Available online at www.sciencedirect.com
ScienceDirect
Bioorganic & Medicinal Chemistry 15 (2007) 6262–6272
Bioorganic & Medicinal Chemistry

Second generation of α-tocopherol analogs-nitric oxide donors: Synthesis, physicochemical, and biological characterization☆

Gloria V. López,[a,e] Fabiana Blanco,[c,e] Paola Hernández,[a] Ana Ferreira,[b] Oscar E. Piro,[f] Carlos Batthyány,[d,e] Mercedes González,[a] Homero Rubbo[d,e,*] and Hugo Cerecetto[a,*]

[a]*Departamento de Química Orgánica, Facultad de Ciencias-Facultad de Química Universidad de la República, Montevideo, Uruguay*
[b]*Cátedra de Inmunología, Facultad de Ciencias-Facultad de Química, Universidad de la República, Montevideo, Uruguay*
[c]*Departamento de Biofísica, Facultad de Medicina Universidad de la República, Montevideo, Uruguay*
[d]*Departamento de Bioquímica, Facultad de Medicina Universidad de la República, Montevideo, Uruguay*
[e]*Center for Free Radical and Biomedical Research, Facultad de Medicina, Universidad de la República, Montevideo, Uruguay*
[f]*Departamento de Física, Facultad de Ciencias Exactas, Universidad Nacional de La Plata-Instituto IFLP(CONICET), La Plata, Argentina*

Received 16 April 2007; revised 6 June 2007; accepted 8 June 2007
Available online 13 June 2007

Abstract—Synthesis, physicochemical, and biological characterization of a series of α-tocopherol mimetics 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 mammal cytotoxic activities. The lipophilic–hydrophilic balance of all products was also evaluated. A new hybrid furoxan, phenol derivative **17**, possesses adequate profile of the studied properties.

1. Introduction

Atherosclerosis is the main cause of morbidity and mortality in the western society. Experimental evidence supports the hypothesis that the oxidation of the low density lipoprotein (LDL) is involved in the initiation and progress of this disease, suggesting that antioxidant supplementation might prevent or retard the development of atherosclerotic plaques.[1] Large and randomized clinical trials have not yet demonstrated a beneficial effect of use of antioxidant vitamins to reduce atherosclerotic risk in humans,[2–8] questioning the oxidation hypothesis. Nonetheless, several factors could explain the failure of vitamin E supplementation to prevent atherosclerosis: (i) the inclusion of patients without biochemical evidence of increased oxidative stress, (ii) the relatively short duration and suboptimal dosages of vitamin E treatment, and (iii) the poor patient compliance added to the lack of monitoring of vitamin E levels.[9] In any case, the development of novel drugs with improving antioxidant capacities in vivo continues being a necessity for atherosclerosis therapy.

On these bases we have recently designed, synthesized, and biologically characterized a large series of hybrid molecules combining vitamin E substructures and ·NO releasing moieties (furoxans and organic nitrates). These hybrid molecules will be capable of both binding specifically to LDL particle and to releasing ·NO, reason by which they will be specifically delivered in vivo into LDL, protecting it from oxidative modifications, and thus, improving the antioxidant effects of NO donors and therefore being useful for the treatment of atherosclerosis (Chart 1).[10] Besides, we have been working on the synthesis and biological properties of novel nitrated lipids which have NO-releasing property, capability to down-regulate iNOS expression during macrophage activation, and ability to modulate inflammation.[11] Herein, as a further development of these research lines we describe the synthesis and biological characterization of novel tocopherol analogs-·NO

Keywords: NO donor; Vitamin E; Antioxidant; LDL oxidation.
☆ Part of this research is presented in the Uruguayan patent of invention: González, M., López, G. V., Cerecetto, H., Batthyány, C., Radi, R, Rubbo, H. UR Patent No. 28445, 2004: Análogos de Tocoferol Dadores de Óxido Nítrico.
* Corresponding authors. Tel.: +598 2 9249561; fax: +598 2 9249563 (H.R.); tel.: +598 2 5258516x216; fax: +598 2 525 07 49 (H.C.); e-mail addresses: hrubbo@fmed.edu.uy; hcerecet@fq.edu.uy

0968-0896/$ - see front matter © 2007 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmc.2007.06.019

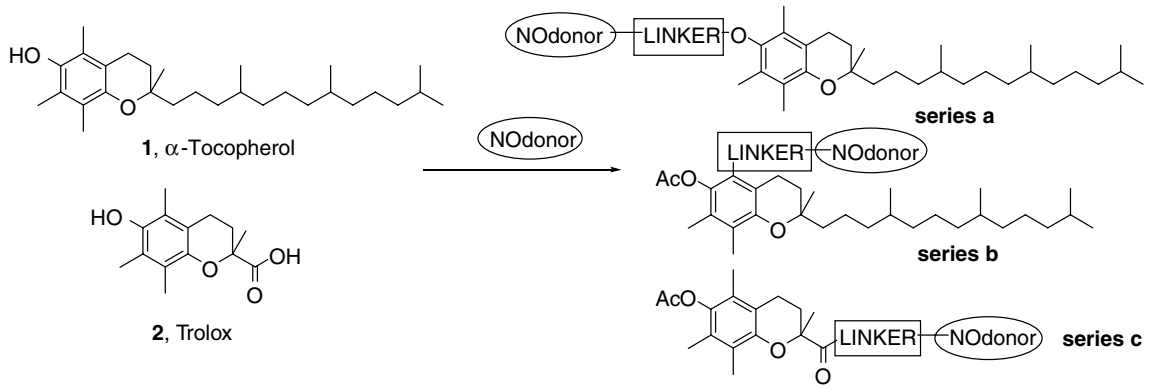


Chart 1. Chemical structures of α-tocopherol, Trolox®, and general structures of previously designed compounds, tetramethylchroman system is linked to the ·NO releasing moieties by adequate functionalities.[10]

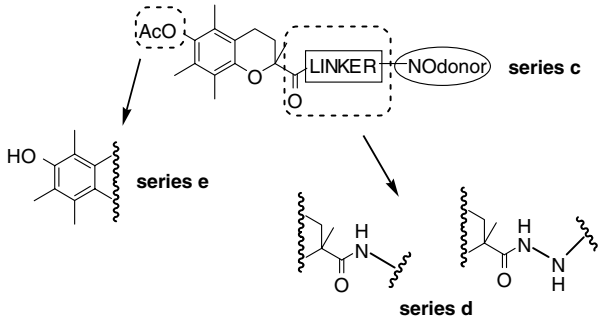
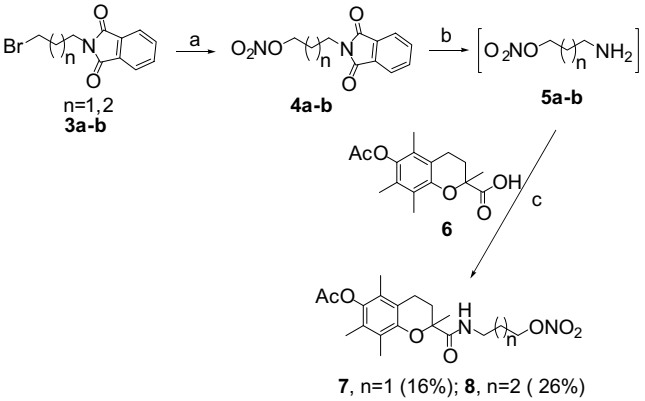


Chart 2. General structures of the newly designed compounds: series d and e.



