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Novel antioxidant agents deriving from molecular combination of Vitamin C and NO-donor moieties

Clara Cena, Konstantin Chegaev, Silvia Balbo, Loretta Lazzarato, Barbara Rolando, Marta Giorgis, Elisabetta Marini, Roberta Fruttero and Alberto Gasco*

Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via Pietro Giuria 9, I-10125 Torino, Italy

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Abstract—In this paper, we describe a new class of products in which NO-donor moieties are linked to either the 3-*O*H (**4a**–**f**) or 2-*O*H group (**7a**–**c**) of ascorbic acid (ASA). Log *P*s and pK_as of these products were experimentally evaluated. All the compounds were tested for their antioxidant activity on lipidic peroxidation induced by Fe^{3+}-ADP/NADPH in lipids of microsomal membranes of rat hepatocytes. Only 3-*O* series displays antioxidant activity and it seems to be principally dependent on the lipophilicity. Both series trigger in vitro NO-dependent vasodilator properties.

1. Introduction

Today there is an interest in multi-target drugs, namely in products which are able to modulate, directly or through metabolites, more than one physiological target. These compounds could represent an alternative to the use of cocktails of single target drugs in the treatment of complex diseases by combining therapeutic mechanisms. Usually, multi-target drugs are obtained by joining two drugs, or a crucial part of them, through metabolically stable or cleavable linkers.[1] A particular family of these products is represented by appropriate drugs linked to nitric oxide (NO) donor moieties.[2] NO-donor antioxidants belong to this family.[3] These products combine NO-dependent pharmacological properties with radical scavenger activities. They are potentially interesting in the treatment of some forms of cardiovascular disease associated with endothelial dysfunction, such as atherosclerosis, hypercholesterolemia, and hypertension. In these pathologies, an increased concentration of reactive oxygen species (ROS), a decreased ability to produce NO by endothelial cells, a decreased sensitivity of the vessel to the actions of NO, and a NO destruction are heavily implicated.[4] *L*-Ascorbic acid (ASA, **1**) (Fig. 1) is a very important, highly hydrophilic radical scavenger. It is able to efficiently trap a number of radicals, including anion superoxide, peroxyl and hydroxyl radicals, and to restore the antioxidant properties of Vitamin E, a highly lipophilic radical scavenger, by reducing the α-tocopheroxyl radical (α-TO•) to α-tocopherol.[5] ASA seems to play a role in preventing endothelial dysfunction. The mechanism of such an action is largely unknown. Several hypotheses have been formulated including an ascorbate-induced decrease in low density lipoproteins (LDL) oxidation, a scavenging of intracellular superoxide, a potentiated release of NO from circulating or tissue *S*-nitrosothiols, a direct reduction of nitrite to NO, an activation of either endothelial NO synthase (e-NOS) or smooth muscle guanylate cyclase.[6] ASA decomposes upon exposure to heat, UV light, metal ions, and other oxidants. A number of pro-drugs and ASA derivatives have been developed in order to increase the stability of these compounds,[7] including 3-*O*- and 2-*O*-alkylascorbic acids, as for example the methyl derivatives **1a**

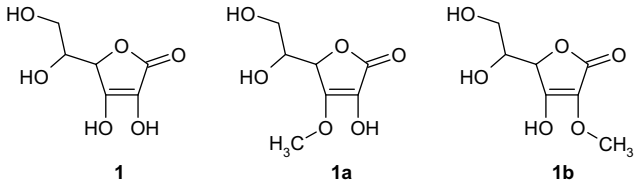

Figure 1. Ascorbic acid (ASA), 3-*O* methyl, and 2-*O* methyl ASA derivatives.

Keywords: Multitarget drugs; NO-donor antioxidants; NO-donor ascorbic acid derivatives.
* Corresponding author. Tel.: +39 0116707670; fax: +39 0116707286; e-mail: alberto.gasco@unito.it

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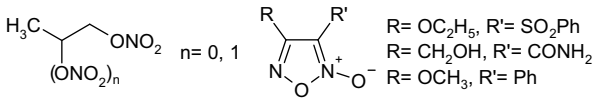


Figure 2. Reference NO-donor compounds.

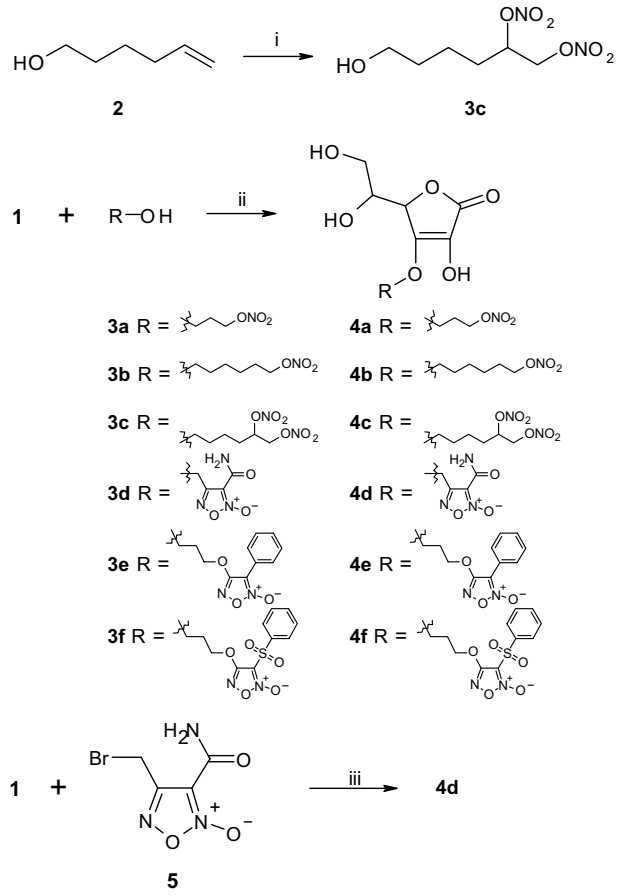
and **1b** (Fig. 1).[8] A number of these latter products displayed inhibitory effects on some lipid peroxidation models and were capable of alleviating myocardial lesions induced by ischemia reperfusion.

