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Development of a New Class of Potential Antiatherosclerosis Agents: NO-Donor Antioxidants

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Abstract—A new class of NO-donor phenol derivatives is described. The products were obtained by joining appropriate phenols with either nitrooxy or 3-phenylsulfonylfuroxan-4-yloxy moieties. All the compounds proved to inhibit the ferrous salt/ascorbate induced lipidic peroxidation of membrane lipids of rat hepatocytes. They were also capable of dilating rat aorta strips precontracted with phenylephrine.

Atherosclerosis is the main cause of morbidity and mortality in the western societies.[1] This pathology is closely correlated with endothelial dysfunction resulting from an increase of plasma lipids, peroxidation of low-density lipoproteins (LDLs) and an impairment of endothelial-derived relaxing factor (EDRF, nitric oxide, $NO^{\bullet}$)-mediated bioactions. Oxidative modifications of high levels of LDLs following oxidative stress, leads to the formation of foam cells, precursors of atherosclerotic plaques. Experimental evidence supports the hypothesis that the treatment of hypercholesterolemic animals and human subjects with appropriate amounts of antioxidants can reduce the LDL oxidation and the expression of atherosclerosis.[1,2] In an atherosclerotic blood vessel, $NO^{\bullet}$ bioactions are impaired by a number of reasons including a possible decreased $NO^{\bullet}$ production, an increased $NO^{\bullet}$ inactivation and a decrease in responsiveness of the target cells to $NO^{\bullet}$. By contrast, vasodilation to exogenous sources of $NO^{\bullet}$ seems to be preserved.[3] On these bases we have recently designed a large series of hybrid structures in which a number of selected antioxidants were linked to NO-donor moieties through appropriate spacers. Here we report the preliminary results of a study on the capacity of inhibiting the ferrous salt/ascorbate induced lipid peroxidation of membrane lipids of rat hepatocytes and on the in vitro vasodilating properties of a first series of such products. As NO-donor moieties, we chose either the nitrooxy function present in simple vasodilating nitric esters **1** (der.s **17**, **20**, **24**) or the 3-phenylsulfonylfuroxan-4-yloxy moiety present in 4-ethoxy-3-phenylsulfonylfuroxan (CHF 2363) **2**, a known NO-dependent vasodilator[4] (der.s **10**, **11**, **15**). As antioxidants, we considered phenols characterized by having different O—H bond dissociation energy (BDE) which is one of the factors that contribute to the antioxidant capacity of the products: *p*-cresol (**25,** BDE, 86.2 ± 0.6 kcal/mol), 2,6-di-*t*-butyl-*p*-methylphenol (**26**, BDE, 81.02 ± 0.13 kcal/mol) and 6-hydroxy-2,2,5,7,8-pentamethylchroman, the phenol substructure present in the vitamin E, (**27,** BDE, 78.2 ± 0.18 kcal/mol).[5]

