Brief Articles

Nitric Oxide Releasing Morpholine Derivatives as Hypolipidemic and Antioxidant Agents

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The synthesis and evaluation of activity of some nitric acid esters of substituted morpholines are presented. All compounds inhibit lipid peroxidation and reduce cholesterol (20−63%) and triglyceride (37−85%) plasma levels. The more potent NO donors **14** and **17** specifically reduce low-density lipoprotein (LDL). These data indicate that proper structural modifications to the hypolipidemic and antioxidant morpholines enabling NO production, besides preserving or enhancing the above activities, offer a remarkable reduction of LDL, considered advantageous for antiatheromatic agents.

Introduction

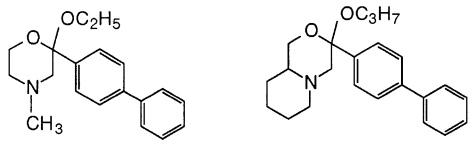
Cardiovascular disease, considered to be the bane of western civilization of the 21st century, and in particular atherosclerosis are associated with increased concentration of plasma lipids, oxidative damage of low-density lipoproteins (LDLs), and dysregulation of endogenous nitric oxide (NO) activity. The oxidation of LDL lipids leads to the formation of foam cells, fatty streaks, and atheromatic plaques.[1] Furthermore, the amount of NO at the site of endothelial injury and NO-mediated vasodilation are dramatically decreased. The reduced levels of endogenous NO have been attributed to the formation of the most toxic peroxynitrite anion radical via the reaction of NO with the superoxide anion radical.[2] Finally, it has been proposed that NO possesses hypocholesterolemic activity due to its ability to interfere with the production of apoprotein B (apo B), the main constituent of LDLs.[3]

We have recently reported[4] the hypocholesterolemic and hypolipidemic effects of some new morpholine derivatives with antioxidant activity (Chart 1). Extending that study in the present investigation, we find it of interest to properly modify the above structures so that the new molecules would preserve the hypolipidemic and antioxidant activity and in addition could act as NO donors. Thus, the combination of antioxidant, hypolipidemic, and NO-releasing potential could favor the inhibition of the major atherogenic mechanisms. The designed molecules are 2-(3-nitrooxypropoxy)-2-arylmorpholine derivatives, and the examined parameters are their ability to generate NO, their effect on plasma lipid levels, and the inhibition of lipid peroxidation.

Results and Discussion

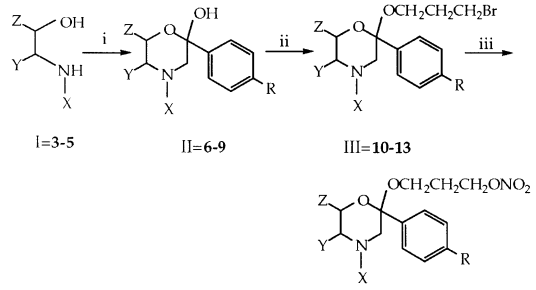
Chemistry. Scheme 1 demonstrates the synthesis of the compounds. The substituted 2-hydroxymorpholines **6**−**9** are formed via a spontaneous cyclization of a hydroxyaminoketone intermediate.[4,5] The acid-catalyzed reaction of the hemiketals **6**−**9** with 3-bromopropan-1-ol gives the corresponding 2-(3-bromopropoxy) derivatives **10**−**13** in an overall yield of 70%. The nitric acid esters **14**−**17** are synthesized (yield 35%) from the respective bromopropoxy derivatives and silver nitrate in acetonitrile, according to the literature.[6,7]

Chart 1



1 **2**

Scheme 1[a]



I=**3-5** II=**6-9** III=**10-13**

IV=**14-17**

3, 6, 10, 14 X=CH_3; Y,Z=H; R=C_6H_5
4, 7, 11, 15 X=CH_3; Y,Z=$(CH_2)_4$; R=C_6H_5
5, 8, 12, 16 X,Y=$(CH_2)_4$; Z=H; R=C_6H_5
3, 9, 13, 17 X=CH_3; Y, Z=H; R=H

[a] (i) 4-Bromoacetylbiphenyl or 4-bromoacetylbenzene, acetone, room temperature, 15 h; (ii) gaseous HBr in Et_2O, acetone, 3-bromopropan-1-ol, reflux, 3−5 h; (iii) silver nitrate, acetonitrile, 80 °C, 2 h.