As a development of our work in the field of NO-donor antioxidants,[9] here we describe a new class of products in which alkyl chains containing NO-donor nitrooxy and furoxan moieties are linked to either the 3-*O*H (**4a**–**f**) or 2-*O*H group (**7a**–**c**) of ASA. The NO-donor moieties we used in our approach were nitrooxy-substituted alkyl moieties which are present in the simple nitric esters as well as the 3-phenylsulfonylfuroxan-4-yloxy substructure present in 4-ethoxy-3-phenylsulfonylfuroxan, the 3-carbamoylfuroxan-4-ylmethyl substructure present in the 4-hydroxymethyl-3-furoxancarboxamide and the 3-phenylfuroxan-4-yloxy substructure present in the 4-methoxy-3-phenylfuroxan (Fig. 2).[9] These reference compounds display extremely modulated in vitro NO-dependent vasodilator properties. Synthesis of the NO-donor derivatives of Vitamin C, determination of their structures through pK_a and NMR analysis, as well as the study of their lipophilic–hydrophilic balance are reported. Antioxidant properties, assessed in the thiobarbituric acid reactive substances (TBARS) assay and the ability to relax rat aorta strips precontracted with phenylephrine of all the products are also discussed.

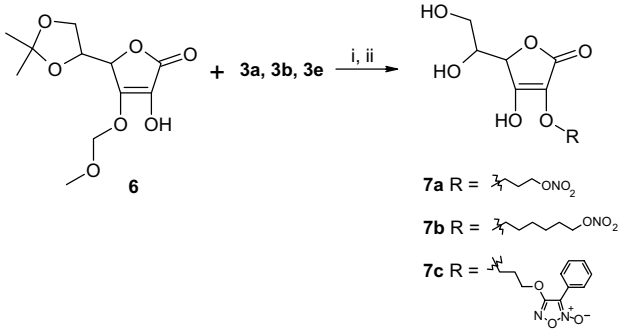
2. Synthesis

All NO-donor alcohols (**3a**–**f**) were previously described in the literature. The synthetic procedure for **3c** was modified with respect to the literature one: the desired product was obtained by treating 5-hexen-1-ol (**2**) with I_2 and $AgNO_3$ (Scheme 1). The 3-*O* substituted compounds **4a**–**f** were prepared by the reactions of corresponding alcohols with ASA under the Mitsunobu conditions (Scheme 1), namely by treating the adduct of PPh_3 and diisopropyl azodicarboxylate (DIAD) in THF solution with ASA, followed by the addition of the appropriate alcohol, resulting in the regioselective formation of the 3-*O* substituted compounds.[10] The products were obtained in a fairly good yields (35 – 43%), with the exception of products **4d** (17%) and **4f** (17%). In the case of **4d**, the yield could be slightly improved (30%) by treating **1** directly with 4-bromomethylfuroxan derivative (**5**) in DMSO solution, in the presence of $NaHCO_3$ (Scheme 1).

To synthesize the final 2-*O* substituted compounds **7a**–**c**, 5,6-*O*-isopropyliden-3-*O*-(methoxymethyl) ascorbic acid (**6**) was treated with the appropriate alcohol under the same Mitsunobu conditions used to prepare the 3-*O* analogues. Removal of the protective groups by treatment with HCl in methanol followed by purification gave rise to the title compounds (Scheme 2).



Scheme 1. Reagents and conditions: (i) I_2, $AgNO_3$, CH_3CN, rt, then reflux; (ii) PPh_3, DIAD, THF dry, −78 °C, then heat up to rt; (iii) $NaHCO_3$, DMSO dry, 50 °C.



Scheme 2. Reagents and conditions: (i) PPh_3, DIAD, −15 °C, then rt; (ii) MeOH, HCl 4 M, rt.

3. Results and discussion

3.1. Ionization constant and NMR studies

Dissociation constants (pK_a) of all the compounds described in the present work (**4a**–**f**, **7a**–**c**), together with those of ASA, **1a** and **1b** taken as references, are col-

Table 1.