Chemistry

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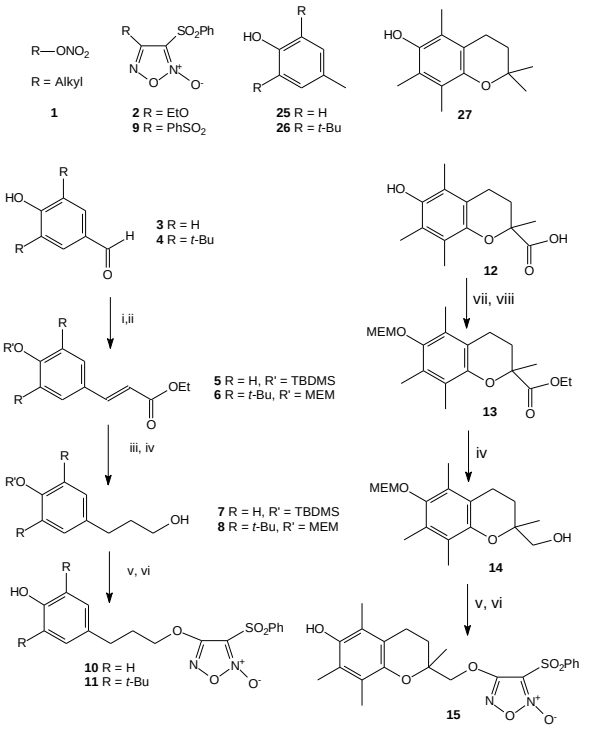
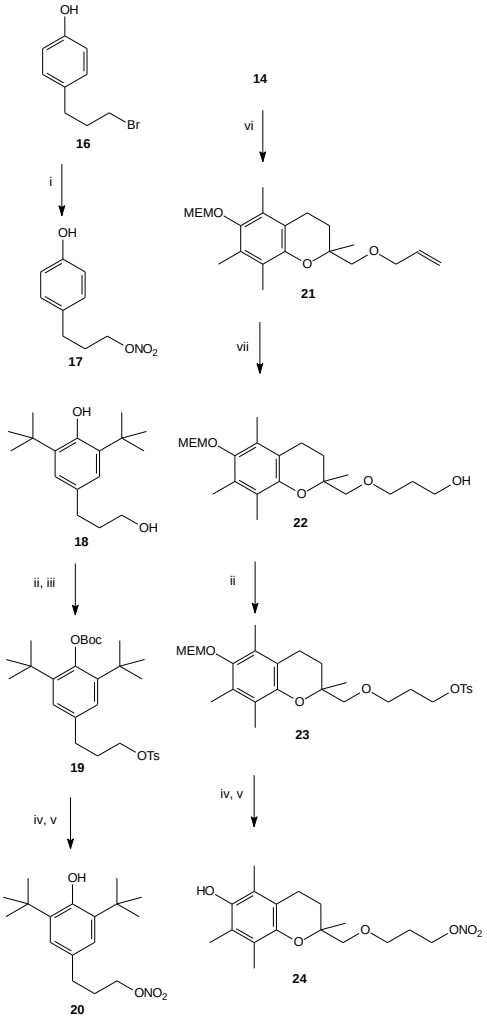
The preparation of the furoxan derivatives follows the synthetic routes that are illustrated in Scheme 1.[6] The hydroxyl groups of the *p*-hydroxybenzaldehyde (**3**) and of its 2,3-di-*t*-butyl derivative **4** were protected with *t*-butyldimethylsilyl chloride (TBDMSCl) and 2-methoxyethoxymethyl chloride (MEMCl) respectively. The resulting products were subjected to the modified Wittig reaction in the presence of the phosphonacetic acid triethyl ester in basic medium to obtain the α,β-unsaturated esters **5** and **6** respectively. Both the products were transformed into the corresponding saturated alcohols **7** and **8** by reduction first with H_2, Pd/C and then with $LiAlH_4$. Selective nucleophilic displacement of the 4-phenylsulfonyl group of the 3,4-bis(phenylsulfonyl)furoxan (**9**) by the intermediates **7** and **8** in dry THF in the presence of NaH followed by acid deprotection of the phenol functions afforded the final products **10**, **11**. The starting material for the preparation of the furoxan **15** was the commercial acid **12**. This product was treated with ethanol in the presence of *p*-toluensulfonic acid (*p*-TSA) to obtain the corresponding ester that was MEM protected on the hydroxy group to afford **13**. This latter compound was reduced with $LiAlH_4$ in dry THF to the corresponding alcohol **14** and then left to react with **9** in basic medium to give, after deprotection of the phenol function, the final product **15**.



Scheme 1. i: **3**, TBDMSCl, NaH, THF; **4**, MEMCl, di-*iso*-propyl ethylamine, $C_2H_4Cl_2$, reflux; ii: $(EtO)_2POCH_2COOEt$/*t*-BuOK, THF, -78 °C; iii: H_2, Pd/C, EtOH; iv: $LiAlH_4$, THF; v: **9**, NaH, THF; vi: HCl/dioxane to obtain **10**, CF_3COOH/CH_2Cl_2 to obtain **11** and **15**; vii: *p*-TSA, EtOH, reflux; viii: MEMCl, NaH, THF.

