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Searching for new NO-donor Aspirin-like molecules: a new class of nitrooxy-acyl derivatives of salicylic acid[§]

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Abstract. A new class of products in which the phenol group of salicylic acid is linked to alkanoyl moieties bearing nitrooxy functions has been synthesised and studied for their polyvalent actions. The products were stable in acid and neutral media, whilst they were hydrolysed in human serum. Their half-lives were dependent on the structure of alkanoyl moieties. The products showed anti-inflammatory activities similar to aspirin when tested in the carrageenan-induced paw edema assay in the rat. Interestingly, unlike aspirin, they showed reduced or no gastrotoxicity in a lesion model in rats at equimolar doses. A number of them were able to inhibit platelet aggregation induced by collagen in human platelet rich plasma (PRP). All the products were capable of relaxing rat aortic strips precontracted with phenylephrine in a concentration-dependent manner. Selected members of this new class of NSAIDs might represent possible safer alternatives to aspirin in different clinical settings.

KEYWORDS: nitric acid esters, NO-aspirins, gastrosparing NO-donor aspirins, salicylic acid derivatives, NO-NSAIDs.

Introduction

Aspirin (**1**) (Chart 1) is a well established drug, belonging to the class of non steroidal anti-inflammatory drugs (NSAIDs). These products are widely used due to their anti-inflammatory, analgesic, antipyretic and platelet antiaggregatory properties. The pharmacological basis of these beneficial effects are related, at least in part, to their ability to inhibit two cyclooxygenase isoenzymes, COX-1 and COX-2, which are involved in the production of prostanoids from arachidonic acid.[1,2] Aspirin is unique among NSAIDs since it modifies both isoforms covalently by acetylating a serine residue (Ser^{530}) positioned in the arachidonic acid-binding channel of the enzyme.[3] It is considered a non selective COX-inhibitor, although it displays a somewhat higher inhibitor potency in COX-1 assays. The major drawback of the drug is a significant gastrotoxicity that is responsible for gastric ulceration, exacerbation of peptic ulcer symptoms, gastrointestinal haemorrhage, erosive gastritis and, in some cases, death.[1,2,4] Nitric oxide (NO)-releasing aspirins are an interesting class of products that were originally designed to reduce gastrotoxicity of the parent aspirin.[5,6] They are hybrid drugs that combine aspirin properties with gastroprotection exerted by NO that they are able to release. NO-induced gastroprotection occurs through a number of mechanisms, including an increase

of gastric microcirculation and mucous and bicarbonate secretion, as well as the inhibition of neutrophil adhesion to vascular endothelium of gastric microcirculation.[7,8] These products were originally obtained by joining, through a simple ester bridge, the carboxylic group of aspirin with moieties containing NO-donor nitrooxy groups (-ONO_2). Prototypes of such structures **2** (NCX4040), **3** (NCX4016), **4** (NCX4060) are reported in Chart 1.

Subsequently, a second family of NO-donor aspirins **5** (Chart 1) was developed,[9] which, despite a close similarity with the first generation molecules, contains furoxan derivatives as NO-donor groups. Unlike the previous NO-donor-aspirins, that seem to require enzymatic metabolism for NO release,[10-12] these products proved to release NO under the action of thiols.[13] Intracellular release induced by glutathione is potentiated by ascorbic acid.[14] Recently, an additional class of NO-donor NSAIDs, including aspirins **6** (Chart 1), has been proposed. They have moieties, containing an ONN(O)N substructure, linked to the carboxy group through a methylene bridge.[15] In serum these compounds are metabolised to a mixture of products, including *N*-diazeniumdiolate anions which are able to release NO spontaneously.

It is becoming more and more evident that NO-donor aspirins display a number of effects exceeding their original intended use. They display a variety of actions, including anti-inflammatory and analgesic effects, antiplatelet and vasodilator properties, beneficial effects in the treatment of restenosis and myocardial ischaemia. In addition, a number of them proved to be more potent than aspirin in inhibiting the proliferation of cancer cell lines, including prostate cancer cells.[16-19] Thus, **3**, the prototype of NO-donor aspirins, was found to enhance the preventive and therapeutic effectiveness of the antitumor immunity elicited by cancer vaccination.[20] Finally a possible use of NO-aspirins in the treatment of Type 2 diabetes has been claimed.[18] The mechanism of action of NO-aspirins is still under investigation, also taking into account that the simple esters of aspirin, and in particular **3**, are often not true aspirin pro-drugs. Some of them are rapidly metabolised, with little or no formation of aspirin, when incubated in serum, plasma and rat liver subcellular fractions.[9,12] This behaviour is in keeping with the knowledge that the loss of the negative charge by the aspirin molecule, due to the esterification of the COOH group (pK_a=3.5), makes the acetoxy moiety extremely susceptible to enzymatic cleavage.[21] Interestingly, it was recently found that neither aspirin nor NO contributes to the antitumor effect of **3**, that, in contrast,

is due to the quinone methide formed after carboxylic ester hydrolysis.[22,23] As a development of our work in this area, we designed a new class of NO-donor "aspirin-like" compounds (Chart 2). These products are formally derived from aspirin by substituting acyl groups containing nitrooxy NO-donor moieties for the acetyl group. They are not pro-drugs of aspirin but true aspirin/NO-donor hybrids. All the NO-donor aspirins known thus far were designed by merging NO-donor moieties at the carboxylic site of aspirin. In this paper we describe the synthesis, stability in different buffer solutions and in human serum of these products, as well as preliminary pharmacological characterisation including in vivo anti-inflammatory, gastrosparing properties, in vitro antiaggregatory activity, and cGMP-dependent vasodilation.

Chemistry

Mononitrooxy-substituted compounds were synthesised according to the pathways reported in Scheme 1. The preparation of the products **29-32** was carried out starting from the bromo-substituted alkanoic acids **7-10**. These starting materials were transformed into the corresponding acyl chlorides **11-14** by $SOCl_2$ in dry CH_2Cl_2, in the presence of a few drops of dry DMF. The acyl chlorides were conjugated to salicylaldehyde in dry CH_2Cl_2, in the presence of dry pyridine to give **15-18**. The action of $AgNO_3$ on these intermediates in refluxing acetonitrile afforded the corresponding mononitrooxy-substituted aldehydes **19-22** that gave rise to the final compounds **29-32**, under the action of $KMnO_4$. For the final compound **32** mild oxidative reagents ($NaClO_2$ and H_2O_2) were utilized, since better yields were obtained. The preparation of the target products **28**, **33** was carried out starting from the nitrooxy-substituted alkanoic acids **23**, **26**. These acids were transformed into the corresponding acyl chlorides **24**, **27** by the action of $SOCl_2$. Coupling of **24** with salicylaldehyde under the same conditions used to prepare the aldehydes **15-18**, gave **25** that yielded the target model **33** after subsequent oxidation with $KMnO_4$. The remaining mononitrooxy derivative **28** was prepared for direct coupling of **27** with salicylic acid in dry THF, in the presence of dry pyridine. The preparation of the dinitrooxy-substituted final products **51-55** is reported in Scheme 2. Two of them required the dinitrooxy-substituted alkanoic acids **35** and **36** as starting materials. The former is a product known in literature[24] while the latter was prepared by action of I_2 and $AgNO_3$ in acetonitrile solution on hept-6-enoic acid (**34**). These products were transformed into the corresponding acyl chlorides **37**, **38** and then coupled

with salicylaldehyde to give **48**, **49**. Oxidation of these intermediates afforded the final target compounds **53**, **54**. All these reactions were carried out under conditions similar to those described for the preparation of the mononitrooxy-substituted product **33** from **23**. To prepare **51**, **52**, **55** the unsaturated acids **39-41** were used as starting materials. Acids **39** and **41** were transformed in the corresponding active intermediates through the reaction with N,N'-dicyclohexylcarbodiimide (DCC); these intermediates were then coupled to salicylaldehyde in dry CH_2Cl_2 solution in the presence of 4-N,N'-dimethylaminopyridine (DMAP) to give **43** and **45**. To prepare aldehyde **44** the best results were obtained transforming **40** into the corresponding acyl chloride **42** and then coupling **42** with salicylaldehyde, under the conditions already described. The unsaturated aldehydes **43-45** were transformed into the corresponding dinitrooxy derivatives **46**, **47**, **50** by action of I_2 and $AgNO_3$ in CH_3CN solution. The aldehydes **47** and **50** afforded the desired final products by usual oxidation with $KMnO_4$, while aldehyde **46** was oxidated using mild conditions ($NaClO_2$ and H_2O_2).