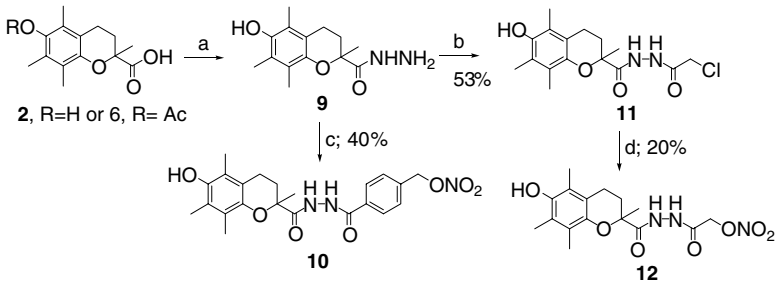
Scheme 1. Syntheses of nitrooxy-derivatives **7** and **8**. Reagents and conditions: (a) $AgNO_3$, CH_3CN, rt, 72 h; (b) NH_2CH_3 (g), THF, rt; (c) CDI, CH_2Cl_2, rt, 24 h.

donors (series d and e, Chart 2), in order to improve the biological and physicochemical properties of previously reported compounds (Chart 1).[10] In the first approach derivatives from series c (Chart 1) possessed mainly as linker the ester function and the phenol protected as acetate. On the one hand, the ester moiety could be metabolized in biological medium faster than amide or hydrazide moieties which are studied in this paper. On the other hand, the compounds' lipophilic properties should be adequate for an optimal LDL interaction. Therefore, herein some structural modifications are analyzed as lipophilicity variations. The hybrid compounds were characterized in terms of their ability to release ·NO, and promote vasodilatation, lipophilicity, and cytotoxic activity against mammal cells. Besides this, the influence of LDL in their solubility in aqueous media was also tested.

2. Results and discussion

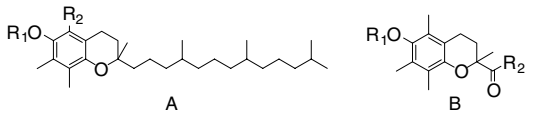
2.1. Chemistry

In order to study the influence of structural variations in the biological properties of parent compounds (series a–c, Chart 1), new derivatives were prepared (series d and e, Chart 2). The preparation of the new organic nitrate derivatives, belonging to series d, follows the synthetic routes that are illustrated in Schemes 1 and 2. On the one hand, amide derivatives **7** and **8** with longer side chain than parent compound 2,5,7,8-tetramethyl-2-(2-nitrooxyethylcarbamoyl)-chroman-6-yl acetate (compound **21**, Table 1) were synthesized.[12] To obtain the final products **7** and **8**, the amines **5a**–**b** were firstly prepared. To this end, the corresponding bromoalkylphthalimide derivative was treated with $AgNO_3$ in acetonitrile at room temperature to afford the corresponding nitrooxy derivatives **4a**–**b**. These products were transformed into the corresponding amines **5a**–**b** which were directly treated with the acetate derivative **6**, previously activated with carbonyldiimidazole (CDI) to afford final amides **7**–**8**. On the other hand, the diacylhydrazides **10** and **12** were obtained from the hydrazide derivative of the acid **2** (**9**). This reactant was treated with 4-nitrooxymethylbenzoic acid[13] in presence of CDI to obtain the final product **10**. At the same time, **9** was treated with 2-chloroacetylchloride in the presence of triethylamine to obtain the corresponding diacylhydrazide **11**. This latter compound was treated with $AgNO_3$ in acetonitrile at room temperature to afford the final product **12** in a moderate yield. Attempts to obtain the acetyl derivatives at 6-position of the chroman system were unsuccessful. The reaction of **2** or **6** in the (a) conditions (Scheme 2) yielded intermediate **9** in all cases. So, the prepared diacylhydrazide derivatives posses free phenolic group like derivatives belonging to series e (Chart 2).



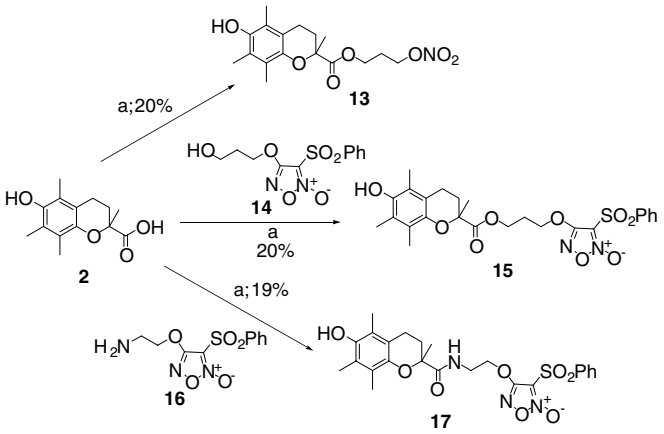
Scheme 2. Syntheses of nitrooxy-derivatives **10** and **12**. Reagents and conditions: (a) i—CDI, THF, rt, 1 h; ii—$NH_2NH_2{\cdot}H_2O$, THF, rt, 24 h; (b) 2-chloroacetylchloride, Et_3N, toluene, rt, 1 h; (c) i—4-nitrooxymethylbenzoic acid, CDI, CH_2Cl_2, rt, 1 h; ii—**9**; (d) $AgNO_3$, CH_3CN, rt, 168 h.

Table 1. ·NO releasing rate, vasorelaxation properties, and mammalian cytotoxicity of tested compounds



Compound	Structure	R_1	R_2	[·NO] (μM)[a,b]	% vasodilatation[d]	Cytotoxicity IC_{50} (μM)	
						THP-1	J774
1	A	H	CH_3	—	[e]	>500	>500
2	B	H	OH	—	[e]	[e]	[e]
6	B	Ac	OH	—	0	[e]	[e]
7	B	Ac	HN(CH2)3ONO2	[c]	30 ± 6	>500	404
8	B	Ac	HN(CH2)4ONO2	[c]	25 ± 3	459	390
10	B	H	HN-NH-CO-C6H4-CH2ONO2	[c]	49 ± 2	[e]	440
12	B	H	HN-NH-CO-CH2ONO2	[c]	14 ± 2	[e]	79
13	B	H	O(CH2)3ONO2	[c]	73 ± 6	188	82
15	B	H	O(CH2)3O-furoxan-SO2Ph	0.74 ± 0.04	85 ± 6	52	22
17	B	H	HN(CH2)2O-furoxan-SO2Ph	0.94 ± 0.07	104 ± 9	54	18
18	A	Ac	CH_3	—	0	[e]	[e]
19	A	Ac	CH_2ONO_2	[c]	43 ± 9	>500	>500
20	B	Ac	O(CH2)3ONO2	[c]	83 ± 25	>500	108
21	B	Ac	HN(CH2)3ONO2	[c]	46 ± 8	>500	>500
22	B	Ac	O(CH2)3O-furoxan-SO2Ph	0.21 ± 0.02	43 ± 4	25	17
23	B	Ac	HN(CH2)2O-furoxan-SO2Ph	0.35 ± 0.02	100 ± 23	38	17
24	A	Ph-furoxan	CH_3	[c]	15 ± 4	[e]	[e]

[a] Determined at 6 μM compound concentration in the presence of 30 μM of cysteine.
[b] All values are means ± SEM ($n = 5$).
[c] No ·NO liberation was observed at 6 μM–1mM compound concentration range in the presence of 30 μM–5 mM of cysteine.
[d] Tested compounds (10 μM of furoxan derivatives or 20 μM of nitrooxy-derivatives) were added to the tissue-organ bath system after contraction of the thoracic aorta ring with NA (1 μM).
[e] No data.



