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Design, synthesis, and biological characterization of potential antiatherogenic nitric oxide releasing tocopherol analogs☆

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Abstract—Synthesis and biological characterization of a series of α-tocopherol analogs with NO-releasing capacity are reported. The selected NO-donor moieties were nitrooxy and furoxan. All products were tested for their in vitro NO-releasing capacities, vasodilating properties, and antiplatelet activity. They were also capable of preventing LDL oxidation.

1. Introduction

Atherosclerosis is a complex disease of diverse etiology, where oxidation, increased deposition, and altered metabolism of lipoproteins are key events associated with lesion development.[1,2] Lipid oxidation was first proposed as an early step in atherogenesis, since modification of low density lipoprotein (LDL) by cells or other mechanisms could cause it to become cytotoxic.[3–5] The oxidation hypothesis suggests that antioxidant supplementation might prevent or retard the development of the atheroma plaque, improving patients prognosis.[6] Although the oxidation hypothesis of atherosclerosis has received considerable experimental support, recent negative results from large scale supplementation studies using antioxidant vitamins have questioned this idea.[7–13] The oxidative hypothesis of atherosclerosis is not necessarily disproved by the failure of these particular antioxidant trails,[6] emphasizing the importance of developing new drugs with improving antioxidant capacities in vivo. Human LDL contains a number of antioxidants that inhibit lipid oxidation, α-tocopherol (**1**, vitamin E, Chart 1) being the most abundant (–6 α-tocopherol molecules per LDL particle). α-Tocopherol is selectively targeted into LDL during its metabolism and as a way to its delivery to all cells, due to the action of the α-tocopherol-transfer protein.[14,15] Besides, nitric oxide (•NO) is a free radical species that has strong biological antioxidant actions.[16] In fact, it has been previously shown that •NO inhibits LDL oxidation by scavenging of lipid propagatory radicals.[17–22] Moreover, •NO is able to diffuse into LDL and that makes it a potentially more effective lipid antioxidant than α-tocopherol.[23–29] Therefore, hybrid molecules combining the vitamin E structure and •NO releasing moieties, to target •NO delivery in vivo specifically into LDL, should be a possible therapeutic strategy to protect LDL from oxidative modifications, contributing to the treatment of atherosclerosis. Furoxans (1,2,5-oxadiazole *N*-oxide derivatives), organic nitrates, thionitrites, and nonoates, represent important classes of NO donors.[30,31] Organic nitrates ($RONO_2$) are the oldest class of NO donors that have been clinically applied. Also, it is well known that furoxans are able to release •NO at physiological pH, in the presence of thiol cofactors.[32,33] Herein, we describe the synthesis and preliminary biological characterization

Keywords: NO donor; Vitamin E; Antioxidant; LDL oxidation.
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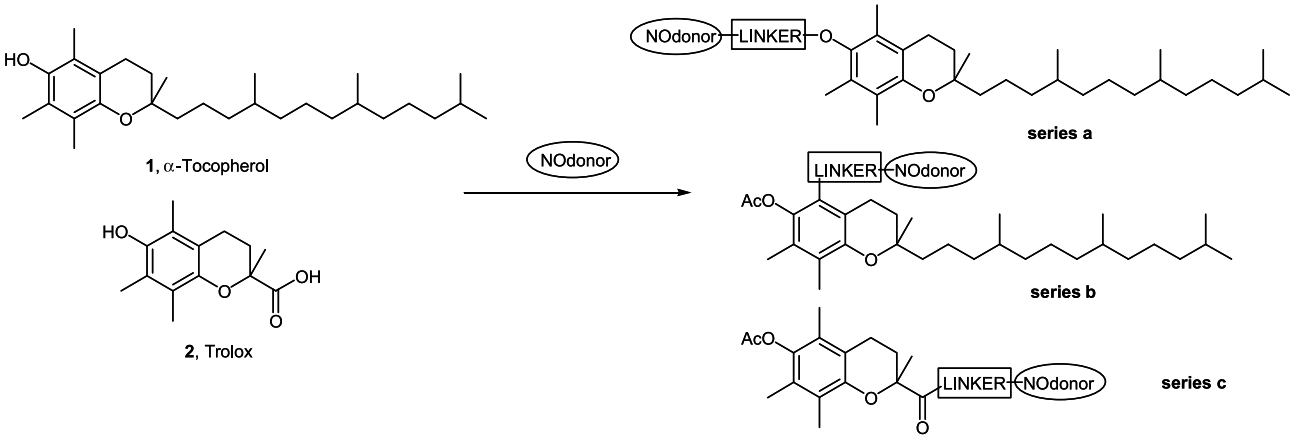


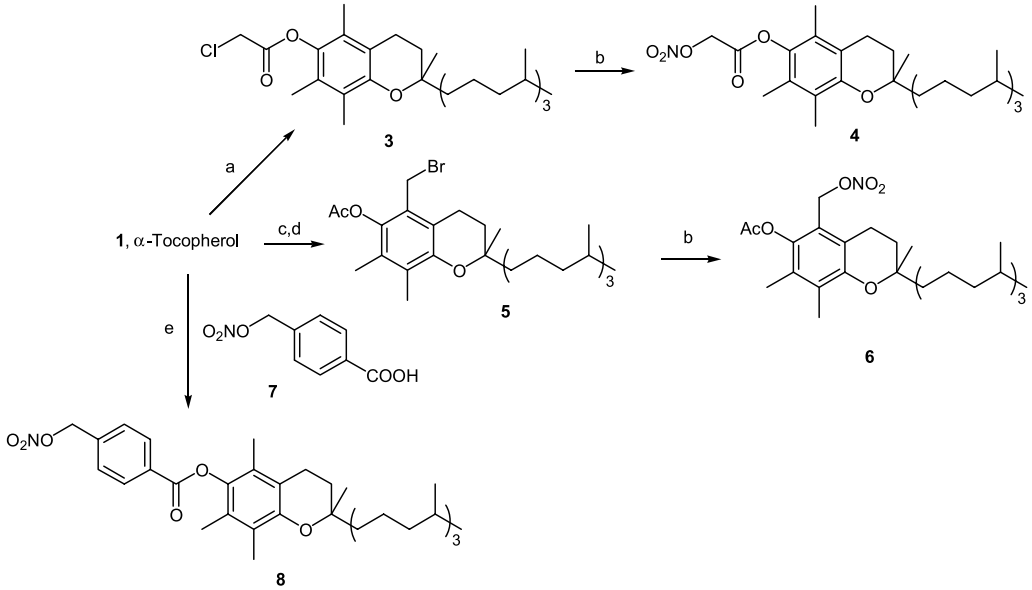
Chart 1. Chemical structure of α-tocopherol and Trolox®, and general structure of the designed compounds; tetramethylchroman system is linked to the ·NO releasing moieties by adequate functionalities.

of novel tocopherol analogs—·NO donors obtained by coupling, through appropriate spacers, either furoxan or nitrooxy groups with a-tocopherol or its analog, trolox (Chart 1). These hybrid compounds release ·NO, due to the presence of a furoxan or nitrooxy substructure, inhibiting platelet aggregation and exhibiting vasorelaxation properties. Moreover, they effectively protect LDL from oxidation, combining the tocopherol substructure with affinity to LDL and antioxidant properties of ·NO-donor.