Results and discussion

Stability in aqueous buffer solutions and in human serum. The stability of all the final products was studied by HPLC in aqueous buffer solutions of pH 1 and 7.4 as well as in human serum (Table 1). Salicylic acid (**56**) and the nitrooxyalkanoic acids were the only transformation products. In an acidic medium, the stability, after 3 hour incubations, ranged from 85% to 97%. At pH 7.4, most of the products showed a stability > 90%, with the only exception of products **28** and **51** for which only 55% and 20% remained unchanged over the same time period. A different situation occurred when the compounds were incubated in serum, in which they can undergo enzymatic metabolism. All of them were hydrolysed, following a first-order kinetic. In Figure 1 the hydrolysis in serum of **31** is reported as an example. The observed pseudo-first-order rate constants (k_{obs}) for the hydrolysis were calculated from the slopes of linear plots of the logarithm of the remaining ester against time; the corresponding half-lives ($t_{1/2}$) were obtained from equation 1 (Table 1).

$$t_{1/2} = 0.693/k_{obs} \qquad \text{eq. 1}$$

Analysis of these parameters shows that they are influenced by the structure of the ester chain. In the mononitrooxy series some products are less stable than aspirin while others as stable as, or more stable than

it. The stability increases with the length of the chain, but when the number of carbons become > 6 (compound **32**) it decreases. The presence of two methyl groups at α-position of the ester function (compound **33**) induces strong stabilisation. A similar situation occurs in the dinitrooxy series, but all of these compounds are definitively more stable than the related mono-substituted analogues. This picture might be justified by a different ability of the models to interact with the esterase enzyme and/or to bind with plasma proteins, following their different lipophilicity and stereo-electronic properties.[25,26]

Antiinflammatory Activity. All the products, included aspirin (**1**) and salicylic acid (**56**) as references, were tested on carrageenan-induced paw edema in conscious rats. The injection of carrageenan into rat hind paw produced an immediate paw swelling, which reached a peak at 4-5 h. Aspirin, administered by intragastric route at 120 mg/kg, just prior to carrageenan injection, significantly reduced (47 ± 2.0%) paw edema at 3 h, when compared with vehicle-treated animals (Figure 2). Also, a number of the new NO-donor products were able to reduce paw edema in a significant or highly significant manner, when administered intragastrically at a dose equimolar with aspirin (120 mg/kg; Figure 2). The most active compounds, which induce antiinflammatory effects comparable to those caused by aspirin, belong both to the class of mononitrooxy-derivatives (compounds **30**, **31**, **33**) and to that of dinitrooxyderivatives (compounds **52-54**).

As aforementioned, the anti-inflammatory effects of NSAIDs can be partly explained by the inhibition of prostanoid production following the inhibition of COX enzymes. More recently, it was found that the irreversible acetylation of COX-2 isoform by aspirin may contribute to its anti-inflammatory activity, through the synthesis of aspirin-triggered lipoxins (ATLs).[27] Since the drug is rapidly deacetylated under the action of esterases present in blood, intestinal mucosa and tissues such as liver and kidney,[28,29] it has been proposed that salicylic acid may also contribute to its anti-inflammatory action.[30] It is known that rodents (rats, guinea pigs) tend to metabolize ester containing drugs, including aspirin, much faster than humans.[25,28] Furthermore, NO may display anti-inflammatory actions of its own when produced in the appropriate amounts.[31-33] In particular, both NO and salicylate are able to inhibit NF-κB, a transcription factor involved in the synthesis of inflammatory cytokines, cytokine receptors and adhesion molecules in a variety of cells.[30,34-37] Actually **3** was shown to inhibit this factor and to suppress the processing of IL-1beta and IL-18,

two inflammatory cytokines, by inhibiting caspase-1 activity.[17,38] Studies dedicated to this question are necessary in order to clarify whether the mechanisms underlying the in vivo antiinflammatory properties of this new class of NSAIDs involve combinations of COX- effects of the native products, of the metabolite salicylic acid, and possibly of NO-release.

Acute Gastric Mucosal Damage. All the compounds, included aspirin (**1**) and salicylic acid (**56**) as references, were assessed for their ulcerogenic properties in conscious rats. The development of gastric lesions was assessed 3 h after intragastric administration of the compounds and lesions were quantified by determining the "lesion index" on the basis of their greatest length in millimetres (Figure 3). Aspirin displays strong gastrotoxicity in spite of its ability to generate ATLs which are endowed with gastroprotective effects as well.[39] In the present study, aspirin, administered at 120 mg/kg, produced macroscopically detectable gastric damage, characterised by mucosal necrosis and haemorrhage (lesion index = 50 ± 3.8). All the NO-donor products described here displayed greatly reduced gastrotoxicity when administered at a dose equimolar with aspirin. There are two effects which contribute to gastrotoxicity of conventional acidic NSAIDs: the systemic effect and the local irritant effect.[40,41] The former is dependent on COX- inhibition, in particular of the COX-1 isoform in gastric epithelial cells. The latter is the result of a number of events including perturbation of physico-chemical properties of phospholipids and "ion trapping" of the drugs into surface epithelial cells. These two events are tightly linked to the pK_a and to the lipophilicity of acid NSAIDs.[42] From this point of view, the new products here developed show acid properties (pK_a = 3.85 ÷ 3.69) close to that of aspirin and greater lipophilicity (log P = 1.60 ÷ 3.22; log $P_{aspirin}$ = 1.14) (Unpublished data). While it plausible to hypothesize a role of NO release in the reduced gastrotoxicity, it must be remembered that these products are metabolised to salicylic acid , which is unable to induce gastric mucosal damage (see Figure 3 and ref. 30).

Platelet antiaggregatory activity. Antiaggregatory effects of the compounds were studied on collagen-induced platelet aggregation of human platelet rich plasma (PRP). It is known that collagen-induced aggregation occurs through a pathway dependent on arachidonic acid cascade.[43] The results expressed as $IC_{50,}$ or, when IC_{50} could not be calculated, as a percentage of inhibition at the maximal concentration tested, are reported in Table 1. Analysis of the data shows that the most active compounds were **29 > 30 >**

28, belonging to the mononitrooxy series and the dinitrooxy substituted model **51.** In order to determine whether NO-mediated stimulation of soluble guanylate cyclase (sGC) was involved in their antiaggergatory action, these products were also tested in parallel in the presence of ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a well known inhibitor of sGC. The ineffectiveness of ODQ on the antiaggregatory activities of compounds **28**-**30** and **51** excludes any involvement of NO-mediated stimulation of sGC in keeping with the known inability of platelets to effect NO release from organic nitrates.[14,44] This strengthens the possibility that platelet COX-1 inhibition is the main underlying mechanism .

Vasodilator activity. It is known that organic nitrates display vasodilator activities. The generally accepted mechanism of this action involves their conversion in vascular smooth muscle cells into NO with consequent activation of the sGC. In turn sGC increases the intracellular levels of cyclic guanosine-3,5-monophosphate (cGMP), with consequent activation of cGMP-dependent protein kinase (cGK-I) and vasodilation.[45] In order to highlight the NO-releasing activity of the compounds described in the present work, their vasodilatory effects 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, in keeping with the presence in the structures of NO-donor nitrooxy functions. The vasodilator potencies, expressed as EC_{50}, are shown in Table 1. In the mononitrooxy series the potency increases with the length of the linear lateral chain. The most active term was the branched product **33** which bears two methyl groups at the α-carbon to ester function. Similar behaviour is shown by the dinitrooxy-substituted compounds, but in this series the branched compound **55** is less active than **54** containing the longest linear lateral chain and as active as **53** which is the homologous immediately inferior to **54**. Length of the chain being equal, the dinitrooxy substituted compounds are always more active than the corresponding mononitroxy analogues, in keeping with the statement that the number of nitrate groups determines reactivity and potency of organic nitrates.[46] When the vasodilator experiments were repeated in the presence of 1 μM ODQ, a decrease in the potencies was observed, in keeping with NO-induced activation of the sGS.

Conclusions

We have been able to successfully prepare a new class of "aspirin-like" products obtained through a novel approach which implies the merging of nitrooxy-acyl moieties at the phenol site of salicylic acid. Generally speaking, these compounds are stable in acidic media and at physiological pH, but they are hydrolysed when incubated in serum. This new class of products displays anti-inflammatory activity in the carrageenan-induced paw edema test, with several compounds being as potent as aspirin; nevertheless, they show reduced or no gastrotoxicity when compared with this lead. Several components may contribute to their in vivo behaviour, including salicylic acid formation, NO release and variable linkers. Furthermore, some of them are able to block platelet aggregation induced by collagen in human plasma rich platelets . All the products trigger cGMP dependent vasodilator actions when tested on pre-contracted rat aorta strips, according to the presence in their structures of nitrooxy NO-donor moieties. Additional pharmacological and biochemical studies, mainly addressing COX-1/COX-2 inhibition, are necessary to highlight action mechanisms of these products and to understand whether selected members of this new class of NSAIDs might represent possible safer alternatives to aspirin in different clinical settings.

Experimental Section

Synthesis. ^{1}H 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) and the results are within ± 0.4% of the theoretical values. Compounds **8**,[47] **10**,[48] **23**,[49] **26,**[50] **35**,[24] **41**,[51] were synthesised according to literature.

General procedure for the preparation of 15, 16, 17, 18, 25, 44, 48, 49.

$SOCl_2$ (6.55 mmol) and a few drops of dry DMF were added to a solution of the appropriate carboxilic acid **7**, **8**, **9**, **10**, **23**, **35**, **36** and **40** (5.46 mmol) in dry CH_2Cl_2 (15 mL), stirred under N_2 at r.t. The stirring was continued for 2 h at room temperature (r.t). The solution of the acyl chloride so obtained was slowly added to a stirred solution of salicylaldehyde (4.37 mmol) and dry Py (8.19 mmol) in dry CH_2Cl_2 (10 mL), kept under N_2 at 0 °C. The reaction was allowed to reach r.t. and then stirred for 2.5 h. Then the mixture was washed with HCl 2M (3 x 10 mL). The combined organic layers were dried, filtered and concentrated under reduced pressure. The crude product so obtained was purified by flash chromatography. Chromatographic eluents and yields of the products were as follows.