Nitrooxy derivatives were synthesized according to the pathway reported in Scheme 2.[7] The simple nitrooxy derivative **17** was obtained by the action of $AgNO_3$ on 4-(3-bromopropyl)phenol (**16**) in acetonitrile at 60 °C. To prepare the final product **20**, the alcoholic group of **18** was treated with *p*-toluensulfonyl chloride (TsCl) in the presence of triethylamine (TEA). The corresponding tosylate, obtained in fair yield, was later protected on the phenol group with di-*t*-butyl dicarbonate $(t\text{-Boc})_2O$, in the presence of 4-*N*,*N*-dimethylaminopyridine (DMAP) to give **19**. This latter product was treated in refluxing benzene with tetrabutylammonium nitrate (Bu_4NNO_3) and then at room temperature with a CH_2Cl_2 solution of trifluoroacetic acid to afford the final compound **20**. The MEM protected 6-hydroxychroman structure **14** was used as a starting material for the preparation of **24**. It was transformed into **21** by reaction with allyl bromide and NaH in *N*,*N*-dimethylformamide (DMF). This latter product was left to react fist with 9-borabicyclo[3.3.1]nonane (9-BBN) in THF and then with hydrogen peroxide and sodium acetate to give the propanol derivative **22**. The corresponding tosylate **23**, obtained under the same conditions used to prepare the tosylate of **18**, was transformed into the final nitrooxyderivative **24** following procedures similar to those used to transform **19** into **20**.



Scheme 2. i: $AgNO_3$, CH_3CN, 60 °C; ii: TsCl, TEA, CH_2Cl_2, rt; iii: $(t\text{-Boc})_2O$, DMAP, CH_2Cl_2, rt; iv: Bu_4NNO_3, benzene, reflux; v:TFA, CH_2Cl_2, rt; vi: allyl bromide, NaH, DMF, rt; vii: a) 9-BBN, THF, rt, b) H_2O_2, NaOAc, 0 °C.

Results and Discussion

All the products described in the present work were

assessed as inhibitors of ferrous salt/ascorbate induced lipidic peroxidation of membrane lipids of rat hepatocytes.[8] The progress of the peroxidation was followed by visible spectroscopy detection of 2-thiobarbituric acid reactive substances (TBARS) which are the final metabolites of the autoxidation. All the products were able to inhibit the lipid oxidation in a dose dependent manner. One example is reported in Figure 1.

The potencies as antioxidants of these compounds (IC_{50}) are collected in Table 1 together with those of the parent phenols. The hybrid products show potencies near to those of the reference phenols, with the only partial exception of the derivative **10**. It is noteworthy that also the NO-donor furoxan **2** displays an antioxidant action, which is close to that of **25.**

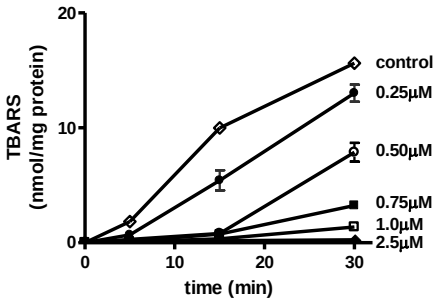


Figure 1.Effect of **15** on time course of lipid peroxidation

The potential antioxidant activity of furoxan derivatives is an interesting aspect of the furoxan chemistry that is worthy of additional investigation.

All the hybrid products behaved as potent *in vitro* vasodilators. They were capable of dilating in a dose dependent manner rat aorta strips precontracted with phenylephrine.[9] One example is reported in Figure 2.

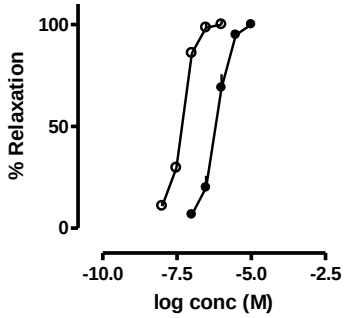


Figure 2. Concentration-response curves for vasodilating activity of compound **15** in the absence (open circle) and in the presence (solid circle) of ODQ.