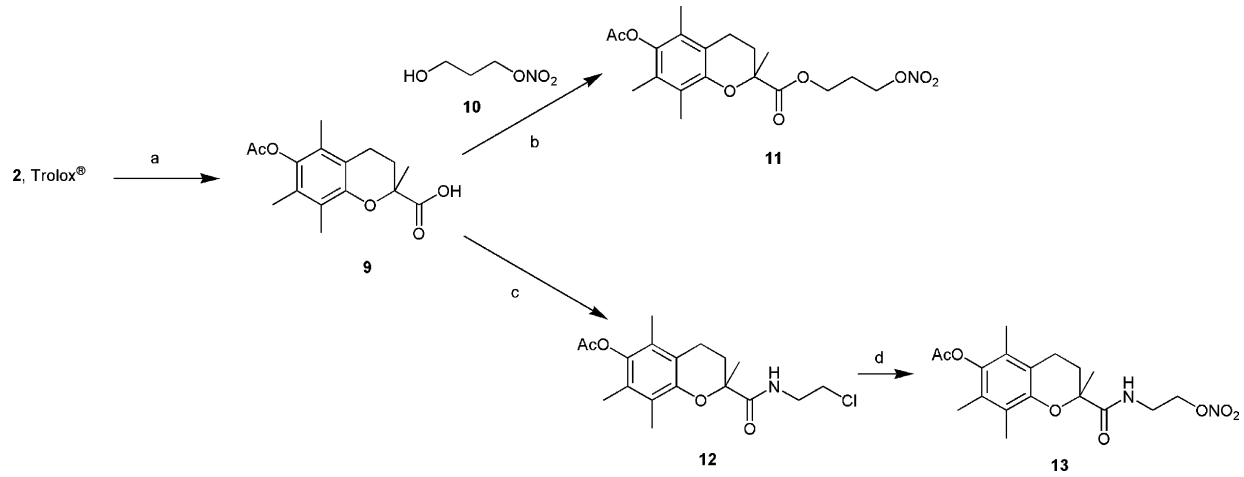
2. Results and discussion

2.1. Chemistry

The preparation of the organic nitrate derivatives follows the synthetic routes that are illustrated in Schemes 1 and 2. Compounds **4**, **6**, and **8** were prepared from α-tocopherol. This reactant was treated with 2-chloroacetyl chloride in the presence of pyridine to obtain the corresponding ester **3**. This latter compound was treated with $AgNO_3$ in acetonitrile at 80 °C to afford the final product **4** in a moderate yield. To prepare compound **6**, the α-tocopherol derivative **5** was prepared as before.[34] This latter compound was transformed into the final nitrooxy derivative **6** following the same procedure used to transform **3** into **4**. The simple nitrooxy derivative, **8**,[35] was synthesized by direct dicyclohexylcarbodiimide (DCC) promoted esterification of **1** as described in Scheme 1. The starting material for the preparation of the nitrooxy derivatives **11** and **13** (Scheme 2) was the acetate derivative of the commercial acid **2** (Trolox, Chart 1). The final product **11** was obtained by treating **9** with 3-(nitrooxy)propanol (**10**) in presence of DCC. To obtain the final product **13**, compound **9** was first transformed into the corresponding acyl chloride, which was directly treated with 2-chloroethylamine to afford the corresponding amide **12**. This latter product was treated with $AgNO_3$ in acetonitrile at 80 °C to afford **13**.

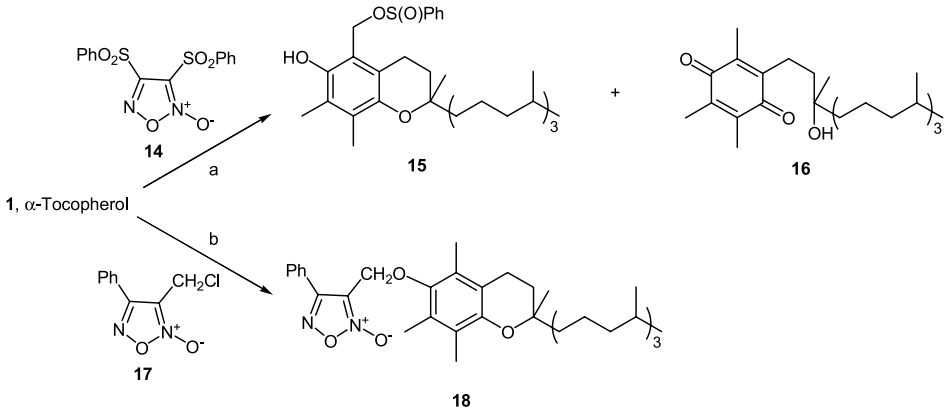


Scheme 1. Synthesis of α-tocopherol analog—nitrooxy derivatives **4**, **6**, and **8**. Reagents and conditions: (a) 2-Chloroacetyl chloride, pyridine, toluene, 15 min; (b) $AgNO_3$, CH_3CN, 80 °C, 35 h; (c) acetic anhydride, pyridine, rt, 3 days; (d) HgO, Br_2, dry CCl_4, rt, 3 h; (e) DCC, CH_2Cl_2, 40 °C, 24 h.

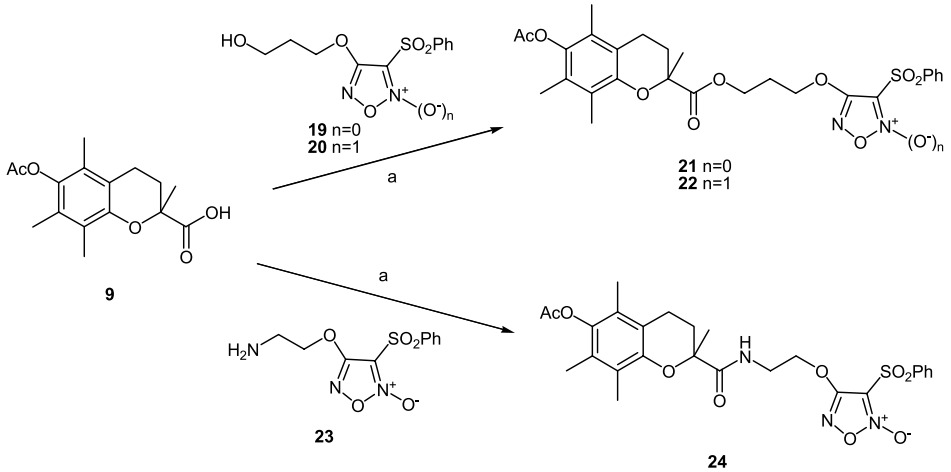


Scheme 2. Synthesis of α-tocopherol analog—nitrooxy derivatives **11** and **13**. Reagents and conditions: (a) Acetic anhydride, pyridine, rt, 2 h; (b) DCC, CH_2C1_2, reflux, 12 h; (c) (i) **9**, $SOC1_2$, DMF, toluene, reflux, 90 min; (ii) 2-chloroethylamine hydrochloride, Et_3N, CH_2C1_2, rt; (d) $AgNO_3$, CH_3CN, 80 °C, 35 h.