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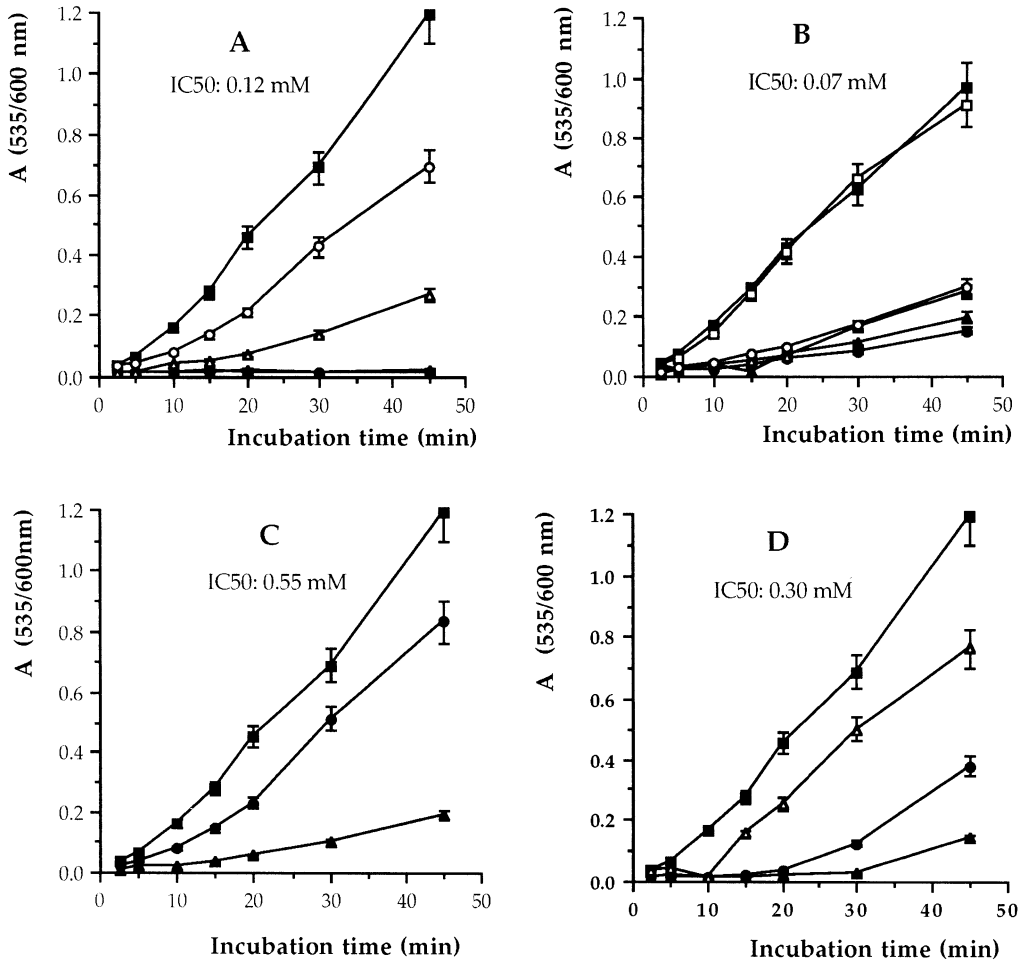

Figure 1. Time courses of lipid peroxidation, as affected by the tested compounds (A) **14**, (B) **15**, (C) **16**, and (D) **17**, and IC_{50} values: control (■); 0.1 mM (○); 0.25 mM (△); 0.5 mM (●); 1 mM (▲); 1 mM 3-nitrooxypropan-1-ol (panel B, □).