The vasodilating potencies (EC_{50}) of all the compounds are collected in Table 1. This action is cyclic GMP (cGMP) dependent since a considerable decrease in the potency was observed when the experiments were repeated in the presence of 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a well known inhibitor of the sGC.[10] A comparison between the IC_{50} and EC_{50} values shows that in this series of products the vasodilating action prevails over the antioxidant one. Also the nitrooxy derivatives proved to be dose dependent antioxidants with a potency similar to that of the reference phenols (Table 1). They too display *in vitro* sGC dependent vasodilating activity but it is less potent than the corresponding furoxan derivatives' activity (Table 1). Work is in progress in order to obtain products with better balanced antioxidant and vasodilating activities. All the products reported in this preliminary paper could be of value as potential antiatherosclerotic agents and they are worthy of *in vivo* further investigations.

Table 1. Antioxidant and vasodilating activity of the reference NO-donors **1-2**, of the hybrids **10**, **11**, **15, 17**, **20** , **24** and of the parent phenols **25-27**

Compd	Antioxidant activity	Vasodilating activity	
			+ ODQ
	IC_{50} (95% CL) μM[a]	EC_{50} ± SE μM[a]	EC_{50} ± SE μM
1(R=*n*-pr)	[b]	41±6	[c]
2	110 (98-122)	0.012 ± 0.002	1.2 ± 0.2
10	47 (45-48)	0.012 ± 0.001	0.36 ± 0.09
11	2.0 (1.9-2.0)	0.11 ± 0.03	4.8 ± 0.5
15	0.49 (0.48-0.50)	0.044 ± 0.004	0.67 ± 0.09
17	143 (133-153)	1.0 ± 0.2	[c]
20	2.0 (1.9-2.1)	40 ± 1	[c]
24	0.15 (0.15-0.16)	1.2 ± 0.1	10 ± 1
25	290 (260-324)	-	-
26	1.7 (1.6-1.9)	-	-
27	0.17 (0.16-0.17)	-	-

[a]Values are means of at least five experiments.
[b]Derivative **1** was inactive when tested at 1mM.
[c]The products **1**, **17** and **20** induced respectively 4.6 ± 0.6, 38 ± 9, 15 ± 5 % of vasodilation when tested at 100 μM.

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6. *Compound **10***: m.p. 92-93 °C (EtOH); *m/z* (EI): 376 (M^+); ^{1}H NMR (DMSO-d_6): δ 9.19 (s, 1H, OH); 8.05-8.03 (m, 2H), 7.93-7.88 (m, 1H), 7.78-7.73 (m, 2H) ($PhSO_2$); 7.00-6.98 (m, 2H), 6.70-6.67 (m, 2H) (PhOH); 4.34 (t, 2H, J = 6.2 Hz, CH_2O); 2.57 (t, 2H, J = 7.5 Hz, CH_2Ph); 2.00 (m, 2H, $CH_2CH_2CH_2$). Anal. ($C_{19}H_{20}N_2O_8S$) C, H, N. *Compound **11***: m.p. 110-111 °C (EtOH); *m/z* (EI): 488 (M^+); ^{1}H NMR (DMSO-d_6):

δ 8.05-8.02 (m, 2H), 7.89-7.86 (m, 1H), 7.77-7.72 (m, 2H) ($PhSO_2$); 6.86 (s, 2H, PhOH); 6.74 (s, 1H, OH); 4.33 (t, 2H, J = 5.9 Hz, CH_2O); 2.57 (t, 2H, J = 7.2 Hz, CH_2Ph); 1.99 (m, 2H, $CH_2CH_2CH_2$); 1.33 (s, 18H, *t*Bu). Anal. ($C_{25}H_{32}N_2O_6S$) C, H, N. *Compound* ***15***: m.p. 68-72 °C dec. (MeOH/H_2O); *m/z* (EI): 460 (M^+); ^{1}H NMR ($CDCl_3$): δ 8.00-7.98 (m, 2H), 7.72-7.68 (m, 1H), 7.53-7.48 (m, 2H) ($PhSO_2$); 4.46 (1H, d, AB system, J = 10.4 Hz, CH_aH_bO); 4.40 (1H, d, AB system, J = 10.4 Hz, CH_aH_bO); 2.71 (m, 2H, 4-CH_2-chromane); 1.94-1.87 (m, 1H), 1.28-1.22 (m, 1H) (3-CH_2-chromane); 2.16 (s, 3H), 2.14 (s, 3H), 2.02 (s, 3H) (5,7,8-CH_3-chromane); 1.41 (s, 3H, 2-CH_3-chromane). Anal. ($C_{22}H_{24}N_2O_7S$) C, H, N.