2-Formylphenyl 4-bromobutanoate (15): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 80%. ^{1}H-NMR ($CDCl_3$) δ 2.34-2.36 (*m*, 2H, -C*H*$_2$CH$_2$Br), 2.89 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 7.1 Hz), 3.57 (*t*, 2H, -C*H*$_2$Br, $^3J_{HH}$ = 6.4 Hz), 7.18 (*d*, 1H, C_6H_4), 7.43 (*t*, 1H, C_6H_4), 7.66 (*t*, 1H, C_6H_4), 7.88 (*d*, 1H, C_6H_4), 10.1 (*s br*, 1H, C*H*O). ^{13}C-NMR ($CDCl_3$) δ 27.4, 32.3, 32.5, 123.5, 126.5, 128.0, 132.0, 134.8, 151.0, 171.0, 188.9. MS (CI) *m/z* 271/273 $(M+1)^+$.

2-Formylphenyl 5-bromopentanoate (16): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 57%. ^{1}H-NMR ($CDCl_3$) δ 1.91-2.05 (*m*, 4H, -C*H*$_2$C*H*$_2$CH$_2$Br), 2.70 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 6.8 Hz), 3.48 (*t*, 2H, -C*H*$_2$Br, $^3J_{HH}$ = 6.3 Hz), 7.18 (*d*, 1H, C_6H_4), 7.40 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.88 (*d*, 1H, C_6H_4), 10.1 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 23.2, 31.9, 32.9, 33.1, 123.5, 126.5, 128.2, 131.7, 135.3, 151.3, 171.4, 188.8. MS (CI) *m/z* 285/287 $(M+1)^+$.

2-Formylphenyl 6-bromohexanoate (17): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 68%. ^{1}H-NMR ($CDCl_3$) δ 1.57-1.65 (*m*, 2H), 1.78-1.83 (*m*, 2H), 1.89-1.97 (*m*, 2H)(-C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$Br), 2.70 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 7.5 Hz), 3.44 (*t*, 2H, -C*H*$_2$Br, $^3J_{HH}$ = 6.7 Hz), 7.19 (*d*, 1H, C_6H_4), 7.39 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.88 (*d*, 1H, C_6H_4), 10.10 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 23.8, 27.6, 32.4, 33.5, 34.0, 123.5, 126.4, 128.1, 131.0, 135.3, 151.5, 171.7, 188.8. MS (CI) *m/z* 299/301 $(M+1)^+$.

2-Formylphenyl 7-bromoheptanoate (18): eluent (PE/EtOAc 95/5 v/v); pale yellow oil; yield 62%. ^{1}H-NMR ($CDCl_3$) δ 1.47-1.55 (*m*, 4H), 1.78-1.96 (*m*, 4H) (-C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$Br), 2.70 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 7.3 Hz), 3.45 (*t*, 2H, -C*H*$_2$Br, $^3J_{HH}$ = 6.7 Hz), 7.19 (*d*, 1H, C_6H_4), 7.42 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H,

C_6H_4), 7.90 (*d*, 1H, C_6H_4), 10.12 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 24.4, 27.8, 28.2, 32.5, 33.8, 33.9, 123.5, 126.4, 128.1, 131.2, 135.3, 151.6, 171.9, 188.7. MS (CI) *m/z* 313/315 $(M+1)^+$.

2-Formylphenyl 2,2-dimethyl-3-(nitrooxy)propanoate (25): in this case the formation of the acyl chloride and the next synthetic step are very slow and one week stirring was required. Eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 45%. ^{1}H-NMR ($CDCl_3$) δ 1.51 (*s*, 6H, -CH_3), 4.69 (*s*, 2H, -CH_2ONO$_2$), 7.15 (*d*, 1H, C_6H_4), 7.43 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.88 (*d*, 1H, C_6H_4), 10.07 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 22.4, 42.7, 77.5, 123.3, 126.8, 128.2, 131.8, 135.3, 151.0, 172.8, 188.5. MS (CI) *m/z* 268 $(M+1)^+$.

2-Formylphenyl pent-4-enoate (44): eluent (PE/EtOAc 95/5 v/v); pale yellow oil immediately used in the next synthetic step; yield 65%.

2-Formylphenyl 5,6-bis(nitrooxy)hexanoate (48): eluent (PE/EtOAc 90/10 – 80/20 v/v); pale yellow oil; yield 56%. ^{1}H-NMR ($CDCl_3$) δ 1.89-1.98 (*m*, 4H, -$CH_2CH_2CH_2$CH-), 2.74-2.78 (*m*, 2H, -OCOCH_2-), 4.49-4.55 (*dd*, 1H, AMX like system, -CH_aH$_b$ONO$_2$), 4.73-4.83 (*dd*, 1H, AMX like system, -CH$_a$$H_bONO_2$), 5.36-5.39 (*m*, 1H, AMX like system,-C*H*ONO$_2$), 7.17 (*d*, 1H, C_6H_4), 7.44 (*t*, 1H, C_6H_4), 7.66 (*t*, 1H, C_6H_4), 7.86 (*d*, 1H, C_6H_4), 10.02 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 19.9, 28.5, 33.0, 71.1, 78.8, 123.5, 126.7, 128.0, 133.0, 135.4, 150.6, 171.1, 189.3. MS (CI) *m/z* 343 $(M+1)^+$.

2-Formylphenyl hept-6-enoate (49): eluent (PE/EtOAc 85/15 v/v); pale yellow oil; yield 58%. ^{1}H-NMR ($CDCl_3$) δ 1.55-1.67 (*m*, 2H), 1.80-1.90 (*m*, 4H)(-$CH_2CH_2CH_2CH_2$CH-), 2.71 (*t*, 2H, -OCOCH_2-, $^3J_{HH}$ = 7.2 Hz), 4.50 (*dd*, 1H, AMX like system, -CH_aH$_b$ONO$_2$), 4.78 (*dd*, 1H, AMX like system, -CH$_a$$H_bONO_2$), 5.29-5.37 (*m*, 1H, AMX like system, -C*H*ONO$_2$), 7.17 (*d*, 1H, C_6H_4), 7.43 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H, C_6H_4), 7.87 (*d*, 1H, C_6H_4), 10.1 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 24.0, 24.3, 29.0, 33.4, 71.2, 79.0, 119.9, 123.5, 128.1, 132.2, 135.4, 151.0, 171.4, 189.0. MS (CI) *m/z* 357 $(M+1)^+$.

General procedure for the preparation of 19, 20, 21, 22.

A solution of the appropriate bromo derivative **15**, **16**, **17** and **18** (18.4 mmol) and $AgNO_3$ (46.0 mmol) in CH_3CN (150 mL) was stirred at 70 °C for 7 h. The mixture was filtered through Celite® and concentrated under reduced pressure. The residue was treated with CH_2Cl_2 (50 mL) and H_2O (50 mL). After separation the aqueous layer was extracted twice with CH_2Cl_2 (50 mL). The combined organic layers were dried,

filtered and concentrated under reduced pressure. The crude product so obtained was purified by flash chromatography. Chromatographic eluents and yields of the products were as follows.

2-Formylphenyl 4-(nitrooxy)butanoate (19): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 84%. ^{1}H-NMR ($CDCl_3$) δ 2.17-2.27 (*m*, 2H, -C*H*$_2$CH$_2$ONO$_2$), 2.82 (*t*, 2H, -OCOC*H*$_2$-, $^{3}J_{HH}$ = 7.1 Hz), 4.61 (*t*, 2H, -C*H*$_2$ONO$_2$, $^{3}J_{HH}$ = 6.2 Hz), 7.18 (*d*, 1H, C_6H_4), 7.44 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H, C_6H_4), 7.87 (*d*, 1H, C_6H_4), 10.0 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 22.1, 30.1, 71.8, 123.5, 126.7, 127.9, 132.7, 134.9, 150.6, 170.8, 189.1. MS (CI) *m/z* 254 $(M+1)^+$.

2-Formylphenyl 5-(nitrooxy)pentanoate (20): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 64%. ^{1}H-NMR ($CDCl_3$) δ 1.88-1.95 (*m*, 4H, -C*H*$_2$C*H*$_2$CH$_2$ONO$_2$), 2.75 (*m*, 2H, -OCOC*H*$_2$-), 4.55 (*m*, 2H, -C*H*$_2$ONO$_2$), 7.19 (*d*, 1H, C_6H_4), 7.43 (*t*, 1H, C_6H_4), 7.66 (*t*, 1H, C_6H_4), 7.89 (*d*, 1H, C_6H_4), 10.1 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 20.9, 26.2, 33.3, 72.7, 123.5, 126.5, 128.0, 132.2, 135.4, 151.0, 171.2, 188.9. MS (CI) *m/z* 268 $(M+1)^+$.

2-Formylphenyl 6-(nitrooxy)hexanoate (21): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 80%. ^{1}H-NMR ($CDCl_3$) δ 1.52-1.62 (*m*, 2H), 1.77-1.89 (*m*, 4H)(-C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$ONO$_2$), 2.68 (*t* , 2H, -OCOC*H*$_2$-, $^{3}J_{HH}$ = 7.4 Hz), 4.49 (*t*, 2H, -C*H*$_2$ONO$_2$, $^{3}J_{HH}$ = 6.5 Hz), 7.17 (*d*, 1H, C_6H_4), 7.41 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.88 (*d*, 1H, C_6H_4), 10.10 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 24.1, 25.1, 26.5, 33.7, 73.0, 123.5, 126.5, 128.1, 131.7, 135.3, 151.3, 171.6, 188.9. MS (CI) *m/z* 282 $(M+1)^+$.

