the longest hydrocarbon chain, displays the strongest interaction with liposomes, whereas compound **24** shows the weakest interaction.

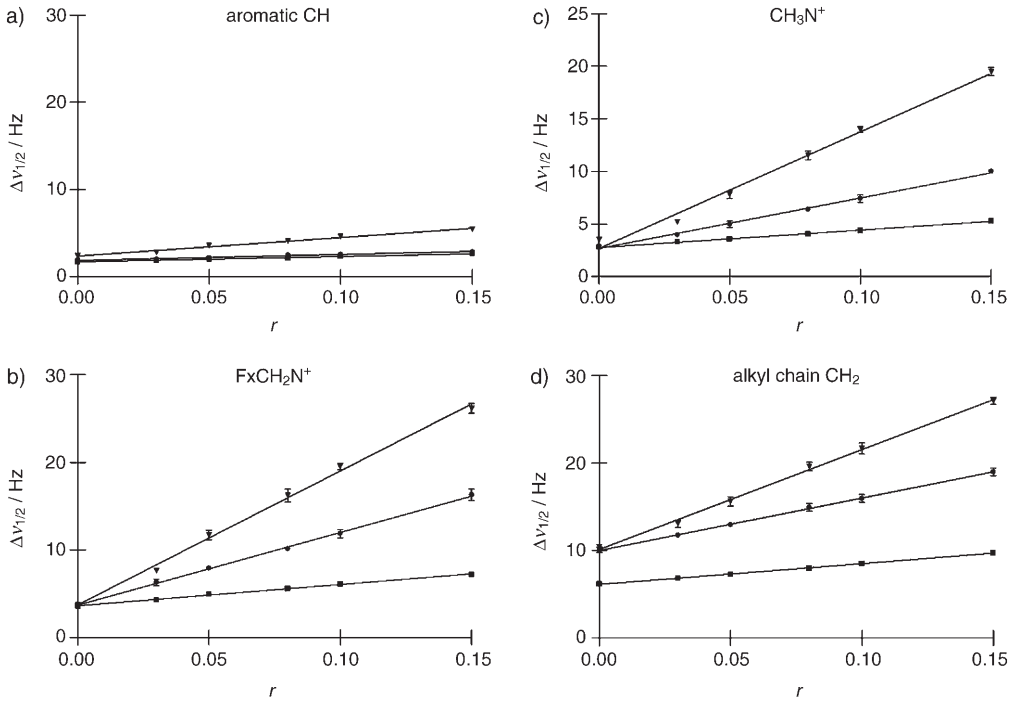

Figure 1. Plots represent the change in $\Delta\nu_{1/2}$ of a) aromatic CH, b) $FxCH_2N^+$, c) CH_3N^+, and d) the envelope of the alkyl chain CH_2 group signals as a function of PPL/ligand concentration ratio (*r*) for compounds **24** (■), **25** (●), and **26** (▼).

Table 1. Slopes of the linear regression obtained by plotting the observed $\Delta\nu_{1/2}$ value (in Hz) versus the concentration ratio of PPL/ligand.[a]

Compound[b]	Aromatic CH	$FxCH_2N^+$	CH_3N^+	Envelope of alkyl chain CH_2 groups
24	6.1 ± 0.5	24.4 ± 1.0	16.6 ± 0.4	23.7 ± 0.7
25	6.7 ± 0.8	82.9 ± 2.8	47.9 ± 1.8	60.0 ± 2.5
26	21.2 ± 1.0	153.6 ± 4.0	110.2 ± 3.6	114.1 ± 3.8

[a] Values were obtained for at least four experiments; $r^2 \geq 0.97$. [b] Compound **23** did not show any relevant interaction with phospholipids.