Compound	Antioxidant action	Vasodilation	Ionization and lipophilicity		
	IC_{50} (95%CL) (μM)	EC_{50} ± SEM (μM)[a] (+1 μM ODQ)	pK_a ± SD	log $D^{1.0}$ ± SD (CLOGP)	log $D^{7.4}$ ± SD
ASA	—[b]	—	4.08 ± 0.01 (3-*O*H) 10.85 ± 0.03 (2-*O*H)	−1.65 ± 0.06 (−1.76)	—[c]
1a	>1 mM	—	7.54 ± 0.01	−1.41 ± 0.04 (−1.74)	−1.61 ± 0.05
4a	454 (414–497)	55 ± 9 (>100)	7.55 ± 0.01	−0.48 ± 0.05 (−0.94)	−0.55 ± 0.04
4b	80 (77–83)	2.1 ± 0.7 (>100)	7.60 ± 0.02	0.86 ± 0.05 (0.40)	0.76 ± 0.02
4c	28 (26–32)	0.72 ± 0.13 (>100)	7.64 ± 0.01	0.78 ± 0.01 (0.21)	0.66 ± 0.02
4d	>1 mM	6.0 ± 0.8 (>100)	7.07 ± 0.01	−0.99 ± 0.04 (−2.96)	−1.44 ± 0.03
4e	17 (16–18)	3.8 ± 0.4 (80.9 ± 11.7)	7.60 ± 0.03	1.53 ± 0.03 (0.74)	1.13 ± 0.05
4f	80 (76–83)	0.034 ± 0.004 (7.0 ± 0.5)	7.62 ± 0.02	1.18 ± 0.01 (0.99)	0.98 ± 0.01
1b	Inactive at 1 mM	—	3.23 ± 0.02	−1.44 ± 0.04 (−1.46)	—[c]
7a	Inactive at 1 mM	—[d]	3.19 ± 0.01	−0.26 ± 0.02 (−0.66)	—[c]
7b	>1 mM	7.1 ± 0.8 (>100)	3.56 ± 0.01	0.84 ± 0.03 (0.68)	−1.93 ± 0.03
7c	392 (373–414)	32 ± 2 (>100)	3.44 ± 0.01	1.71 ± 0.04 (1.02)	−1.35 ± 0.04

[a] EC_{50} values are means of at least five experiments.
[b] Maximal inhibition of lipid peroxidation in the range 10–50 μM: 77% at 50 μM (see discussion).
[c] Log *D* could not be measured by shake-flask method (log $D < -2$).
[d] EC_{50} could not be calculated because at the maximal concentration tested (100 μM), 50% of tissue relaxation was not achieved.

lected in Table 1. The values were determined by potentiometric titration with GlpKa apparatus (Sirius Analytical Instruments Ltd.). Most of the compounds showed good aqueous solubility and titrations were performed in water. Products **4e** and **4f** required titrations in the presence of methanol as a cosolvent and pK_a values were obtained by extrapolation to zero content of the cosolvent. The pK_a measurements showed that between the two enol hydroxyl groups of the ASA's lactone ring, the 3-*O*H is significantly more acidic than 2-*O*H (Table 1). This difference is partly retained in 3-*O*CH_3 and 2-*O*CH_3 derivatives. The pK_a values for the other compounds of the table are consistent with the assigned structures. In fact, 3-*O* substituted compounds have pK_a near that of the 3-*O*CH_3 model, while 2-*O* substituted compounds are near that of 2-*O*CH_3. This means that the NO-donor moieties linked to the lateral 3-*O* and 2-*O* alkyl chains of **4a**–**f** and **7a**–**c**, scantly influence the pK_a values of these compounds. The only exception is the amide derivative **4d**, in which the electron-withdrawing furoxan substructure is joined to the 3-*O* position through a methylene bridge. The assigned structures were also confirmed by ^{13}C NMR spectra. Recently, ^{13}C NMR spectra of a series of 3-*O* and 2-*O* alkyl derivatives of 5,6-*O*-isopropylidene-*L*-ASA have been critically discussed.[11] In the 3-*O* alkyl derivatives C-3 carbons display characteristic upfield chemical shifts with respect to C-3 carbons in 2-*O* alkyl substituted compounds, while the C-2 carbon chemical shifts are scarcely influenced regardless of the site of alkylation. The same situation occurs for the *O*-alkyl substituted compounds described in the present work (see Section 5).

3.2. Lipophilicity studies

Distribution coefficients (log *D*) between *n*-octanol and water were measured by shake flask technique at room temperature at pH 1 and pH 7.4. The results are reported in Table 1. The 3-*O* substituted compounds, according to their pK_a values, at pH 1 are present essentially as neutral forms (N), and consequently the tabulated log $D^{1.0}$ represent the logarithms of the partition coefficients of these species (log P^N). The measured values are in a good agreement with the calculated values using CLOGP algorithm,[12] with the only partial exception of **4d**. At pH 7.4 all the products are ionized for about 40%, and consequently their log $D^{7.4}$ are lower than their log $D^{1.0}$ of about 0.2 U. A partly different situation occurs for the 2-*O* substituted compounds. According to their pK_a values, they are largely present in the unionized form at pH 1 and in the ionized form (I) at pH 7.4, and consequently log $D^{1.0} \cong$ log P^N and log $D^{7.4} \cong$ log P^I. Generally speaking, all the NO-donor derivatives of ASA described in the present work are definitely more lipophilic than ascorbic acid.

3.3. Antioxidant properties

All the final compounds were assessed as inhibitors of Fe^{3+}-ADP/NADPH-induced peroxidation of membrane lipids of rat hepatocytes. ASA and its 3-*O*CH_3 and 2-*O*CH_3 derivatives were also considered for comparison. The 2-thiobarbituric acid (TBA) assay was used to follow the progress of the autooxidation. The procedure involves the detection of the final metabolites of the lipid autooxidation, namely 2-thiobarbituric acid reactive substances (TBARS) by visible spectroscopy.[13] A number of products under study were able to inhibit, in a concentration-dependent manner, the progress of the reaction (see an example in Fig. 3). The behavior of ASA is worthy of a special comment. The complex role of this product in different lipid peroxidation systems resulting either in antioxidant or pro-oxidant actions has been extensively reported.[14] Under the conditions we set up to carry out our study, ASA was able to inhibit lipidic peroxidation in a concentration-dependent manner in the range from 10 to 50 μM, showing 77% maximal inhibition. By contrast, at higher concentrations it showed a pro-oxidant effect. **1a** and **1b** derivatives were inactive when tested at 1 mM concentration. This is in agreement with the known fact that monoalkylation of the 3-*O* or 2-*O* position of ASA notably reduces the electron-donating activity of the resulting