Table 1. In Vitro Nitric Oxide Release, Effect of the Examined Compounds on Plasma Total Cholesterol (TC), Triglyceride (TG), and Low-Density Lipoprotein (LDL) Levels, and clogP Values[a]

compd	% release of nitric oxide (μmol/μmol)	% decrease compared to controls[b] (56 μmol/kg, ip)			clogP
		TC	TG	LDL	
14	17.3	63***	85***	47***	4.63
14[c]		40**	54**	36**	
15	1.0	23**	38***	1^{NS}	6.07
16	4.6	20*	37**	25***	5.55
17	27.2	20*	45***	43***	2.75
SNAP	56.7				
probucol		18**	11^{NS}	18^{NS}	10.75

[a] All determinations are performed at least in duplicate and SD is always within ±10% of the absorbance values. Asterisks indicate statistical significance (Student's *t*-test) as follows: (***) $P < 0.005$; (**) $P < 0.05$; (*) $P < 0.1$. NS = not significant. [b] Each group is composed of four to six rats. Results are from two to three independent experiments. [c] 168 μmol/kg, po.

The structures of the novel compounds **10**−**17** are confirmed by IR, ^{1}H NMR, and elemental analysis.

Nitric Oxide Release. The ability of the nitric acid esters and of the known NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) to yield NO, expressed as micromoles of the formed NO per 100 μmol of the tested compound, is presented in Table 1. They are found to spontaneously produce from 1 μmol (compound **15**) to 27 μmol (compound **17**) of NO per 100 μmol. Despite the limited number of the tested compounds, an inverse correlation between NO generating ability and lipophilicity, calculated as clogP,[8] (Table 1) could be observed and attributed to the fact that a lower lipophilicity favors the nonenzymatic in vitro release of NO, since it facilitates the hydrolysis of the nitric ester group.

Hypolipidemic Activity. The plasma levels of total cholesterol, triglyceride, and LDL−cholesterol after the administration of the studied compounds and of probucol, as a reference, to hyperlipidemic rats are shown in Table 1. At the end of the experiment, all animals appeared normal both macroscopically and by autopsy. After a single ip dose of 56 μmol/kg, compounds **14**−**17** considerably reduced total cholesterol and triglyceride concentrations. Compound **14** is also effective after an oral administration of 168 μmol/kg. However, the effect on LDL is negligible-to-moderate for the poor NO donors **15** and **16**, while a very significant (compound **14**) and almost selective (compound **17**) reduction is observed for the more potent NO donors. The above effect on LDL, considered advantageous for hypocholesterolemic agents, could be explained by the ability of the liberated NO to affect apo B production. It has been reported[3] that the NO-generating compounds sodium nitroprusside and *S*-nitroso-*N*-acetylpenicillamine inhibit the diet-induced elevation of LDL−cholesterol in rabbits and suppress net apo B production in HepG2 cells, respectively, probably by counteracting the depletion of NO caused by hypercholesterolemia via a cellular and sterol-independent mechanism.

Lipid Peroxidation. The time course of lipid peroxidation, as affected by different concentrations of the examined compounds, and their IC_{50} values after 45 min of incubation are given in Figure 1.

Compounds **14**−**17** inhibit almost completely (85−100%) the peroxidation reaction at 1 mM. The most active antioxidant is compound **14**, also found to be a potent NO donor. This compound inhibits lipid peroxidation completely at 0.5 mM, and by about 40% at 0.1 mM, after 45 min of incubation. The 2-ethoxy analogue of **14**, without a nitric ester group but with almost the same lipophilicity (clogP = 4.47) inhibits lipid peroxidation by 68% at 0.5 mM and has no inhibitory effect at lower concentrations.[4] On the other hand, the best NO donor but least lipophilic compound **17**, although a potent antioxidant (68% inhibition at 0.5 mM), is less active than **14**. It could be assumed, therefore, that the NO-releasing ability reinforces the antioxidant character of these compounds, in balance with a suitable lipophilicity permitting their access to the lipid domains. Concerning the poorer NO donors **15** and **16**, there is no improvement in antioxidant activity of these nitric esters (Figure 1) compared with their 2-propoxy counterparts,[4] further supporting the assumption that the in situ liberated NO, and not the nitric ester group, enhances the antioxidant potential of these compounds.