2-Formylphenyl 7-(nitrooxy)heptanoate (22):eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 79%. ^{1}H-NMR ($CDCl_3$) δ 1.47-1.52 (*m*, 4H), 1.75-1.83 (*m*, 4H)(-C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$ONO$_2$), 2.69 (*t*, 2H, -OCOC*H*$_2$-, $^{3}J_{HH}$ = 7.4 Hz), 4.47 (*t*, 2H, -C*H*$_2$ONO$_2$, $^{3}J_{HH}$ = 6.6 Hz), 7.17 (*d*, 1H, C_6H_4), 7.41 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.88 (*d*, 1H, C_6H_4), 10.10 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 24.4, 25.4, 26.6, 28.4, 33.9, 73.2, 123.5, 126.4, 128.1, 131.4, 135.4, 151.5, 171.8, 188.9. MS (CI) *m/z* 296 $(M+1)^+$.

2-Formylphenyl but-3-enoate (43).

To a solution of vinyl acetic acid (3.0 mL; 35.3 mmol) in dry CH_2Cl_2 (50 mL), stirred under inert atmosphere DCC, (2.18 g; 31.7 mmol) was added. After 1 h salicylaldehyde (3.0 mL; 28.2 mmol) and DMAP (0.43 g; 3.53 mmol) were added. The reaction was completed after 2 h. The mixture was filtered and the filtrate was washed with H_2O (20 mL) and brine (20 mL). The organic layer was dried, filtered and

concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/EtOAc 95/5 v/v) to give the title compound as yellow oil. Yield 67%. ^{1}H-NMR ($CDCl_3$) δ 3.46 (*d,* 2H, -C*H*$_2$CH=CH$_2$), 5.28-5.36 (*m*, 2H, -CH$_2$CH=C*H*$_2$), 6.02-6.11 (*m*, 1H, -CH$_2$C*H*=CH$_2$), 7.20 (*d*, 1H, C_6H_4), 7.41 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.89 (*d*, 1H, C_6H_4), 10.11 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 38.9, 119.7, 123.4, 126.5, 128.0, 129.2, 131.2, 135.3, 151.5 169.8, 188.7. MS (CI) *m/z* 191 $(M+1)^+$.

2-Formylphenyl (2,2-dimethyl)-hex-5-enoate (45). The title compound was prepared in the same manner as **43**. Eluent (PE/EtOAc 98/2 v/v); yellow oil; yield 47%. ^{1}H-NMR ($CDCl_3$) δ 1.40 (*s*, 6H, -C*H*$_3$) 1.80-1.86 (*m*, 2H, -C*H*$_2$CH$_2$CH-), 2.12-2.20 (*m*, 2H, -CH$_2$C*H*$_2$CH-), 5.10 (*dd*, 1H, AMX like system, -CH=C*H*$_a$H$_b$), 4.86 (*dd*, 1H, AMX like system, -CH=CH$_a$*H*$_b$), 5.85 (*m*, 1H, AMX like system, -C*H*=CH$_2$), 7.14 (*d*, 1H, C_6H_4), 7.38 (*t*, 1H, C_6H_4), 7.63 (*t*, 1H, C_6H_4), 7.92 (*d*, 1H, C_6H_4), 10.15 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 24.8, 29.4, 39.6, 42.8, 115.0, 123.3, 126.2, 128.3, 129.9, 135.3, 138.0, 152.5, 175.9, 188.3. MS (CI) *m/z* 247 $(M+1)^+$.

General procedure for the preparation of 36, 46, 47, 50.

Iodine (9.79 mmol) was added portion-wise to a stirred solution of the appropriate unsatured compounds **34**, **43**, **44** and **45** (9.79 mmol) and $AgNO_3$ (1.66 g, 9.79 mmol) in CH_3CN (100 mL) kept at -15 °C. At the end of the addition the stirring was continued for 1 h. Then $AgNO_3$ (19.6 mmol) was added and the mixture was heated at 70 °C until the disappearance of the starting material, as checked by TLC. After cooling the mixture was filtered through Celite®. The filtrate was concentrated under reduced pressure, dissolved in water (50 mL) and extracted with EtOAc (4 x 50 mL). The combined organic layers were dried, filtered and concentrated under reduced pressure. The crude product so obtained, when necessary, was purified by flash chromatography. Chromatographic eluents and yields of the products were as follows.

6,7-Dinitrooxyheptanoic acid (36): yellow oil; yield 88%. ^{1}H-NMR ($CDCl_3$) δ 1.50-1.55 (*m*, 2H), 1.67-1.80 (*m*, 4H)(-C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$OCO-), 2.41 (*t*, 2H, -OCOC*H*$_2$-, $^{3}J_{HH}$ = 7.1 Hz), 4.48 (*dd*, 1H, AMX like system, -C*H*$_a$H$_b$ONO$_2$), 4.76 (*dd*, 1H, AMX like system, -CH$_a$*H*$_b$ONO$_2$), 5.26-5.33 (*m*, 1H, AMX like system, -C*H*ONO$_2$), 9.66 (*s br*, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 24.0, 24.3, 27.4, 33.5, 71.2, 78.6, 179.5. MS (CI) *m/z* 253 $(M+1)^+$.

2-Formylphenyl 3,4-bis(nitrooxy)butanoate (46): eluent (PE/EtOAc 90/10 v/v); the orange solid was treated with *i*Pr_2O to obtain the title compound as pale yellow solid; yield 15%. ^{1}H-NMR ($CDCl_3$) δ 3.18 (*d*, 2H, -OCOC*H*$_2$-), 4.71-4.77 (*dd*, 1H, AMX like system, -C*H*$_a$H$_b$ONO$_2$), 4.95-5.00 (*dd*, 1H, AMX like system, -CH$_a$*H*$_b$ONO$_2$), 5.81-5.84 (*m*, 1H, AMX like system, -C*H*ONO$_2$), 7.18 (*d*, 1H, C_6H_4), 7.49 (*t*, 1H, C_6H_4), 7.67 (*t*, 1H, C_6H_4), 7.86 (*d*, 1H, C_6H_4), 9.96 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 34.0, 70.3, 74.9, 123.4, 127.2, 127.6, 134.3, 135.6, 149.4, 167.1, 189.6. MS (CI) *m/z* 315 $(M+1)^+$.

2-Formylphenyl 4,5-bis(nitrooxy)pentanoate (47): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 53%. ^{1}H-NMR ($CDCl_3$) δ 2.10-2.27 (*m*, 2H, -CH$_2$C*H*$_2$CH-), 2.87 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 6.9 Hz), 4.57 (*dd*, 1H, AMX like system, -C*H*$_a$H$_b$ONO$_2$), 4.86 (*dd*, 1H, AMX like system, -CH$_a$*H*$_b$ONO$_2$), 5.52 (*m*, 1H, AMX like system, -C*H*ONO$_2$), 7.17 (*d*, 1H, C_6H_4), 7.46 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.85 (*d*, 1H, C_6H_4), 9.98 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 24.2, 29.2, 71.1, 78.0, 123.5, 126.8, 127.8, 133.7, 135.5, 150.1, 170.6, 189.5. MS (CI) *m/z* 329 $(M+1)^+$.

2-(2,2-Dimethyl)-formylphenyl 5,6-bis(nitrooxy)hexanoate (50): eluent (PE/EtOAc 90/10 v/v); pale yellow oil; yield 55%. ^{1}H-NMR ($CDCl_3$) δ 1.37 (*s*, 6H, -C*H*$_3$), 1.82-1.95 (*m*, 4H, -C*H*$_2$C*H*$_2$CH-), 4.48-4.54 (*dd*, 1H, AMX like system, -C*H*$_a$H$_b$ONO$_2$), 4.77-4.82 (*dd*, 1H, AMX like system, -CH$_a$*H*$_b$ONO$_2$), 5.30-5.37 (*m*, 1H, AMX like system, -C*H*ONO$_2$), 7.11 (*d*, 1H, C_6H_4), 7.41 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H, C_6H_4), 7.87 (*d*, 1H, C_6H_4), 10.0 (*s*, 1H, -C*H*O). ^{13}C-NMR ($CDCl_3$) δ 24.6, 25.1, 35.2, 42.4, 71.2, 79.6, 123.4, 126.6, 128.3, 132.2, 135.3, 151.1, 175.4, 188.8. MS (CI) *m/z* 371 $(M+1)^+$.

2-{[3-(Nitrooxy)propanoyl]oxy)}benzoic acid (28). $SOCl_2$ (2.43 mL, 33.3 mmol) and a few drops of dry DMF were added to a solution of 3-(nitrooxy)propionic acid (3.0 g, 22.2 mmol) in dry THF (20 mL), stirred under N_2 at r.t.; the stirring was continued for 3 h at r.t. The solution of the acyl chloride so obtained was slowly added to a stirred solution of salycilic acid (3.07 g, 22.2 mmol) and dry Py (2.7 mL, 33.3 mmol) in dry THF (40 mL), kept under N_2 at 0 °C. The mixture was allowed to reach r.t. and the stirring was continued overnight. The mixture was diluted with Et_2O (90 mL) and washed twice with HCl 2M (60 mL). The organic layer was dried, filtered and concentrated under reduced pressure. The crude product was partially purified by flash chromatography (CH_2Cl_2/MeOH 97/3 v/v). The crude solid so obtained was crystallized by toluene. Yield 46%. mp 86-88 °C (from toluene). ^{1}H-NMR ($CDCl_3$) δ 3.09 (*t*, 2H, -

OCOC*H*$_2$-, $^3J_{HH}$ = 6.4 Hz), 4.87 (*t*, 2H, -C*H*$_2$ONO$_2$, $^3J_{HH}$ = 6.4 Hz), 7.16 (*d*, 1H, C_6H_4), 7.39 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H, C_6H_4), 8.16 (*d*, 1H, C_6H_4), 10.0 (*s vvbr*, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 32.2, 67.6, 121.8, 123.9, 126.6, 132.7, 135.2, 150.8, 168.3, 169.8. MS (CI) *m/z* 256 $(M+1)^+$.