The preparation of furoxan derivatives follows the synthetic routes that are illustrated in Schemes 3 and 4. α-Tocopherol (**1**) was treated with either 3,4-bis(phenylsulfonyl)furoxan, **14**, or 3-chloromethyl-4-phenylfuroxan, **17**, yielding compounds **15**, **16**, and **18**, respectively (Scheme 3). In the case of the reaction of **1** with furoxan **14**, the expected product (Fig. 1) was not obtained even in mild conditions.



Scheme 3. Synthesis of α-tocopherol analog—furoxan derivative **18**. Reagents and conditions: (a) K_2CO_3 (1.2 eq.), 18:crown:6, THF, rt, 24 h; (b) K_2CO_3 (0.5 eq.), 18:crown:6, THF, reflux, 9 h.



Scheme 4. Synthesis of tocopherol analog—foroxan derivatives **22** and **24**, and tocopherol analog—forazan derivative **21**. Reagents and conditions: (a) (i) **9**, $SOCl_2$, DMF, toluene, reflux, 90 min; (ii) **19**, **20**, or **23**, Et_3N, CH_2C1_2, rt.

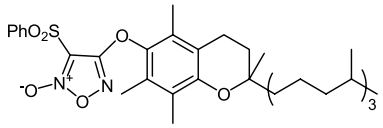


Figure 1. Expected product of the reaction between furoxan **14** and α-tocopherol, **1**.

Indeed (6-hydroxy-2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)chroman-5-yl)methyl benzenesulfinate, **15**,[36] was generated as the main product. α-Tocopheryl quinone, **16**, was also isolated as the result of oxidation promoted by furoxan **14**. Compound **15** could be obtained by the reaction between highest reactive 5a-methyl group (Mills–Nixon effect)[37] of the ortho-quinone methide **I** with the nucleophile $PhSO_2^-$ (benzenesulfonic anion) resulted from furoxan **14** decomposition (Fig. 2).[38]

The final products **21**, **22**, and **24** (Scheme 4) were prepared by treatment of the appropriate alcohols **19** and **20**, and amine **23**, respectively, with the corresponding acid chloride of trolox acetate **9**, following a procedure similar to those used to transform **9** into **12**. Furazan derivative **21** was included in the experiments to know the relevance of the *N*-oxide moiety in the studied bio activities.[16]

2.2. ·NO releasing capacity

The ability of nitrooxy derivatives **4**,**6**, **8**, **11**, and **13**, furoxans **18**, **22**, and **24**, and furazan **21** to release ·NO was determined at different concentrations in the presence of cysteine (5- to 20-fold molar excess) following hemoglobin oxidation.[39] Furoxan reactants **14**, **17**, **20**, and **23** were included to compare their ·NO releasing behavior with the new compounds. The new furoxan derivatives, **22** and **24**, generate high levels of ·NO (Table 1). Initial rates of NO donation, expressed as

Table 1. ·NO releasing rate of the tested compounds

Compound	$[\cdot NO]_{release}$[a,b] ($k_{NO\ releasing\ rate}$ 10^{-3} (min^{-1}))
4	—[c]
6	—[c]
8	—[c]
11	—[c]
13	—[c]
14	3.7 ± 0.5
17	—[c]
19	—[c]
20	1.17 ± 0.06
21	—[c]
22	0.21 ± 0.02 (7 ± 1)
23	1.2 ± 0.1
24	0.35 ± 0.02 (13.3 ± 0.7)

[a] Determined at 6 μM compound concentration in the presence of 30 μM of cysteine.
[b] All values are mean ± SEM ($n = 5$).
[c] No ·NO liberation was observed at 6–100 μM compound concentration in the presence of 30–500 μM of cysteine.

μM min^{-1}, were linearly dependent on the concentration of the single compounds (Fig. 3). In contrast, furoxan derivative **18**, furazan derivative **21**, and nitrooxy derivatives were unable to release ·NO (Table 1) in the conditions assayed. The potential anti-aggregatory effects of these compounds were studied on ADP-induced platelet aggregation model.[40] The anti-aggregatory potency of furoxan derivatives correlated with their ability to produce ·NO (data not shown).

2.3. Vasorelaxation properties

All the hybrid α-tocopherol analogs—·NO donor derivatives had the ability to induce rat aortic rings vasorelaxation (Figs. 4A–D). These results indicate that organic nitrate derivatives release ·NO. As observed for platelet aggregation, the vasoactive properties of

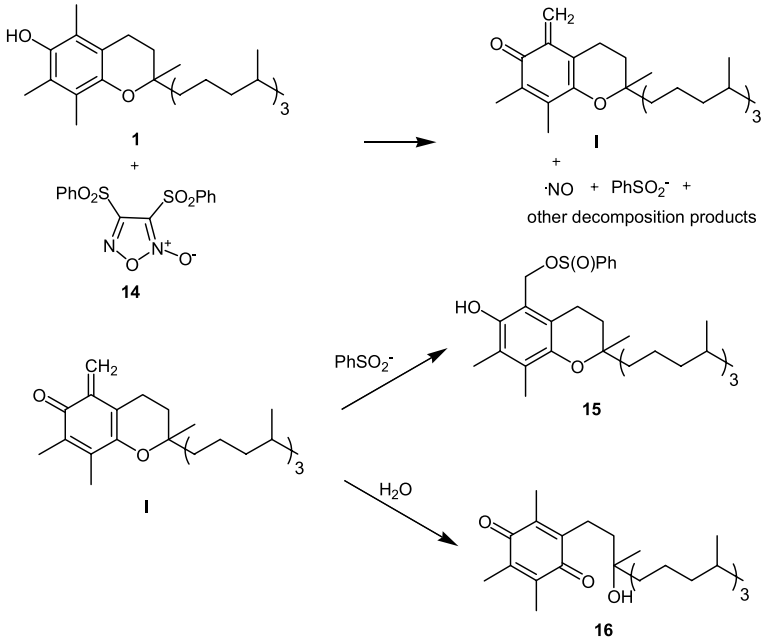


Figure 2. Speculative proposal for the generation of derivatives **15** and **16**.