Scheme 3. Syntheses of nitrooxy-derivative **13** and furoxan derivatives **15** and **17**. Reagents and conditions: (a) i—CDI, CH_2Cl_2, 1 h; ii—3-(nitrooxy)propanol or **14** or **16**, rt, 24 h.

In addition, in order to study the relevance of free phenolic group of the chroman ring, series e, deprotected compounds **13**, **15**, and **17** analogs to previously prepared compounds **20**, **22**, and **23**, respectively (Table 1),[10] were prepared following the synthetic routes that are illustrated in Scheme 3. The nitrooxy-derivative **13** was prepared by treatment of **2**, previously activated with CDI, with 3-(nitrooxy)propanol.[14] Meanwhile, furoxan derivatives **15** and **17** were prepared by treatment of **2** with the appropriate alcohol **14** and amine **16**, respectively, following a procedure similar to those used to obtain **13**. All new compounds were characterized by NMR (^{1}H-, ^{13}C-, COSY, HMQC, and HMBC experiments), IR, and MS. The purity was established by TLC and microanalysis. Single crystals of the acetate of derivative **13** (compound **20**, Table 1) adequate for structural X-ray diffraction studies[15] were obtained by slow evaporation from an Et_2O solution. Figure 1 shows the ORTEP[15a] molecular drawing of compound **20**. It may be noted a distinguished structural feature, namely, the terminal 3-nitrooxypropyl group folds back capping the fused rings to conform a globular molecule. These crystallographic data were used to perform the theoretical studies described soon after (Table 4).

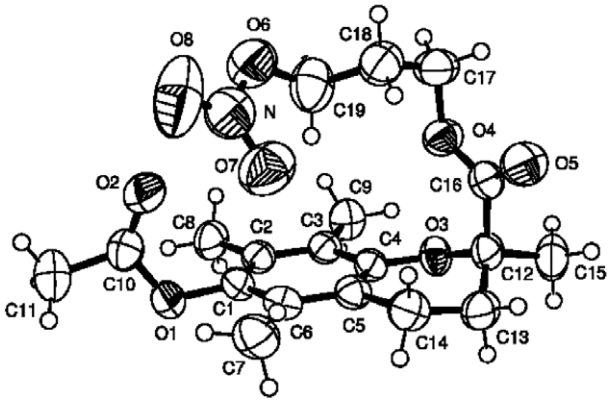


Figure 1. Molecular plot of 3-nitrooxypropyl 6-acetoxy-2,5,7,8-tetramethylchroman-2-carboxylate (**20**) showing the labeling of the non-H atoms and their displacement ellipsoids at the 50% probability level.

2.2. Biological characterization

2.2.1. In vitro NO releasing capability. The ability of all new final products to release ·NO was determined at different concentrations in the presence of cysteine (5- to 20-fold molar excess) following hemoglobin oxidation.[16]

The new furoxan derivatives, **15** and **17**, released higher levels of ·NO in comparison with the previously characterized parent compounds (**22** and **23**, respectively). In contrast, nitrooxy derivatives were unable to release ·NO in the assayed conditions (Table 1).

2.2.2. Vasodilating properties. All hybrid compounds were assessed for their capacity to induce vasorelaxation using rat aortic rings. Results showed that nitrooxy and furoxan derivatives promoted vasorelaxation of noradrenaline pre-contracted vessels (Table 1). Considering that the vasoactive property of one compound could be indicative of its ·NO-releasing capability,[17] our results could indicate that all compounds are able to release ·NO even though we could not detect it for the nitrooxy derivatives. In order to confirm this phenomena, vasorelaxation studies were performed in presence of 1*H*-[1,2,4]-oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ). In fact, the vasorelaxant properties of the tested compounds are cGMP-dependent because the well-known inhibitor of the soluble guanylate cyclase ODQ caused a significant reduction in the vasodilator potencies (see results for compounds **20**, **22**, and **23**, Table 2). These results suggest a participation of ·NO in the vasodilating action of the nitrooxy and furoxan derivatives. Additionally, furoxan derivatives **15** and **17**, which were found to release ·NO, showed the greatest aortic vasodilating

Table 2. ·Vasorelaxation properties with and without ODQ

Compound	% vasodilatation[a]	
	Without ODQ	+1 μM ODQ
20	83 ± 25	10 ± 2
22	43 ± 4	6 ± 4
23	100 ± 23	39 ± 16

[a] See Table 1.

effects. The absence of vasodilatation activity of derivatives **6** and **18** (Table 1), analogs possessing similar structural core, demonstrated the relevance of the included moieties in our compounds.

2.2.3. In vitro mammalian cytotoxicity. Unspecific mammalian cytotoxicity of all the final products and some reactants was evaluated in vitro at 1–500 μM, using human macrophage-like cells (phorbol ester differentiated THP-1 cells) and mouse J774 macrophages.[18] In general, mouse J774 macrophages were more sensitive than THP-1 cells for this type of compounds. Relatively speaking, furoxan derivatives are more toxic than nitrooxy derivatives as denote the IC_{50} values summarized in Table 1. Apparently, the diacylhydrazide moiety does not produce more cytotoxicity than the other linkers, ester or amide (compare derivative **10** to **7**, **8**, and **21** cytotoxic behaviors; or compare derivative **12** to **13** and **20** mammalian cytotoxicity). Derivatives belonging to series d, amides **7** and **8**, were more cytotoxic drugs than the parent compound **21**. Their cytotoxic potential seems to depend on the long of the amide chain (see results on J774 cells, Table 1), while their experimental lipophilicities, expressed as R_M, are comparable between **7**, **8** and the parent compound **21** (Table 3).

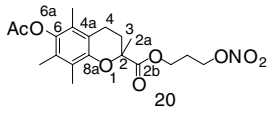
Table 3. Lipophilicity data for α-tocopherol and hybrid compounds

Compound	R_M	*c*log *P*
1	1.7	8.1
7	0.2	4.0
8	0.1	4.4
10	−0.02	3.6
12	−0.7	0.9
13	—	3.6
15	0.6	9.4
17	0.3	8.6
18	1.7	8.6
19	2.0	10.1
20	0.4	4.4
21	0.3	3.9
22	0.8	9.8
23	0.4	9.4
24	1.7	10.5

2.3. Lipophilicity studies

To further study the influence of physicochemical properties in biological activity the lipophilicity was theoretically and experimentally determined, together with the variation of compound solubility in aqueous milieu, in both the presence and the absence of LDL. For theoretical analysis the structure of each derivative was optimized using a quantum mechanical methodology (AM1). Then, the equilibrium geometries at ground state were calculated applying HF/3-21G*. The crystallographic structure of the corresponding acetate of derivative **13** (**20**, Table 1) was used as template to compare the experimental and theoretical structural data (bond length and dihedral angles, Table 4). We determined and examined the logarithm of the partition coefficient between octanol and water of the un-ionized molecules (*c*log *P*, Table 3) using Villar method implemented in Spartan modelling package.[19] Lipophilicity was also determined experimentally by reverse phase TLC experiments on precoated TLC-C_{18}. The R_f values were converted into R_M values via the following relationship: $R_M = \log[(1/R_f) - 1]$.[20] R_M values are summarized in