General procedure for the preparation of 29, 30, 31, 33, 52, 53, 54, 55

$KMnO_4$ (4.38 mmol) was added to a stirred solution of the appropriate aldehyde **19**, **20**, **21**, **25**, **47**, **48**, **49** and **50** (2.92 mmol) in acetone (20 mL) kept at 0 °C. The reaction was allowed to reach r.t. and was completed after 3 h. Oxalic acid was added and the mixture was filtered and the filtrate was diluted with CH_2Cl_2 (20 mL). The organic layer was washed with H_2O (20 mL) and then was dried, filtered and concentrated under reduced pressure. The crude product was purified by crystallization.

2-{[4-(Nitrooxy)butanoyl]oxy}benzoic acid (29): m.p. 70.5 – 71.5 °C (from PE/toluene 50/50 v/v); white solid; yield 52%. ^{1}H-NMR (DMSO-d_6) δ 2.05 (*qi*, 2H, -C*H*$_2$CH$_2$ONO$_2$), 2.71 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 6.0 Hz), 4.63 (*t*, 2H, -C*H*$_2$ONO$_2$, $^3J_{HH}$ = 6.0 Hz), 7.21 (*d*, 1H, C_6H_4), 7.39 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H, C_6H_4), 7.94 (*d*, 1H, C_6H_4), 13.13 (*s*, 1H, -COO*H*). ^{13}C-NMR (DMSO-d_6) δ 21.4, 29.7, 72.6, 123.7, 123.8, 126.1, 131.3, 133.8, 150.0, 165.5, 170.9 . MS (CI) *m/z* 270 $(M+1)^+$.

2-{[5-(Nitrooxy)pentanoyl]oxy}benzoic acid (30): m.p. 48.5-50.5 °C (from PE/toluene 70/30 v/v); white solid; yield 56%. ^{1}H-NMR ($CDCl_3$) δ 1.89 (*m*, 4H, -C*H*$_2$C*H*$_2$CH$_2$ONO$_2$), 2.66 (*m*, 2H, -OCOC*H*$_2$-), 4.47 (*m*, 2H, -C*H*$_2$ONO$_2$), 7.12 (*d*, 1H, C_6H_4), 7.37 (*t*, 1H, C_6H_4), 7.63 (*t*, 1H, C_6H_4), 8.12 (*d*, 1H, C_6H_4), 12.1 (*s br*, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 20.9, 26.3, 33.5, 73.0, 122.2, 124.1, 126.4, 132.7, 135.2, 151.3, 170.5, 171.7. MS (CI) *m/z* 284 $(M+1)^+$.

2-{[6-(Nitrooxy)hexanoyl]oxy}benzoic acid (31): m.p. 68.0-70.0 °C (from PE/toluene 75/25 v/v); white solid; yield 82%. ^{1}H-NMR (DMSO-d_6) δ 1.41-1.77 (*m*, 6H, -C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$ONO$_2$), 2.60 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 7.3 Hz), 4.55 (*t*, 2H, -C*H*$_2$ONO$_2$, $^3J_{HH}$ = 6.0 Hz), 7.20 (*d*, 1H, C_6H_4), 7.39 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H, C_6H_4), 7.94 (*d*, 1H, C_6H_4), 13.10 (*s*, 1H, -COO*H*). ^{13}C-NMR (DMSO-d_6) δ 23.5, 24.4, 25.7, 33.1, 73.6, 123.7, 124.1, 126.0, 131.3, 133.7, 150.0, 165.6, 171.5. MS (CI) *m/z* 286 $(M+1)^+$.

2-{[2,2-Dimethyl-3-(nitrooxy)propanoyl]oxy}benzoic acid (33): m.p. 95.0-96 °C (from PE/toluene 75/25 v/v); white solid; yield 61%. ^{1}H-NMR ($CDCl_3$) δ 1.47 (*s*, 6H, -C*H*$_3$), 4.67 (*s*, 2H, -C*H*$_2$-), 7.10 (*d*, 1H,

C_6H_4), 7.37 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 8.13 (*d*, 1H, C_6H_4), 12.1 (*s* vvbr, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 22.3, 42.5, 77.5, 122.2, 123.8, 126.5, 132.6, 135.1, 150.9, 170.2, 172.8. MS (CI) *m/z* 284 $(M+1)^+$.

2-{[4,5-Bis(nitrooxy)pentanoyl]oxy}benzoic acid (52): m.p. 92.5-93.0 °C (from PE/toluene 45/55 v/v); white solid; yield 89%. ^{1}H-NMR ($CDCl_3$) δ 2.13-2.25 (*m*, 2H, -CH_2CH_2CH-), 2.83 (*t*, 2H, -OCOCH_2-, $^3J_{HH}$ = 6.0 Hz), 4.54 (*dd*, 1H, AMX like system, -C$H_aH_bONO_2$), 4.84 (*dd*, 1H, AMX like system, -$CH_aH_bONO_2$), 5.50 (*m*, 1H, AMX like system, -C*H*ONO_2), 7.13 (*d*, 1H, C_6H_4), 7.40 (*t*, 1H, C_6H_4), 7.66 (*t*, 1H, C_6H_4), 8.14 (*d*, 1H, C_6H_4), 11.0 (*s vvbr*, 1H, COO*H*). ^{13}C-NMR ($CDCl_3$) δ 24.6, 29.8, 71.4, 78.2, 122.0, 124.2, 126.9, 133.0, 135.6, 151.3, 169.9, 171.2. MS (CI) *m/z* 345 $(M+1)^+$.

2-{[5,6-Bis(nitrooxy)hexanoyl]oxy}benzoic acid (53): m.p. 101.5-102.5 °C (from PE/toluene 50/50 v/v); white solid; yield 72%. ^{1}H-NMR (DMSO-d_6) δ 1.73-1.86 (*m*, 4H, -CH_2CH_2CH-), 2.64 (*t*, 2H, -OCOCH_2-, $^3J_{HH}$ = 6.0 Hz), 4.73 (*dd*, 1H, AMX like system, -C$H_aH_bONO_2$), 4.96 (*dd*, 1H, AMX like system, -$CH_aH_bONO_2$), 5.46 (*m*, 1H, AMX like system, -C*H*ONO_2), 7.19 (*d*, 1H, C_6H_4), 7.39 (*t*, 1H, C_6H_4), 7.64 (*t*, 1H, C_6H_4), 7.93 (*d*, 1H, C_6H_4), 13.3 (*s br*, 1H, -COO*H*). ^{13}C-NMR (DMSO-d_6) δ 19.5, 27.5, 32.8, 71.7, 80.0, 123.7, 123.9, 126.0, 131.3, 133.7, 150.0, 165.5, 171.2 . MS (CI) *m/z* 359 $(M+1)^+$.

2-{[6,7-Bis(nitrooxy)heptanoyl]oxy}benzoic acid (54): the crude product was purified by preparative HPLC (Lichrospher 250-25 C_{18}, CH_3CN/H_2O/TFA 60/40/0.1, flow 39 mL/min, λ 224 nm, injection 2 mL, solution 100 mg/mL) to give the title compound as white solid; yield 89%; m.p. 92.5-93.0 °C (from PE/toluene 45/55 v/v). ^{1}H-NMR ($CDCl_3$) δ 1.51-1.65 (*m*, 2H), 1.74-1.86 (*m*, 4H), (-$CH_2CH_2CH_2$CH-), 2.67 (*t*, 2H, -OCOCH_2-, $^3J_{HH}$ = 6.0 Hz), 4.47 (*dd*, 1H, AMX like system, -C$H_aH_bONO_2$), 4.74 (*dd*, 1H, AMX like system, -$CH_aH_bONO_2$), 5.30 (*m*, 1H, AMX like system, -C*H*ONO_2), 7.13 (*d*, 1H, C_6H_4), 7.38 (*t*, 1H, C_6H_4), 7.65 (*t*, 1H, C_6H_4), 8.11 (*d*, 1H, C_6H_4), 8.49 (*s br*, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 23.9, 24.3, 29.0, 33.6, 71.1, 78.9, 121.8, 124.0, 126.4, 132.5, 135.3, 151.1, 170.0, 172.2. MS (CI) *m/z* 373 $(M+1)^+$.