An intermediate situation occurs for **25**. Compound **23** does not display any detectable interaction with liposomes. The NMR method also makes it possible to monitor the relative mobility changes of distinct structural portions within the same molecule by comparing the $\Delta\nu_{1/2}$ slope of suitable signals. In this respect, in all derivatives, the strongest interactions, as indicated by the slopes, are observed for the CH_2 and CH_3 groups directly linked to the ammonium nitrogen atom as well as for the envelope of the CH_2 signals of the hydrophobic chain. In contrast, the aromatic CH signals show the lowest degree of interaction. Owing to the exchange with D_2O, it was not possible to observe the carbamoyl NH_2 protons. The picture shown above is in keeping with the classical model of interaction of amphiphilic compounds, which implies their electrostatic absorption on the ionic surface of the bilayer and their alkyl chains anchored to the hydrophobic region of the liposome.

Antioxidant and vasodilator properties

The final products were assessed as inhibitors of ferrous salt/ascorbate-induced lipidic peroxidation of membrane lipids of rat hepatocytes. The progress of the autoxidation was followed with UV/Vis spectroscopy to detect the 2-thiobarbituric acid reactive species (TBARS), which are the final products of the reaction. The products proved to inhibit the progress of the reaction in a concentration-dependent manner. The potencies of the products as antioxidants (IC_{50} values) are reported in Table 2. They follow the sequence **26** > **25** > **24** ⩾ **23**, which parallels their degree of accumulation in phospholipid vesicles. Consequently, the more the product is anchored to the membranes the more efficiently it scavenges radicals. Similar results were obtained in the study of LDL lipid peroxidation. In this case, oxidation was initiated by the addition of $CuSO_4$ and was followed spectrophotometrically by continuously recording the

Table 2. Antioxidant properties and vasodilating activity of compounds **23**–**26**.

Compound	Vasodilating activity[a] EC_{50} ± SE [μM]	+ 1 μM ODQ	Antioxidant activity[b] IC_{50} (95% CL) [μM]
23	7.9 ± 0.4	> 100	118 (111–126)
24	1.3 ± 0.2	> 100	19 (18–21)
25	0.90 ± 0.11	> 100	4.4 (4.2–4.7)
26	0.46 ± 0.09	18 ± 1	2.4 (2.3–2.5)

[a] Values are the average of at least nine experiments. [b] TBARS assay; values are the average of at least five experiments.

increase of the absorption at $\lambda = 234$ nm, a characteristic of conjugated lipid hydroperoxides. A typical example of such measurement is shown in Figure 2a). The lag phase, namely the period in which no oxidation occurs, was determined as a function of the concentration of the products (Figure 2b). Again, the ability to inhibit LDL oxidation follows the order