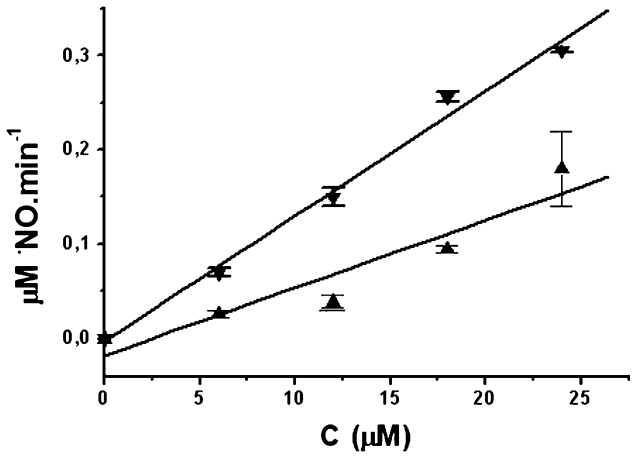


Figure 3. Correlation curves between initial ·NO formation rate (μM ·NO min^{-1}) and tested compound concentration (*C*, μM): (▲) **22**, (▼) **24**. The reaction was followed by detecting the increase of absorbance (ΔA) at $\lambda = 401$ nm over the first 10 min. The reaction was started by adding the tested compounds (0–24 μM) to a 10 μM HbO_2 and cysteine (30–100 μM) solution in 50 mM phosphate buffer, pH 7.4, 37 °C.

these novel compounds correlated with the extent of ·NO released. Thus, derivatives **22** and **24** showed the greatest aortic vasodilating effects, whereas the parent compounds (α-tocopherol acetate and trolox acetate **9**) were unable to produce vasodilatation in the same conditions (Fig. 4A). Derivative **24** dose dependently ($EC_{50} = 1.0 \pm 0.1$ μM) vasodilated aortic rings by a ·NO release-dependent mechanism (Fig. 4B). As oxyhemoglobin has been reported to scavenge ·NO, decreasing the biological activity of ·NO and related compounds,[41,42] we examined the effect of the hemoprotein on **24**-elicited vasorelaxation. The structural motive present in derivative **18** and, in particular, the substitutions on furoxan moiety promote a partial lack of vasodilating activity (Fig. 4A). The *N*-oxide relevance in the observed vasodilating activity was confirmed with the furazan analog **21** (Fig. 4D). In fact, at 10.0 μM furoxan **24** was found to be about 10 times more potent than the deoxygenated analog, furazan **21** in causing vasorelaxation. On the other hand, the organic nitrates were drastically less active than compound **24** in its vasodilating properties (Fig. 4D; compare compound