Table 4. Crystallographic and AM1/3-21G* optimized structure of compound **20**



Distances and angles[a,b]	Crystallographic data	AM1/3-21G* optimized geometry	Relative error $\lvert(d_{PM3/6.31G^*} - d_{crist})/d_{crist}\rvert$
O1–C2	1.428	1.433	0.004
O1–C8a	1.384	1.380	0.003
C2–C2a	1.524	1.541	0.011
C2–C2b	1.525	1.536	0.007
C2–C3	1.520	1.533	0.009
C3–C4	1.508	1.531	0.015
C4–C4a	1.506	1.516	0.007
C4a–C8a	1.393	1.401	0.006
C6–C6a	1.414	1.409	0.004
O1–C2–C3	110.19	109.78	0.004
C2–C3–C4	111.88	110.48	0.013
C3–C4–C4a	112.82	111.72	0.010
C4–C4a–C8a	120.19	119.94	0.002
C4a–C8a–O1	122.11	122.53	0.003
C8a–O1–C2	117.63	118.59	0.008
C2b–C2–C3	110.37	109.43	0.009
C2b–C2–C2a	108.46	107.71	0.007
C2a–C2–O1	105.14	109.62	0.043
C2b–C2–O1	111.12	106.66	0.040

[a] Distances in Å and angles in °.
[b] Numbers according to figure.

Table 3. As it was expected, the presence of free OH in the nitrooxy-derivative **13** increased its theoretical hydrophilicity. Regarding furoxans belonging to series e, **15** and **17**, like in the case of derivative **13** the free phenol transforms these compounds less hydrophobic (R_M and *c*log*P*, Table 3). But, the most developed hydrophilic derivatives were the diacylhydrazides (**10** and **12**, series e); their low lipophilicity could be the result of the free OH added to the presence of the NHNH system. Taken together, these results showed that the lipophilicity was similar to that of α-tocopherol when the derivative was obtained by direct modification of **1**, but derivatives obtained by Trolox's structural modifications showed a wide range of lipophilicity, demonstrating that this methodology is a best way to modulate the physicochemical property of the developed compounds.

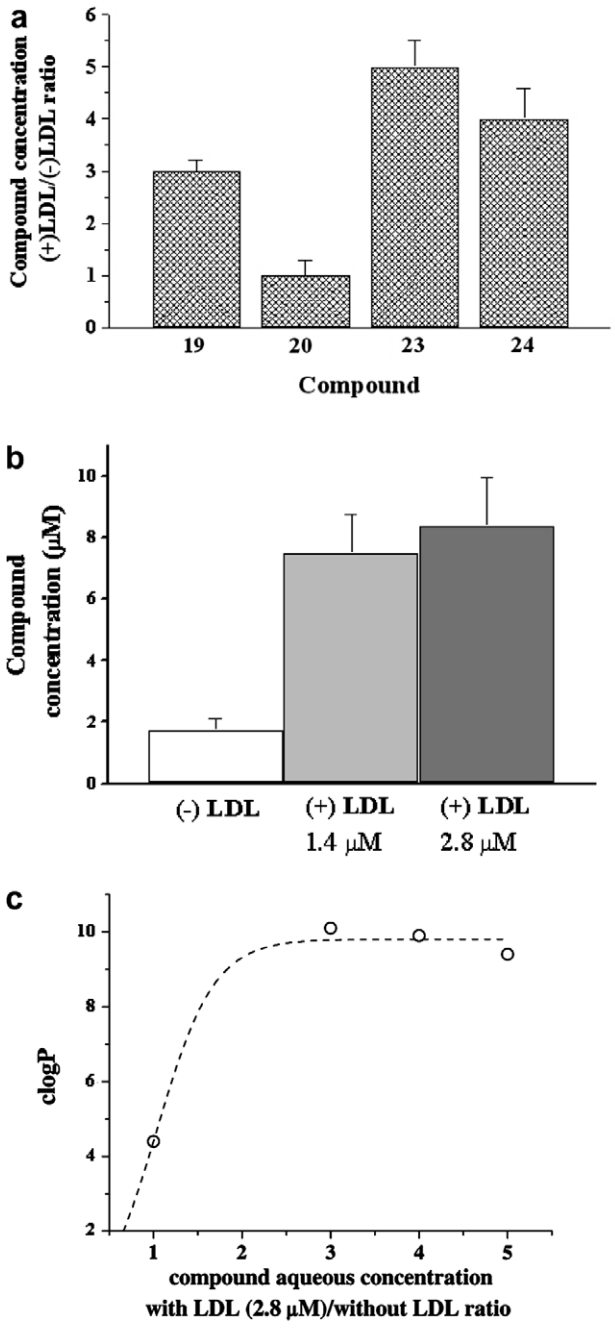


Figure 2. (a) Compounds **19**, **20**, **23**, and **24** aqueous concentration in presence of LDL (2.8 μM)/absence of LDL ratio. (b) Compound **23** aqueous, phosphate buffer, pH 7.4, solubility in absence and increasing amount of LDL. (c) *c*log*P* versus aqueous concentration with and without LDL ratio (the dotted line means tendency).

Besides this, in order to know how lipophilicity may influence in compound-LDL interaction, we preliminarily evaluated solubility variations in aqueous milieu of four representative compounds, in both in the presence and in the absence of LDL. The selected derivatives belong to α-tocopherol-nitrooxy family, compound **19**, and α-tocopherol–furoxan family, compound **24**, or belong to Trolox-nitrooxy family, compound **20**, and Trolox–furoxan family, compound **23**. That is, a methanolic solution of the studied compounds was evaporated in order to form a thin layer of the compound in the tube surface. Then 5 mM phosphate buffer, pH 7.4, was added, followed by LDL. The mixture was treated with methanol and analyzed by RP-HPLC. Our results (Fig. 2a) showed that compound **19**, the nitrooxy-derivative of α-tocopherol, was three times more soluble in aqueous solution in the presence of LDL, meanwhile the solubility of hybrid compound **20**, the nitrooxy-derivative of Trolox, did not change in the presence of LDL. On the other hand, compound **23**, the furoxan derivative of Trolox, was five times more soluble in the presence of LDL. Besides, compound **24**, the furoxan derivative of α-tocopherol, was four times more soluble in aqueous solution in the presence of LDL. For compound **23**, a little difference in the aqueous solubility promoted by LDL was observed when the amount of LDL was duplicated (Fig. 2b).

The aqueous solubility changes in presence and absence of LDL well correlated with compounds, calculated lipophilicity *c*log*P* (Fig. 2c). In particular, compounds, **19** and **24**, the α-tocopherol modified derivatives, possess an adequate lipophilicity due to the unmodified phytyl lateral chain. In contrast, the furoxan derivatives **15**, **17**, **22**, and **23** possess adequate lipophilicity to interact with LDL probably as the result of the presence of the lipophilic sulfone moiety.

3. Conclusion

Two new series, **d** and **e**, of α-tocopherol analogs-nitric oxide donor were successfully developed. Our results for the complete series of the studied compounds, indicate that the nitrooxy derivatives **7**, **8**, **10**, **19**, and **21** exhibit less toxicity than furoxan derivatives, and that the NO-release capability could be associated with cytotoxic effects (in general, compounds with the highest vasoactive properties possess the highest cytotoxic effects, see Table 1). Interestingly, the nitrooxy derivative with the best 'NO-like' profile, compound **20**, was more toxic for mouse than for human macrophages. The best developed furoxan was derivative **17** possessing excellent vasoactivity and being the lowest cytotoxicity among its

family. These findings emphasize the necessity of performing further in vivo studies of these hybrid compounds as drugs.