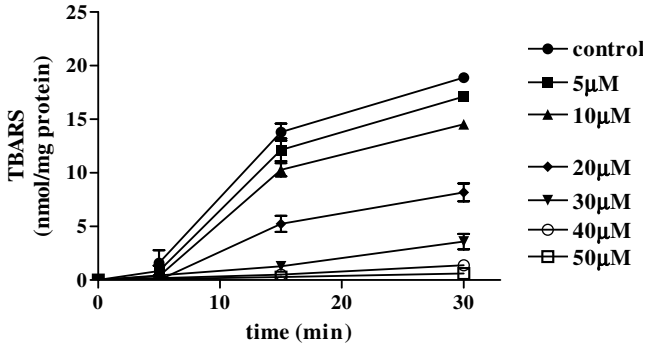


Figure 3. Effect of compound **4e** on time course of lipid peroxidation.

products.[14c] The potencies of the NO-donor ASA derivatives expressed as IC_{50}, namely the molar concentration able to reduce autooxidation by 50%, are collected in Table 1. Analysis of the data shows that 2-*O* substituted compounds do not display any antioxidant activity, with the exception of the feebly active phenylfuroxan derivative (**7c**), which is the most lipophilic member of the group. This behavior probably largely depends on the low lipophilicity of the products, due to their almost complete ionization under physiological pH conditions used to carry out the antioxidant experiments. Actually, it is known that 2-*O*-alkyl ascorbic acids require long lipophilic alkyl moieties to inhibit lipid peroxidation.[8a] The antioxidant behavior of 3-*O* substituted compounds (**4a**–**f**) is quite different from that of the 2-*O* isomers. The products are more lipophilic owing to their higher pK_a values. At pH 7.4, they are ionized for about 40% with the only exception of **4d** which is ionized for 68%. The most active product of the organic nitrate series is the dinitrooxy derivative **4c** followed by mononitrooxy derivatives **4b** and **4a**. Their activities parallel their lipophilicities. In the furoxan series the two active products are the lipophilic compounds **4e** and **4f**, while the hydrophilic amide derivative **4d** is inactive. The important role that the lipophilicity seems to exert on the antioxidant activity in this class of products is in accordance with the finding that the antioxidant potency of 3-*O*-alkyl ascorbic acids, assessed on lipid peroxidation induced in rat microsomes by Fe^{2+} and Fe^{3+}-ADP, displays a parabolic dependence on lipophilicity.[14c] Actually we calculated that the optimal CLOGP of this latter products ranges from 3 to 4. Consequently, their log *D* should range from 2.60 to 3.70, assuming for these products the same pK_a as the parent 3-*O*CH_3 derivative and neglecting the partition of the ionized forms. The low lipophilicity of the 3-*O* NO-donor ascorbic acid derivatives described in the present work indicates that they should be positioned on the ascending branch of parabolic dependence. In the conditions set up to evaluate antioxidant properties of NO-donor ASA derivatives, none of the reference NO-donor substructures (Fig. 2) displayed lipid peroxidation inhibition at 500 μM concentration.

3.4. Vasodilator activity

It is known that organic nitrates and furoxan derivatives display vasodilator activities. The generally accepted mechanism of this action involves their conversion into NO in vascular smooth muscle cells with consequent activation of the soluble guanylate cyclase (sGC). In the case of nitrates this conversion prevalently follows an enzymatic pathway,[15a] while for furoxans it should occur under the action of intracellular thiols.[15b] The vasodilator activities of the new compounds described in the present work were evaluated on denuded rat aorta strips pre-contracted with phenylephrine. All the products were capable of relaxing the contracted tissue in a concentration-dependent manner. The vasodilator potencies, expressed as EC_{50}, are reported in Table 1. In the 3-*O* substituted nitric esters series, the most active product is the dinitrooxy compound **4c**, followed by the nitrooxyhexyl derivative **4b** and its analogue **4a** with a shorter alkyl chain. In the furoxan family, the phenylsulfonyl substituted furoxan **4f** is a very potent vasodilator, followed by **4e** and then by **4d**, which bear 3-phenylfuroxan-4-yloxy and 3-carbamoylfuroxan-4-yl substructures, respectively. Certainly, the very high NO-donor capacity of the 4-phenylsulfonylfuroxan-3-yloxy substructure present in **4f**,[15b] probably combined with its high lipophilicity, plays paramount roles in the high vasodilator potency of the product. In the 2-*O* substituted series, the mononitrooxyhexyl derivative **7b** was the most potent vasodilator, followed by the phenylfuroxan derivative **7c** and by the mononitrooxypropyl derivative **7a**. The members of the 3-*O* series resulted to be more potent than their isomers of the 2-*O* series. When the vasodilator 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 sGC, a decrease in the potencies was observed, which is in keeping with NO-activation of the sGC.

4. Conclusions

A new class of 2-*O*-alkyl and 3-*O*-alkyl ascorbic acids bearing on the alkyl chains NO-donor nitrooxy and furoxan moieties was synthesized. The antioxidant properties of these compounds were evaluated on lipid peroxidation induced in rat microsomes by Fe^{3+}-ADP/NADPH using TBARS detection and taking as references ASA and its *O*-methyl derivatives **1a** and **1b**. Unlike the 2-*O* series the 3-*O* one displays antioxidant activities, which seem to be principally dependent on the higher lipophilicity of the products belonging to this class. All the products were able to relax rat aorta strips precontracted with phenylephrine in a dose-dependent manner. The vasodilator action was decreased by the presence of ODQ, and this is in keeping with a NO activation of the sGC. Selected members of 3-*O* series are worthy of additional *in vivo* study since they are potentially useful in the treatment of some forms of the cardiovascular disease associated with endothelial dysfunction.