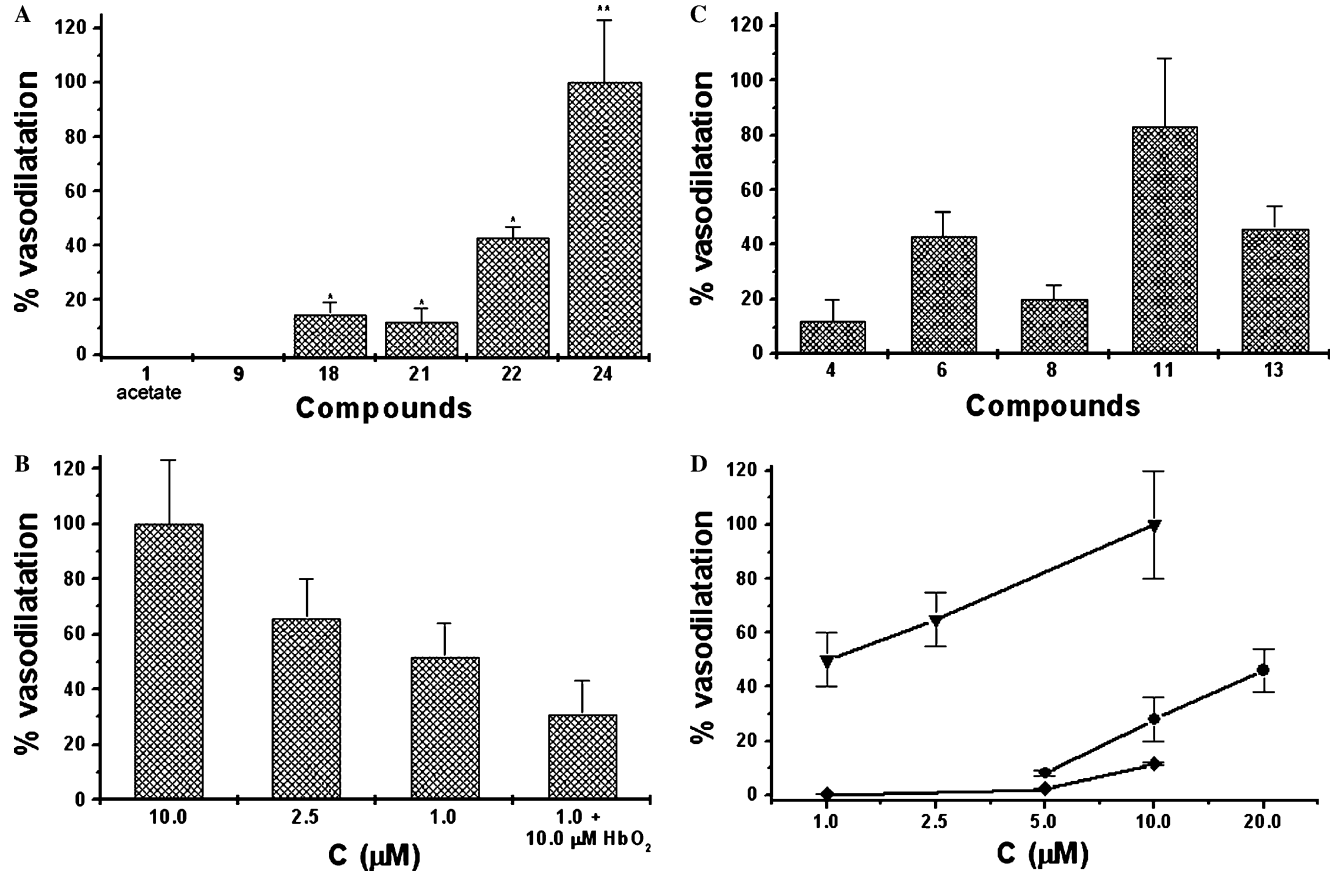


Figure 4. (A) Percentage of vasodilating activity of α-tocopherol acetate (**1** acetate), **9**, **18**, **21**, **22**, and **24**. Tested compounds (10 μM) were added to the tissue–organ bath system after contraction of the thoracic aorta ring with NA (1 μM). $^{*}p < 0.01$ and $^{**}p < 0.05$. (B) Percentage of vasodilating activity of **24** at different concentrations (1–10 μM) in the absence of HbO_2 and at 1 μM concentration in the presence of HbO_2 (10 μM) added to the bath 10 min before the tested compound; (C) percentage of vasodilating activity of **4**, **6**, **8**, **11**, and **13**. Tested compounds (20 μM) were added as in (A). $^{*}p < 0.01$ and $^{**}p < 0.05$; (D) percentage of vasodilating capacity in rat aortic model of α-tocopherol analogs—furoxan **24** (▼), furazan **21** (♦), and organic nitrate **13** (●) derivatives at different compound concentrations (1.0–20.0 μM). Tested compounds were added as previously.

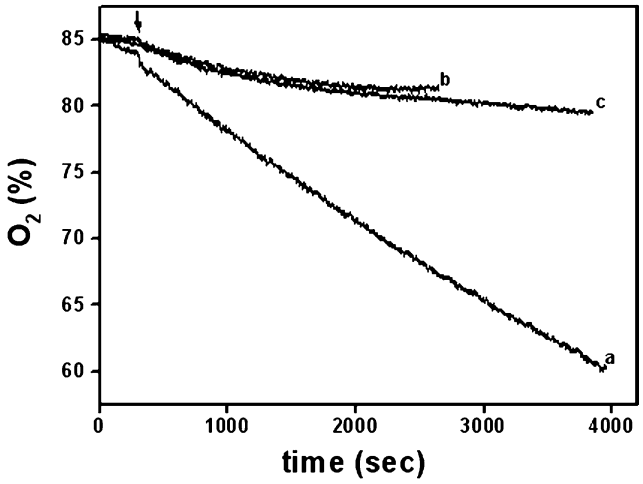


Figure 5. Compound **24** incubated with LDL inhibits oxygen uptake during its ABAP-mediated oxidation. (a) Oxidation of LDL (8 μM) in 50 mM phosphate buffer by 5 mM ABAP was monitored using a Clark type oxygen electrode at 37 °C. (b) Control trace. (c) Oxidation of LDL previously incubated with **24** in 50 mM phosphate buffer.

24 to its analog **13**), emphasizing the special behavior of furoxan moiety in the ·NO-release capacity.

In summary, the tocopherol derivatives belonging to series **a** (see Chart 1) displayed low vasodilating activity. However, derivative **6**, series **b**, showed modest vasodilating activity at 20 μM. On the other hand, derivatives belonging to series **c**, **11**, **22**, **24**, and in a less extent **13**, displayed a good vasodilating activity.

2.4. Antioxidant properties

To test the capacity of these compounds to protect LDL from oxidative modifications, we studied the effect of ·NO donors on azo-compound (ABAP, 2,2′-azobis(2-amidopropane))-mediated LDL oxidation.[43] We selected furoxan derivative, **24**, due to its high ·NO-releasing ability. As expected, compound **24** protected LDL from azo compound-mediated oxidation. In fact, when LDL was incubated with derivative **24**, it decreased ABAP-mediated oxygen consumption (Fig. 5).

3. Conclusion

Our results show that the new tocopherol analogs, **6**, **11**, **13**, **22**, and **24**, represent a new class of ·NO donors having different capacity to release ·NO. These tocopherol derivatives exhibited vasorelaxation properties with derivatives belonging to series **c** (Chart 1), **11**, **22**, **24**, and in a less extent **13** displayed greater vasodilating effects. This suggests that these compounds stimulate ·NO signaling pathways in vascular tissue. Moreover, the observed LDL-protective activity of derivative **24** suggest the potential use of these compounds for prevention of atherosclerosis disease. These 'site-specific' observations are significant in view of the fact that the protective effect of typical ·NO donors or antioxidants decrease with time and distances of the biological targets, that is, LDL. Our observations emphasize the necessity of performing further studies to analyze the LDL protective activity of these compounds in vivo.

4. Experimental

4.1. Chemistry

Argon and nitrogen were purchased from AGA S.A. (Montevideo, Uruguay). Other chemicals were purchased from Sigma (St. Louis, MO, USA) or Aldrich (Milwaukee, WI, USA) at the highest purity available. Compounds **5**,[34] **7**,[35] **8**,[35] **10**,[44] **14**,[45] **17**,[46] **19**,[47] **20**,[47] and **23**[48] were synthesized according to literature methods. Elemental analyses were obtained from vacuum-dried samples (over phosphorous pentoxide at 3–4 mmHg, 24 h at room temperature) and performed on a Fisons EA 1108 CHNS-O analyzer and were within 0.4% of theoretical values. Infrared spectra were recorded on a Bomen, Hartman & Braun FTIR spectrophotometer, using potassium bromide tablets. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker DPX-400 instrument, with $CDCl_3$ as solvent and tetramethylsilane as the internal reference. Electron impact (EI) and electrospray (ES+) mass spectra were obtained at 70 eV on a Shimadzu GC-MS QP 1100 EX or on a Hewlett Packard 1100 MSD spectrometer, respectively. TLC was carried out on Alugram® Sil G/UV_{254} or aluminum oxide on polyester plates. Column chromatography (CC) was carried out on silica gel (Merck, 60–230 mesh) or aluminum oxide (Merck, 70–230 mesh). All solvents were dried and distilled prior to use.

4.2. 2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl 2-chloroacetate (3)

To a stirred solution of α-tocopherol (500 mg, 1.2 mmol) and pyridine (0.1 mL) in toluene (2.0 mL), was added dropwise over 15 min a solution of 2-chloroacetyl chloride (0.1 mL) in toluene (2 mL). The reaction mixture was filtered, and then the filtrate was washed with brine. The organic layer was dried with sodium sulfate and the solvent evaporated in vacuo. The product was purified by column chromatography (SiO_2, hexane:ethyl ether (9:1)). Yellow oil, yield 81%. ^{1}H NMR: δ = 4.34 (s, 2H), 2.62 (t, J = 6.6 Hz, 2H), 2.12 (s, 3H), 2.05 (s, 3H), 2.01 (s, 3H), 1.88–1.74 (m, 2H), 1.61–1.48 (m, 3H), 1.47–1.35 (m, 4H), 1.36–1.21 (m, 11H), 1.19–1.04 (m, 6H), 0.90–0.86 (m, 12H). ^{13}C NMR: δ = 166.39, 150.19, 140.61, 126.84, 125.12, 123.70, 118.00, 75.61, 40.97, 40.74–12.19 (other methylic, methylenic, and methynic carbons). IR: υ_{max} = 2951, 2926, 2868, 1779, 1757, 1460, 1414, 1377, 1235, 1148, 1109, 1067 cm^{-1}. Anal. ($C_{31}H_{51}ClO_3$) C, H.