In reference to the compounds' lipophilicity, we were able to develop derivatives with a widespread of lipophilic–hydrophilic balance that could allow the drugs to biodistribute differentially in different biological compartments.

An in-depth study of aqueous stability of the different derivatives from series **c** and **d** was not performed. However, when derivatives **7**, **12**, and **20** were incubated in different aqueous solutions some relevant features could be extracted. In general, hydrazide derivatives were more stable in biological medium than ester or amide derivatives. For example after 48 h of incubation of compounds **7**, **12**, and **20** in aqueous solution, neutral pH and at 37 °C, hydrolysis product (Trolox, **2**) was not detected (TLC analysis). While in an acidic pH, Trolox was detected after 24 h in the case of derivative **20** and after 48 h for derivative **7** being derivative **12** stable in these conditions even after 96 h of incubation. These observations along with the consideration that diacylhydrazide derivative **10** has relevant vasoactive properties and relatively low cytotoxic activity pointed out that it could be a potential leader for a new series of compounds. Thus, the aqueous stability studies promoted us to complete the biological characterization of derivative **10** and perform the design, synthesis, and biological characterization of new hydrazide derivatives analogs to **10** with potential application in the treatment of atherosclerosis.

Our observations emphasize the necessity of performing further studies to analyze the synthesized compounds as in vivo LDL protective agents, a task that we are currently undertaking.

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) or Aldrich (Milwaukee, WI) at the highest purity available. Compounds **6**,[10] **14**,[21] **16**,[22] **18**–**23**[10] were synthesized according to literature methods. Elemental analyses were obtained from vacuum-dried samples (over phosphorus pentoxide at 3–4 mm Hg, 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 (unless otherwise indicated) and tetramethylsilane as the internal reference. Electron impact (EI) and electrospray (ES+) mass spectra were obtained at 20 or 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.

4.1.1. *N*-3-Nitrooxypropylphthalimide (4a). To a stirred solution of silver nitrate (1.9 g, 10.1 mmol) in acetonitrile (20 mL) protected from light was added compound **3a** (2.0 g, 7.5 mmol). The reaction mixture was allowed to stir for 3 days at room temperature. The mixture was concentrated under reduced pressure and the residue treated with water. The precipitated AgCl was removed by filtration, and filtrate was extracted with ethyl acetate. The combined organic layers were dried with sodium sulfate and evaporated in vacuo to give **4a** as a white solid (1.8 g, 96%) which was used without further purification. The transformation was confirmed by ^{1}H NMR, ^{13}C NMR, and MS. ^{1}H NMR: δ 7.88 (dd, 2H, J = 3.1 Hz, 5.3 Hz), 7.76 (dd, 2H, J = 3.1 Hz, 5.3 Hz), 4.54 (t, 2H, J = 6.3 Hz), 3.86 (t, 2H, J = 6.7 Hz), 2.15 (q, 2H, J = 6.5 Hz). ^{13}C NMR: δ 168.59, 134.54, 132.36, 123.80, 70.99, 34.98, 26.59. MS (EI, 20 eV): m/z (%) 250 ($M^{+\cdot}$, 1), 204 (34), 188 (11), 174 (35), 160 (100).

4.1.2. *N*-4-Nitrooxybutylphthalimide (4b). The title compound was prepared from **3b** (2.0 g, 7 mmol) following a similar procedure used for the synthesis of **4a** as a white solid (1.8 g, 98%) which was used without further purification. The transformation was confirmed by ^{1}H NMR, ^{13}C NMR, and MS. ^{1}H NMR: δ 7.87 (dd, 2H, J = 3.1 Hz, 5.2 Hz), 7.75 (dd, 2H, J = 3.1 Hz, 5.2 Hz), 4.52 (t, 2H, J = 5.7 Hz), 3.76 (t, 2H, J = 6.3 Hz), 1.83 (m, 4H). ^{13}C NMR: δ 168.71, 134.44, 132.42, 123.70, 72.78, 37.52, 25.32, 24.67. MS (EI, 20 eV): m/z (%) 264 ($M^{+\cdot}$, 1), 218 (4), 202 (1), 188 (19), 160 (100).

4.1.3. 2,5,7,8-Tetramethyl-2-(3-nitrooxypropylcarbamoyl)-chroman-6-yl acetate (7). To a stirred solution of **4a** (312 mg, 1.2 mmol) in THF was bubbled NH_2CH_3 (g), obtained from dropping a concentrated aqueous solution of methylamine hydrochloride over solid sodium hydroxide. The reaction was followed until the disappearance of the reactant. The mixture volume was reduced in vacuo. The solution containing amine **5a** was added to a solution of **6** (292 mg, 1 mmol) previously activated with CDI (394 g, 1.2 mmol) in THF (12 mL). After 24 h of stirring at room temperature the mixture was diluted with ethyl acetate and washed with brine. The organic layer was dried and the solvent evaporated under reduced pressure. The product was purified by CC (SiO_2, hexane/ethyl ether (1:1)). Colorless oil (63 mg, 16%). ^{1}H NMR: δ 6.52 (br s, 1H), 4.36–4.31 (m, 1H), 4.24–4.19 (m, 1H), 3.45–3.37 (m, 1H), 3.32–3.24 (m, 1H), 2.66–2.49 (m, 2H), 2.41–2.36 (m, 1H), 2.33 (s, 3H), 2.18 (s, 3H), 2.05 (s, 3H), 1.96 (s, 3H), 1.91–1.82 (m, 3H), 1.54 (s, 3H). ^{13}C NMR: δ 174.81, 169.83, 148.46, 142.35, 142.16, 127.96, 126.21, 122.76, 118.66, 79.26, 70.89, 36.11, 29.70, 27.58, 25.07 (br s), 20.89, 13.37, 12.49, 12.40. IR: ν_{max} 2932, 1755, 1671, 1628, 1550, 1456, 1370, 1281, 1213, 1113, 1080. MS (EI, 70 eV): m/z (%) 332 ($M^{+\cdot}$ –ONO_2, 15), 289 (20), 247 (2), 232 (2), 189 (5), 176 (3). Anal. ($C_{19}H_{26}N_2O_7$) C, H, N.

4.1.4. 2,5,7,8-Tetramethyl-2-(4-nitrooxybutylcarbamoyl)-chroman-6-yl acetate (8). The title compound was prepared from **4b** (330 mg, 1.2 mmol) following a similar procedure used for the synthesis of **7a**. The product was purified by CC (SiO_2, hexane/ethyl ether (1:1)) as a colorless oil (106 mg, 26%). ^{1}H NMR: δ 6.43 (br s, 1H), 3.96–3.94 (m, 1H), 3.52–3.34 (m, 3H), 2.71–2.48 (m, 3H), 2.32 (s, 3H), 2.15 (s, 3H), 2.03 (s, 3H), 1.96 (s, 3H), 1.90–1.75 (m, 4H), 1.68–1.60 (m, 1H), 1.57 (s, 3H). ^{13}C NMR: δ 172.27, 169.94, 148.84, 141.70, 127.42, 125.96, 122.42, 118.91, 79.65, 48.15 (2C), 30.69, 25.47 (br s), 23.02 (2C), 21.30, 20.89, 13.36, 12.57, 12.47. IR: ν_{max} 3015, 2928, 1750, 1618, 1456, 1370, 1217, 1113, 1078. MS (EI, 70 eV): *m/z* (%) 345 ($M^{+\cdot}$–ONO_2, 18), 303 (5), 247 (100), 232 (6), 205 (65), 189 (9), 175 (5), 161 (5). Anal. ($C_{21}H_{28}N_2O_7$) C, H, N.

