Water-Soluble Nitric-Oxide-Releasing Acetylsalicylic Acid (ASA) Prodrugs

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Water-soluble nitric-oxide-releasing aspirin pro-drugs

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Summary

A new series of water-soluble (R-benzoyloxy) methyl esters of aspirin bearing at the benzoyl ring both alkyl chains containing nitric oxide (NO)-releasing nitrooxy groups and solubilizing moieties, was synthesized, and the products evaluated as true aspirin pro-drugs. Most products are solid substances and all possess good water solubility. They are quite stable in acid solutions (pH=1) and less stable at physiological pH. In human serum they are immediately metabolized by esterases, producing a mixture of aspirin (ASA), salicylic acid (SA), and of the related NO-donor benzoic acids, with other minor products. Due to aspirin release, the pro-drugs are capable of inhibiting collagen-induced platelet aggregation of human platelet-rich plasma. The simple NO-donor benzoic acids 28 and 48, studied as representative models of the whole class of benzoic acids formed following metabolism of the prodrugs in serum, did not trigger antiaggregatory activity when tested at 300 µM concentration. Only 28 displays quite potent NO-dependent vasodilatatory action. For two selected pro-drugs, 38 and 49, in vivo gastrotoxicity and anti-inflammatory properties are also reported: their anti-inflammatory activities are similar to that of aspirin when tested in the carrageenan-induced paw edema assay in rats. The gastrotoxicity the two pro-drugs was lower than that of aspirin in a lesion model in rats.

Introduction

Nitric oxide (NO)-donor aspirins (NO-ASA) are a class of products that has received particular attention in recent years.¹² The pharmacological rationale for developing them was that NO displays gastrosparing properties and consequently could be expected to reduce the well-known gastrotoxicity of ASA, without reducing its antithrombotic and anti-inflammatory properties.³⁵ NO-ASAs have the general structure 1 (Chart 1) in which the carboxylic group of acetylsalicylic acid
(ASA) is linked through an enzymatically-labile ester bridge to a substructure containing a NO-donor moiety, belonging to the nitrate, furoxan, NONOate class. Examples of such hybrids are reported in the literature. The action of these substances is complex, and there is no clear evidence to date that they are true pro-drugs of ASA, namely that they are capable of releasing ASA in the plasma or other tissues. The absence of a negative charge, which conversely is present on ASA at physiological pH (ASA, pK_a=3.5), renders their acetyloxy group highly susceptible to enzymatic cleavage. These products should thus be considered pro-drugs of salicylic acid (SA) rather than of ASA. We recently showed that (nitrooxyacyloxy)methyl esters of ASA and related carbonates are true ASA pro-drugs (2, 3 Chart 1). In these structures the deacetylation rate constant is lower than the hydrolytic rate constant of the carbonyloxymethyloxycarbonyl moiety, and consequently the products are able to release ASA in amounts that depend on the structure of the NO donor acyl moiety. One problem with these double esters is their poor water solubility; many are oily in nature. This study describes a new series of (nitrooxyacyloxy)methyl esters of ASA, which are true ASA pro-drugs and that possess good water solubility and a solid physical state. In these products, the NO-donor nitrooxyacyloxy moiety comprises the benzoyloxy scaffold, bearing a nitrooxyalkyloxy chain at the para position, and a solubilizing substructure at the meta position (Chart 2). In most of the derivatives (38-44) the solubilizing substructure is a metabolically-labile aminoacyloxy group (I, Chart 2); in the case of compound 49 (Chart 2), the morpholinomethyl solubilizing substructure is directly jointed to the aromatic ring. The synthesis, solubility, stability in different media, release of ASA, together with in vitro platelet antiaggregatory profile of all these new pro-drugs are discussed. The in vivo gastrotoxicity and anti-inflammatory properties are also reported for two selected compounds, 38 and 49.

Chemicals. The products bearing aminoacyloxy groups were obtained as reported in Schemes 1 - 3. The commercially-available 3,4-dihydroxybenzaldehyde (4) was bromoalkylated with 1,3-dibromopropane in acetonitrile solution to give 5 that, in turn, was transformed by action of AgNO_3 into the related nitrooxy analogue 7. This intermediate was reacted under inert atmosphere with the appropriate tert-butoxycarbonyl (BOC) protected amino acid in CH_2Cl_2 solution, in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 4-(dimethylamino)pyridine (DMAP), to afford the related aminoacyloxy substituted aldehydes 9, 11-14. The corresponding acids 15, 17-20 were obtained by oxidation with KMnO_4 in acetone solution. The acid 16 and its intermediates 10, 8 were obtained in the same manner as its inferior homologue 15, the only difference being that the starting material was the chloro substituted compound 6. Coupling of these acid derivatives 15-20 with ASA chloromethyl ester 29, in the presence of Cs_2CO_3, gave the oxymethyloxy esters 30-35, and subsequent removal of BOC-protection in
dioxane containing HCl yielded the desired compounds 38-43. The final para-nitroxybutyloxy product 39 and the its intermediates 16, 10, 8, were obtained in the same manner as its inferior homologue 38 and related intermediates. For the preparation of the dinitrooxy substituted pro-drug 44 3,4-dihydroxybenzaldehyde (4) was alkylated with allylbromide to give the unsaturated aldehyde 21. This latter was treated with (BOC)-protected β-alanine to give 22, following the general conditions described above for the preparation of the (BOC)-protected aminoacyloxy substituted aldehydes. This intermediate was transformed by action of iodine and AgNO₃ into the dinitrooxyester 23, which, in turn was oxidized with KMnO₄ to the corresponding acid 24. The final compound 44 was obtained from 24 through the intermediate 36, using the procedures described above to prepare the aminoacyloxy substituted pro-drugs from the related acids.

Scheme 2 reports the synthesis of the simple NO-donor benzoic acids 28. In particular, the ester derivative 27 was obtained starting from the methyl ester of 3,4-dihydroxybenzoic acid 25, in the same manner as its aldehyde analogue 7. 27 was then hydrolyzed to the corresponding carboxylic acid 28 with NaOH in a THF/H₂O mixture heated to 40 °C.

Scheme 4 reports the synthetic pathway followed to obtain the target product 49 containing the morpholino moiety as solubilizing group. The ester 45 treated with 1,3-dibromopropane in acetonitrile solution gave rise to the bromocompound 46. This product, by action of AgNO₃ in acetonitrile solution, was transformed into the nitrooxy analogue 47, in turn hydrolyzed to the related acid 48, which was coupled with 29 to give the desired final structure.

All the basic products were successfully transformed into the related hydrochlorides, with the sole exception of the morpholino derivative 49, which was characterized as an oxalate. Most of these salts are solid compounds having well-defined melting points (Table 1). Products 39 and 41, which are both superior homologues of 38, the former at the aminoacyloxy chain, the latter at the nitrooxyalkyloxy moiety, are oily. Compound 44, formally derived from 38 by introducing a second nitrooxy group α-positioned to the first, is a foam.

**Water solubility.** The water solubility values of all compounds described here are given in Table 1. All products display good water solubility, in most cases higher than that of ASA (3 g/l, The Merck Index Fourteenth Edition). For the solid hydrochlorides, the higher the solubility, the lower the melting point.

**Hydrolysis studies.** Most of the pro-drugs described here (compounds 38-44) contain three substructures susceptible to hydrolysis: the acetyloxy group, the aminoacyloxy group, and the carbonyloxymethyloxycarbonyl moiety. The possible routes whereby these products can hydrolyze
are reported in Scheme 5. Hydrolysis was assessed by high performance liquid chromatography (HPLC) in pH 1 and pH 7.4 buffered solutions, as well as in human serum. In human serum, hydrolysis is catalyzed by carboxylesterases, ubiquitous enzymes that display broad substrate specificity: the same ester can often be hydrolyzed by more than one enzyme.\textsuperscript{12} In human serum, the fate of all compounds was very similar: the observed half-lives were < 1 min (see Table 1). The metabolites were monitored for 6 h. After 10 minutes, only ASA, SA, IV, and traces of II and III were present: this means that the aminoacyloxy and the carbonyloxymethyloxycarbonyl moieties hydrolyze simultaneously, and much more faster than the related acetyloxy functions. After 6 h, only SA and IV, accompanied by traces of ASA, were present. Figure 1 shows the concentration over time of the metabolites of compound 38, as an example of the performance of the class as a whole. This is an interesting result, and shows that these products are true ASA pro-drugs. Maximal ASA production is reached for all compounds after 2-5 min. These values, expressed as % of the initial concentration of the compounds, are reported in Table 1. They are similar for all products, and fall in the range 50-57%. Table 1 also gives the areas under the ASA release curves, measured after ten minutes’ incubation (AUC\textsubscript{0-10min}). They do not entirely parallel the corresponding max ASA % values, which suggests that the substitution pattern at the benzoyl ring exerts some influence on the hydrolysis of the carbonyloxymethyloxycarbonyl moieties. The products are more stable in physiological solution (pH=7.4 phosphate buffer) than in serum: after 3 hours’ incubation, the sole metabolite formed was III. The hydrolysis strictly followed first-order kinetic, and the observed pseudo-first-order constants (k\textsubscript{obs}) were calculated from the slopes of linear plots of the logarithm of the remaining ester against time: the corresponding half-lives (Table) were obtained from the eq 1.

\[ t_{1/2} = \frac{0.693}{k_{obs}} \text{ (1)} \]

The most stable pro-drugs are compounds 39 (86 min), 38 (57 min), and 44 (20 min), bearing the β-aminopropionyloxy chain at the 3-position of the benzoyl ring. The other compounds show \( t_{1/2} \) values in the range 10-15 min. In pH=1 buffered solution, all products remained 70-90% unchanged after 3h (Table 1); during this time, only III had formed. Unlike the products discussed above, the pro-drug 49 bears the water-solubilizing morpholinomethyloxy group directly linked to the benzoyl scaffold, rather than through a vulnerable ester bridge. Consequently, only the acetyloxy and the carbonyloxymethyloxycarbonyl moieties undergo hydrolysis, with a consequent decrease in the number of possible metabolites (see Scheme 5). This product is more stable in serum than the pro-drugs discussed previously, due to the lack of the very vulnerable aminoacyloxy function. Like the other pro-drugs, its deacetylation rate constant
is lower than the hydrolytic rate constant of the carbonyloxymethyloxycarbonyl moiety, which enables ASA to be present among the metabolites. The hydrolysis strictly follows a first order kinetics. Its half-life, calculated from the observed pseudo-first-order rate constant, is 2.2 min. Figure 2 shows the concentrations of the metabolites over time for this product. The maximum concentration of aspirin is reached after 6 min, and the amount of aspirin released (% max and AUC values) is lower than the values measured for all other pro-drugs (see Table 1). After 6 h, only 48, SA, and traces of ASA were present. The compound is very stable, not only in pH=1 buffered solution, but also at physiological pH.