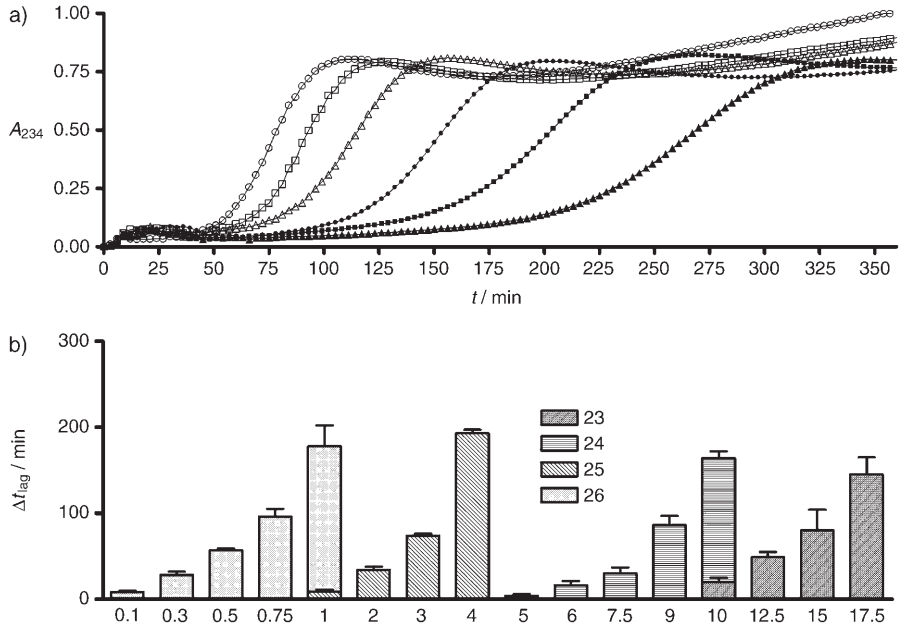

Figure 2. a) Kinetics of copper ion induced LDL oxidation showing the effect of compound **26** on LDL oxidation (see text for details); control ○, **26** 0.10 μM □, **26** 0.30 μM △, **26** 0.50 μM ●, **26** 0.75 μM ■, **26** 1.0 μM ▲. b) Bar graph showing the effects of compounds **23**–**26** on oxidation resistance in the copper-induced oxidation assay; *x*-axis values indicate compound concentration in μM.

26 > **25** > **24** ≫ **23**. Interestingly, these products were also able to lower the oxidation rate during the propagation phase with respect to the controls. This effect, at the maximal concentrations tested, was particularly evident for compound **25** followed by **26**, while it was feeble for **24** and **23**.

The compounds were also assessed for their ability to relax rat aorta strips precontracted with phenylephrine. They were capable of relaxing the contracted strips in a dose-dependent manner. Their vasodilator potencies, expressed as EC_{50} values, are collected in Table 2. The significant variation in the vasodilating potency of these products are again ranked in the sequence **26** > **25** > **24** > **23**. The vasorelaxing activities are cGMP-dependent. In fact, when the experiments were repeated in the presence of 1 μM ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a well-known inhibitor of soluble guanylate cyclase (sGC), a substantial decrease in potency was observed. This is consistent with the involvement of NO-mediated activation of soluble guanylate cyclase in vasodilator action. There is evidence that NO release from furoxan derivatives is usually thiol-dependent.[7] Because these quaternary salts should not penetrate the cells, the results suggest that the bioconversion involved in NO release is better implemented in the membrane of vascular smooth cells than in the interstitial space.

In conclusion, we were able to synthesize selected members of a new class of amphiphilic quaternary ammonium salts endowed with NO-donor and antioxidant properties. We showed that the more the products accumulate on phospholipid layers, the more efficient they are as antioxidants in TBARS and LDL peroxidation tests. The same trend is observed for their NO-mediated properties, as evaluated on denuded rat aorta strips. These structures could be interesting tools for pharmacological investigations.

Experimental Section

Chemistry: Melting points were determined with capillary apparatus (Büchi 540). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 instrument, with Si-$(CH_3)_4$ as an internal standard. The following abbreviations were used to indicate the peak multiplicity: s = singlet; d = doublet; t = triplet, q = quartet, m = multiplet. Low-resolution mass spectra were recorded with a Finnigan-Mat TSQ-700 instrument. Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230–400 mesh ASTM) using the indicated eluents. Petroleum ether 40–70 °C (PE) was used as co-eluent. The progress of the reactions was followed by thin layer chromatography (TLC) on 5× 20 cm plates with a layer thickness of 0.2 mm. Anhydrous magnesium sulfate was used as a drying agent for the organic phases. Organic solvents were removed under vacuum at 35–40 °C. Analysis (C, H, N) of the new compounds dried at 20 °C, pressure < 10 mm Hg for 24 h, was performed by REDOX (Monza), and the results are within ±0.4% of the theoretical values. Structures **1**[11] and **10**[12] were synthesized according to published methods.

***N*-[2-(4-Benzyloxy-3,5-dimethoxyphenyl)ethyl] acetamide (2):** Ac_2O (1.0 mL, 10.6 mmol) was added dropwise at 0 °C to a stirred solution of **1** (2.0 g, 7.0 mmol) and Et_3N (1.5 mL, 10.6 mmol) in dry CH_2Cl_2. Stirring was continued for 1 h. After this time the reaction mixture was diluted with CH_2Cl_2 and sequentially washed with water, 1 N HCl, $NaHCO_3$ (saturated), and brine. The organic layer was dried, and the solvent was removed. The product was purified by flash chromatography (eluent CH_2Cl_2/EtOAc 6:4).