4.1.5. 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carbohydrazide (9). To a stirred solution of **2** (250 mg, 1 mmol) in dry THF (13 mL) was added CDI (175 mg, 1.1 mmol). The reaction mixture was allowed to stir for 1 h at room temperature. Then, it was slowly added to a solution of hydrazine monohydrate (1 mL) in dry THF (6 mL) and allowed to stir for 24 h. The mixture volume was reduced in vacuo and water (5 mL) was added. The product precipitated as a crystalline white solid (174 mg, 66%), filtered and dried at room temperature in a desicator. It was used without further purification. The hydrazide formation was confirmed by ^{1}H NMR and ^{13}C NMR. ^{1}H NMR: δ 8.48 (s, 1H), 7.46 (s, 1H), 4.34 (br s, 2H), 2.56–2.38 (m, 2H), 2.25–2.19 (m, 1H), 2.09 (s, 3H), 2.07 (s, 3H), 1.99 (s, 3H), 1.77–1.67 (m, 1H), 1.39 (s, 3H). ^{13}C NMR: δ 173.09, 146.70, 144.81, 123.55, 122.18, 121.04, 117.83, 77.88, 30.41, 25.38, 20.94, 13.57, 12.89, 12.62.

4.1.6. 6-Hydroxy-*N*′-(4-(nitrooxymethyl)benzoyl)-2,5,7,8-tetramethylchroman-2-carbohydrazide (10). To a stirred solution of 4-nitrooxymethyl benzoic acid[13] (112 mg, 0.6 mmol) in dry dichloromethane (10 mL) was added CDI (115 mg, 0.7 mmol). The reaction mixture was allowed to stir for 1 h at room temperature. Then, it was slowly added **9** (150 mg, 0.6 mmol) in portions. The reaction mixture was heated at reflux for 12 h and the solvent evaporated under reduced pressure. The product was purified by CC (SiO_2, hexane/ethyl acetate (6:4)). Colorless oil (106 mg, 40%).^{1}H NMR: δ 9.18 (d, J = 6.1 Hz, 1H), 8.88 (br s, 1H), 7.85 (d, J = 8.1 Hz, 2H), 7.48 (d, J = 8.1 Hz, 2H), 5.48 (s, 2H), 4.35 (br s, 1H), 2.75–2.59 (m, 2H), 2.43–2.34 (m, 1H), 2.28 (s, 3H), 2.21 (s, 3H), 2.12 (s, 3H), 2.03–1.96 (m, 1H), 1.63 (s, 3H). ^{13}C NMR: δ 171.46, 163.46, 146.28, 144.31, 137.12, 132.66, 129.28, 128.14, 122.75, 122.06, 119.27, 117.92, 78.41, 73.94, 29.95, 24.64, 20.64, 12.62, 12.43, 11.68. MS (ES+): *m/z* 443 ($M^{+\cdot}$). Anal. ($C_{22}H_{25}N_3O_7$) C, H, N.

4.1.7. *N*′-(2-Chloroacetyl)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carbohydrazide (11). To a stirred mixture of **9** (132 mg, 0.5 mmol), triethylamine (0.1 mL), and toluene (8 mL) at 0 °C was slowly added 2-chloroacetylchloride (0.1 mL) in toluene (2 mL). Then, it was allowed to stir for 1 h at room temperature. The reaction mixture was filtered and the filtrate washed with brine. The organic layer was dried with sodium sulfate and evaporated under reduced pressure. The product was purified by CC (SiO_2, ethyl acetate/hexane (6:4)). White solid (90 mg, 53%). ^{1}H NMR: δ 8.77 (d, J = 5.2 Hz, 1H), 8.73 (d, J = 5.2 Hz, 1H), 4.33 (s, 1H), 4.14 (s, 2H), 2.72–2.63 (m, 2H), 2.43–2.38 (m, 1H), 2.23 (s, 3H), 2.20 (s, 3H), 2.12 (s, 3H), 2.00–1.94 (m, 1H), 1.58 (s, 3H). ^{13}C NMR: δ 171.62, 162.80, 146.30, 144.26, 122.62, 121.99, 119.29, 118.02, 78.52, 41.33, 29.94, 24.72, 20.62, 12.59, 12.39, 11.67.

4.1.8. 6-Hydroxy-*N*′-(2-nitrooxyacetyl)-2,5,7,8-tetramethylchroman-2-carbohydrazide (12). To a stirred solution of silver nitrate (85 mg, 0.5 mmol) in acetonitrile (2.0 mL) protected from light was added dropwise compound **10** (101 mg, 0.3 mmol) in acetonitrile. The reaction mixture was allowed to stir for 7 days at room temperature. The mixture was concentrated under reduced pressure and the residue 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 CC (SiO_2, hexane/ethyl acetate (1:1)). Yellow solid (22 mg, 20%). mp (°C): 55.0–57.0. ^{1}H NMR: δ 9.24 (d, J = 4.9 Hz, 1H), 8.96 (d, J = 4.9 Hz, 1H), 4.18 (s, 2H), 3.76 (br s, 1H), 2.71–2.64 (m, 1H), 2.54–2.46 (m, 1H), 2.11–2.06 (m, 1H), 2.04 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.72–1.64 (m, 1H), 1.51 (s, 3H). ^{13}C NMR: δ 173.05, 163.58, 143.36 (2C), 141.61, 141.48, 140.83 (2C), 76.34, 41.38, 38.55, 27.17, 21.27, 12.80, 12.63, 12.47. IR: ν_{max} 3300, 2928, 1644, 1541, 1375, 1310, 1211, 756. MS (ES+): *m/z* 367 ($M^{+\cdot}$). Anal. ($C_{16}H_{21}N_3O_7$) C, H, N.

4.1.9. 3-Nitrooxypropyl 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate (13). The title compound was prepared from **2** (500 mg, 2 mmol) and 3-nitrooxypropanol[13] (670 mg, 5.5 mmol) following a similar procedure used for the synthesis of **11**. The reaction mixture was stirred for 24 h at room temperature. The product was purified by CC (SiO_2, hexane/ethyl ether (6:4)). White solid (141 mg, 20%). mp (°C): 84.0–86.0. ^{1}H NMR: δ 4.29–4.22 (m, 1H), 4.19 (s, 1H, –OH), 4.17–4.07 (m, 3H), 2.69–2.52 (m, 2H), 2.49–2.45 (m, 1H), 2.19 (s, 3H), 2.18 (s, 3H), 2.08 (s, 3H),1.95–188 (m, 3H), 1.64 (s, 3H). ^{13}C NMR: δ 174.45, 146.10, 145.83, 122.81, 121.75, 118.98, 117.25, 77.73, 69.63, 60.99, 31.14, 26.66, 25.93, 21.46, 12.53, 12.14, 11.57. IR: ν_{max} 3489, 2926, 1738, 1630, 1450, 1279, 1194, 1138, 1109, 858. MS (EI, 70 eV): *m/z* (%) 307 (M^+-46, 17), 289 (3), 231 (14), 217 (10), 203 (57), 189 (14), 175 (15), 161 (9), 147 (5), 133 (4), 119 (6), 105 (9), 91 (18), 77 (100). Anal. ($C_{17}H_{23}NO_7$) C, H, N.