2-{[2,2-Dimethyl-5,6-bis(nitrooxy)hexanoyl]oxy}benzoic acid (55): m.p. 72.0-73.0 °C (from PE/toluene 70/30 v/v), white solid; yield 49%. ^{1}H-NMR ($CDCl_3$) δ 1.40 (*s*, 6H, -CH_3), 1.78-1.93 (*m*, 4H, -CH_2CH_2CH-), 4.45-4.52 (*dd*, 1H, AMX like system, -C$H_aH_bONO_2$), 4.73-4.79 (*dd*, 1H, AMX like system, -$CH_aH_bONO_2$), 5.27-5.34 (*m*, 1H, AMX like system, -C*H*ONO_2), 7.07 (*d*, 1H, C_6H_4), 7.37 (*t*, 1H, C_6H_4), 7.64

(*t*, 1H, C_6H_4), 8.11 (*d*, 1H, C_6H_4), 11.75 (*s vvbr*, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 24.6, 25.0, 25.6, 35.2, 42.1, 71.2, 79.4, 122.3, 123.7, 126.3, 132.4, 135.0, 151.2, 170.2, 175.4. MS (CI) *m/z* 387 $(M+1)^+$.

2-{[7-(Nitrooxy)heptanoyl]oxy}benzoic acid (32). To a solution of **22** (2.6 g, 8.80 mmol) in CH_3CN (20 mL) kept at 0 °C were added a solution of KH_2PO_4 (0.80 g) in H_2O (10 mL) and H_2O_2 30% (1.1 mL, 9.68 mmol) and dropwise a solution of $NaClO_2$ 80% (1.40 g, 12.3 mmol) in H_2O (12 mL). After 2 h the reaction was completed. Na_2SO_3 was added to destroy the excess of H_2O_2. After acidification with HCl 6M the mixture was diluted with H_2O (100 mL) and extracted twice with CH_2Cl_2 (100 mL). The organic layer was dried, filtered and concentrated under reduced pressure. The crude product was crystallized from PE/toluene 70/30 v/v to give the title compound as white solid. Yield 71%; m.p. 47.0-49.0 °C (from PE/toluene 70/30 v/v). ^{1}H-NMR ($CDCl_3$) δ 1.36-1.54 (*m*, 4H), 1.74-1.81 (*m*, 4H)(-C*H*$_2$C*H*$_2$C*H*$_2$C*H*$_2$CH$_2$ONO$_2$) 2.64 (*t*, 2H, -OCOC*H*$_2$-, $^3J_{HH}$ = 7.3 Hz), 4.44 (*t*, 2H, -C*H*$_2$ONO$_2$, $^3J_{HH}$ = 6.6 Hz), 7.12 (*d*, 1H, C_6H_4), 7.36 (*t*, 1H, C_6H_4), 7.63 (*t*, 1H, C_6H_4), 8.10 (*d*, 1H, C_6H_4), 11.53 (*s*, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 24.2, 25.4, 26.6, 28.6, 33.9, 73.3, 122.3, 124.0, 126.2, 132.4, 135.0, 151.2, 170.3, 172.1. MS (CI) *m/z* 312 $(M+1)^+$.

2-{[3,4-Bis(nitrooxy)butanoyl]oxy}benzoic acid (51): the title compound was prepared in the same manner as **32;** m.p. 128-129 °C (from toluene); yield 68%. ^{1}H-NMR ($CDCl_3$) δ 3.15 (*d*, 2H, -OCOC*H*$_2$-), 4.69-4.76 (*dd*, 1H, AMX like system, -C*H*$_a$H$_b$ONO$_2$), 4.92-4.98 (*dd*, 1H, AMX like system, -CH$_a$*H*$_b$ONO$_2$), 5.77-5.84 (*m*, 1H, AMX like system, -C*H*ONO$_2$), 7.16 (*d*, 1H, C_6H_4), 7.41 (*t*, 1H, C_6H_4), 7.67 (*t*, 1H, C_6H_4), 8.15 (*d*, 1H, C_6H_4), 10.90 (s*vv br*, 1H, -COO*H*). ^{13}C-NMR ($CDCl_3$) δ 34.0, 70.2, 74.8, 121.5, 123.7, 126.9, 132.8, 135.3, 138.4, 150.5, 167.4. MS (CI) *m/z* 331 $(M+1)^+$.

Evaluation of stability in acqueous buffer solutions and in human serum.

Hydrolysis in acidic medium (pH 1) and in phosphate buffer (pH 7.4). A solution of each compound (10 mM) in acetonitrile was added to a HCl 0.1 M or to phosphate buffer pH 7.4 (50 mM) preheated at 37 °C; the final concentration of the compound was 250 μM. The resulting solution was maintained at 37 ± 0.5 °C and at appropriate time intervals a 20 μL aliquote of reaction solution was analysed by RP-HPLC.

Hydrolysis in human serum. A solution of each compound (10 mM) in acetonitrile was added to human serum (Sigma) preheated at 37 °C; the final concentration of the compound was 250 μM. The resulting solution was incubated at 37 ± 0.5 °C and at appropriate time intervals 500 μL of reaction mixture was

withdrawn and added to 750 μL of acetonitrile containing 0.1% trifluoroacetic acid in order to deproteinize the serum. The sample was sonicated, vortexed and then centrifuged for 10' at 2150 g, the clear supernatant was filtered by 0.45 μm PTFE filters (Alltech) and analysed by RP-HPLC.

The reverse-phase HPLC procedure allowed separation and quantitation of remaining compound and of salicylic acid. HPLC analyses were performed with a HP 1100 chromatograph system (Agilent Technologies, Palo Alto, CA, USA) equipped with a quaternary pump (model G1311A), a membrane degasser (G1379A), a diode-array detector (DAD) (model G1315B) integrated in the HP1100 system. Data analysis was done using a HP ChemStation system (Agilent Technologies). The analytical column was a Nucleosil 100-5C18 Nautilus (250 × 4.6 mm, 5 μm particle size) (Macherey-Nagel). The mobile phase consisting of acetonitrile/water (55/45) with 0.1% trifluoroacetic acid and the flow-rate was 1.2 mL/min. The injection volume was 20 μL (Rheodyne, Cotati, CA). The column effluent was monitored at 226 nm (for compounds) and 240 nm (for salicylic acid) referenced against a 600 nm wavelength. Quantitation was done by comparison of peak areas with standards chromatographed under the same conditions.

Antiinflammatory activity. Male Wistar rats, weighing 180-200 g (Harlan, S. Pietro al Natisone, Italy) were individually housed in hanging stainless-steel cages with grid floors, at constant room temperature (25 ± 1 °C) and humidity (60 ± 5%), with an artificial 12:12 h light/dark cycle. Edema was induced in conscious rats by intraplantar injection into the right hindpaw of 0.1 ml of 1% carrageenan, suspended in 1% carboxymethylcellulose (CMC). Immediately after carrageenan injection, compounds or vehicle (CMC, 1%) were administered intragastrically to different groups of rats in a volume of 10 ml/kg. Salicylic acid and NO-aspirin derivatives were administered at a dose equimolar with aspirin 120 mg/kg. Groups of 6-8 animals were used. Paw volume was measured with a water plethysmometer (Basile, Comerio, Italy) immediately before carrageenan injection and 3 hour afterwards. The edema reduction in treated animals was expressed as percentage inhibition of the edema observed in vehicle-treated animals, considered as 100. The results obtained are presented as mean ± SEM. Statistical analysis was performed with ANOVA followed by Dunnett test.

Gastrotoxicity. Male Wistar rats, weighing 180-200 g (Harlan, S. Pietro al Natisone, Italy) were individually housed in hanging stainless-steel cages with grid floors, at constant room temperature (25 ± 1 °C) and humidity (60 ± 5%), with an artificial 12:12 h light/dark cycle. They were deprived of food but not of water 24 h before the experiments. Groups of rats (n = 8-10) were given aspirin 120 mg/kg by intragastric route or equimolar doses of the compounds under study (vehicle CMC 1%). Rats were sacrificed 3 h after the administration of the compounds. Immediately after the sacrifice, the stomachs were removed, opened along the lesser curvature and examined for the assessment of mucosal lesions, the stomachs were laid on a flat surface under a stereomicroscope. The glandular mucosa was examined and each individual hemorrhagic lesion was measured along its greatest length (<1 mm: rating = 1; 1-2 mm: rating = 2; >2 mm: rating according to their greatest length). The lengths of the lesions were summed to give an overall total, designated as the lesion index, for each stomach. The results obtained are presented as mean ± SEM. Statistical analysis was performed with ANOVA followed by Newman-Keuls test.

Inhibition of human platelet aggregation in vitro. Venous blood samples were obtained from healthy volunteers who had not taken any drug for at least two weeks. Volunteers, who were treated according to Helsinki protocol for biomedical experimentation, gave their informed consent to the use of blood samples for research purposes. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 200 g for 20 minutes. Aliquots (500 μL) of PRP were added into aggregometer (Chrono-log 4902D) cuvettes and aggregation was recorded as increased light transmission under continuous stirring (1000 rpm) at 37 °C for 10 minutes after addition of the stimulus. Collagen at submaximal concentration (0.8-1.5 μg/mL) was used as platelet activator in PRP. Compounds under study were preincubated with PRP 10 min before addition of the stimulus (collagen). Vehicle alone (0.5% DMSO) added to PRP did not affect platelet function in control samples. The role of NO and sGC in the inhibitory effect was investigated using the sGC inhibitor, ODQ (50 μM). At least 5 experiments for each compound were performed.

The antiaggregatory activity of tested compounds is evaluated as % inhibition of platelet aggregation compared to control samples. For most active compounds IC_{50} values could be calculated by non-linear regression analysis, otherwise % inhibition at maximal concentration tested (300 μM) is reported.