General method for the preparation of amides 3–5: CDI (1.2 g, 7.3 mmol) was added to a stirred solution of the appropriate fatty acid (6.7 mmol) in dry CH_2Cl_2 followed by the addition of a solution of **1** (1.6 g, 5.6 mmol) in dry CH_2Cl_2. Stirring was continued for 2 h. After this time the reaction mixture was diluted with CH_2Cl_2 and sequentially washed with water, 1 N HCl, $NaHCO_3$ (saturated), and brine. The organic layer was dried, and the solvent was removed. The product was purified by flash chromatography with the indicated eluent.

***N*-[2-(4-Benzyloxy-3,5-dimethoxyphenyl)ethyl]ethylamine (6) oxalate:** A solution of **2** (4.8 g, 14.5 mmol) was added to a stirred suspension of $LiAlH_4$ (1.7 g, 43.4 mmol) in dry THF kept under nitrogen. The reaction mixture was held at reflux for 5 h and then cooled at 0 °C. After the sequential addition of H_2O (1.7 mL), NaOH (15%, 1.7 mL), and H_2O (5.1 mL), the mixture was filtered on a Celite pad. The residue was washed with MeOH, and the filtrate was evaporated under vacuum. The oil obtained was partially purified by flash chromatography (eluent CH_2Cl_2/MeOH sat. NH_3 95:5). The pure product was prepared by adding a saturated solution of $H_2C_2O_4$ in acetone to a saturated solution of product in acetone and filtering the resulting white solid.

General method for the preparation of amides 7–9: To a stirred solution of the appropriate protected amide derivatives **3–5** (1.8 mmol) in MeOH, 20% $Pd(OH)_2$/C was added in a ratio 5% *w*/*w* with respect to the starting amide. The reaction flask was connected to a balloon filled with hydrogen, and the mixture was stirred at room temperature for 3 h. Then the catalyst was filtered on a Celite pad, and the solvent was removed to give the title compounds as pink solids. Analytically pure samples were obtained by crystallization from iPr_2O.

4-({Ethyl-[2-(4-hydroxy-3,5-dimethoxyphenyl)ethyl]amino}methyl)furoxan-3-carboxyamide (11): The benzyl group of **6** (2.4 g, 7.5 mmol) was removed using the same procedure used for the synthesis of **7–9**. The resulting amine was immediately used for further reaction. Compound **10** (2.0 g, 9.0 mmol) was added to a stirred solution of the amino derivative in acetone, followed by the addition of 1 N $KHCO_3$ (10 mL, 10 mmol). The reaction mixture was stirred at room temperature for 2 h, concentrated, and then diluted with EtOAc. The organic solution was washed with water, brine, dried, and evaporated. The oily residue was purified by flash chromatography (eluent CH_2Cl_2/EtOAc 6:4).

General method for the preparation of the furoxan derivatives 12–14: Reduction of amide group of compounds **7–9** was performed by using the same procedure used for the synthesis of **6**. The crude amines were immediately used for further reaction. To a stirred solution of the appropriate amino derivative (4.1 mmol) in acetone, **10** (1.0 g, 4.5 mmol) was added, followed by the addition of 1 N $KHCO_3$ (5 mL, 5 mmol). The reaction mixture was stirred at room temperature for 2 h, concentrated, and then diluted with EtOAc. The organic solution was washed with water, brine, dried, and evaporated. The oily residue was purified by flash chromatography with the indicated eluent.

General method for the preparation of Boc-protected derivatives 15–18: DMAP (0.23 g, 1.9 mmol) was added at 0 °C to a stirred solution of amine **11–14** (1.9 mmol) and Boc_2O (0.6 g, 2.8 mmol) in dry CH_2Cl_2. The mixture was stirred at 0 °C for 1.5 h, then imidazole (0.13 g, 1.9 mmol) was added to destroy the excess Boc_2O. The reaction was stirred at room temperature for an additional 15 min, then the mixture was poured into H_2O and extracted with CH_2Cl_2. The organic phase was washed with $NaHCO_3$ (saturated), brine, dried, and evaporated. The brown oil obtained was purified by flash chromatography with the indicated eluent.