5. Experimental

^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 at 300 and 75 MHz, respectively, using

$SiMe_4$ as the internal standard. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. Melting points were determined with a capillary apparatus (Büchi 540). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230-400 mesh ASTM); PE stands for 40–60 petroleum ether. 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 the drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Preparative HPLC was performed on a LiChrospher® C_{18} column (250×25 mm, 10 μm) (Merck Darmstadt, Germany) with a Varian ProStar mod-210 with Varian UV detector mod-325. Elemental analyses (C, H, N) were performed by REDOX (Monza). Compounds **1a**,[16] **1b**,[16] **3a**,[17] **3b**,[17] **3e**,[18] **3f**,[19] **5**[20], and **6**[8a] were synthesized according to the literature. The compound **3d** was a gift from Sanofi-Aventis Deutschland GmbH.

5.1. 6-Hydroxyhexane-1,2-diyl dinitrate (3c)

To a stirred solution of **2** (2.0 mL, 17 mmol) and $AgNO_3$ (9.0 g, 53 mmol) in CH_3CN, I_2 (4.3 g, 17 mmol) was added in one portion. The reaction was stirred at room temperature (rt) until all I_2 dissolved, then it was heated at reflux for 5 h. The precipitate was filtered off and the reaction mixture was concentrated and diluted with EtOAc (100 mL). The organic phase was washed twice with H_2O (100 mL), brine, dried and evaporated. The resulting dark yellow oil was purified by flash chromatography (eluent 7/3 PE/EtOAc) to give the title compound as a yellow oil, 55%. ^{1}H NMR ($CDCl_3$): δ 1.51–1.86 (m, 7H, $HOCH_2(CH_2)_3$), 3.67 (t, 2H, $J = 5.9$ Hz, $HOCH_2$), 4.48 (dd, 1H), 4.76 (dd, 1H) (AMX like, CH_2ONO_2), 5.28–5.35 (m, 1H, $CHONO_2$); ^{13}C NMR (CD_3OD): δ 21.4, 29.1, 31.9, 62.2, 71.3, 79.2. MS: *m/z* 225 $(M+H)^+$.

Lit. data:[21] pale yellow oil. ^{1}H NMR (500 MHz, Acetone-d_6): δ 1.51–1.58 (m, 4H), 1.80–1.88 (m, 2H), 3.50 (t, 1H), 3.55 (d, 2H), 4.72 (dd, 1H), 5.00 (dd, 1H), 5.47–5.53 (m, 1H).

5.2. General procedure for the preparation of products 4a–f

To a stirred solution of PPh_3 (1.2 eq.) in THF dry under positive N_2 pressure, DIAD (1.2 eq.) was added dropwise at −78 °C. After 10 min, the precipitate of Mitsunobu betaine was formed. Stirring was continued for 10 min, then a solution of ASA (1.0 eq.) in DMF dry was added to the above mixture at −78 °C. The cooling bath was removed and a solution of the corresponding alcohol (1.0 eq.) in THF dry was added. The mixture was allowed to reach rt and was stirred overnight. The reaction mixture was concentrated, diluted with EtOAc and the organic phase was washed with H_2O, brine, dried, and evaporated. The resulting oil was purified by flash chromatography with eluents indicated.

5.2.1. 5-(1,2-Dihydroxyethyl)-3-hydroxy-4-(3-nitrooxypropoxy)-5*H*-furan-2-one (4a). The resulting oil was purified first by flash chromatography (eluent 95/5 CH_2Cl_2/MeOH) and further by HPLC (RP-18, eluent 1/1 MeOH/H_2O, 39 mL/min, 100 mg/injection) to give the title compound as a pale yellow oil, 35%. ^{1}H NMR (CD_3OD): δ 2.16–2.24 (m, 2H, $CH_2CH_2CH_2$), 3.67 (d, 2H, $J = 6.9$ Hz, $CH(OH)CH_2(OH)$), 3.86 (t, 1H, $J = 6.6$ Hz, $CH(OH)CH_2(OH)$), 4.61–4.82 (m, 4H, $CH_2CH_2CH_2$), 4.82 (d, 1H, $J = 1.6$ Hz, $CHOC{=}O$); ^{13}C NMR (CD_3OD): δ 28.5, 63.4, 68.8, 70.5, 71.2, 76.6, 121.0, 151.2, 172.9. MS: *m/z* 280 $(M+H)^+$. Anal. ($C_9H_{13}NO_9 \cdot 0.75\ H_2O$) C, H, N: C calcd 36.93, found 36.82, H calcd 4.99, found 4.79, N calcd 4.79, found 4.51.

5.2.2. 5-(1,2-Dihydroxyethyl)-3-hydroxy-4-(6-nitrooxyhexyloxy)-5*H*-furan-2-one (4b). The resulting oil was purified by flash chromatography (eluent 95/5 CH_2Cl_2/MeOH) to give the title compound as a pale yellow oil, 43%. ^{1}H NMR (CD_3OD): δ 1.47–1.49 (m, 4H), 1.72–1.80 (m, 4H) ($CH_2(CH_2)_4CH_2$), 3.65 (d, 2H, $J = 6.9$ Hz, $CH(OH)CH_2(OH)$), 3.84 (t, 1H, $J = 6.6$ Hz, $CH(OH)CH_2(OH)$), 4.47–4.54 (m, 4H, $CH_2(CH_2)_4CH_2$), 4.77 (d, 1H, $J = 1.5$ Hz, $CHOC{=}O$); ^{13}C NMR (CD_3OD): δ 26.2, 26.4, 27.8, 30.6, 63.5, 70.6, 72.5, 74.6, 76.7, 120.5, 152.2, 173.2. MS *m/z* 322 $(M+H)^+$. Anal. ($C_{12}H_{19}NO_9 \cdot 0.25\ H_2O$) C, H, N: C calcd 44.24, found 43.97, H calcd 6.03, found 5.89, N calcd 4.30, found 4.27.