Pharmacological studies.

Platelet Antiaggregatory Activity. Antiaggregatory activity of the new NO-aspirins was assessed through collagen-induced platelet aggregation of human platelet rich plasma (PRP), taking ASA as reference standard. The inhibitory effect of a compound was tested by adding it to PRP 10 min before addition of the stimulus. All products displayed a concentration-dependent inhibitory effect. Their antiaggregatory potencies, expressed as IC₅₀, are in Table 1. As previously mentioned, compounds IV and 48 are the metabolites formed rapidly from the pro-drugs under study, under the action of serum esterases. The antiaggregatory action of 28 and 48, chosen as prototypes of these acid metabolites, was likewise evaluated. No antiaggregatory activity was observed when the products were tested at 300 µM concentration, meaning that NO does not play a significant role in the antiaggregatory effect of these pro-drugs, and that this activity is largely due to their capacity to produce ASA. The relationship between AUC₀₋₁₀min and IC₅₀ values for the entire series of pro-drugs studied is depicted in Figure 3.

Vasodilator activity. All the pro-drugs can be expected to display vasodilator properties in vivo, due to the rapid formation of the relative NO-donor benzoic acid metabolites (IV and 48) under the action of plasma esterases. The vasodilator activity of two selected acids, 28 and 48, chosen as prototypes of these metabolites, was evaluated on endothelium-denuded rat-aorta strips, precontracted with phenylephrine. The first compound (28), which contains the meta-hydroxybenzoic acid moiety that is common to all the aminoacyloxy-substituted pro-drugs, was found to relax the contracted tissue in a concentration-dependent manner. Its potency, expressed as EC₅₀, is in Table 1. When the experiments were repeated in the presence of 1 µ ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a known inhibitor of soluble guanylate cyclase (sGC), the potency decreased (Table 1), in keeping with NO-induced activation of sGC being the mechanism underlying the vasodilator effect. Conversely, the second compound (48) was inactive as a vasodilator, after testing it at up to 100 µM concentration. This product contains the same para-
nitrooxy alkyl chain as 28, but the water solubilizing group is covalently attached to the benzoic acid scaffold. The lipophilic-hydrophilic balance of the two products differs (28, logD7.4 = -0.80; 48, logD7.4 = -1.30) as do their steric and electronic properties. The former at physiological pH exists principally as an anionic species (anion form >99%; pK_a COOH = 4.41) while the latter exists as a mixture of anion and zwitterion species (anion form = 54%, zwitterion form = 46%; pK_a COOH = 3.90, pK_a MORPHOLINE = 7.33). These diverse physico-chemical profiles should give the two products different capacities both to reach and to interact with the target enzyme involved in NO production, thus affording different vasodilator behaviour. This does not rule out that 48 could release NO in a more complex biological environment: experiments incubating this compound in liver homogenate pointed to a time-dependent production of nitrite and nitrate (NO_x), the oxidised metabolites of NO derived from biotransformation of organic nitrates (data not shown).

Anti-inflammatory activity. The two NO-donor ASA pro-drugs, 38 and 49, and ASA itself, as reference drug, were tested for their anti-inflammatory activity by the carrageenan-induced paw edema test in conscious rats (six per group). The intraplantar injection of carrageenan produced an immediate increase in the paw volume, the inflammatory response peaking 4-5 h hours after carrageenan injection. The intragastric administration of 120 mg/kg ASA, just prior to carrageenan injection, led to a significant reduction in edema (57.4 ± 6.8 %, P < 0.01 versus vehicle-treated controls) assessed 3 h after ASA administration. Both pro-drugs, 38 and 49, administered at doses equimolar to 120 mgkg⁻¹ ASA, caused a significant reduction in paw edema (54.9 ± 6.6 % and 51.5 ± 4.3 % respectively, P > 0.01 versus vehicle-treated controls). The degree of reduction achieved following the administration of the two pro-drugs was comparable to that caused by ASA (Figure 4A).

Acute Gastric Mucosal Damage. The two NO-donor ASA pro-drugs, 38 and 49, and ASA itself, as reference drug, were administered intragastrically to conscious rats (six per group) and after 3 h the lesion index of each rat stomach was assessed. ASA, at a dose of 120 mg/kg, resulted in the development of macroscopically-detectable lesions located in the gastric mucosa, and characterized by necrosis and hemorrhage, the lesion index being 55.3 ± 10.4. In contrast, neither pro-drug, in a dose equimolar to 120 mgkg⁻¹ ASA, exerted macroscopically-detectable damaging effects on the gastric mucosa (Figure 4B).

Conclusions

We have developed a new class of (benzoyloxy)methyl esters of ASA, bearing, at the benzyol ring, both alkyl chains containing NO-releasing nitrooxy groups and aminoacyloxy solubilizing moieties.
The products were characterized as salts, and most of them are solid compounds with well-defined melting points. The entire class display good water solubility and acid stability. The products are rapidly metabolized in physiological conditions. In human serum, under the action of esterases, the aminoacyloxy and the carbonyloxymethyloxycarbonyl moieties simultaneously hydrolyze, much more rapidly than do the related acetyloxy functions. The resulting mixture of metabolites includes significant amounts of ASA, and consequently these compounds may be considered true NO-donor ASA-prodrugs. All the products display good antiaggregatory properties as tested on collagen-induced platelet aggregation of human platelet-rich plasma; this activity is roughly related to the amount of ASA released in the first ten minutes’ incubation. The benzoic acid derivative 28, containing the meta-hydroxybenzoic acid moiety and studied as prototype of the acid metabolites deriving from the aminoacyloxy substituted pro-drugs, is endowed with NO-dependent vasodilator activity, unlike 48, in which the solubilizing group remains covalently attached to the benzoic acid scaffold. The related pro-drugs 38, 49 display in vivo anti-inflammatory properties and reduced gastrotoxicity as tested on rats.

As a whole, based on their pharmacological and physico-chemical profiles, the pro-drugs described here represent an improved class of NO-donor ASA pro-drugs in comparison to the related less soluble pro-drugs previously described.\textsuperscript{10} For this reason they could be more suitable for clinical applications.

**Experimental.**

**Chemistry.**

\textsuperscript{1}H and \textsuperscript{13}C NMR spectra were recorded on a Bruker Avance 300 at 300 and at 75 MHz, respectively, using SiMe\textsubscript{4} as internal standard. The following abbreviations are used to indicate peak multiplicity: s=singlet, d=doublet, t=triplet, m=multiplet, br s=broad signal. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. Melting points were determined with a capillary apparatus (Buchi 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 monitored by thin layer chromatography (TLC) on 5 cm × 20 cm plates, with layer thickness 0.25 mm. Anhydrous sodium sulfate was used as the drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Elemental analyses (C, H, N) were performed by REDOX (Monza), and the results are within 0.4% of the theoretical values. Compounds 29\textsuperscript{13}, 45\textsuperscript{14} were synthesized as indicated in the literature.
4-(3-Bromopropoxy)-3-hydroxybenzaldehyde (5)

A solution of 4 (5.00 g, 36.22 mmol), 1,3-dibromopropane (7.35 mL, 72.44 mmol) and KHCO₃ (4.35 g, 43.46 mmol) in CH₂CN (100 mL) was refluxed for 4 hours. The mixture was then poured into H₂O (50 mL) and extracted with EtOAc (50 mL); the organic layer was washed twice with a saturated solution of NaHCO₃ (50 mL) and extracted twice with 2N NaOH (50 mL). The last aqueous layers were acidified with 6M HCl and then extracted with EtOAc (3 × 50 mL). The combined organic layers were dried with MgSO₄, filtered, and concentrated under reduced pressure to give the title compound as a pale yellow semisolid; yield: 47 %. ¹H-NMR (CDCl₃) δ 2.43 (qi, 2H, -OCH₂CH₂-), 3.60 (t, 2H, -CH₂Br), 4.32 (t, 2H, -OCH₂CH₂-), 5.88 (s, 1H, OH), 7.01 (d, 1H, C₆H₃), 7.42 (m, 1H, C₆H₃), 7.44 - 7.46 (m, 1H, C₆H₃), 9.85 (s, 1H, -CHO). ¹³C-NMR (CDCl₃) δ 29.3, 31.8, 66.9, 111.2, 114.4, 124.6, 130.8, 146.2, 150.9, 191.1. MS (Cl) m/z 259/261 (M+1)+.

4-(4-Chlorobutoxy)-3-hydroxybenzaldehyde (6)

A solution of 4 (1.00 g, 7.24 mmol), 1-bromo-4-chlorobutane (2.50 mL, 21.7 mmol) and KHCO₃ (870 mg, 8.69 mmol) in CH₂CN (20 mL) was refluxed for 14 hours. The mixture was then poured into H₂O (30 mL) and extracted with EtOAc (30 mL); the organic layer was washed twice with a saturated solution of NaHCO₃ (30 mL) and extracted with 2N NaOH (3 × 30 mL). The last aqueous layers were acidified with 6M HCl and then extracted with EtOAc (3 × 30 mL). The combined organic layers were dried with MgSO₄, filtered, and concentrated under reduced pressure to give the title compound as a white solid; yield: 60 %. m.p. 58 - 59 °C (PE/Toluene 1/1 v/v). ¹H-NMR (CDCl₃) δ 1.95 - 2.12 (m, 4H, -OCH₂CH₂-), 3.65 (t, 2H, -CH₂Cl), 4.20 (t, 2H, -OCH₂CH₂-), 5.93 (s, 1H, OH), 6.97 (d, 1H, C₆H₃), 7.41 - 7.46 (m, 2H, C₆H₃), 9.84 (s, 1H, -CHO). ¹³C-NMR (CDCl₃) δ 26.9, 29.6, 45.0, 68.9, 111.4, 114.8, 125.0, 131.2, 146.7, 151.6, 191.6. MS (Cl) m/z 229/231 (M+1)+.