4.1.10. 3-(3-Phenylsulfonyl-*N*^{2}-oxide-1,2,5-oxadiazole-4-yl)oxypropyl 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylate (15). The title compound was prepared from **2** (100 mg, 0.4 mmol) and alcohol **14** (163 mg, 0.6 mmol) following the procedure used for the synthesis of **13**. Product was purified by CC (SiO_2, hexane/ethyl acetate (7:3)). Colorless oil (43 mg, 20%). ^{1}H NMR: δ 8.05 (dd, J = 8.4 Hz, 0.8 Hz, 2H), 7.75 (t, J = 7.2 Hz, 1H), 7.62 (t,

J = 8.0 Hz, 2H), 4.40–4.34 (m, 1H), 4.29 (br s, 1H, –OH), 4.19–4.11 (m, 2H), 4.08–4.02 (m, 1H), 2.67–2.46 (m, 3H), 2.18 (s, 3H), 2.15 (s, 3H), 2.08–2.07 (m, 2H), 2.02 (s, 3H), 1.93–1.85 (m, 1H), 1.49 (s, 3H). ^{13}C NMR: δ 174.49, 159.02, 146.16, 145.81, 138.49, 135.99, 130.09, 128.89, 122.78, 121.92, 119.08, 117.25, 108.98, 77.58, 67.70, 60.74, 31.20, 28.32, 26.00, 21.42, 12.56, 12.17, 11.55. IR: ν_{max} 3568, 2928, 1734, 1616, 1553, 1450, 1420, 1375, 1261, 1169, 1140, 1086. MS (EI, 70 eV): m/z (%) 500 ($M^{+\cdot}$ −32, 15), 455 (2), 391 (2), 307 (11), 276 (16), 248 (4), 231 (23), 217 (5), 205 (100), 189 (40), 175 (19), 161 (19), 147 (8), 91 (14). Anal. ($C_{25}H_{28}N_2O_9S$) C, H, N, S.

4.1.11. 6-Hydroxy-2,5,7,8-tetramethyl-*N*-[2-(3-phenylsulfonyl-*N*^{2}-oxide-1,2,5-oxadiazole-4-yl)oxyethyl]chroman-2-carboxamide (17). The title compound was prepared from **2** (100 mg, 0.4 mmol) and amine **16** (97 mg, 0.6 mmol) following the procedure used for the synthesis of **13**. The crude product was purified by CC (SiO_2, hexane/ethyl acetate (7:3)). Colorless oil that crystallized at 4 °C (39 mg, 19%).^{1}H NMR (acetone-d_6): δ 8.09 (dd, J = 8.2 Hz, 1.2 Hz, 2H), 7.86 (t, J = 7.5 Hz, 1H), 7.71 (t, J = 7.9 Hz, 2H), 7.38 (br s, 1H), 6.57 (s, 1H), 4.54–4.51 (m, 2H), 3.81–3.65 (m, 2H), 2.69–2.53 (m, 2H), 2.33–2.27 (m, 1H), 2.16 (s, 6H), 2.11 (s, 3H), 1.89–1.82 (m, 1H), 1.64 (s, 3H). ^{13}C NMR (acetone-d_6): δ 174.81, 159.63, 146.76, 144.55, 138.61, 136.13, 130.14, 129.01, 122.73, 122.09, 120.20, 117.97, 105.01, 78.20, 70.47, 38.05, 30.14, 23.90, 20.60, 12.25, 11.78, 11.30. IR: ν_{max} 3438, 2928, 1665, 1613, 1551, 1451, 1358, 1258, 1169, 1086. MS (ES^+): m/z (%) 517 ($M^{+\cdot}$, 5), 293(3), 275(33), 205(62), 189(22), 175(9), 161(8), 77(81). Anal. ($C_{24}H_{27}N_3O_8S$) C, H, N, S.

4.2. X-ray diffraction

Crystal data and refinement results for compound **20**, $C_{19}H_{25}NO_8$, are summarized in the Supplementary Table S1. Diffraction data were collected on an Enraf-Nonius CAD4 diffractometer with EXPRESS[15b] and reduced with XCAD4.[15c] The data were corrected analytically for absorption with PLATON.[15d] The structure was solved by direct and Fourier methods with SHELXS[15e] program and its non-H atom refined by full-matrix least-squares with SHELXL[15f] program. The hydrogen atoms were positioned stereo-chemically and refined with the riding model. The methyl H-atom positions were optimized by treating them as rigid groups which were allowed to rotate around the corresponding C–C bond during the refinement. Atomic fractional coordinates and equivalent isotropic displacement parameters, intra-molecular bond distances and angles, and other crystallographic data are also given as supporting information. The structure has been deposited with the Cambridge Crystallographic Data Centre, reference number CCDC-640896.

4.3. Lipophilicity determination

4.3.1. Experimental. Reversed phase TLC experiments were performed on precoated TLC plates SIL RP-18W/UV254 (Macherey-Nagel, Düren, Germany) and eluted with acetone/water (50:50, v/v). The plates were developed in a closed chromatographic tank and dried, and the spots were located under UV light.

4.3.2. Theoretical. Quantum-chemical calculations were performed for the lowest energy conformation of the compounds using the Spartan'04 suite of programs.[23] The compounds were built with standard bond lengths and angles using the Spartan'04 1.0.1 version, and the geometry of each molecule was fully optimized by applying the semiempirical AM1 method in gas phase from the most stable conformer obtained using molecular mechanics (MMFF) methods. Then, the equilibrium geometry was determined using DFT/6-31G* and Villar Log$P$ calculated at AM1 level.

4.4. Aqueous solubility changes in presence of LDL

A methanolic solution containing studied compound (**19**, **20**, **23** or **24**) (0.2 μmol) was evaporated in order to form a film in the tube surface. Then phosphate buffer (5 mM, pH 7.4) (200 μL) and aqueous solution of LDL (200 or 100 μL) (final concentration 2.8 μM or 1.4 μM) were added. The mixture was homogenized by vortex for 10 s and the aqueous supernatant treated with MeOH (HPLC grade) in order to denature LDL. The amount of studied compounds was determined in the aqueous solution by RP-HPLC (C_{18}, 4.6 × 250 mm, MeOH/H_2O isocratic, 1 mL min^{-1}, detection λ = 260 nm). The same experiment without LDL was repeated for each compound as control. Every value is the average of at least three determinations.

4.5. Biology

4.5.1. $^{\cdot}$NO release evaluation. The rate of $^{\cdot}$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.[24] 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 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 $\Delta\varepsilon = \varepsilon_{401MetHb} - \varepsilon_{401HbO2}$ 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 (1-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}.

4.5.2. Vasorelaxation assays. Wistar Kyoto rats (250–300 g) were heparinized (100 U/mL i/p) and anesthetized (40 mg/kg pentobarbital ip), 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 Krebs–Henseleit 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 submaximal 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 controls. Drug vehicle (DMSO) also served as control and did not affect the experiments.The effect of 1 μM ODQ on relaxation was evaluated by its addition to the bath at least 10 min before compound addition.