5.2.3. 4-(5,6-Bisnitrooxyhexyloxy)-5-(1,2-dihydroxyethyl)-3-hydroxy-5*H*-furan-2-one (4c). The resulting oil was purified by flash chromatography (eluent 95/5 CH_2Cl_2/MeOH) to give the title compound as a pale yellow oil, 37%. ^{1}H NMR (CD_3OD): δ 1.57–1.63 (m, 2H), 1.79–1.85 (m, 4H) ($(O_2NO)CH(CH_2)_3CH_2O$), 3.66 (d, 2H, $J = 7.0$ Hz, $CH(OH)CH_2(OH)$), 3.82–3.85 (m, 1H, $CH(OH)CH_2(OH)$), 4.52–4.63 (m, 3H, $(O_2NO)CH$-$CH(ONO_2)(CH_2)_3CH_2O$), 4.79 (d, 1H, $J = 1.5$ Hz, $CHOC{=}O$), 4.90–4.94 (m, 1H, $(O_2NO)CHH$ (overlapped with OH signal)), 5.41–5.44 (m, 1H, $CH(ONO_2)$); ^{13}C NMR (CD_3OD): δ 22.2, 29.7, 30.4, 63.4, 70.6, 72.1, 72.9, 76.7, 81.1, 120.6, 151.9, 173.1. MS *m/z* 383 $(M+H)^+$. Anal. ($C_{12}H_{18}N_2O_{12} \cdot 1.25\ H_2O$) C, H, N:: C calcd 35.61, found 36.21, H calcd 5.10, found 4.88, N calcd 6.92, found 6.31.

5.2.4. 4-[2-(1,2-Dihydroxyethyl)-4-hydroxy-5-oxo-2,5-dihydrofuran-3-yloxymethyl]-furoxan-3-carboxylic acid amide (4d). The resulting oil was purified by flash chromatography (eluent 95/5 CH_2Cl_2/MeOH) to give the title compound as a white solid, mp 186–187 °C (MeOH), 17%. ^{1}H NMR (DMSO-d_6 + CD_3OD): δ 3.63 (d, 2H, $J = 6.3$ Hz, $CH(OH)CH_2(OH)$), 3.86 (t, 1H, $J = 6.9$ Hz, $CH(OH)CH_2(OH)$), 4.87 (s, 1H, $CHOC{=}O$), 5.77, 5.87 (dd, 2H, $J = 14.3$ Hz, CH_2O); ^{13}C NMR (DMSO-d_6): 61.5, 63.4, 68.4, 74.4, 110.1, 120.4, 149.1, 155.0, 155.6, 169.9. MS *m/z* 318 $(M+H)^+$. Anal. ($C_{10}H_{11}N_3O_9 \cdot 0.5\ H_2O$) C, H, N: C calcd 36.82, found 36.74, H calcd 3.71, found 3.49, N calcd 12.88, found 13.25.

5.2.5. 5-(1,2-Dihydroxyethyl)-3-hydroxy-4-[3-(3-phenylfuroxan-4-yloxy)propoxy]-5*H*-furan-2-one (4e). The resulting oil was purified by flash chromatography (eluent gradient from 9/1 to 7/3 CH_2Cl_2/EtOAc then 95/5 CH_2Cl_2/MeOH) to give the title compound as a color-

less oil which turns into a white foam during desiccation, 39%. ^{1}H NMR (CD_3OD): δ 2.34–2.42 (m, 2H, $CH_2CH_2CH_2$), 3.63 (d, 2H, J = 6.0 Hz, CH(OH)-CH_2(OH)), 3.85 (t, 1H, J = 7.2 Hz, CH(OH)CH_2(OH)), 4.65–4.78 (m, 5H, $CH_2CH_2CH_2$ + CHOC=O), 7.49–7.57 (m, 3H), 8.11–8.13 (m, 2H) (C_6H_5); ^{13}C NMR (CD_3OD): δ 30.3, 63.5, 68.7, 69.1, 70.6, 76.7, 108.8, 121.0, 123.9, 127.4, 130.0, 131.6, 151.4, 163.8, 172.9. MS m/z 395 $(M+H)^+$. Anal. ($C_{17}H_{18}N_2O_9 \cdot 0.75\ H_2O$) C, H, N: C calcd 50.06, found 50.14, H calcd 4.82, found 4.58, N calcd 6.87, found 6.57.

5.2.6. 4-[3-(3-Phenylsulfonylfuroxan-4-yloxy)propoxy]-5-(1,2-dihydroxyethyl)-3-hydroxy-5*H*-furan-2-one (4f). The resulting oil was purified by flash chromatography (eluent gradient from 98/2 to 95/5 CH_2Cl_2/MeOH) to give the title compound as a white solid, mp 144–147 °C (dec.), 17%. ^{1}H NMR (CD_3OD): δ 2.26–2.34 (m, 2H, $CH_2CH_2CH_2$), 3.64 (d, 2H, J = 6.9 Hz, CH(OH)CH_2(OH)), 3.82–3.87 (m, 1H, CH(OH)-CH_2(OH)), 4.58 (t, 2H), 4.68 (t, 2H) ($CH_2CH_2CH_2$), 4.81 (d, 1H, J = 1.3 Hz CHOC=O), 7.67–7.72 (m, 2H), 7.80–7.85 (m, 1H), 8.04–8.06 (m, 2H) (C_6H_5); ^{13}C NMR (CD_3OD): δ 30.1, 63.4, 68.7, 69.0, 70.6, 76.7, 111.8, 121.0, 129.6, 131.0, 137.0, 139.5, 151.4, 160.4, 172.9. MS m/z 459 $(M+H)^+$. Anal. ($C_{17}H_{18}N_2O_{11}S \cdot 0.5\ H_2O$) C, H, N: C calcd 43.68, found 43.91, H calcd 4.10, found 3.89, N calcd 5.99, found 5.78.