4-(Allyloxy)-3-hydroxybenzaldehyde (21)

A solution of 4 (2.00 g, 14.48 mmol), allyl bromide (1.20 mL, 14.48 mmol) and KHCO₃ (1.74 g, 17.37 mmol) in CH₂CN (20 mL) was refluxed for 4 hours. The mixture was then poured into H₂O (50 mL) and extracted with EtOAc (50 mL); the organic layer was washed twice with a saturated solution of NaHCO₃ (50 mL) and extracted twice with 2N NaOH (50 mL). The last aqueous layers were acidified with 6M HCl and then extracted with EtOAc (3 × 50 mL). The combined organic layers were dried with MgSO₄, filtered, and concentrated under reduced pressure to give the title compound as a pale yellow semisolid; yield: 40 %. m.p. 57 - 60 °C (iPr₂O). ¹H-NMR (CDCl₃) δ 4.69 (d, 2H, -OCH₂-), 5.34-5.46 (m, AMX-like system, 2H, -CH=CH₂), 5.99-6.13 (m, AMX-like system, 2H, -CH=CH₂ + -OH), 6.96 (d, 2H, C₆H₃), 7.38-7.46 (m, 2H, C₆H₃), 9.83 (s, 1H, -CHO). ¹³C-NMR
(CDCl₃) δ 69.9, 111.5, 114.4, 119.2, 124.4, 130.6, 131.8, 146.3, 150.9, 191.2. MS (Cl) m/z 179 (M+1)*.

Methyl 4-(3-bromopropoxy)-3-hydroxybenzoate (31)

A solution of methyl 3,4-dihydroxybenzoate (2.10 g, 12.5 mmol), 1,3-dibromopropane (3.80 mL, 37.5 mmol) and KHCO₃ (1.50 g, 14.53 mmol) in CH₃CN (20 mL) was refluxed for 5 hours. The mixture was then poured into H₂O (50 mL) and extracted twice with EtOAc (30 mL); the combined organic layers were washed with brine (10 mL), dried with MgSO₄, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/EtOAc 9/1 v/v to PE/EtOAc 7/3 v/v) to give the title compound as a white solid; yield: 25%. m.p. 106.5-107.5 °C (iPr₂O).

1H-NMR (CDCl₃) δ 2.39 (qi, 2H, -OCH₂C₂H₅), 3.58 (t, 2H, -C₂H₂Br), 3.88 (s, 3H, -OCH₃), 4.26 (t, 2H, -OCH₂C₂H₅), 5.75 (sbr, 1H, OH), 6.90 (d, 1H, C₆H₃), 7.57-7.61 (m, 2H, C₆H₃).

13C-NMR (CDCl₃) δ 29.4, 31.8, 50.0, 66.7, 110.8, 115.9, 122.8, 132.1, 145.3, 149.4, 166.8.

MS (CI) m/z 289/291 (M+1)*.

Methyl 4-(3-bromopropoxy)-3-(morpholin-4-ylmethyl)benzoate (46)

To a solution of 27 (2.00 g, 7.90 mmol) in CH₃CN (12 mL) K₂CO₃ (1.66 g, 12.0 mmol) and 1,3-dibromopropane (3.96 mL, 39.0 mmol) were added and the mixture was heated under reflux for 6 h. The cooled reaction mixture was then poured into H₂O (50 mL) and extracted with EtOAc (3 x 50 mL). The combined organic layers were washed with H₂O (25 mL), brine (25 mL), dried with MgSO₄, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/EtOAc 60/40 v/v) to give the title compound (1.12 g) as a colourless oil; yield: 38%. 1H-NMR (CDCl₃) δ 2.36 (qi, 2H, -OCH₂C₂H₅), 2.48 (t, 4H, -CH₂NCH₂-N), 3.64-3.73 (m, 6H, -CH₂OCH₂CH₂-OCH₂Br), 3.89 (s, 3H, -OCH₃), 4.19 (t, 2H, -OCH₂), 6.90 (d, 1H, C₆H₃), 7.94 (d, 1H, C₆H₃), 8.01 (s, 1H, Arom). 13C-NMR (CDCl₃) δ 29.8, 32.2, 51.9, 53.7, 56.7, 65.5, 67.0, 110.8, 122.4, 126.1, 130.6, 132.3, 160.6, 166.9.

General procedure for the preparation of nitrooxy derivatives (7, 8, 47)

A solution of the appropriate bromo/chloro derivative (1.93 mmol) and AgNO₃ (0.82 g, 4.82 mmol) in CH₃CN (15 mL) was stirred at 70 °C for 14h. Brine was then added to precipitate the excess of AgNO₃, the mixture was filtered through Celite® and concentrated under reduced pressure. The residue was treated with EtOAc (50 mL) and H₂O (50 mL). After separation, the aqueous layer was extracted twice with EtOAc (10 mL). The combined organic layers were dried with MgSO₄, filtered, and concentrated under reduced pressure. The crude product thus obtained was purified, if necessary, by flash chromatography. Chromatographic eluents and yields of the products were as follows.
3-(4-Formyl-2-hydroxyphenoxy)propyl nitrate (7)
The crude product was used without any purification as a yellow oil; yield 89 %. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 2.33 (qi, 2H, -OCH\(_2\)CH\(_2\)), 4.27 (t, 2H, -OCH\(_2\)CH\(_2\)), 4.67 (t, 2H, -CH\(_2\)ONO\(_2\)), 5.76 (sbr, 1H, -OH), 6.97 (d, 1H, C\(_6\)H\(_5\)), 7.42 (m, 1H, C\(_6\)H\(_5\)), 7.44-7.47 (m, 1H, C\(_6\)H\(_5\)), 9.85 (s, 1H, -CHO). \(^13\)C-NMR (CDCl\(_3\)) \(\delta\) 26.9, 65.3, 69.6, 111.1, 114.7, 124.4, 131.1, 146.1, 150.5, 190.8. MS (Cl) \(m/\zeta\) 242 (M+1)*.

4-(4-Formyl-2-hydroxyphenoxy)butyl nitrate (8)
The crude product was used without any purification as yellow solid; yield 63 %. m.p. 108-108.5 \(^0\)C (iPr\(_2\)O). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 1.95-2.04 (m, 4H, -OCH\(_2\)CH\(_2\)), 4.20 (t, 2H, -OCH\(_2\)CH\(_2\)), 4.55 (t, 2H, -CH\(_2\)ONO\(_2\)), 5.74 (s, 1H, OH), 6.96 (d, 1H, C\(_6\)H\(_5\)), 7.41-7.46 (m, 2H, C\(_6\)H\(_5\)), 9.85 (s, 1H, -CHO). \(^13\)C-NMR (CDCl\(_3\)) \(\delta\) 23.7, 25.5, 68.2, 72.5, 111.0, 114.4, 124.5, 130.8, 146.1, 150.9, 191.0. MS (Cl) \(m/\zeta\) 256 (M+1)*.

Methyl 3-hydroxy-4-(3-nitrooxypropoxy)benzoate (27)
The crude product was crystallized with toluene to give the title compound as a pale yellow solid; yield 68 %. m.p. 76.5-77 \(^0\)C (toluene). \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 2.28 (qi, 2H, -OCH\(_2\)CH\(_2\)), 3.88 (s, 3H, -OCH\(_3\)), 4.23 (t, 2H, -OCH\(_2\)CH\(_2\)), 4.67 (t, 2H, -CH\(_2\)ONO\(_2\)), 5.50 (sbr, 1H, OH), 6.87 (d, 1H, C\(_6\)H\(_5\)), 7.59-7.61 (m, 2H, C\(_6\)H\(_5\)). \(^13\)C-NMR (CDCl\(_3\)) \(\delta\) 26.9, 52.0, 65.0, 69.7, 110.8, 116.1, 122.8, 123.9, 145.2, 149.2, 166.7. MS (Cl) \(m/\zeta\) 272 (M+1)*.

Methyl 3-(morpholin-4-ylmethyl)-4-[3-(nitrooxy)propoxy]benzoate (47)
Eluent: PE/EtOAc 60/40 v/v; colourless oil; yield: 75%. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\) 2.27 (qi, 2H, -OCH\(_2\)CH\(_2\)), 2.49 (m, 4H, -CH\(_2\)NCH\(_2\)), 3.53 (s, 2H, -NCH\(_2\)Ar), 3.70 (m, 4H, -CH\(_2\)OCH\(_2\)), 3.89 (s, 3H, -OCH\(_3\)), 4.15 (t, 2H, -OCH\(_2\)CH\(_2\)), 4.72 (t, 2H, -CH\(_2\)ONO\(_2\)), 6.87 (d, 1H, C\(_6\)H\(_5\)), 7.94 (d, 1H, C\(_6\)H\(_5\)), 8.02 (s, 1H, C\(_6\)H\(_5\)). \(^13\)C-NMR (CDCl\(_3\)) \(\delta\) 27.0, 51.9, 53.7, 56.7, 63.9, 67.0, 69.8, 110.7, 122.6, 126.1, 130.6, 132.4, 160.4, 166.9.

General procedure for the preparation of N-BOC-protected derivatives (9-14, 22)
To a solution of BOC-protected aminoacid (9.38 mmol) in dry CH\(_2\)Cl\(_2\) (60 mL), stirred under inert atmosphere, EDC-HCl (3.96 g; 20.66 mmol) and DMAP (0.12 g; 0.92 mmol) were added. After 30 min the appropriate phenol derivative (1.70 g; 7.05 mmol) was added. The reaction was completed after 3 hours The reaction mixture was washed with H\(_2\)O (50 mL), dried, filtered, and concentrated under reduced pressure. The crude product thus obtained was purified by flash chromatography. Chromatographic eluents and yields of the products were as follows.