4.5.3. Cytotoxicity to macrophages test. J774.1 murine macrophage-like cells (ATCC, USA) were maintained by passage in DMEM containing 4 mM L-glutamine, and supplemented with 10% heat-inactivated fetal calf serum (FCS). THP-1 human monocyte-like cells (generously donated by Dr. C. Rosales, UNAM, México) were maintained in RPMI 1640 medium containing 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 1% sodium piruvate, 10 mM Hepes, and supplemented with 10% heat-inactivated FCS. J774 and THP-1 cells were seeded (1×10^5 cells/well) in 96-well microplates with 100 μL of RPMI medium supplemented with fetal serum. In the case of THP-1 cells, phorbol ester was added to the culture medium (100 ng/mL) to induce macrophage differentiation. Cells were allowed to attach for 48 h in a humidified 5% CO_2/95% air atmosphere at 37 °C and, then, exposed to hybrid compounds (1–500 μM) for 24 h. Afterwards, cell viability was assessed by measuring the mitochondrial-dependent reduction of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) to formazan. For that purpose, MTT was added to cells to a final concentration of 0.2 mg/mL and cells were incubated at 37 °C for 3 h. After removing the media, formazan crystals were dissolved in DMSO, and the absorbance at 560 nm was read using a microplate spectrophotometer. Results are expressed as IC_{50} (compound concentration that reduced 50% control absorbance at 560 nm). Every IC_{50} is the average of at least three determinations.

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

Acknowledgments

We gratefully acknowledge Universidad de la República and PEDECIBA for financial support. This work was supported by grants from FOGARTY-NIH to H.R.; G.V.L. and F.B. thank PEDECIBA for scholarship. The X-ray diffraction experiments were carried out at LANADI (CONICET/UNLP). O.E.P. is a research fellow of CONICET.

Supplementary data

Crystal data, diffraction data collection details, and refinement results for derivative **20** (Table S1). Listings of atomic coordinates and equivalent isotropic displacement parameters (Table S2), bond distances and angles (Table S3), anisotropic displacement parameters (Table S4), and hydrogen atoms' positions and isotropic displacement parameters (Table S5). Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2007.06.019.

References and notes

1. Steinberg, D.; Witztum, J. L. *Circulation* **2002**, *105*, 2107.
2. Rapola, J. M.; Virtamo, J.; Auca, J. K.; Heinonen, O. P.; Albanes, D.; Taylor, P. R.; Huttunen, J. K. *JAMA* **1996**, *275*, 693.
3. de Gaetano, G. *Lancet* **2001**, *357*, 89.
4. Salonen, J. T.; Nyyssonen, K.; Salonen, R.; Lakka, H. M.; Kaikkonen, J.; Porkkala-Sarataho, E.; Voutilainen, S.; Lakka, T. A.; Rissanen, T.; Leskinen, L.; Tuomainen, T. P.; Valkonen, V. P.; Ristonmaa, U.; Poulsen, H. E. *J. Intern. Med.* **2000**, *248*, 377.
5. Stephens, N. G.; Parsons, A.; Schofield, P. M.; Kelly, F.; Cheeseman, K.; Mitchinson, M. J. *Lancet* **1996**, *347*, 781.
6. Boaz, M.; Smetana, S.; Weinstein, T.; Matas, Z.; Gafter, U.; Iaina, A.; Knecht, A.; Weissgarten, Y.; Brunner, D.; Fainaru, M.; Green, M. S. *Lancet* **2000**, *356*, 1213.
7. Marchioli, R. *Lancet* **1999**, *354*, 447.
8. Yusuf, S.; Dagenais, G.; Pogue, J.; Bosch, J.; Sleight, P. *N. Engl. J. Med.* **2000**, *342*, 154.
9. Robinson, I.; de Serna, D. G.; Gutierrez, A.; Schade, D. S. *Endocr. Pract.* **2006**, *12*, 576.
10. López, G. V.; Batthyány, C.; Blanco, F.; Botti, H.; Trostchansky, A.; Migliaro, E.; Radi, R.; González, M.; Cerecetto, H.; Rubbo, H. *Bioorg. Med. Chem.* **2005**, *13*, 5787.
11. Trostchansky, A.; Souza, J. M.; Ferreira, A.; Ferrari, M.; Blanco, F.; Trujillo, M.; Castro, D.; Cerecetto, H.; Baker, P. R.; O'Donnell, V. B.; Rubbo, H. *Biochemistry* **2007**, *46*, 4645.
12. Cerecetto, H.; Lopez, G. V. *Mini Rev. Med. Chem.* **2007**, *7*, 315.
13. Scaramuzzino, G. WO 03094923, 2003; *Chem. Abstr.* **2003**, *139*, 386419.
14. Kawashima, Y.; Ikemoto, T.; Horiguchi, A.; Hayashi, M.; Matsumoto, K.; Kawarasaki, K.; Yamazaki, R.; Okuyama, S.; Hatayama, K. *J. Med. Chem.* **1993**, *36*, 815.
15. (a) Johnson C. K. *ORTEP-II.A Fortran Thermal-Ellipsoid Plot Program*. Report ORNL-5138; Oak Ridge National Laboratory: TN, USA, 1976.; (b) CAD4 Express Software. Enraf-Nonius, Delft, The Netherlands, 1994.; (c) Harms, K.; Wocadlo, S. XCAD4–CAD4 Data Reduction; University of Marburg: Marburg, Germany, 1995.; (d) Spek, A. L. PLATON, A Multipurpose Crystallographic Tool; Utrecht University: Utrecht, The Netherlands, 1998; (e) Sheldrick, G. M. *SHELXS-97. Program for Crystal Structure Resolution*; University of Göttingen: Göttingen, Germany, 1997; (f) Sheldrick, G. M. *SHELXL-97. Program for Crystal Structures Analysis*; University of Göttingen: Göttingen, Germany, 1997.
16. Feelisch, M.; Noack, E. A. *Eur. J. Pharmacol.* **1987**, *139*, 19.
17. Wang, P. G.; Xian, M.; Tang, X.; Wu, X.; Wen, Z.; Cai, T.; Janczuk, A. J. *Chem. Rev.* **2002**, *102*, 1091.
18. Muelas, S.; Di Maio, R.; Cerecetto, H.; Seoane, G.; Ochoa, C.; Escario, J. A.; Gómez-Barrio, A. *Folia Parasitol.* **2001**, *48*, 105.
19. Hehre, W. J.; Shusterman, A. J.; Huang, W. W. *A Laboratory Book of Computational Organic Chemistry*; Wavefunction Inc.: Irvine, CA, 1996.

20. Tsantili-Kakoulidou, A.; AntoniadovUyza, A. *J. Chromatogr.* **1988**, *445*, 317.
21. Lolli, M. L.; Cena, C.; Medana, C.; Lazzarato, L.; Morini, G.; Coruzzi, G.; Namarini, S.; Fruttero, R.; Gasco, A. *J. Med. Chem.* **2001**, *44*, 3463.
22. Sorba, G.; Medana, C.; Fruttero, R.; Cena, C.; Di Stilo, A.; Galli, U.; Gasco, A. *J. Med. Chem.* **1997**, *40*, 463.
23. *Spartan'04*; Wavefunction, Inc. 18401 Von Karman Avenue, Suite.
24. Gibson, Q. H.; Roughton, F. J. *J. Physiol.* **1957**, *136*, 507.