The reduction of blood lipids and the inhibition of LDL oxidation are the prime therapeutic approaches to the treatment of atherosclerosis.[9] NO, crucial for the homeostatic control of the vasculature, can also protect LDL from oxidation[10] mainly by inhibiting chain propagation reactions.[11] Thus, the introduction of a nitric ester moiety into the structure of the substituted morpholines further improves their hypolipidemic and antioxidant profile and could be regarded as a useful template for the design of new antiatheromatic agents.

Experimental Section

Materials. All chemicals are of the highest commercially available purity. 2-Thiobarbituric acid and diagnostic kits for total cholesterol, LDL, and triglyceride determination are purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents are purchased from Aldrich-Chemie (Steinheim, Germany). Commercial 2-piperidinemethanol (Aldrich) corresponds to the racemate.

For the in vivo experiments, male Fischer-344 rats (200−280 g) are used.

Synthesis. Melting points (mp) are obtained on a MELTEMPII (Laboratory Devices) apparatus and are uncorrected. Infrared (IR) spectra are recorded on a Perkin-Elmer 597 infrared spectrophotometer. Proton nuclear magnetic resonance (^{1}H NMR) spectra are obtained with a Bruker AW 80 MHz and a Bruker 400 MHz spectrometer. Chemical shifts are reported in parts per million (δ) relative to tetramethylsilane (TMS), and signals are given as follows: s, singlet; d, doublet; t, triplet; m, multiplet. Elemental analyses are performed with a Perkin-Elmer 2400 CHN analyzer.

General Procedure for the Preparation of 2-(3-Bromopropoxy)morpholines 10−13 and Their Nitrooxy Derivatives 14−17 (Racemates). The synthesis of the 2-hydroxymorpholine derivatives **6**−**9** has been described previously.[4,5] The synthesis of the 2-(3-bromopropoxy) derivatives **10**−**13** is carried out by refluxing a mixture of the corresponding 2-hydroxy analogues (1 mmol) and 3-bromopropan-1-ol (30 mmol) in acidified acetone (50 mL) for 3−6 h. The products are isolated as salts and recrystallized from acetone and ether. Conversion into 2-(3-nitrooxypropoxy) derivatives **14**−**17** is performed by reaction of the free base of the 3-bromopropoxy analogues (10 mmol) with silver nitrate (11 mmol) in acetonitrile (80 mL). The mixture is heated (80 °C) for 2 h,[6,7] then filtered. Acetonitrile is evaporated in vacuo, and dichloromethane is added to the residue. The mixture is washed with water and dried (K_2CO_3), and the final product is isolated as a hydrobromide and recrystallized from acetone and ether.

2-(4-Biphenyl)-2-(3-bromopropoxy)-4-methylmorpholine, Hydrobromide, 10. White solid, yield 81%, mp 154−156 °C. IR: 2700, 1600 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 1.8−2.2 (m, 2H, $OCH_2CH_2CH_2Br$), 2.4−2.6 (s, 2H, C3−*H*), 2.8 (s, 3H, $N-CH_3$), 3.1−3.8 (m, 6H, C5−*H*, $OCH_2CH_2CH_2$ Br), 4.2−4.4 (t, 2H, C6−*H*), 7.2−7.8 (m, 9H$_{arom}$). Anal. ($C_{20}H_{25}Br_2NO_2$) C, H, N.

2-(4-Biphenyl)-2-(3-bromopropoxy)-4-methyloctahydro-1,4-benzoxazine, Hydrobromide, 11. White solid, yield 65%, mp 155−156 °C. IR: 2700, 1600 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 1.8−2.0 (m, 8H, alicyclic (CH_2)$_4$), 2.1 (m, 2H, $OCH_2CH_2CH_2$-Br), 2.4 (s, 3H, C3−*H*), 2.8 (s, 3H, $N-CH_3$), 3.0−3.3 (m, 1H, C5−*H*), 3.5−3.7 (m, 3H, $O(CH_2)_2CH_2Br$, C6−*H*), 3.8 (t, 2H, $OCH_2(CH_2)_2Br$), 7.3−7.6 (m, 3H$_{arom}$), 7.7−7.9 (m, 6H$_{arom}$), 11.5 (s, 1H, $^+$N−*H*). Anal. ($C_{24}H_{31}Br_2NO_2$) C, H, N.