7. *Compound* ***17***: oil; *m/z* (EI): 197 (M^+); ^{1}H NMR ($CDCl_3$): δ 7.05-7.02 (m, 2H, Ph), 6.80-6.78 (m, 2H, Ph); 5.92 (br, 1H, OH), 4.42 (t, 2H, J = 6.5 Hz, CH_2O); 2.65 (t, 2H, J = 8.0 Hz, CH_2Ph); 2.04-1.95 (m, 2H, $CH_2CH_2CH_2O$). Anal. ($C_9H_{11}NO_4$) C, H, N. *Compound* ***20***: m.p.78-79 °C; *m/z* (EI): 309 (M^+); ^{1}H NMR ($CDCl_3$): δ 6.96 (s, 2H, Ph), 5.10 (s, 1H, OH), 4.47 (t, 2H, J = 6.5 Hz, CH_2O); 2.65 (t, 2H, J = 7.7 Hz, CH_2Ph); 2.06-2.00 (m, 2H, $CH_2CH_2CH_2O$); 1.43 (s, 18H, *t*Bu). Anal. ($C_{17}H_{27}NO_4$) C, H, N. *Compound* ***24***: oil; *m/z* (EI): 339 (M^+); ^{1}H NMR ($CDCl_3$): δ 4.57 (t, 2H, J = 6.5 Hz, CH_2ONO_2); 4.19 (s, 1H, OH); 3.63-3.58 (m, 2H, OCH_2CH_2); 3.47 (1H, d, AB system, J = 9.8 Hz, CH_aH_bO) 3.41 (1H, d, AB system, J = 9.8 Hz, CH_aH_bO); 2.61 (m, 2H, 4-CH_2-chromane); 2.15 (s, 3H); 2.11 (s, 3H); 2.09 (s, 3H) (5,7,8-CH_3-chromane); 2.03-1.92 (m, 3H) (3-CH*H*-chromane and $CH_2CH_2ONO_2$); 1.79-1.72 (m, 1H, 3-CH*H*-chromane); 1.57 (s, 3H, 2-CH_3-chromane); Anal. ($C_{17}H_{25}NO_6$) C, H, N.
8. Microsomal membranes from male Wistar rats (200-250 g) were prepared by differential centrifugation (8000 × *g*, 20 min; 120000 × *g*, 1h) in a HEPES/Sucrose buffer (10 mM, 250 mM, pH = 7.4) and stored at –80°C. Incubation was performed at 37°C in a Tris-HCl/KCl buffer (100 mM, 150 mM, pH = 7.4) containing microsomal membranes (2 mg prot/mL), ascorbic acid (100 μM) and DMSO solutions of the tested compounds. 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 $FeSO_4$ 2.5 μM. Aliquots were taken from the incubation mixture at 5, 15 and 30 min and treated with trichloroacetic acid (TCA) 10% 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.[11] 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 non linear regression analysis.
9. The experiments were assessed according to the procedure described in *J. Med. Chem.* **1997**, *40*, 463-469 with little modification: the aortic strips were contracted with 1μM L-phenylephrine; the effect of 1 μM ODQ on relaxation was evaluated in a separate series of experiments in which it was added to the bath at least 5 min before the contraction; responses were recorded by isometric transducer connected to the MacLab System PowerLab.
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