5-Formyl-2-[[(nitrooxy)propoxy]-phenyl-3-[(tert-butoxycarbonyl)amino]propanoate (9)
Eluent: PE/EtOAc 80/20 v/v; yellow solid; yield: 70 %. m.p. 80.7-83 °C (iPr2O). 1H-NMR (CDCl3) δ 1.46 (s, 9H, t-Bu), 2.25 (qi, 2H, -OCH2CH2­), 2.84 (t, 2H, -CH2CH2NH­), 3.52-3.54 (m, 2H, -CH2CH2NH­), 4.21 (t, 2H, -OCH2CH2­), 4.62 (t, 2H, -CH2ONO2­), 5.11 (sbr, 1H, -NH), 7.08 (d, 1H, C6H), 7.62 (m, 1H, C6H), 7.75-7.79 (m, 1H, C6H), 9.87 (s, 1H, -CHO). 13C-NMR (CDCl3) δ 26.8, 28.4, 34.5, 36.1, 64.9, 69.4, 79.7, 112.7, 123.5, 130.2, 130.4, 140.1, 154.9, 155.8, 170.0, 189.9. MS (Cl) m/z 413 (M+1)+.

5-Formyl-2-[(nitrooxy)butoxy]-phenyl-3-[(tert-butoxycarbonyl)amino]propano (10)

Eluent: PE/EtOAc 80/20 v/v; yellow oil; yield: 48 %. 1H-NMR (CDCl3) δ 1.46 (s, 9H, t-Bu), 1.89-1.978 (m, 4H, -OCH2CH2CH2­), 2.83 (t, 2H, -CH2CH2NH­), 3.50-3.56 (m, 2H, -CH2CH2NH­), 4.13 (t, 2H, -OCH2CH2­), 4.53 (t, 2H, -CH2ONO2­), 5.02 (m, 1H, NH), 7.06 (d, 1H, C6H), 7.61-7.62 (m, 1H, C6H), 7.75-7.78 (m, 1H, C6H), 9.88 (s, 1H, -CHO). 13C-NMR (CDCl3) δ 23.6, 25.3, 28.4, 34.5, 36.2, 68.1, 72.5, 79.6, 112.7, 123.5, 130.1, 130.2, 140.1, 155.2, 155.8, 170.1, 189.9. MS (Cl) m/z 427 (M+1)+.

5-Formyl-2-[(nitrooxy)propoxy]-phenyl-3-[(tert-butoxycarbonyl)amino]acetate (11)

Eluent: PE/EtOAc 80/20 v/v; white solid; yield: 50 %. m.p. 97-98 °C (iPr2O). 1H-NMR (CDCl3) δ 1.47 (s, 9H, t-Bu), 2.25 (qi, 2H, -OCH2CH2­), 4.17-4.22 (m, 4H, -CH2NH- + -OCH2CH2­), 4.64 (t, 2H, -CH2ONO2­), 5.10 (m, 1H, NH), 7.07 (d, 1H, C6H), 7.61-7.62 (m, 1H, C6H), 7.76-7.79 (m, 1H, C6H), 9.87 (s, 1H, -CHO). 13C-NMR (CDCl3) δ 26.8, 28.3, 42.1, 64.9, 69.6, 80.3, 112.7, 123.3, 130.3, 139.9, 154.9, 155.7, 168.4, 189.8. MS (Cl) m/z 399 (M+1)+.

5-Formyl-2-[(nitrooxy)propoxy]-phenyl-3-[(tert-butoxycarbonyl)amino]butanoate (12)

Eluent: PE/EtOAc 70/30 v/v; white solid; yield: 85 %. m.p. 71.5-72.5 °C (iPr2O). 1H-NMR (CDCl3) δ 1.46 (s, 9H, t-Bu), 1.95 (qi, 2H, -CH2CH2NH­), 2.25 (qi, 2H, -OCH2CH2­), 2.66 (t, 2H, -COCH2CH2­), 3.23-3.30 (m, 2H, -CH2CH2NH­), 4.20 (t, 2H, -OCH2CH2­), 4.62 (t, 2H, -CH2ONO2­), 4.70 (sbr, 1H, NH), 7.07 (d, 1H, C6H), 7.60-7.61 (m, 1H, C6H), 7.75-7.78 (m, 1H, C6H), 9.88 (s, 1H, -CHO). 13C-NMR (CDCl3) δ 25.4, 26.8, 28.4, 31.1, 39.8, 64.8, 69.5, 79.4, 112.7, 123.6, 130.1, 130.3, 140.2, 155.0, 156.0, 170.9, 190.0. MS (Cl) m/z 427 (M+1)+.

5-Formyl-2-[(nitrooxy)propoxy]-phenyl-2-[(tert-butoxycarbonyl)amino]propanoate (13)

Eluent: PE/EtOAc 70/30 v/v; white solid; yield: 65 %. 93.5 °C darkening/fuming, m.p. 102 °C(iPr2O). 1H-NMR (CDCl3) δ1.47 (s, 9H, t-Bu), 1.57 (d, 3H, -CHCH3­), 2.24 (qi, 2H, -OCH2CH2­), 4.19 (t, 2H, -OCH2CH2­), 4.58-4.66 (m, 3H, -CHCH3 + -CH2ONO2­), 5.05-5.08 (m, 1H, NH), 7.07 (d, 1H, C6H), 7.61-7.62 (m, 1H, C6H), 7.76-7.79 (m, 1H, C6H), 9.88 (s, 1H, -CHO). 13C-NMR (CDCl3) δ 18.5, 26.8, 28.3, 49.2, 64.7, 69.4, 80.1, 110.7, 112.7, 123.4, 130.3, 140.0, 155.0, 171.2, 189.8. MS (Cl) m/z 413 (M+1)+.
5-Formyl-2-[(nitrooxypropoxy)-phenyl-2-(tert-butoxycarbonylamino)-3-(tert-butyl-dimethylsilyloxy)butanoate (14)

Eluent: PE/EtOAc 90/10 v/v; colourless oil; yield: 29 %. $^1$H-NMR (CDCl$_3$) $\delta$ 0.11 (s, 3H, CH$_3$Si-), 0.12 (s, 3H, CH$_3$Si-), 0.90 (s, 9H, tBuSi-), 1.31 (s, 3H, -CHCH$_3$), 1.47 (s, 9H, iBuO-), 2.25 (q, 2H, -OCH$_2$CH$_2$), 4.16 (t, 2H, -OCH$_2$CH$_2$), 4.45-4.49 (m, 1H, -CHNH-), 4.60-4.72 (m, 3H, -CH$_2$ONO$_2$ + -CHO$_2$Si-), 5.25-5.30 (m, 1H, -NH-), 7.07 (d, 1H, C$_6$H$_5$), 7.60-7.61 (m, 1H, C$_6$H$_5$), 7.74-7.78 (m, 1H, C$_6$H$_3$), 9.87 (s, 1H, -CHO). $^{13}$C-NMR (CDCl$_3$) $\delta$ -4.9, -4.1, 18.0, 21.0, 25.7, 26.7, 28.3, 59.5, 64.7, 68.7, 69.6, 80.0, 112.8, 122.8, 130.2, 140.4, 155.0, 156.1, 169.2, 189.8.

2-(Allyloxy)-5-formylphenyl 3-[(tert-butoxycarbonylamino)propanoate (22)

Eluent: PE/EtOAc 90/10 v/v to 80/20; colourless oil; yield: 56 %. $^1$H-NMR (CDCl$_3$) $\delta$ 1.46 (s, 9H, t-Bu), 2.82 (t, 2H, -CH$_2$CH$_2$NH-), 3.53-3.58 (m, 2H, -CH$_2$CH$_2$NH-), 4.67-4.68 (m, 2H, -OCH$_2$-), 5.17 (svbr, 1H, -NH), 5.32-5.43 (m, AMX like system, 2H, -CH=CH$_2$), 5.95-6.08 (m, AMX like system, 1H, -CH=CH$_2$), 7.07 (d, 1H, C$_6$H$_5$), 7.61-7.62 (m, 1H, C$_6$H$_5$), 7.73-7.76 (m, 1H, C$_6$H$_3$), 9.87 (s, 1H, -CHO). $^{13}$C-NMR (CDCl$_3$) $\delta$ 28.4, 31.1, 33.8, 34.7, 36.2, 69.6, 79.5, 113.2, 118.9, 123.5, 130.1, 131.6, 140.2, 155.0, 190.0. MS (CI) m/z 350 (M+1)$^+$. 

2-[2,3-Bis(nitrooxy)propyl]-5-formylphenyl 3-[(tert-butoxycarbonylamino)propanoate (23)

Iodine (0.70 g, 2.78 mmol) was added portionwise to a stirred solution of 22 (0.97g, 2.78 mmol) plus AgNO$_3$ (0.47 g, 2.78 mmol) in CH$_3$CN (10 mL) kept at -15 °C. At the end of the addition, stirring was continued for 1h. AgNO$_3$ (1.18 g, 6.95 mmol) was then added and the mixture heated to 70 °C for 16 h. After cooling, the mixture was filtered through Celite®. The filtrate was concentrated under reduced pressure, dissolved in water (40 mL) and extracted with EtOAc (3 x 20 mL). The combined organic layers were washed with brine (20 mL), dried with MgSO$_4$, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/EtOAc 80/20 v/v) to give the title compound as a yellow oil; yield: 31 %. $^1$H-NMR (CDCl$_3$) $\delta$ 1.46 (s, 9H, t-Bu), 2.84 (t, 2H, -CH$_2$CH$_2$NH-), 3.51-3.56 (m, 2H, -CH$_2$CH$_2$NH-), 4.38 (d, 2H, -OCH$_2$CH-), 4.69-4.90 (m, AMX-like system, 2H, -CHCH$_2$ONO$_2$), 5.03 (svbr, 1H, -NH), 5.62-5.66 (m, AMX-like system, 1H, -CHCH$_2$ONO$_2$), 7.08 (d, 1H, C$_6$H$_5$), 7.64-7.65 (m, 1H, C$_6$H$_3$), 7.76-7.81 (m, 1H, C$_6$H$_3$), 9.90 (s, 1H, -CHO). $^{13}$C-NMR (CDCl$_3$) $\delta$ 14.2, 27.9, 28.4, 34.5, 36.0, 60.5, 65.8, 68.4, 70.0, 113.0, 123.9, 130.0, 131.3, 140.2, 153.9, 155.9, 179.9, 189.8. MS (CI) m/z 473 (M+1)$^+$. 