5.3. Alternative procedure for the preparation of 4d

To a stirred solution of ASA (0.20 g, 1.1 mmol) in DMSO dry (10 mL) under positive N_2 pressure, $NaHCO_3$ (0.14 g, 1.7 mmol) was added at rt and the reaction mixture was stirred for 1 h. Then **5** (0.22 g, 1 mmol) was added and the reaction mixture was heated at 50 °C overnight. The next day the reaction mixture was poured into H_2O (30 mL) and extracted with EtOAc (3×20 mL). Organic phases were combined, washed with brine, dried and evaporated. The resulting solid was crystallized twice from MeOH to give the title compound as a white solid, 30%.

5.4. General procedure for the preparation of products 7a–c

(i) To a stirred solution of PPh_3 (1.2 eq.) in THF dry under positive N_2 pressure, DIAD (1.2 eq.) was added dropwise at −15 °C. After 15 min, a precipitate of Mitsunobu betaine was formed. Stirring was continued for 10 min, then **6** (1.1 eq.) was added, followed by an addition of the corresponding alcohol (1.0 eq.) (solid or in THF dry solution). The mixture was stirred at −15 °C for 30 min, then the cooling bath was removed, the reaction was allowed to reach rt and was stirred for additional 2 h. Then the reaction mixture was concentrated, diluted with EtOAc and the organic phase washed with H_2O, $NaHCO_3$ sat. sol., brine, dried, and evaporated. The resulting oil was purified by flash chromatography with the eluents indicated. Intermediate protected products were immediately used for the deprotection reaction.

(ii) To a stirred solution of the protected 2-*O* substituted ASA derivatives (0.80–1.0 g) in MeOH (10 mL), 4 M HCl (2 mL) was added and the reaction was stirred at rt until complete (TLC control). The reaction mixture was concentrated, diluted with H_2O and extracted with EtOAc. The organic phase was washed with brine, dried and evaporated. The title products were purified as indicated.

5.4.1. 5-(1,2-Dihydroxyethyl)-4-hydroxy-3-(3-nitrooxypropoxy)-5*H*-furan-2-one (7a). (i) The intermediate protected compound was purified by flash chromatography (eluent 98/2 CH_2Cl_2/ EtOAc), 76%. (ii) The resulting oil was purified by flash chromatography (eluent EtOAc) and lyophilized to give the title compound as colorless oil, 81%. ^{1}H NMR (CD_3OD): δ 2.04–2.13 (m, 2H, $CH_2CH_2CH_2$), 3.67 (d, 2H, J = 6.9 Hz, CH(OH)-CH_2(OH)), 3.92 (t, 1H, J = 6.3 Hz, CH(OH)CH_2(OH)), 4.04–4.12 (m, 2H), 4.67 (t, 2H, J = 6.6 Hz) ($CH_2CH_2CH_2$), 4.86 (s, 1H, CHOC=O (overlapped with OH signal)); ^{13}C NMR (CD_3OD): δ 28.3, 63.4, 69.1, 70.5, 71.4, 76.9, 121.8, 161.7, 172.8. MS: m/z 280 $(M+H)^+$. Anal. ($C_9H_{13}NO_9 \cdot 0.75\ H_2O$) C, H, N: C calcd 36.93, found 36.50, H calcd 4.99, found 4.51, N calcd 4.78, found 4.67.

5.4.2. 5-(1,2-Dihydroxyethyl)-4-hydroxy-3-(6-nitrooxyhexyloxy)-5*H*-furan-2-one (7b). (i) The intermediate protected compound was purified by flash chromatography (eluent 99/1 CH_2Cl_2/ EtOAc), 87%. (ii) The resulting oil was dissolved in H_2O and water was washed twice with Et_2O and then extracted with EtOAc. The organic solvent was removed to give colorless oil which was lyophilized to give the title product as an extremely hydroscopic pale yellow solid, 45%. ^{1}H NMR (CD_3OD): δ 1.40–1.53 (m, 4H), 1.76–1.78 (m, 4H) ($CH_2(CH_2)_4CH_2$), 3.67 (d, 2H, J = 6.9 Hz, CH(OH)CH_2(OH)), 3.89–4.05 (m, 3H), 4.49 (t, 2H, J = 6.6 Hz) (CH(OH)CH_2(OH), $CH_2(CH_2)_4CH_2$), 4.84 (d, 1H, J = 1.8 Hz, CHOC=O (overlapped with OH signal)); ^{13}C NMR (CD_3OD): δ 26.3, 26.5, 27.4, 29.5, 63.5, 70.5, 72.9, 74.7, 76.8, 122.1, 161.2, 173.0. MS m/z 322 $(M+H)^+$. Anal. ($C_{12}H_{19}NO_9 \cdot 0.5\ H_2O$) C, H, N: C calcd 43.64, found 43.83, H calcd 6.10, found 6.02, N calcd 4.24, found 4.32.