4.3. 2,5,7,8-Tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl 2-nitrooxyacetate (4)

To a stirred solution of silver nitrate (340 mg, 2 mmol) in acetonitrile (2.0 mL) protected from light, was added dropwise compound **3** (100 mg, 0.2 mmol) in acetoni-

trile. The reaction mixture was allowed to stir for 24 h at room temperature, and then for 35 h at 80 °C. The mixture was concentrated under reduced pressure and the residue was treated with water. The precipitated AgCl was removed by filtration, and the filtrate was extracted with ethyl ether. The combined organic layers were dried with sodium sulfate and evaporated in vacuo. The product was purified by column chromatography (SiO_2, hexane/ethyl ether (95:5)). Yellow oil, yield 42%. ^{1}H NMR: δ = 5.20 (s, 2H), 2.61 (t, J = 6.7 Hz, 2H), 2.11 (s, 3H), 2.04 (s, 3H), 1.99 (s, 3H), 1.88–1.74 (m, 2H), 1.62–1.51 (m, 3H), 1.46–1.37 (m, 3H), 1.34–1.21 (m, 12H), 1.19–1.06 (m, 6H), 0.90–0.86 (s, 12H). ^{13}C NMR: δ = 164.98, 150.36, 140.10, 126.78, 125.09, 123.83, 118.08, 75.66, 67.29, 39.78–12.19 (other methylic, methylenic, and methynic carbons). IR: υ_{max} = 2951, 2928, 2868, 1778, 1659, 1458, 1412, 1377, 1288, 1186, 1109, 1070, 841 cm^{-1}. MS (IE, 70 eV): m/z (%) = 533 (M^+, 1), 486 (37), 458 (9), 430 (100), 261 (7), 221 (28), 193 (7), 165 (42), 149 (8), 69 (6), 57 (11). Anal. ($C_{31}H_{51}NO_6$) C, H, N.

4.4. 5-Nitrooxymethyl-2,7,8-trimethyl-2-(4,8,12-trimethyltridecyl)chroman-6-yl acetate (6)

The title compound was prepared from **5** (136 mg, 0.25 mmol) following the procedure used for the synthesis of **4**. Yellow oil, yield 28%. ^{1}H NMR: δ = 5.41 (s, 2H), 2.78 (t, J = 6.7 Hz, 2H), 2.36 (s, 3H), 2.15 (s, 3H), 2.04 (s, 3H), 1.87–1.75 (m, 2H), 1.61–1.51 (m, 3H), 1.46–1.21 (m, 15H), 1.18–1.03 (m, 6H), 0.90–0.86 (s, 12H). ^{13}C NMR: δ = 170.05, 150.31, 142.07, 131.24, 129.19, 128.49, 119.26, 76.11, 68.03, 39.78–12.72 (other methylic, methylenic, and methynic carbons). MS (IE, 70 eV): m/z (%) = 533 (M^+, 2), 445 (12), 71 (13), 57 (29), 43 (100). Anal. ($C_{31}H_{51}NO_6$) C, H, N.

4.5. 6-Acetoxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (9)

A mixture of Trolox® (500 mg, 2 mmol), pyridine (5.1 mL), and acetic anhydride (4.3 mL) was stirred for 2 h at room temperature. The reaction mixture was diluted with water (10.0 mL), and extracted several times with ethyl ether. The combined organic layers were washed with saturated copper sulfate solution and brine. It was dried with sodium sulfate and the solvent evaporated in vacuo to give **9** as a white solid, which was used without further purification. The acetylation process was confirmed by ^{1}H NMR.

4.6. 3-Nitrooxypropyl 6-acetoxy-2,5,7,8-tetramethylchroman-2-carboxylate (11)

To a stirred solution of **9** (100 mg, 0.34 mmol) in dry dichloromethane (2.0 mL) was added DCC (71 mg, 0.34 mmol). The reaction mixture was allowed to stir for 1 h in ice-water bath. Then, alcohol **12** (42 mg, 0.34 mmol) was added and allowed to stir at room temperature. The reaction mixture was heated at reflux for 12 h, filtered, and then solvent evaporated under reduced pressure. The product was purified by column chromatography (SiO_2, hexane/ethyl ether (1:1)). Colorless oil, yield 28%. ^{1}H NMR: δ = 4.28 (br s, 1H), 4.14 (br s, 2H), 4.06 (br s, 1H), 2.69–2.45 (m, 3H), 2.33 (s, 3H), 2.18 (s, 3H), 2.05 (s, 3H), 1.95 (s, 3H), 1.91–1.85 (m, 3H), 1.65 (s, 3H). ^{13}C NMR: δ = 174.14, 169.74, 149.86, 141.92, 127.72, 125.64, 123.31, 117.47, 77.86, 69.65, 61.13, 31.96, 23.02, 21.26, 20.85, 14.46, 13.24, 12.35, 12.11. IR (KBr): υ = 2934, 1755, 1632, 1456, 1369, 1331, 1280, 1213, 1179, 1140, 1111 cm^{-1}. MS (IE, 70 eV): m/z (%) = 395 (M^+, 8), 353 (17), 307 (9), 247 (11), 231 (10), 205 (35), 164 (3), 135 (3), 107 (3), 43 (100). Anal. ($C_{19}H_{25}NO_8$) C, H, N.

4.7. 2-(2-Chloroethylcarbamoyl)-2,5,7,8-tetramethylchroman-6-yl acetate (12)

To a stirred solution of trolox acetate **9** (150 mg, 0.5 mmol) in dry toluene (1.5 mL), thionyl chloride (0.06 mL, 0.8 mmol), and a drop of dry DMF were added. The mixture was heated at reflux for 90 min and cooled to room temperature. Solvent and thionyl chloride excess was evaporated in vacuo and the residue was dissolved in dry dichloromethane (1.0 mL). Then, solutions of 2-chloroethylamine hydrochloride (58 mg, 0.34 mmol) and triethylamine (0.3 mL) in dichloromethane were added dropwise. The reaction mixture was stirred at room temperature until the disappearance of the reactant. It was washed with 10% sodium bicarbonate solution and then with water. The organic layer was dried with sodium sulfate and the solvent was evaporated in vacuo. The product was purified by column chromatography (SiO_2, hexane/ethyl ether (7:3)). Yellow oil, yield 28%. ^{1}H NMR: δ = 6.92 (br s, 1H), 3.62–3.49 (m, 4H), 2.68–2.58 (m, 2H), 2.35 (s, 4H), 2.20 (s, 3H), 2.07 (s, 3H), 1.99 (s, 4H), 1.55 (s, 3H). ^{13}C NMR: δ = 174.80, 169.86, 148.36, 142.08, 127.80, 126.03, 122.94, 118.47, 79.00, 44.19, 41.13, 29.56, 27.75/26.71, 20.87, 20.67, 13.35, 12.49, 12.38. MS (IE, 70 eV): m/z (%) = 355 (M^++2, 3), 353 (M^+, 8), 313 (9), 311 (27), 247 (11), 205 (71), 189 (7), 175 (3), 161 (3), 91 (8), 63 (9), 43 (100). Anal. ($C_{18}H_{24}ClNO_4$) C, H, N.