General procedure for the preparation of protected quaternary ammonium iodides 19–22: The appropriate amino derivative **15–18** (2.2 mmol) was dissolved in CH_3I (10 mL), and the resulting solution was held at reflux for 24 h. Then CH_3I was evaporated under a dry stream of N_2. The solid residue was triturated with dry Et_2O (15 mL) and filtered. Products were crystallized from the indicated solvent.

General procedure for the preparation of quaternary ammonium chlorides 23–26: The appropriate protected quaternary salt **19–22** (1.2 mmol) was slowly dissolved in HCl (concd, 10 mL). The reaction mixture was stirred at room temperature until reaction completion (monitored by TLC), and then the solvent was evaporated. The residue was dissolved in MeCN, and freshly prepared AgCl (from $AgNO_3$ 1.0 g and brine) was added. The reaction was stirred for 30 min, then was filtered through a Celite pad, and the solvent was removed. The oil obtained was purified by flash chromatography with the eluent indicated to give the compound as a glass like oil, which solidified when treated with dry CH_2Cl_2. The precipitate was filtered, washed first with CH_2Cl_2, then with Et_2O, and dried in a desiccator. Compounds **23–26** were amorphous solids with a complex thermal behavior above 110 °C.

[3-(Aminocarbonyl)furoxan-4-yl]methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)ethyl-ethyl-methyl-ammonium chloride (23): Eluent CH_2Cl_2/MeOH 8:2; white powder (yield: 78%); ^{1}H NMR (300 MHz, CD_3OD, TMS): $\delta = 1.51$ (t, 3H, CH_2CH_3), 3.11–3.18 (m, 2H, CH_2), 3.26 (s, 3H, N^+CH_3), 3.70–3.74 (m, 4H, $2CH_2$), 3.84 (s, 6H, OCH_3), 5.12 (s, 2H, FxCH_2), 6.64 ppm (s, 2H, C_6H_2); ^{13}C NMR (75 MHz, CD_3OD, TMS): $\delta = 8.3$, 29.5, 48.7, 54.6, 56.7, 59.7, 64.0, 107.2, 111.6, 127.0, 135.7, 149.4, 150.1, 157.7 ppm; elemental analysis: calcd (%) for $C_{17}H_{25}ClN_4O_6 \cdot 1.75\,H_2O$ (448.39 g mol^{-1}): C 45.54, H 6.41, N 12.50, found: C 45.52, H 6.17, N 12.46.

[3-(Aminocarbonyl)furoxan-4-yl]methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)ethyl-hexyl-methyl-ammonium chloride (24): Eluent CH_2Cl_2/MeOH 8:2; white powder (yield: 79%); ^{1}H NMR (300 MHz, CD_3OD, TMS): $\delta = 0.94$ (t, 3H; CH_2CH_3), 1.39 (m, 6H; $(CH_2)_3CH_3$), 1.89–1.91 (m, 2H; $NCH_2CH_2CH_2$), 3.16 (m, 2H), 3.57 (m, 2H), 3.73 (m, 2H) ($C_5H_{11}CH_2N^+$, $C_6H_2(CH_2)_2$), 3.28 (s, 3H; N^+CH_3), 3.85 (s, 6H; OCH_3), 5.14 (s, 2H; FxCH_2), 6.64 ppm (s, 2H; C_6H_2); ^{13}C NMR (75 MHz, CD_3OD, TMS): $\delta = 14.3$, 23.5, 23.5, 27.0, 29.8, 32.4, 49.5, 55.1, 56.9, 64.4, 64.8, 107.3, 111.8, 127.3, 135.9, 149.6, 150.2, 157.9 ppm; elemental analysis: calcd (%) for $C_{21}H_{33}ClN_4O_6 \cdot H_2O$ (490.98 g mol^{-1}): C 51.37, H 7.19, N 11.41, found: C 51.55, H 7.04, N 11.41.