General procedure for the preparation of carboxylic acid from aldehyde (15-20, 24)

KMnO$_4$ (0.52 g, 3.27 mmol) was added to a solution of the appropriate aldehyde (2.18 mmol) in acetone (50 mL), and stirred at 0 °C. The reaction was allowed to reach r.t.; it was complete after 1h (TLC detection, eluent CH$_2$Cl$_2$/MeOH 95/5 v/v). Oxalic acid was added and the mixture was filtered and the filtrate was diluted with CH$_2$Cl$_2$ (100 mL). The organic layer was washed twice
with H$_2$O (50 mL), then dried with MgSO$_4$, filtered, and concentrated under reduced pressure to give the desired product.


White solid; yield 96 %. m.p. 123-126 °C (Toluene). $^1$H-NMR (DMSO-d$_6$) δ 1.46 (s, 9H, t-Bu), 2.15 (qi, 2H, -OCH$_2$CH$_2$), 2.73 (t, 2H, -CH$_2$CH$_2$NH-), 3.10-3.17 (m, 2H, -CH$_2$CH$_2$NH-), 4.17 (t, 2H, -OCH$_2$CH$_2$), 4.61 (t, 2H, -CH$_2$ONO$_2$), 6.97-5.00 (m, 1H, -NH), 7.23 (d, 1H, C$_6$H$_3$), 7.70 (m, 1H, C$_6$H$_3$), 7.83-7.87 (m, 1H, C$_6$H$_3$), 12.8 (svvbr, 1H, -COOH). $^{13}$C-NMR (DMSO-d$_6$) δ 26.0, 28.1, 33.9, 36.0, 64.9, 70.5, 77.8, 113.0, 123.3, 124.0, 128.7, 138.9, 153.5, 155.5, 166.3, 169.3. MS (CI) m/z 429 (M+1)$^+$. 


White solid; yield 90 %. m.p. 102 °C (toluene). $^1$H-NMR (DMSO-d$_6$) δ 1.39 (s, 9H, t-Bu), 1.79-1.83 (m, 4H, -OCH$_2$CH$_2$CH$_2$-), 2.71 (t, 3H, -CH$_2$CH$_2$NH-), 3.26-3.33 (m, 2H, -CH$_2$CH$_2$NH-), 4.11 (t, 2H, -OCH$_2$CH$_2$-), 4.57 (t, 2H, -CH$_2$ONO$_2$), 6.97-7.01 (m, 1H, NH), 7.21 (d, 1H, C$_6$H$_3$), 7.67-7.68 (m, 1H, C$_6$H$_3$), 7.82-7.85 (d, 1H, C$_6$H$_3$), 12.8 (sbr, 1H, -COOH). $^{13}$C-NMR (DMSO-d$_6$) δ 22.7, 24.6, 28.1, 33.9, 36.0, 67.7, 73.3, 77.7, 113.0, 123.0, 123.9, 128.7, 138.8, 153.7, 155.4, 166.3, 169.2. MS (CI) m/z 443 (M+1)$^+$. 

3-[[[(tert-Butoxycarbonyl)amino]acetoxy]-4-[[nitrooxy]propoxy]benzoic acid (17)

White solid; yield 50 %. m.p. 158.5-159 °C (Toluene). $^1$H-NMR (DMSO-d$_6$) δ 1.40 (s, 9H, t-Bu), 2.09-2.16 (m, 2H, -OCH$_2$CH$_2$-), 3.99-4.01 (m, 2H, -CH$_2$NH-), 4.16 (t, 2H, -OCH$_2$CH$_2$-), 4.66 (t, 2H, -CH$_2$ONO$_2$), 7.26 (d, 1H, C$_6$H$_3$), 7.61-7.62 (m, 1H, C$_6$H$_3$), 7.84-7.87 (m, 1H, C$_6$H$_3$), 12.0 (sbr, 1H, -COOH). $^{13}$C-NMR (DMSO-d$_6$) δ 26.9, 28.9, 42.5, 65.7, 71.5, 79.3, 114.1, 124.2, 124.4, 129.8, 139.7, 154.4, 156.7, 167.1, 169.6. MS (CI) m/z 415 (M+1)$^+$. 


White solid; yield 75 %. m.p. 138-140 °C (Toluene). $^1$H-NMR (CDCl$_3$) δ 1.46 (s, 9H, t-Bu), 1.95 (qi, 2H, -CH$_2$CH$_2$NH-), 2.23 (qi, 2H, -OCH$_2$CH$_2$-), 2.66 (t, 2H, -COCH$_2$CH$_2$-), 3.24-3.30 (m, 2H, -CH$_2$CH$_2$NH-), 4.18 (t, 2H, -OCH$_2$CH$_2$-), 4.61 (t, 2H, -CH$_2$ONO$_2$), 4.70 (sbr, 1H, NH), 6.99 (d, 1H, C$_6$H$_3$), 7.79 (s, 1H, C$_6$H$_3$), 8.00 (d, 1H, C$_6$H$_3$). $^{13}$C-NMR (CDCl$_3$) δ 25.4, 26.8, 28.4, 29.7, 31.1, 64.6, 69.5, 82.3, 112.3, 122.4, 125.1, 129.8, 139.4, 154.4, 169.6, 170.9. MS (CI) m/z 443 (M+1)$^+$. 


White solid; yield 80 %. m.p. 133-134 °C (Toluene). $^1$H-NMR (DMSO-d$_6$) δ $^1$H-NMR (CDCl$_3$) δ 1.41-1.43 (m, 12H, t-Bu + -CHCH$_3$), 2.13 (qi, 2H, -OCH$_2$CH$_2$-), 4.16 (t, 2H, -OCH$_2$CH$_2$-), 4.24-4.30 (m, 1H, -CHCH$_3$), 4.65 (t, 2H, -CH$_2$ONO$_2$), 7.25 (d, 1H, C$_6$H$_3$), 7.51-7.57 (m, 2H, C$_6$H$_3$ + NH), 7.84-7.87 (m, 1H, C$_6$H$_3$), 12.91 (sbr, 1H, -COOH). $^{13}$C-NMR (DMSO-d$_6$) δ 16.6, 25.9, 28.1,
49.0, 64.7, 70.4, 78.3, 113.0, 123.3, 123.5, 128.8, 138.7, 153.5, 155.2, 166.2, 171.3. MS (Cl) m/z 429 (M+1)+

3-[3-(tert-butoxycarbonylamino)(tert-butyl-dimethylsilyloxy)butoxy]-4-(nitrooxypropoxy)benzoic acid (20)

Colourless oil; yield 71 %. 1H-NMR (CDCl3) δ 0.12 (s, 3H, CH3Si-), 0.13 (s, 3H, CH3Si-), 0.84 (s, 9H, tBuSi-), 1.35 (d, 3H, -CHCH3), 1.48 (s, 9H, tBuO-), 2.22 (qi, 2H, -OCH2CH2-), 4.16 (t, 2H, -OCH2CH2-), 4.48-4.52 (m, 1H, -CHNH-), 4.61-4.69 (m, 3H, -CH2ONO2 + -CHOH), 5.31 (d, 1H, -NH), 6.99 (d, 1H, C6H5), 7.81-7.82 (m, 1H, C6H5), 7.98-8.02 (m, 1H, C6H5), 9.86 (sbr, 1H, -CHO). 13C-NMR (CDCl3) δ -4.9, -4.1, 18.4, 21.0, 25.7, 26.7, 28.3, 59.5, 64.6, 68.7, 69.7, 80.0, 112.4, 122.4, 124.5, 130.0, 139.5, 154.4, 156.1, 169.2, 170.6. MS (Cl) m/z 574 (M+1)+

4-[2,3-bis(nitrooxy)propyl]-3-[[3-(tert-butoxycarbonylamino)propanoyl]oxy]benzoic acid (24)

Pale yellow oil; yield 90 %. 1H-NMR (DMSO-d6) δ 1.36 (s, 9H, t-Bu), 2.70 (q, 2H, -CH2CH2NH-), 4.41-4.49 (m, 2H, -OCH2CH2-), 4.81-5.01 (m, AMX like system, 2H, -CHCH2NO2), 5.75-5.85 (m, AMX-like system, 1H, -CHCH2NO2), 6.98 (t, 1H, -NH), 7.24 (d, 1H, C6H5), 7.38 (sbr, 1H, -COOH), 7.69-7.70 (m, 1H, C6H5), 7.81-7.85 (m, 1H, C6H5). 13C-NMR (DMSO-d6) δ 29.3, 35.0, 36.9, 50.7, 66.8, 70.5, 78.4, 114.2, 116.2, 125.1, 129.6, 139.8, 153.7, 163.4, 167.1, 169.6. MS (Cl) m/z 490 (M+1)+

3-Hydroxy-4-(3-nitrooxypropoxy)benzoic acid (28)

To a solution of 27 (0.56 g, 2.06 mmol) in THF (10 mL) and H2O (10 mL), NaOH pellets (165 mg; 4.13 mmol) were added. The mixture was heated to 40 °C for 4 h. The residue was then treated with HCl 6N and extracted twice with EtOAc (20 mL). The combined organic layers were dried with MgSO4, filtered, and concentrated under reduced pressure to give the title compound as a yellow solid; yield 59 %. m.p. 166.5-169.5 °C (toluene). 1H-NMR (DMSO-d6) δ 2.14 (qi, 2H, -OCH2CH2-), 4.12 (t, 2H, -OCH2CH2-), 4.74 (t, 2H, -CH2NO2), 7.00 (d, 1H, C6H5), 7.35-7.42 (m, 2H, C6H5), 9.27 (svbr, 1H, OH), 12.58 (svbr, 1H, -COOH). 13C-NMR (DMSO-d6) δ 26.0, 64.4, 70.8, 112.4, 116.1, 121.4, 123.4, 146.2, 150.4, 167.0. MS (Cl) m/z 258 (M+1)+