3-(4-Biphenyl)-3-(3-bromopropoxy)octahydro-1,4-pyrido[2,1-*c*]oxazine, Hydrobromide, 12. White solid, yield 68%, mp 166−168 °C (dec). IR: 2700, 1600 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 1.5−1.7 (m, 2H), 1.8 (t, 2H), 2.0−2.2 (m, 2H) (total 6H, $CH(CH_2)_3CH_2N$), 2.3−2.5 (m, 2H, $OCH_2CH_2CH_2Br$), 3.0−3.2 (m, 2H, $(CH(CH_2)_3CH_2N$), 3.3−3.4 (m, 1H, C5−*H*), 3.5−3.7 (m, 4H, $O(CH_2)_2CH_2Br$, C3−*H*), 3.9−4.0 (m, 3H), 4.3 (t, 1H) (total 4H, $OCH_2(CH_2)_2Br$, C6−*H*), 7.2−7.7 (m, 9H$_{arom}$), 11.5 (s, 1H, $^+$N−*H*). Anal. ($C_{23}H_{29}Br_2NO_2\cdot0.65H_2O$) C, H, N.

2-Phenyl-2-(3-bromopropoxy)-4-methylmorpholine, Hydrobromide, 13. White solid, yield 75%, mp 177−179 °C. IR: 2700, 1600 cm^{-1}. ^{1}H NMR (DMSO-d_6): δ 1.8−2.5 (m, 4H, $OCH_2CH_2CH_2Br$, C3−*H*), 2.9 (s, 3H, $N-CH_3$), 3.0−3.4 (m, 6H, C5−*H*, $OCH_2CH_2CH_2Br$), 4.1−4.2 (m, 2H, C6−*H*), 7.3−7.6 (m, 5H$_{arom}$). Anal. ($C_{14}H_{21}Br_2NO_2$) C, H, N.

2-(4-Biphenyl)-2-(3-nitrooxypropoxy)-4-methylmorpholine, Hydrobromide, 14. White solid, yield 35%, mp 161−162 °C. IR: 2700, 1630 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.9−2.2 (m, 2H, $OCH_2CH_2CH_2ONO_2$), 2.9 (s, 3H, $N-CH_3$), 3.2−3.5 (m, 6H, C3−*H*, C5−*H*, $OCH_2(CH_2)_2ONO_2$), 4.1−4.2 (t, 2H, C6−*H*), 4.8 (t, 2H, CH_2-ONO_2), 7.4−7.8 (m, 9H$_{arom}$). Anal. ($C_{20}H_{25}BrN_2O_5\cdot0.9H_2O$) C, H, N.

2-(4-Biphenyl)-2-(3-nitrooxypropoxy)-4-methyloctahydro-1,4-benzoxazine, Hydrobromide, 15. White solid, yield 32%, mp 132−134 °C. IR: 2700, 1630 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.8−2.2 (m, 10H, alicyclic (CH_2)$_4$), $OCH_2CH_2CH_2ONO_2$), 2.8 (s, 3H, $N-CH_3$), 3.2−3.4 (m, 4H, C3−H, $OCH_2(CH_2)_2ONO_2$), 3.6−3.7 (m, 1H, C5−*H*), 3.8 (m, 1H, C6−*H*), 4.9 (t, 2H, CH_2-ONO_2), 7.2−7.9 (9H$_{arom}$, m). Anal. ($C_{24}H_{31}BrN_2O_5\cdot0.8H_2O$) C, H, N.