[3-(Aminocarbonyl)furoxan-4-yl]methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)ethyl-methyl-octyl ammonium chloride (25): Eluent CH_2Cl_2/MeOH 85:15; white powder (yield: 63%); ^{1}H NMR (300 MHz, CD_3OD, TMS): $\delta = 0.89$ (t, 3H; CH_2CH_3), 1.32–1.39 (m, 10H; $(CH_2)_5CH_3$), 1.90–1.92 (m, 2H; $NCH_2CH_2CH_2$), 3.16 (m, 2H), 3.57 (m, 2H), 3.75 (m, 2H) ($C_7H_{15}CH_2N^+$, $C_6H_2(CH_2)_2$), 3.28 (s, 3H; N^+CH_3), 3.85 (s, 6H; OCH_3), 5.14 (s, 2H; FxCH_2), 6.64 ppm (s, 2H; C_6H_2); ^{13}C NMR (75 MHz, CD_3OD, TMS): $\delta = 14.5$, 23.5, 23.7, 27.3, 29.8, 30.2, 30.2, 32.9, 49.5, 55.1, 56.9, 64.4, 64.8, 107.4, 111.8, 127.2, 135.9, 149.6, 150.3, 157.9 ppm; elemental analysis: calcd (%) for $C_{23}H_{37}ClN_4O_6 \cdot 1.5\,H_2O$ (528.05 g mol^{-1}): C 52.32, H 7.64, N 10.61, found: C 52.37, H 7.31, N 10.66.

[3-(Aminocarbonyl)furoxan-4-yl]methyl-2-(4-hydroxy-3,5-dimethoxyphenyl)ethyl-decyl-methyl ammonium chloride (26): Eluent CH_2Cl_2/MeOH 9:1; white powder (yield: 70%); ^{1}H NMR (300 MHz, CD_3OD, TMS): $\delta = 0.90$ (t, 3H; CH_2CH_3), 1.30–1.39 (m, 14H; $(CH_2)_7CH_3$), 1.90–1.92 (m, 2H; $NCH_2CH_2CH_2$), 3.15 (m, 2H), 3.57 (m, 2H), 3.75 (m, 2H) ($C_7H_{15}CH_2N^+$, $C_6H_2(CH_2)_2$), 3.18 (s, 3H; N^+CH_3), 3.85 (s, 6H; OCH_3), 5.13 (s, 2H; FxCH_2), 6.64 ppm (s, 2H; C_6H_2); ^{13}C NMR (75 MHz, CD_3OD, TMS): $\delta = 14.5$, 23.5, 23.7, 27.3, 29.8, 30.2, 30.4, 30.6, 30.6, 33.0, 49.5, 55.1, 56.9, 64.4, 64.9, 107.4, 111.8, 127.3, 135.8, 149.5, 150.2, 157.9 ppm; elemental analysis: calcd (%) for $C_{25}H_{41}ClN_4O_6 \cdot {}^1/_2\,H_2O$ (538.08 g mol^{-1}): C 55.80, H 7.87, N 10.41, found: C 55.47, H 7.73, N 10.11.

Preparation of liposomes: L-α-Phosphatidylcholine dioleoyl (DOPC) was purchased from Sigma Aldrich. DOPC was dried from a chloroform solution to a thin layer and then hydrated with deuterated water to obtain multilamellar vesicles (MLV) with a lipid concentration of ~12 mM. The liposomes were prepared by the extrusion method:[13] MLV preparation was extruded five times through polycarbonate filters (100 nm) to obtain large unilamellar vesicles (LUV). The liposomal stock suspension was stored at 4 °C. The lipid concentration of liposomes was determined as reported in reference [13]. The size distribution of liposomes was measured by photon correlation spectroscopy using a Coulter Model N4SD submicron particle analyzer (Coulter Electronics, Florida, USA).

Critical micellar concentration (CMC): The critical micellar concentration of compounds **24**–**26** in aqueous solution (HPLC-grade water) was measured at 24.0 ± 0.1 °C using a ring tensiometer (Digital Tensiometer K 10, Krüss, Hamburg, Germany). Surface-tension measurements were performed at varying compound concentrations ranging from 20 to 2 mM. Each measurement was repeated three times.