5.4.3. 5-(1,2-Dihydroxyethyl)-4-hydroxy-3-[3-(3-phenylfuroxan-4-yloxy)propoxy]-5*H*-furan-2-one (7c). (i) The intermediate protected compound was purified by flash chromatography (eluent 98/2 CH_2Cl_2/EtOAc), 80%. (ii) The resulting oil was dissolved in H_2O and the water was washed twice with CH_2Cl_2 and then extracted with EtOAc. The organic solvent was removed to give a colorless oil which was lyophilized to give the title compound as a white solid, mp 153–156 °C (dec.), 52%. ^{1}H NMR (CD_3OD): δ 2.24–2.32 (m, 2H, $CH_2CH_2CH_2$), 3.67 (d, 2H, J = 6.9 Hz, CH(OH)CH_2(OH)), 3.91 (t, 1H, J = 7.2 Hz, CH(OH)CH_2(OH)), 4.22 (t, 2H, J = 6.0 Hz), 4.69 (t, 2H, J = 6.0 Hz) ($CH_2CH_2CH_2$), 4.84 (s, 1H, CHOC=O), 7.48–7.54 (m, 3H), 8.10–8.13 (m, 2H) (C_6H_5); ^{13}C NMR (CD_3OD): δ 30.1, 63.6, 68.9, 69.3, 70.5, 76.9, 108.9, 122.0, 123.9, 127.0, 129.6, 131.6, 161.6, 163.8, 172.8. MS m/z 395 $(M+H)^+$. Anal.

($C_{17}H_{18}N_2O_9$) C, H, N: C calcd 51.78, found 51.55, H calcd 4.60, found 4.62, N calcd 7.10, found 7.18.

5.5. Ionization and lipophilicity studies

Potentiometric titrations were performed using the GLpK_a apparatus (Sirius Analytical Instruments Ltd., Forrest Row, East Sussex, UK). Ionization constants were determined by at least four separate titrations for each compound: different aqueous solutions (ionic strength adjusted to 0.15 M with KCl) of the compounds (20 mL, about 1 mM) were initially acidified to pH 1.8 with 0.5 N HCl; the solutions were then titrated with standardized 0.5 N KOH to pH 10.5. The low aqueous solubility of **4e** and **4f** required titrations in the presence of methanol as a cosolvent: at least five different hydro-organic solutions (ionic strength adjusted to 0.15 M with KCl) of the compounds (20 mL, about 1 mM in 15–50 wt% methanol) were initially acidified to pH 1.8 with 0.5 N HCl; the solutions were then titrated with standardized 0.5 N KOH to pH 10.5. The initial estimates of the p$_s K_a$ values (the apparent ionization constants in the water–methanol mixtures) were obtained and aqueous pK_a values were determined by extrapolation to zero content of the cosolvent according to the Yasuda-Shedlovsky procedure.[22] All the titrations were performed under argon at 25.0 ± 0.1 °C.

The apparent partition coefficients (log D^{pH}) were measured by the shake-flask procedure at pH 1.0 and 7.4 (HCl and phosphate buffer solutions with ionic strength adjusted to 0.15 M with KCl, respectively). *n*-Octanol was added to the buffers and the two phases were mutually saturated by shaking for 4 h. The compounds were solubilized in the buffered aqueous phase at a concentration of about 0.1 mM and an appropriate amount of *n*-octanol was added. The two phases were shaken for about 20 min, by which time the partitioning equilibrium of solutes was reached, and then centrifuged (10,000 rpm, 10 min). The concentration of the solutes in the aqueous phase was measured by UV spectrophotometer (UV-2501PC, Shimadzu) at λ_{max}. For each compound at least seven log *D* values at different pHs were measured.

5.6. Antioxidant activity

Microsomal membranes from male Wistar rats (180–200 g) were prepared by differential centrifugation (8000*g*, 20 min; 120,000*g*, 1 h) in a HEPES/Sucrose buffer (10 mM/250 mM, pH 7.4), followed by resuspension in a Tris–HCl/KCl buffer (100 mM/150 mM, pH 7.4), recentrifugation (120,000*g*, 1 h), and were stored at −80 °C. Incubation was performed at 37 °C in a Tris–HCl/KCl buffer containing microsomal membranes (2 mg prot/mL), a mixture of ADP (1 mM) and $FeCl_3$ (50 μM), and DMSO solutions of the tested compounds. The addition of DMSO alone (maximal amount 5%) did not change significantly the extent of peroxidation in the control experiments. Lipid peroxidation was initiated by adding NADPH (0.4 mM).[23,24] Aliquots were taken from the incubation mixture at 5, 15, and 30 min and treated with trichloroacetic acid (TCA) 10% p/V. Lipid peroxidation was assessed by spectrophotometric (543 nm) determination of the TBARS consisting mainly of malondialdehyde (MDA), and TBARS concentrations (expressed in nmol/mg protein) were obtained by interpolation with a MDA standard curve. The antioxidant activity of tested compounds was evaluated as the % of inhibition of TBARS production with respect to control samples, using the plateau values obtained after 30 min of incubation. IC_{50} values were calculated by nonlinear regression analysis of at least five experiments.

5.7. Vasodilator activity

Thoracic aortas were isolated from male Wistar rats weighing 180–200 g. 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 endothelium was removed and the vessels were helically cut: three strips were obtained from each aorta. The tissues were mounted under 1.0 g tension in organ baths containing 30 ml of 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 then contracted with 1 μM L-phenylephrine. When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. Results are expressed as EC_{50} ± SEM (μM). The effects of 1 μM ODQ on relaxation were evaluated in separate series of experiments in which it was added to the organ bath 5 minutes before the contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab®. The addition of the drug vehicle, DMSO, had no appreciable effect on contraction level.

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