3-(Morpholin-4-ylmethyl)-4-[3-(nitrooxy)propoxy]benzoic acid (48)

A solution of 29 (0.56 mmol) in MeOH (5 mL) and 1N NaOH (2 mL) was stirred at room temperature for 4 h. The solvent was then removed under reduced pressure, the residue dissolved in H2O (10 mL), the pH adjusted to 7, and the product extracted in continuous cycle for 18 h with EtOAc. The EtOAc layer was dried with MgSO4, filtered, and concentrated under reduced pressure
to give the desired compound. Colourless oil; yield: 67%. $^1$H-NMR (CDCl$_3$) $\delta$ 2.28 (qi, 2H, -OCH$_2$CH$_2$-), 2.79 (m, 4H, -CH$_2$NCH$_2$-), 3.77 (m, 4H, -CH$_2$OCH$_2$-), 3.85 (s, 2H, -NCH$_2$Ar), 4.21 (t, 2H, -OCH$_2$-), 4.74 (m, 2H, -CH$_2$ONO$_2$), 7.07 (d, 1H, C$_6$H$_5$), 7.98 (d, 1H, C$_6$H$_5$), 8.04 (s, 1H, C$_6$H$_3$). $^{13}$C-NMR (CDCl$_3$) $\delta$ 28.0, 54.2, 57.1, 66.1, 66.9, 71.9, 112.2, 123.7, 126.4, 133.0, 134.7, 161.8, 171.0.

General procedure for the preparation of aspirin esters (30-37, 49)

To a solution of 29 (0.58 g, 2.52 mmol) in DMF (8 mL) the appropriate carboxylic acid derivative (2.52 mmol) and Cs$_2$CO$_3$ (0.41 g, 1.26 mmol) were added. The mixture was stirred at room temperature for 24 hours, then poured into H$_2$O (30 mL) and extracted with EtOAc (3 × 5 mL). The combined organic layers were dried with MgSO$_4$, filtered, and concentrated under reduced pressure. The crude product thus obtained was purified by flash chromatography. Chromatographic eluents and yields of the products were as follows.

$\{2-$(Acetyloxy)benzoyl$\}$oxy)methyl 3-($(3$-$tert$-$butoxycarbonyl)amino$)$propanoyloxy-4-$[$(nitrooxy)$]$propoxy$]$benzoate (30)

Eluent: PE/EtOAc 90/10 to 70/30 v/v; colourless oil; yield: 70 %. $^1$H-NMR (CDCl$_3$) $\delta$ 1.45 (s, 9H, t-Bu), 2.23 (qi, 2H, -OCH$_2$CH$_2$-), 2.35 (s, 3H, CH$_3$CO-), 2.82 (t, 2H, -CH$_2$CH$_2$NH-), 3.49-3.55 (m, 2H, -CH$_2$CH$_2$NH-), 4.18 (t, 2H, -OCH$_2$CH$_2$-), 4.60 (t, 2H, -CH$_2$ONO$_2$), 5.06 (m, 1H, -NH), 6.16 (s, 2H, -OCH$_2$O-), 6.99 (d, 1H, C$_6$H$_5$), 7.11 (d, 1H, C$_6$H$_5$), 7.33 (t, 1H, C$_6$H$_4$), 7.60 (t, 1H, C$_6$H$_4$), 7.79-7.80 (m, 1H, C$_6$H$_3$), 7.98-8.01 (m, 1H, C$_6$H$_3$), 8.07-8.10 (m, 1H, C$_6$H$_4$). $^{13}$C-NMR (CDCl$_3$) $\delta$ 21.0, 26.8, 28.4, 34.5, 36.0, 64.7, 69.4, 79.6, 79.8, 112.4, 121.9, 122.0, 124.0, 124.9, 126.2, 129.9, 132.3, 134.7, 139.2, 151.1, 154.3, 155.7, 163.1, 164.0, 169.7, 171.2. MS (Cl) $m/\ell$ 621 (M+1)$^+$. $\{2-$(Acetyloxy)benzoyl$\}$oxy)methyl-3-($(3$-$tert$-$butoxycarbonyl)amino$)$propanoyloxy-4-$[$(nitrooxy)$]$butoxy$]$benzoate (31)

Eluent: PE/EtOAc 90/10 to 70/30 v/v; colourless oil; yield: 45 %. $^1$H-NMR (CDCl$_3$) $\delta$ 1.45 (s, 9H, t-Bu), 1.82-2.03 (m, 4H, -OCH$_2$CH$_2$CH$_2$-), 2.35 (s, 3H, CH$_3$CO-), 2.81 (t, 2H, -CH$_2$CH$_2$NH-), 3.52 (q, 2H, -CH$_2$CH$_2$NH-), 4.11 (t, 2H, -OCH$_2$CH$_2$-), 4.51 (t, 2H, -CH$_2$ONO$_2$), 5.05-5.15 (m, 1H, NH), 6.16 (s, 2H, -OCH$_2$O-), 6.98 (d, 1H, C$_6$H$_5$), 7.11 (d, 1H, C$_6$H$_5$), 7.32 (t, 1H, C$_6$H$_4$), 7.60 (t, 1H, C$_6$H$_4$), 7.79-7.80 (m, 1H, C$_6$H$_3$), 7.97-8.00 (m, 1H, C$_6$H$_3$), 8.07-8.10 (m, 1H, C$_6$H$_4$). $^{13}$C-NMR (CDCl$_3$) $\delta$ 21.0, 23.6, 25.2, 28.4, 34.5, 36.1, 67.9, 72.5, 79.8, 112.3, 121.6, 122.0, 124.0, 124.8, 126.2, 129.9, 132.2, 134.7, 139.3, 151.1, 154.6, 162.5, 163.1, 164.1, 169.7. MS (Cl) $m/\ell$ 635 (M+1)$^+$. $\{2-$(Acetyloxy)benzoyl$\}$oxy)methyl-3-($(3$-$tert$-$butoxycarbonyl)amino$)$acetox$]$-4-$[$(nitrooxy)$]$propoxy$]$benzoate (32)
Eluent: PE/EtOAc 80/20 to 60/40 v/v; colourless oil; yield: 70 %. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\): 1.46 (s, 9H, t-Bu), 2.23 (q, 2H, -OCH\(_2\)CH\(_2\)-), 2.35 (s, 3H, CH\(_3\)CO-), 4.11-4.20 (m, 4H, -CH\(_2\)NH- + -OCH\(_2\)CH\(_2\)-), 4.63 (t, 2H, -CH\(_2\)ONO\(_2\)), 5.06 (sbr, 1H, NH), 6.16 (s, 2H, -OCH\(_2\)O-), 6.98 (d, 1H, C\(_6\)H\(_5\)), 7.11 (d, 1H, C\(_6\)H\(_5\)), 7.32 (t, 1H, C\(_6\)H\(_4\)), 7.60 (t, 1H, C\(_6\)H\(_4\)), 7.80 (m, 1H, C\(_6\)H\(_5\)), 7.98-8.01 (m, 1H, C\(_6\)H\(_3\)), 8.07-8.09 (m, 1H, C\(_6\)H\(_4\)). \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\): 21.0, 26.9, 28.3, 42.1, 64.6, 69.7, 76.6, 79.8, 85.4, 112.4, 121.9, 124.0, 124.7, 126.1, 130.0, 132.3, 134.6, 139.1, 151.1, 154.4, 155.7, 163.1, 164.0, 168.3, 169.7, 189.8. MS (Cl) m/z 607 (M+1)+.

\([2-(Acetyloxy)benzoyl]oxy)methyl-3-\((3-[tert-butoxycarbonyl]amino)butanoyl]oxy-4-[3-(nitrooxy)propoxy]benzoate (33)\)

Eluent: PE/EtOAc 80/20 to 60/40 v/v; colourless oil; yield: 72 %. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\): 1.45 (s, 9H, t-Bu), 1.93 (q, 2H, -CH\(_2\)CH\(_2\)NH-), 2.25 (q, 2H, -OCH\(_2\)CH\(_2\)-), 2.35 (s, 3H, CH\(_3\)CO-), 2.64 (t, 2H, -COCH\(_2\)CH\(_2\)-), 3.22-3.28 (m, 2H, -CH\(_2\)CH\(_2\)NH-), 3.56 (sbr, 1H, NH), 4.17 (t, 2H, -OCH\(_2\)CH\(_2\)-), 4.60 (t, 2H, -CH\(_2\)ONO\(_2\)), 6.16 (s, 2H, -OCH\(_2\)O-), 6.98 (d, 1H, C\(_6\)H\(_5\)), 7.11 (d, 1H, C\(_6\)H\(_5\)), 7.32 (t, 1H, C\(_6\)H\(_5\)), 7.57-7.62 (m, 1H, C\(_6\)H\(_4\)), 7.77-7.78 (m, 1H, C\(_6\)H\(_5\)), 7.96-8.00 (m, 1H, C\(_6\)H\(_5\)), 8.06-8.10 (m, 1H, C\(_6\)H\(_3\)). \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\): 21.0, 25.4, 26.8, 28.4, 31.1, 39.8, 64.7, 69.5, 79.4, 79.9, 112.4, 122.0, 122.1, 124.0, 125.0, 126.1, 129.8, 132.2, 134.7, 139.5, 151.1, 154.5, 156.0, 163.1, 164.1, 169.7, 170.8. MS (Cl) m/z 635 (M+1)+.