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 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} ± SE (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. Addition of the drug vehicle (DMSO) had no appreciable effect on contraction level.

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Supporting information available. Elemental analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

Figure 1

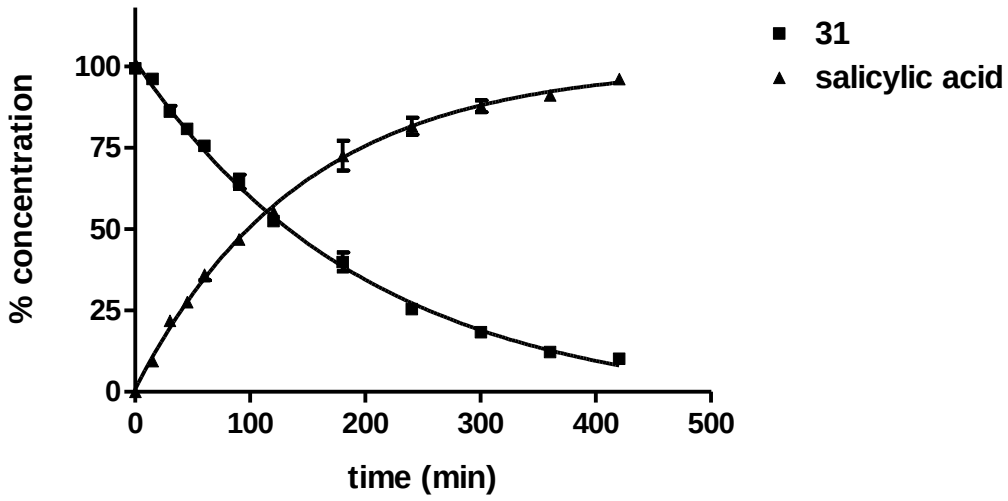


Figure 1. Time courses for compound **31** and salicylic acid in human serum at 37 °C. Values are mean ± SEM (SEM ≤ 2; number of determinations ≥ 4).

Figure 2

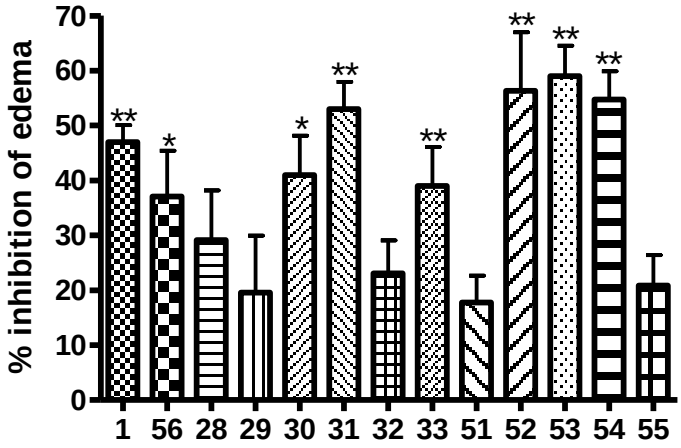


Figure 2. Antiinflammatory effects of aspirin (**1**), salicylic acid (**56**) and of NO-donor aspirin-like molecules (**28**-**32**, **51**-**55**) on carrageenan-induced paw edema in conscious rats. The aspirin-like compounds were administered by intragastric route at a dose equimolar with aspirin, 120 mg/kg, at the same time as carrageenan, and their effects were evaluated 3 h later. Results are expressed as % of inhibition of edema observed in vehicle-treated group. This edema was considered arbitrarily as 100. **P* < 0.05; ***P* < 0.01 vs vehicle (ANOVA, followed by Dunnett test). Values are mean ± SEM (*n* = 8-10 rats per group).

Figure 3

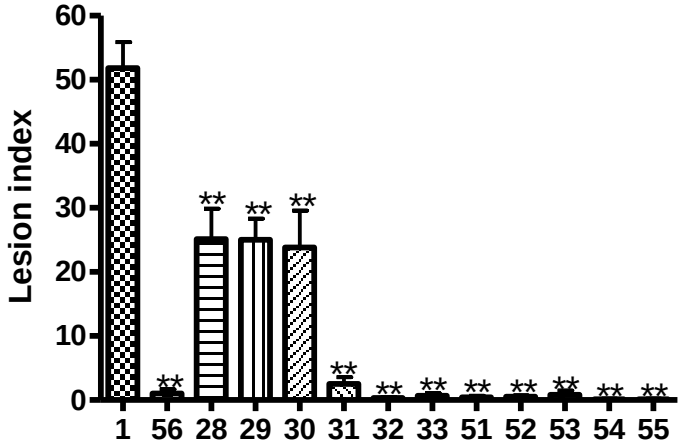
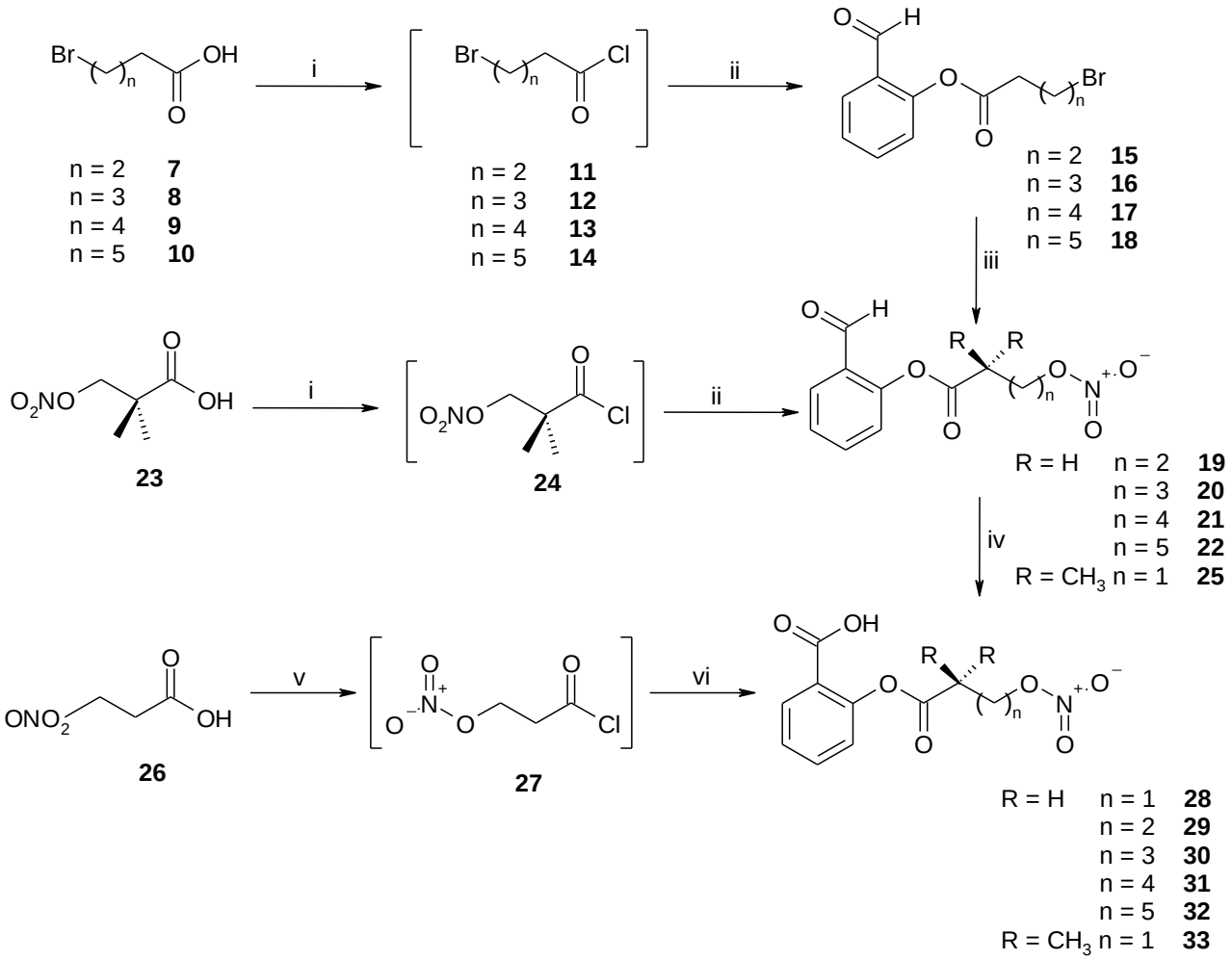