4.8. 2,5,7,8-Tetramethyl-2-(2-nitrooxyethylcarbamoyl)-chroman-6-yl acetate (13)

The title compound was prepared from **12** (50 mg, 0.14 mmol) following a similar procedure used for the synthesis of **4**. The reaction mixture was stirred for 24 h at 80 °C. The product was purified by column chromatography (SiO_2, hexane/ethyl ether (7:3)). Yellow oil, yield 18%. ^{1}H NMR: δ = 7.20 (br s, 1H), 4.53–4.52 (m, 2H), 4.43–4.39 (m, 1H), 4.35–4.30 (m, 1H), 2.64–2.50 (m, 2H), 2.44–2.40 (m, 1H), 2.33 (s, 3H), 2.17 (s, 3H), 2.05 (s, 3H), 1.95 (s, 3H), 1.96–1.85 (m, 1H), 1.64 (s, 3H). ^{13}C NMR: δ = 173.8, 164.2, 149.8, 141.1, 127.6, 125.5, 123.6, 117.4, 77.60, 70.61, 61.02, 30.66, 25.69, 21.12, 20.88, 13.28, 12.38, 12.13. IR: υ_{max} = 2924, 2851, 1754, 1638, 1458, 1370, 1279, 1213, 1198, 1140, 1109. MS (IE, 70 eV): m/z (%) = 381(M^+ + 1, 20), 339 (41), 293 (27), 247 (31), 231 (27), 205 (100). Anal. ($C_{18}H_{24}N_2O_7$) C, H, N.

4.9. 4-Phenyl-3-[2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)chroman-6-oxyl]methyl-l,2,5-oxadiazole N^2-oxide (18)

Catalytic amounts of 18-crown-6 were added to a stirred solution of **1** (100 mg, 0.23 mmol) in dry THF (5.0 mL), **6** (58 mg, 0.28 mmol), and potassium carbonate (17 mg, 0.12 mmol). The mixture was heated at reflux until the disappearance of the reactants. Then, the solvent was evaporated in vacuo and the product was purified by column chromatography (SiO_2, hexane/ethyl ether (8:2)). Yellow oil, yield 65%. ^{1}H NMR: δ = 7.78–7.76 (m, 2H), 7.58–7.51 (m, 3H), 4.81 (s, 2H), 2.58–2.56 (m, 2H), 2.14 (s, 3H), 2.10 (s, 3H), 2.08 (s, 3H), 1.99–1.88 (m, 2H), 1.87–1.34 (m, 15H), 1.30 (s, 3H), 1.18–1.00 (m, 6H), 0.92–0.87 (s, 12H). ^{13}C NMR: δ = 157.88, 149.04, 147.71, 131.49, 129.49, 128.43, 128.09, 126.80, 126.28, 123.76, 118.23, 112.94, 75.38, 62.52, 40.98–12.24 (other methylic, methylenic, and methynic carbons). IR: v_{max} = 2926, 1601, 1458, 1248, 1082 cm^{-1}. MS (ES$^+$): *m/z* (%) = 560 ($M^{+\cdot}-CH_2(CH_3)_2$, 5), 417 (7), 342 (10), 247 (2). Anal. ($C_{38}H_{56}N_2O_4$) C, H, N.

4.10. 3-(3-Phenylsulfonyl-l,2,5-oxadiazole-4-oxyl)propyl 6-acetyloxy-2,5,7,8-tetramethylchroman-2-carboxylate (21)

The title compound was prepared from **9** (100 mg, 0.34 mmol) and alcohol **19** (69 mg, 0.27 mmol) following the procedure used for the synthesis of **12**. The product was purified by column chromatography (SiO_2 hexane/ethyl ether (8:2)). Yellow oil, yield 7%. ^{1}H NMR: δ = 8.07 (d, *J* = 8.6 Hz, 2H), 7.75 (t, *J* = 7.5 Hz, 1H), 7.62 (t, *J* = 7.7 Hz, 2H), 4.33–4.28 (m, 1H), 4.14–4.09 (m, 2H), 4.07 (br s, 1H), 2.66–2.61 (m, 1H) 2.49–2.43 (m, 2H), 2.3l (s, 3H), 2.16 (s, 3H), 2.03 (s+m, 5H), 1.90 (m, 4H), 1.64 (s, 3H). ^{13}C NMR: δ = 174.12, 169.95, 149.84 (two carbons), 149.17, 141.59, 138.39, 135.79, 130.04, 129.28, 127.72, 125.59, 123.30, 117.42, 70.09, 66.22, 60.82, 30.83–12.15 (other methylic, methylenic, and methynic carbons). IR: v_{max} = 2934, 1752, 1574, 1213, 1198 cm^{-1}. MS (ES$^+$): *m/z* (%) = 581 ($M^{+\cdot}$+23, 40), 559 ($M^{+\cdot}$+l, 30). Anal. ($C_{27}H_{30}N_2O_9S$) C, H, N, S.

4.11. 3-(3-Phenylsulfonyl-N^2-oxide-l,2,5-oxadiazole-4-oxyl)propyl 6-acetyloxy-2,5,7,8-tetramethyl-chroman-2-carboxylate (22)

The title compound was prepared from **9** (100 mg, 0.34 mmol) and alcohol **20** (102 mg, 0.34 mmol) following the procedure used for the synthesis of **12**. The product was purified by column chromatography (SiO_2 hexane/ethyl ether (8:2)). Yellow oil, yield 9%. ^{1}H NMR: δ = 8.04 (d, *J* = 8.6 Hz, 2H), 7.75 (t, *J* = 7.5 Hz, 1H), 7.61 (t, *J* = 8.2 Hz, 2H), 4.42–4.36 (m, 1H), 4.17–4.11 (m, 2H), 4.02 (br s, 1H), 2.62–2.46 (m, 3H), 2.29 (s, 3H) 2.16 (s, 3H), 2.02 (s+m, 5H), 1.88 (s, 4H), 1.65 (s, 3H). ^{13}C NMR: δ = 174.23, 169.90, 155.05, 149.89, 141.80, 138.56, 135.92, 130.01, 128.91, 127.70, 125.89, 123.26, 117.47, 110.84, 80.20, 67.68, 60.84, 30.87–12.43 (other methylic, methylenic, and methynic carbons). IR: v_{max} = 2932, 1752, 1553, 1213, 1171 cm^{-1}. MS (ES$^+$): *m/z* (%) = 597 ($M^{+\cdot}$+23, 10), 575 ($M^{+\cdot}$+l, 5), 513 (5). Anal. ($C_{27}H_{30}N_2O_{10}S$) C, H, N, S.