\([2-(Acetyloxy)benzoyl]oxy)methyl-3-\((3-[tert-butoxycarbonyl]amino)propanoyl]oxy-4-[3-(nitrooxy)propoxy]benzoate (34)\)

Eluent: PE/EtOAc 80/20 to 70/30 v/v; colourless oil; yield: 50 %. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\): 1.41-1.43 (s, 19H, t-Bu), 1.57 (d, 3H, -CH\(_3\)H\(_5\)), 2.21 (q, 2H, -OCH\(_2\)CH\(_2\)-), 2.35 (s, 3H, CH\(_3\)CO-), 4.16 (t, 2H, -OCH\(_2\)CH\(_2\)-), 4.60-4.64 (m, 3H, -CH\(_2\)ONO\(_2\) + -CH\(_3\)H\(_5\)), 5.03-5.07 (m, 1H, NH), 6.16 (s, 2H, -OCH\(_2\)O-), 6.98 (d, 1H, C\(_6\)H\(_5\)), 7.11 (d, 1H, C\(_6\)H\(_4\)), 7.32 (t, 2H, C\(_6\)H\(_4\)), 7.59 (t, 1H, C\(_6\)H\(_4\)), 7.79-7.80 (m, 1H, C\(_6\)H\(_3\)), 7.98-8.01 (m, 1H, C\(_6\)H\(_3\)), 8.07-8.10 7.79-7.80 (m, 1H, C\(_6\)H\(_3\)). \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\): 18.6, 21.0, 26.7, 28.3, 64.6, 69.5, 79.8, 112.4, 121.9, 122.0, 124.0, 124.7, 126.1, 130.0, 132.3, 134.7, 139.2, 151.1, 154.4, 163.0, 164.0, 169.7, 171.2. MS (Cl) m/z 621 (M+1)+.

\([2-(Acetyloxy)benzoyl]oxy)methyl-3-\((3-[tert-butoxycarbonyl]amino)(tert-butyldimethylsilyloxy)butyloxy)-4-(3-nitrooxypropoxy)benzoate (35)\)

Eluent: PE/EtOAc 70/30 v/v; colourless oil; yield: 35 %. \(^1\)H-NMR (CDCl\(_3\)) \(\delta\): 0.09 (s, 3H, CH\(_3\)Si-), 0.10 (s, 3H, CH\(_3\)Si-), 0.88 (s, 9H, tBuSi-), 1.32 (d, 3H, -CH\(_3\)H\(_5\)), 1.46 (s, 9H, rBuO-), 2.18 (q, 2H, -OCH\(_2\)CH\(_2\)-), 2.32 (s, 3H, CH\(_3\)CO-), 4.13 (t, 2H, -OCH\(_2\)CH\(_2\)-), 4.44-4.47 (m, 1H, -CH\(_2\)NH-), 4.60-4.67 (m, 3H, -CH\(_2\)ONO\(_2\) + -CH\(_3\)H\(_5\)), 5.24 (d, 1H, -NH-), 6.15 (s, 2H, -OCH\(_2\)O-), 6.97 (d, 1H, C\(_6\)H\(_3\)), 7.10 (d, 1H, C\(_6\)H\(_4\)), 7.30 (t, 1H, C\(_6\)H\(_4\)), 7.54-7.60 (m, 1H, C\(_6\)H\(_4\)), 7.69-7.70 (m, 1H, C\(_6\)H\(_3\)), 7.95-7.99 (m, 1H, C\(_6\)H\(_5\)), 8.04-8.07 (m, 1H, C\(_6\)H\(_5\)). \(^{13}\)C-NMR (CDCl\(_3\)) \(\delta\): -4.9, -4.1, 18.0, 20.9, 25.8,
26.9, 28.4, 59.7, 64.9, 68.8, 69.7, 80.1, 112.7, 122.0, 122.3, 124.1, 124.6, 126.1, 129.8, 132.2, 134.6, 139.8, 151.2, 154.6, 156.1, 163.0, 164.0, 169.1, 169.4.

{(2-(Acetyloxy)benzoyl)oxy}methyl-3-[(3-[tert-butoxycarbonyl]amino)propanoyl]oxy-4-[2,3bis(nitrooxy)propoxy]benzoate (36)

Eluent: PE/ EtOAc 80/20 to 60/40 v/v; colourless oil; yield: 47 %. $^1$H-NMR (CDCl$_3$) δ 1.45 (s, 9H, t-Bu), 2.34 (s, 3H, CH$_3$CO-), 2.82 (t, 2H, -CH$_2$CH$_2$NH-), 3.50-3.53 (m, 2H, -CH$_2$CH$_2$NH-), 4.30 (d, 2H, -OCH$_2$CH-), 4.65-4.86 (m, AMX-like system, 2H, -CHCH$_2$ONO$_2$), 5.09 (s, br, 1H, -NH), 5.59-6.2 (m, 1H, -CHCH$_2$ONO$_2$), 6.16 (s, 2H, -OCH$_2$O-), 6.97 (d, 1H, C$_6$H$_5$), 7.11 (d, 1H, C$_6$H$_5$), 7.32 (t, 1H, C$_6$H$_5$), 7.59 (t, 1H, C$_6$H$_5$), 7.80-7.81 (m, 1H, C$_6$H$_5$), 7.96-8.00 (m, 1H, C$_6$H$_5$), 8.07 (d, 1H, C$_6$H$_5$). $^{13}$C-NMR (CDCl$_3$) δ 21.0, 28.3, 31.2, 34.4, 65.6, 68.5, 76.1, 79.6, 79.9, 110.7, 121.9, 123.1, 124.0, 125.2, 126.2, 129.8, 132.2, 134.8, 139.4, 151.1, 153.4, 155.8, 163.1, 163.8, 169.7, 170.0. MS (Cl) m/z 682 (M+1)$^+$. 

{(2-(Acetyloxy)benzoyl)oxy}methyl- 3-hydroxy-4-(3-nitrooxypropoxy)benzoate (37)

Eluent: PE/EtOAc 90/10 to 70/30 v/v; white solid; yield: 37 %. m.p.: 84.5-85.5 ºC. $^1$H-NMR (CDCl$_3$) δ 2.26 (q, 2H, -OCH$_2$CH$_2$-), 2.35 (s, 3H, CH$_3$CO-), 4.22 (t, 2H, OCH$_2$CH$_2$-), 4.65 (t, 2H, -CH$_2$ONO$_2$), 5.62 (s, 1H, OH), 6.16 (s, 2H, -OCH$_2$O-), 6.85 (d, 1H, C$_6$H$_5$), 7.12 (d, 1H, C$_6$H$_5$), 7.32 (t, 1H, C$_6$H$_5$), 7.56-7.67 (m, 3H, C$_6$H$_5$ + C$_6$H$_5$), 8.07-8.10 (m, 1H, C$_6$H$_5$). $^{13}$C-NMR (CDCl$_3$) δ 21.0, 26.9, 65.1, 69.6, 79.8, 110.8, 116.5, 122.1, 122.5, 125.5, 124.0, 126.1, 132.3, 134.6, 145.4, 149.8, 151.0, 163.1, 164.7, 169.8. MS (Cl) m/z 450 (M+1)$^+$. 

{(2-(Acetyloxy)benzoyl)oxy}methyl 3-(morpholin-4-ylmethyl)-4-[3-(nitrooxy)propoxy]benzoate oxalate (49)

The crude product was purified by flash chromatography (PE/EtOAc 60/40 v/v) to give [(2-(acetyloxy)benzoyl)oxy]methyl 4-(morpholin-4-ylmethyl)-3-[3-(nitrooxy)propoxy]benzoate (0.38 g) as a colourless oil. To a solution of [(2-(acetyloxy)benzoyl)oxy]methyl 4-(morpholin-4-ylmethyl)-3-[3-(nitrooxy)propoxy]benzoate in EtOAc (3 mL) was added a solution of H$_2$C$_2$O$_4$ (0.06 g, 1 eq) in EtOAc (2 mL) and the title compound (0.17 g) was obtained by filtration as a white solid; yield: 20%. m.p. 135-139 ºC (dec.). $^1$H-NMR (DMSO-d$_6$) δ 2.17-2.25 (m, 5H, -OCH$_2$CH$_2$- + -COCH$_3$), 2.71 (m, 4H, -CH$_2$NCH$_2$-), 3.64 (m, 4H, -CH$_2$OCH$_2$-), 3.83 (s, 2H, -NCH$_2$Ar), 4.20 (t, 2H, -OCH$_2$-), 4.74 (t, 2H, -CH$_2$ONO$_2$), 6.14 (s, 2H, -OCH$_2$O-), 7.19 (d, 1H, Arom), 7.27 (d, 1H, Arom), 7.42 (t, 1H, Arom), 7.73 (t, 1H, Arom), 7.96-8.05 (m, 3H, Arom), 8.06 (s, 1H, Arom). $^{13}$C-NMR (DMSO-d$_6$) δ 20.9, 26.3, 52.7, 54.9, 65.2, 65.3, 71.3, 80.5, 112.2, 120.5, 122.2, 124.5, 126.7, 131.8, 132.1, 133.2, 135.4, 150.5, 161.6, 162.6, 163.1, 164.3, 169.3. 

General procedure for the preparation of aminoacid derivatives (38-44)
A solution of the appropriate BOC-protected intermediate (2.00 mmol) in dry dioxane HCl 2.36 M (12.6 mL, 29.0 mmol), was stirred under inert atmosphere for 3 h. The mixture was concentrated under reduced pressure.

**Hydrochloridric salt of [{2-(acetyloxy)benzoyl oxy}methyl-3-[3-aminopropanoyl]oxy]-4-[3-(nitrooxy)propoxy]benzoate (38)**

The crude product was triturated with dry EtO to give the title compound as a white solid; yield: 89 %. m.p.: 94.5-95.5 °C. 1H-NMR (DMSO-d6) δ 2.16 (qii, 2H, -OCH2CH2-), 2.25 (s, 3H, CH3CO-), 3.01-3.06 (m, 2H, -CH2CH2NH2), 3.11-3.13 (m, 2H, -CH2CH2NH2), 4.21 (t, 2H, -OCH2CH2-), 4.63 (t, 2H, -CH2ONO2), 6.14 (s, 2H, -OCH2O-), 7.26-7.34 (m, 2H, C6H3 + C6H4), 7.44 (t, 1H, C6H4), 7.71-7.77 (m, 1H, C6H4), 7.82-7.83 (m, 1H, C6H3), 7.93-8.00 (m, 2H, C6H3 + C6H4), 8.22 (svvbr, 2H, -NH2). 13C-NMR (CDCl3) δ 21.0, 26.6, 31.0, 35.5, 65.0, 70.1, 80.0, 112.4, 121.7, 122.0, 124.0, 124.8, 126.2, 129.9, 132.3, 134.7, 139.1, 151.0, 154.4, 163.1, 164.1, 169.0, 169.7.