NMR experiments: ^{1}H NMR spectra were recorded at 297 K in D_2O at 300 MHz on a Bruker Avance 300 NMR spectrometer: no significant differences were found compared with the spectra recorded in CD_3OD. The concentration of each compound was 10 mM: to this solution, a fixed PPL amount was sequentially added so that the PPL/ligand concentration ratio in the final solutions ranged from 0 to 0.15. This low ratio avoided any interference from the phospholipid proton signals. To be sure that signal broadening resulted from partitioning in the liposome and not from other effects, an evaluation of the compounds' CMC was performed. Compounds **24** and **25** displayed CMC values >20 mM, whereas the CMC of **26** was 9.6 mM. Therefore, only the latter displayed a CMC value close to the solute concentration used for NMR experiments (10 mM); however, this did not influence the signal width. In fact, the experiment carried out at the same PPL/ligand concentration ratio, by increasing concentrations of the compounds (10, 12.5, 20 mM) at constant PPL concentration (1 mM), afforded the same results as the original experiment. The only point that deviated from the linear correlation was that related to the experiment performed at the highest concentration (33 mM).

Antioxidant activity: Microsomal membranes from male Wistar rats (200 g) were prepared by differential centrifugation (8000 *g*, 20 min; 120000 *g*, 1 h) in a HEPES/sucrose buffer (10 mM, 250 mM, pH 7.4) and stored at −80 °C. Incubation was performed at 37 °C in a Tris-HCl/KCl buffer (100 mM, 150 mM, pH 7.4) containing microsomal membranes (2 mg mL^{-1}), ascorbic acid (100 μM), and either H_2O solutions of the tested compounds or H_2O alone. Lipid peroxidation was initiated by the addition of $FeSO_4$ (2.5 μM). Aliquots were taken from the incubation mixture at 5, 15, and 30 min and treated with trichloroacetic acid (TCA, 10% *w*/*v*). Lipid peroxidation was assessed by spectrophotometric ($\lambda = 543$ nm) determination of the TBARS consisting mainly of malondialdehyde (MDA), and TBARS concentrations (expressed in nmol mg^{-1} protein) were obtained by interpolation with an MDA standard curve. The antioxidant activity of tested compounds was evaluated the percent inhibition of TBARS production with respect to control samples treated with H_2O alone. IC_{50} values were calculated by nonlinear regression analysis.

Vasodilator activity: Thoracic aortas were isolated from male Wistar rats weighing 180–200 g. The endothelium was removed, and the vessels were helically cut; three strips were obtained from each aorta. All animals were treated humanely in accordance with recognized guidelines on experimentation. As few animals as possible were used. The purposes and the protocols of our studies have been approved by the Ministero della Salute, Rome, Italy. The tissues were mounted under 1.0 g tension in organ baths containing 30 mL Krebs bicarbonate buffer with the following composition (mM): NaCl 111.2, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 12.0, glucose 11.1, maintained at 37 °C and gassed with 95% O_2 5% CO_2 (pH 7.4). The aortic strips were allowed to equilibrate for 120 min, and were then contracted with L-phenylephrine (1 μM). When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. Results are expressed as $EC_{50} \pm SE$ (μM). The effects of 1 μM ODQ on relaxation were evaluated in a separate series of experiments in which ODQ was added 5 min before contraction. Responses were recorded by an isometric transducer connected to the MacLab system PowerLab. Addition of the drug vehicle, DMSO, had no appreciable effect on contraction level.