4.12. 6-Acetyloxy-2,5,7,8-tetramethyl-*N*-[2-(3-phenylsulfonyl-N^2-oxide-l,2,5-oxadiazole-4-oxyl)ethyl]chroman-2-carboxamide (24)

The title compound was prepared from **9** (100 mg, 0.34 mmol) and amine **25** (97 mg, 0.34 mmol) following the procedure used for the synthesis of **21**. The crude product was purified by column chromatography (SiO_2, hexane/ethyl ether (2:8)). Colorless oil that crystallized at 4 °C, yield 88%. ^{1}H NMR: δ = 8.04 (d, *J* = 7.6 Hz, 2H), 7.75 (t, *J* = 7.6 Hz, 1H), 7.58 (t, *J* = 8.0 Hz, 2H), 6.95 (br s, 1H), 4.48 (br s, 2H), 3.78–3.73 (m, 2H), 2.67–2.59 (m, 2H), 2.36 (s, 3H), 2.23 (s, 3H), 2.06 (s, 3H), 2.00 (br s, 5H), 1.57 (s, 3H). ^{13}C NMR: δ = 175.22, 169.92, 159.15, 148.32, 142.16, 138.40, 136.05, 130.07, 128.90, 127.98, 126.00, 123.18, 118.46, 110.80, 79.08, 70.64, 38.46, 29.70, 25.0/24.1, 20.90, 20.71, 13.40, 12.53(2C). IR: v_{max} = 3434, 2932, 1754, 1676, 1213, 1171 cm^{-1}. MS (ES$^+$): *m/z* (%) = 560 ($M^{+\cdot}$+l, 30), 465 (32), 463 (35), 283 (60). Anal. ($C_{26}H_{29}N_3O_9S$) C, H, N, S.

4.13. ·NO release evaluation

The rate of ·NO release was determined by measuring the oxidation of oxyhemoglobin (HbO_2) to methemoglobin (MetHb) at λ = 401 nm, at 37 °C using a Shimadzu spectrophotometer.[37] The reaction was started by adding the tested compounds (6–100 μM in 50 mM phosphate buffer) to a 10 μM HbO_2 solution in 50 mM phosphate buffer, pH 7.4, in the presence of 5- to 20-fold molar excess of cysteine. The initial rates were calculated from the slope of the straight line portion of each curve. Every NO-releasing rate is the average of at least five determinations. The molar extinction coefficient $\Delta\varepsilon = \varepsilon_{401MetHb} - \varepsilon_{401HbO_2}$ was determined by quantitative oxidation of five different concentrations (1–10 μM) of HbO_2 in pH 7.4 phosphate buffer with a 20 μM solution of NOC-7 (l-hydroxy-2-oxo-3-(*N*-3-methyl-aminopropyl)-3-methyl-1-triazene). The slope ($\Delta\varepsilon$) of the straight line (r = 0.999) obtained plotting the increase of the absorbance ΔA at λ = 401 nm against the HbO_2 concentrations was 57 ± 2 mM^{-1} cm^{-1}. The ability of these compounds to inhibit platelet aggregation in vitro was evaluated as previously described.[38]

4.14. Vasorelaxation assays

Wistar rats (250–300 g) were anesthetized (40 mg kg^{-1} pentobarbital i.p.), and descending thoracic aorta was excised and cut in rings (4 mm in length) (experimental procedures approved by Comisión Honoraria de Experimentación Animal, Universidad de la República). Tissues were mounted under 2 g of passive tension in a Radnoti tissue–organ bath system containing 30 mL of tyrode solution, maintained at 37 °C and gassed with 95% O_2–5% CO_2, pH 7.4. Aortic rings were allowed to equilibrate for 1 h and a dose-dependent contraction was obtained by their incubation in the presence of 1 μM noradrenaline (NA). Tested compounds were added after plateau, using α-tocopherol and Trolox as con-

trols. Drug vehicle (DMSO) also served as control and did not affect the experiments.The effect of 10 μM HbO_2 on relaxation was evaluated by its addition to the bath at least 10 min before compound addition.

4.15. Antioxidant properties

Human LDL (8 μM) obtained by ultracentrifugation from healthy plasma donors as before,[49] was incubated for 15 min at 37 °C in 50 mM potassium phosphate buffer, pH 7.4, in the presence of 2 mM tested compound, added in DMSO (<1% final). Then, the free compound was removed by size exclusion HPLC. The LDL fraction with compound of interest was then incubated in the presence of 5 mM ABAP and LDL oxidation was monitored by oxygen consumption as before.[43] Controls were done using the same experimental conditions, but without LDL and compound, and just without the compound. The presence of the compounds in LDL fraction was checked by RP-HPLC with UV detection after extraction of the compounds from LDL with methanol and using the compound as the standard for all chromatographic purposes.

4.16. Data analysis

Data are expressed as mean ± SEM or 95% confidence interval. Statistical comparisons were carried out with Fisher, ANOVA, Dunnett, or Student tests.

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36. *Compound* 15: ^{1}H NMR: δ = 7.80 (d, J = 7.8 Hz, 2H), 7.66 (t, J = 7.4 Hz, 1H), 7.53 (t, J = 7.6 Hz,, 2H), 6.63 (s, 1H, –OH), 4.53 (d, J = 13.0 Hz, 1H), 4.48 (d, J = 14.3 Hz, 1H),

2.24 (s, 3H), 2.20–2.06 (m, 5H), 1.58–1.08 (m, 26H), 0.89–0.85 (s, 12H). ^{13}C NMR: δ = 147.21, 146.73, 138.51, 134.45, 129.56, 129.02, 128.17, 126.52, 117.94, 112.18, 75.31, 55.94 (CH_2-OS(O)-Ar), 39.77–12.62 (other methylic, methylenic and methynic carbons). MS (IE, 70 eV): m/z(%) = 571(M^+ + l, 3), 570(M^+, 8), 428(50), 203(19), 189(7), 176(18), 165(71), 77(30), 57(44), 43(100). Anal. ($C_{35}H_{54}O_4S$) C, H, S.

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