3-(4-Biphenyl)-3-(3-nitrooxypropoxy)octahydro-1,4-pyrido[2,1-*c*]oxazine, Hydrobromide, 16. White solid, yield 34%, mp 153−155 °C. IR: 2700, 1630 cm^{-1}. ^{1}H NMR ($CDCl_3$): δ 1.5−1.7 (m, 2H), 1.8 (t, 2H), 2.0−2.1 (m, 2H) (total 6H, $CH(CH_2)_3CH_2N$), 2.3−2.5 (m, 2H, $OCH_2CH_2CH_2ONO_2$), 2.9−3.1 (m, 2H, C3−*H*), 3.1−3.3 (m, 2H, $CH(CH_2)_3CH_2N$), 3.4−3.5 (m, 1H, C5−*H*), 3.6−3.7 (d, 2H, C6−*H*), 3.8−4.3 (m, 2H, $OCH_2(CH_2)_2ONO_2$), 4.9 (t, 2H, CH_2-ONO_2), 7.2−7.7 (m, 9H$_{arom}$), 11.5 (s, 1H, $^+$N−*H*). Anal. ($C_{23}H_{29}BrN_2O_5$) C, H, N.

2-Phenyl-2-(3-nitrooxypropoxy)-4-methylmorpholine, Hydrobromide, 17. White solid, yield 35%, mp 145−147 °C. IR: 2700, 1630 cm^{-1}. ^{1}H NMR ($CDCl_3$) δ 1.8−2.2 (m, 2H, $OCH_2CH_2CH_2ONO_2$), 2.9 (s, 3H, $N-CH_3$), 3.0−3.4 (m, 6H, C3−*H*, C5−*H*, $OCH_2(CH_2)_2ONO_2$), 4.1−4.2 (m, 2H, C6−*H*), 4.8 (t, 2H, CH_2-ONO_2), 7.3−7.6 (m, 5H$_{arom}$). Anal. ($C_{14}H_{21}BrN_2O_5$) C, H, N.

In Vitro Nitric Oxide Determination. Compounds are dissolved in water (100 μM) and incubated overnight at room temperature in the presence of cadmium. Aliquots are taken from each sample and added to an equal volume of *N*-naphthylaminoethylamine (0.2%) and sulfonamide (2%) solution in 3 N hydrochloric acid (Griess reagent). Nitric oxide is

estimated spectrophotometrically (540 nm) according to a commercial kit (Oxford Biomedical Research, product NB88).

In Vitro Lipid Peroxidation. The incubation mixture contains heat-inactivated rat hepatic microsomal fraction (corresponding to 2.5 mg of hepatic protein per milliliter or 4 mM fatty acid residues[12,13]), ascorbic acid (0.2 mM) in Tris-HCl/KCl buffer (50 mM, 150 mM, pH 7.4), and the studied compounds in dimethyl sulfoxide (DMSO) at concentrations of 0.1−1.0 mM. The peroxidation reaction is started with $FeSO_4$ solution (10 μM), and aliquots are taken from the incubation mixture (37 °C) at various time intervals for 45 min. Lipid peroxidation is assessed by spectrophotometric (535 against 600 nm) determination of the 2-thiobarbituric acid reactive material consisting mainly of malondialdehyde, an end product of polyunsaturated lipid peroxidation.[12,14] All compounds and solvents are tested and not found to interfere with the assay.

In Vivo Evaluation of Hypolipidemic Activity. An aqueous solution of Triton WR 1339 is administered ip to rats (200 mg/kg),[15] and 1 h later the examined compounds (56 μmol/kg) dissolved in saline or saline only are given ip. Furthermore, compound **14** is given orally (168 μmol/kg) 3 h prior to Triton WR 1339 injection. In all cases, 24 h after the administration of Triton, blood is taken from the aorta and used for the determination of plasma total cholesterol, LDL, and triglyceride concentrations.

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References

(1) Berliner, J. A.; Navab, M.; Fogelman, A. M.; Frank, J. S.; Demer, L. L.; Edwards, P. A.; Watson, A. D.; Lusis, A. J. Atherosclerosis: basic mechanisms. Oxidation, inflammation and genetics. *Circulation* **1995**, *91* (9), 2488−2496.