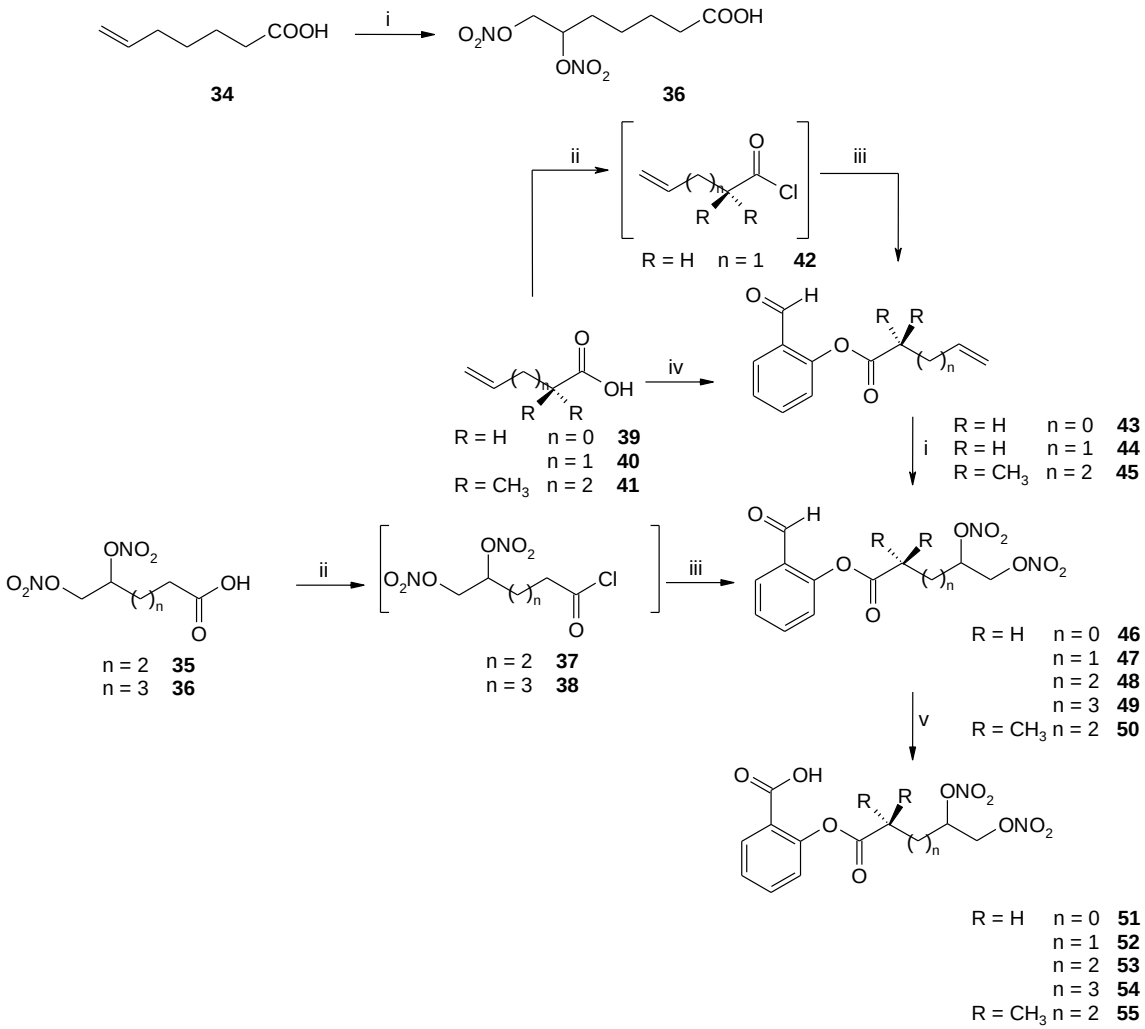
Figure 3. Gastric ulcerogenic effects of aspirin (**1**), salicylic acid (**56**) and of NO-donor aspirin-like molecules (**28**-**32**, **51**-**55**) in conscious rats. The aspirin-like compounds were administered by intragastric route at a dose equimolar with aspirin, 120 mg/kg, and the stomachs were examined 3 h later. Gastric lesions were measured along the greatest length and the cumulative length in millimeters was designated as the "lesion index" for each stomach. All the compounds tested produced significantly less gastric damage than aspirin (**$P < 0.01$; ANOVA and Newman-Keuls test). Values are mean ± SEM (n = 8-10 rats per group).

Scheme 1[a]



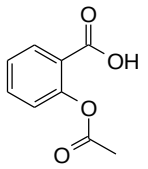
[a] Reagents and conditions: i) $SOCl_2$, dry CH_2Cl_2, dry DMF; ii) salicylaldehyde, dry CH_2Cl_2, dry Py; iii) $AgNO_3$, CH_3CN, 70 °C; iv) $KMnO_4$, Acetone for n = 1-4 and R = H, CH_3; $NaClO_2$ 80%, H_2O_2 30%, CH_3CN for n = 5 and R = H; v) $SOCl_2$, dry THF, dry DMF; vi) salicylic acid, dry THF, dry Py.

Scheme 2[a]

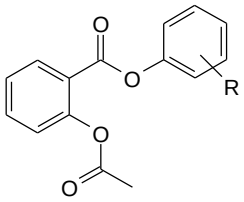


[a] Reagents and conditions: i) I_2, $AgNO_3$, CH_3CN r.t. then $AgNO_3$, CH_3CN reflux; ii) $SOCl_2$, dry CH_2Cl_2, dry DMF; iii) salicylaldehyde, dry CH_2Cl_2, dry Py; iv) DCC, dry CH_2Cl_2 then salicylaldehyde, dry CH_2Cl_2, DMAP; v) $KMnO_4$, Acetone for n = 1-3 and R = H, CH_3; $NaClO_2$ 80%, H_2O_2 30%, CH_3CN for n = 0 and R = H.

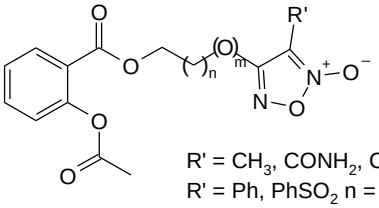
Chart 1. Examples of NO-donors-aspirin



1

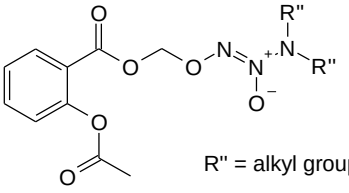


2 NCX4040 R = p-CH_2ONO_2
3 NCX4016 R = m-CH_2ONO_2
4 NCX4060 R = o-CH_2ONO_2



R' = CH_3, $CONH_2$, CN n = 0, m = 0
R' = Ph, $PhSO_2$ n = 2, m = 1

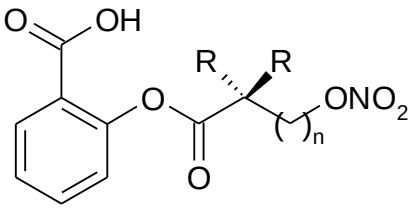
5



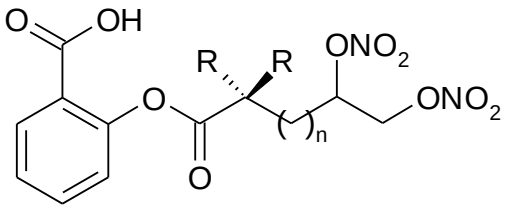
R'' = alkyl groups

6

C**hart 2.** NO-donor “aspirin- like” compounds.



R = H n = 1, 2, 3, 4, 5
R = CH_3 n = 1



R = H n = 0, 1, 2, 3
R = CH_3 n = 2

Table 1. Stability in aqueous buffers and human serum, antiaggregatory and vasodilator activity of the products **28-33** and **51-55**.

Compound	Stability			Antiaggregatory activity		Vasodilator activity
	Aqueous buffers % unchanged at 3 h		Human serum			
	pH 1.0	pH 7.4	$t_{1/2}$ (h) ± SD	IC_{50} (CL 95%) μM [+ 50 μM ODQ]	% inhibition ± SEM at 300 μM [a]	EC_{50} (μM) ± SEM [+ 1 μM ODQ]
1	> 90%	> 90%	1.06 ± 0.08	54 (49-60)		///
28	> 90%	55%	0.26 ± 0.01	162 (129-204) [186 (172-200)]		37 ± 2 [b]
29	> 97%	> 95%	0.47 ± 0.01	30 (24-37) [24 (17-34)]		23 ± 6 [b]
30	> 97%	> 95%	0.76 ± 0.01	97 (85-110) [91 (76-110)]		21 ± 2 [b]
31	> 97%	> 95%	2.03 ± 0.11	a)	27 ± 10	14 ± 1 [b]
32	> 97%	> 95%	1.19 ± 0.08	inactive		8.1 ± 1.4 [b]
33	> 97%	> 95%	9.03 ± 0.22	a)	26 ± 6	6.2 ± 0.9 [b]
51	> 85%	20%	0.28 ± 0.01	126 (98-160) [119 (105-134)]		9.2 ± 0.9 [b]
52	> 85%	> 95%	4.09 ± 0.08	a)	15 ± 8	8.2 ± 1.2 [b]
53	> 85%	> 95%	4.87 ± 0.07	a)	6.7 ± 5.8	5.8 ± 0.7 [b]
54	> 85%	> 95%	6.25 ± 0.17	inactive		3.1 ± 0.7 [b]
55	> 97%	> 95%	15.2 ± 0.40	inactive		5.3 ± 0.6 [b]

a) Due to the low activity of the compound, IC_{50} could not be calculated. In this case the percent of inhibition is reported at 300 μM.

b) In the presence of 1 μM ODQ, EC_{50} values were >100 μM.

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SYNOPSIS TOC

SEARCHING FOR NEW NO-DONOR ASPIRIN-LIKE MOLECULES: A NEW CLASS OF NITROOXY-ACYL DERIVATIVES OF SALICYLIC ACID

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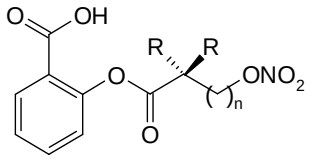

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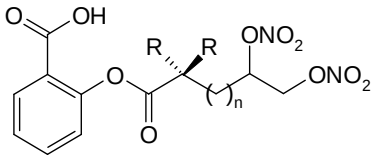

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