LDL isolation and oxidation: Venous blood was taken from normal volunteers, after overnight fasting, in polypropylene tubes containing K-EDTA (1 mg EDTA (mL blood)$^{-1}$), and plasma was collected after centrifugation. The LDL fraction was isolated by ultracentrifugation through a KBr discontinuous gradient and collected as the fraction floating at a density of 1.019–1.063 g mL^{-1}. The determination of the lag phase was carried out as previously described.[14] EDTA was removed by rapid filtration through disposable desalting columns (Econo-Pac 10 DG (Bio-Rad)). Filtered LDL were diluted with PBS (10 mM phosphate, pH 7.4) to give a final concentration of 0.25 mg LDL (mL buffer)$^{-1}$ (50 μg LDL protein mL^{-1}) and transferred to a 1-cm cuvette. The formation of conjugated dienes was measured spectrophotometrically in a Varian Cary 50 Bio spectrophotometer equipped with thermostatic control (37 °C) and an automatically exchangeable six-position cuvette holder operating at 234 nm. Oxidation was initiated by the addition of $CuSO_4$ at a final concentration of 2.5 μM. When the effect of antioxidants on the lag phase was measured, aqueous solutions of the antioxidants (10 μL) were added to the LDL solution immediately before the addition of $CuSO_4$.

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[1] H. Ogita, J. K. Liao, *Endothelium* **2004**, *11*, 123–132.
[2] J. K. Keaney, J. A. Vita, *Prog. Cardiovasc. Dis.* **1995**, *38*, 129–154.
[3] A. Cherubini, G. B. Vigna, G. Zuliani, C. Ruggiero, U. Senin, R. Fellin, *Curr. Pharm. Des.* **2005**, *11*, 2017–2032.
[4] D. Pratico, *Atherosclerosis* **2005**, *181*, 215–224.
[5] G. A. Dillon, A. J. Vita in *Contemporary Cardiology, Vol. 4* (Eds.: J. Loscalzo, A. J. Vita), Humana Press, Totowa, **2000**, pp. 207–226.
[6] a) C. Cena, D. Boschi, G. C. Tron, K. Chegaev, L. Lazzarato, A. Di Stilo, M. Aragno, R. Fruttero, A. Gasco, *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5971–5974; b) D. Boschi, G. C. Tron, L. Lazzarato, K. Chegaev, C. Cena, A. Di Stilo, M. Giorgis, M. Bertinaria, R. Fruttero, A. Gasco, *J. Med. Chem.* **2006**, *49*, 2886–2897.
[7] a) H. Bohn, J. Brendel, P. A. Martorana, K. Schönafinger, *Br. J. Pharmacol.* **1995**, *114*, 1605–1612; b) A. Gasco, K. Schoenafinger in *Nitric Oxide*

Donors (Eds P. G. Wang, T. B. Cai, N. Taniguchi), Wiley-VCH, Weinheim, **2005**, pp. 131 – 175.
[8] P. van Balen, C. Marca Marinet, G. Caron, G. Bouchard, M. Reist, P.-A. Carrupt, R. Fruttero, A. Gasco, B. Testa, *Med. Res. Rev.* **2004**, *24*, 299 – 324.
[9] J. K. Seydel, H-P Cordes, M. Wiese, H. Chi, N. Croes, R. Hanpft, H. Lüllmann, K. Mohr, M. Patten, Y. Padberg, R. Lüllmann-Rauch, S. Vellguth, W. R. Meindl, H. Schönenberger, *Quant. Struct.-Act. Relat.* **1989**, *8*, 266 – 278.
[10] I. K. Pajeva, M. Wiese, H-P Cordes, J. K. Seydel, *J. Cancer Res. Clin. Oncol.* **1996**, *122*, 27 – 40.
[11] D. Bebbington, N. J. T. Monck, S. Gaur, A. M. Palmer, K. Benwell, V. Harvey, C. S. Malcolm, R. H. P. Porter, *J. Med. Chem.* **2000**, *43*, 2779 – 2782.
[12] A. Di Stilo, S. Visentin, C. Cena, A. M. Gasco, G. Ermondi, A. Gasco, *J. Med. Chem.* **1998**, *41*, 5393 – 5401.
[13] C. Ottiger, H. Wunderli-Allenspach, *Eur. J. Pharm. Sci.* **1997**, *5*, 223 – 231.
[14] H. Esterbauer, G. Striegl, H. Puhl, M. Rotheneder; *Free Radical Res. Commun.* **1989**, *6*, 67 – 75.

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