(2) Sumi, D.; Hayashi, T.; Thakur, N. K.; Jayachandran, M.; Asaf, Y.; Kano, H.; Matsui, H.; Iguchi, A. A HMG-CoA reductase inhibitor possesses a potent anti-atherosclerotic effect other than serum lipid lowering effects—the relevance of endothelial nitric oxide synthase and superoxide anion scavenging action. *Atherosclerosis* **2001**, *155* (2), 347−357.

(3) Kurowska, E. M.; Carroll, K. K. Hypocholesterolemic properties of nitric oxide. In vivo and in vitro studies using nitric oxide donors. *Biochim. Biophys. Acta* **1998**, *1932* (1), 41−50.

(4) Chrysselis, M. C.; Rekka, E. A.; Kourounakis, P. N. Hypocholesterolemic and hypolipidemic activity of some novel morpholine derivatives with antioxidant activity. *J. Med. Chem.* **2000**, *43* (4), 609−612.

(5) Rekka, E.; Kourounakis, P. Synthesis, physicochemical properties and biological studies of some substituted 2-alkoxy-4-methylmorpholines. *Eur. J. Med. Chem.* **1989**, *24*, 179−185.

(6) Benedini, F.; Bertolini, G.; Cereda, R.; Dona, G.; Gromo, G.; Levi, S.; Mizrahi, J.; Sala, A. New antianginal nitroesters with reduced hypotensive activity. Synthesis and pharmacological evaluation of 3-[(nitrooxy)alkyl]-*2H*-1,3-benzoxazine-4(*3H*)-ones. *J. Med. Chem.* **1995**, *38*, 130−136.

(7) Iqbal, N.; Knaus, E. E. Synthesis and smooth muscle calcium channel antagonist effect of dialkyl 1,4-dihydro-2,6-dimethyl-4-aryl-3,5-pyridinedicarboxylates containing a nitrooxy or nitrophenyl moiety in the 3-alkyl ester substituent. *Arch. Pharm. (Weinheim, Ger.)* **1996**, *329*, 23−26.

(8) Hansch, C.; Leo, A. *Substituent Constants for Correlation Analysis in Chemistry and Biology*; J. Wiley & Sons: New York, 1991.

(9) Chrysselis, M. C.; Rekka, E. A.; Kourounakis, P. N. Antioxidant therapy and cardiovascular disorders. *Expert Opin. Ther. Pat.* **2001**, *11* (1), 33−43.

(10) Hogg, N.; Kalyanaraman, B.; Joseph, J.; Struck, A.; Parthasarathy, S. Inhibition of low density lipoprotein oxidation by nitric oxide. Potential role in atherogenesis. *FEBS Lett.* **1993**, *334*, 170−174.

(11) Rubbo, H.; Darley-Usmar, V.; Freeman, B. A. Nitric oxide regulation of tissue free radical injury. *Chem. Res. Toxicol.* **1996**, *9*, 809−820.

(12) Rekka, E.; Kolstee, J.; Timmerman, H.; Bast, A. The effect of some H_2-receptor antagonists on rat hepatic microsomal cytochrome P-450 and lipid peroxidation in vitro. *Eur. J. Med. Chem.* **1989**, *24*, 43−54.

(13) Eichenberger, K.; Bohni, P.; Winterhalter, K. H.; Kawato, S.; Richter, C. Microsomal lipid peroxidation causes an increase in the order of the membrane lipid domain. *FEBS Lett.* **1982**, *142*, 59−62.

(14) Rekka, E. A.; Kourounakis, A. P.; Kourounakis, P. N. Investigation of the effect of chamazulene on lipid peroxidation and free radical processes. *Res. Commun. Mol. Pathol. Pharmacol.* **1996**, *92*, 361−364.

(15) Kuroda, M.; Tanzawa, K.; Tsujita, Y.; Endo, A. Mechanism for elevation of hepatic cholesterol synthesis and serum cholesterol levels in Triton WR-1339-induced hyperlipidemia. *Biochim. Biophys. Acta* **1977**, *89*, 119−125.

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