**Hydrochloridric salt of [{2-(acetyloxy)benzoyl oxy}methyl-3-[3-aminopropanoyl]oxy]-4-[3-(nitrooxy)butoxy]benzoate (39)**

The crude product was purified by flash chromatography (H2O/CH3CN/HCl 60/40/0.1 v/v/v) to give the title compound as a colourless oil; yield: 50 %. 1H-NMR (DMSO-d6 + CDCl3) δ 1.78-1.94 (m, 4H, -OCH2CH2CH2-), 2.28 (s, 3H, CH3CO-), 3.09 (t, 2H, -CH2CH2NH-), 3.21 (t, 2H, -CH2CH2NH-), 4.13 (t, 2H, -OCH2CH2-), 4.58 (t, 2H, -CH2ONO2), 6.14 (s, 2H, -OCH2O-), 7.17-7.21 (m, 2H, C6H3 + C6H4), 7.42 (t, 1H, C6H4), 7.81 (t, 1H, C6H4), 7.79-7.80 (m, 1H, C6H3), 7.94-8.03 (m, 2H, C6H3 + C6H4), 8.34 (svvbr, 2H, NH2). 13C-NMR (DMSO-d6 + CDCl3) δ 21.4, 23.8, 25.5, 31.9, 35.4, 68.7, 73.8, 80.6, 113.7, 121.4, 122.7, 124.7, 125.0, 126.9, 130.3, 132.3, 135.5, 139.6, 151.2, 155.4, 163.5, 164.3, 169.0, 169.7.

**Hydrochloridric salt of [{2-(acetyloxy)benzoyl oxy}methyl-3-[2-amino)acetoxy]-4-[3-(nitrooxy)propoxy]benzoate (40)**

The crude product was triturated with dry EtO to give the title compound as a white solid; yield: 40 %. m.p.: 60.5-62.5 °C. 1H-NMR (CDCl3) δ 2.16 (m, 2H, -OCH2CH2-), 2.31 (s, 3H, CH3CO-), 3.99 (m, 2H, 4.20 (t, 2H) (-CH2NH2 + OCH2CH2-), 4.53 (t, 2H, -CH2ONO2), 6.16 (s, 2H, -OCH2O-), 6.83 (d, 1H, C6H3), 7.08 (d, 1H, C6H4), 7.27 (t, 1H, C6H4), 7.55 (t, 1H, C6H4), 7.71 (s, 1H, C6H3), 7.86 (d, 1H, C6H3), 8.03 (d, 1H, C6H4), 8.69 (svvbr, 3H, NH3+). 13C-NMR (CDCl3) δ 20.9, 26.5, 40.4, 65.2, 70.5, 79.9, 112.5, 121.5, 122.0, 124.0, 124.3, 126.2, 130.2, 132.2, 134.7, 138.5, 151.0, 154.2, 158.4, 163.0, 163.9, 165.7, 169.7.

**Hydrochloridric salt of [{2-(acetyloxy)benzoyl oxy}methyl-3-[4-amino)butanoyl]oxy]-4-[3-(nitrooxy)propoxy]benzoate (41)**
The crude product was purified by flash chromatography (H₂O/CH₃CN/HCl 70/30/0.1 v/v/v) to give the title compound as a colourless oil; yield: 42 %. ¹H-NMR (CDCl₃) δ 2.14-2.18 (m, 4H, -OCH₂CH₂- + -CH₂CH₂NH₂), 2.32 (s, 3H, CH₃CO-), 2.74 (t, 2H, COCH₂CH₂-), 3.10-3.20 (m, 2H, -CH₂CH₂NH₂), 4.07 (t, 2H, -OCH₂CH₂-), 4.56 (t, 2H, -CH₂ONO₂), 6.12 (s, 2H, -OCH₂O-), 6.90 (d, 1H, C₆H₃), 7.09 (d, 1H, C₆H₄), 7.29 (t, 1H, C₆H₄), 7.56 (t, 1H, C₆H₄), 7.75-7.76 (m, 1H, C₆H₃), 7.90-7.94 (m, 1H, C₆H₃), 8.02-8.06 (m, 1H, C₆H₄), 8.30 (svvbr, 3H, NH₃⁺). ¹³C-NMR (CDCl₃) δ 21.0, 22.6, 26.7, 30.6, 39.2, 65.0, 70.0, 79.9, 112.4, 121.7, 122.0, 124.0, 124.8, 126.2, 129.8, 132.2, 134.7, 139.4, 151.0, 154.5, 163.1, 164.1, 169.7, 170.4.

**Hydrochloridric salt of [[2-(acetyloxy)benzoyl]oxy]methyl-3-[(2-amino)propanoyl]oxy-4-[3-(nitrooxy)propoxy]benzoate (42)**

The crude product was purified by flash chromatography (H₂O/CH₃CN/HCl 80/20/0.1 v/v/v to 60/40/0.1 v/v/v) to give the title compound as a white solid; yield: 44 %. m.p.: 65.5-69.0 °C. ¹H-NMR (CDCl₃) δ 1.78 (d, 3H, -CHCH₃), 2.09-2.11 (m, 2H, -OCH₂CH₂-), 2.32 (s, 3H, CH₃CO-), 4.03 (m, 2H, -OCH₂CH₂-), 4.40 (m, 1H, -CHCH₃), 4.53 (t, 2H, -CH₂ONO₂), 6.12 (s, 2H, -OCH₂O-), 6.84 (d, 1H, C₆H₃), 7.10 (d, 1H, C₆H₄), 7.29 (t, 1H, C₆H₄), 7.65 (t, 1H, C₆H₄), 7.75 (s, 1H, C₆H₃), 7.86 (d, 1H, C₆H₃), 8.02 (d, 1H, C₆H₄), 8.84 (svvbr, 3H, NH₃⁺). ¹³C-NMR (CDCl₃) δ 16.1, 21.0, 26.6, 65.1, 70.1, 79.9, 112.4, 121.6, 122.0, 124.0, 124.4, 126.2, 130.3, 132.2, 134.7, 138.5, 151.0, 154.2, 163.0, 163.9, 167.8, 169.7.

**Hydrochloridric salt of [[2-(acetyloxy)benzoyl]oxy]methyl3-[(2-amino-3-hydroxybutoxy]-4-(3-nitrooxypropoxy)benzoate (43).**

The crude product was triturated with dry Et₂O to give the title compound as a white solid; yield: 70 %. m.p.: 87.5-88.5 °C. ¹H-NMR (CDCl₃) δ 1.40 (d, 3H, -CHCH₃), 2.08 (m, 2H, -OCH₂CH₂-), 2.24 (s, 3H, CH₃CO-), 3.95 (t, 2H, -OCH₂CH₂-), 4.26 (m, 1H, -CHCH₃), 4.35 (m, 1H, -OH), 4.44 (t, 2H, -CH₂ONO₂), 6.01 (s, 2H, -OCH₂O-), 6.90 (d, 1H, C₆H₃), 7.00 (d, 1H, C₆H₄), 7.22 (t, 1H, C₆H₄), 7.47 (t, 1H, C₆H₄), 7.73 (s, 1H, C₆H₃), 7.82 (d, 1H, C₆H₃), 7.95 (d, 1H, C₆H₄), 8.83 (svvbr, 3H, NH₃⁺). ¹³C-NMR (CDCl₃) δ 13.2, 19.5, 19.9, 25.5, 64.1, 65.2, 69.1, 79.0, 120.5, 120.9, 122.9, 125.2, 129.3, 131.1, 133.7, 137.4, 150.0, 153.2, 162.0, 162.2, 162.9, 165.1, 168.7.

**Hydrochloridric salt of [[2-(acetyloxy)benzoyl]oxy]methyl-3-[(3-[aminopropanoyl]oxy]-4-[2,3-bis(nitrooxy)propoxy]benzoate (44).**

The crude product was triturated with dry Et₂O to give the title compound as a foam; yield: 79 %. ¹H-NMR (DMSO-d₆) δ 2.26 (s, 3H, CH₃CO-), 3.02-3.13 (m, 4H, -CH₂CH₂NH₂), 4.46-4.62 (m, AMX-like system, 2H, -OCH₂CH₂-), 4.85-5.06 (m, AMX-like system, 2H, -CHCH₂ONO₂), 5.82-5.87 (m, 1H, -CHCH₂ONO₂), 6.15 (s, 2H, -OCH₂O-), 7.28 (d, 1H, C₆H₃), 7.36-7.47 (m, 2H, C₆H₃ + C₆H₄), 7.71-7.74 (m, 1H, C₆H₄), 7.76-7.77 (m, 1H, C₆H₃), 7.95-8.00 (m, 2H, C₆H₃ + C₆H₄), 8.33 (svvbr,
\[
\begin{align*}
2H, -NH_2). \ ^{13}C\text{-NMR (CDCl}_3 \delta 20.6, 31.0, 34.3, 66.2, 69.7, 77.4, 80.3, 113.8, 121.5, 121.9, 124.2, 124.5, 126.3, 129.6, 131.5, 135.1, 138.8, 150.1, 153.7, 163.4, 166.4, 168.1, 169.0.
\end{align*}
\]

**Solubility study.**
The solubility of the compounds in water was determined at 25 ± 1 °C, adding excess amounts of the compounds to water in test tubes; the mixtures were sonicated for 10 min and kept under magnetic stirring for 30 min. This time was chosen to minimize hydrolysis during the test. After filtration, an aliquot of the filtrate was diluted with an appropriate amount of water, and analysed by HPLC for the quantitation of the compounds, as reported in the stability study experimental section.

**Stability Studies**

**Evaluation of stability in aqueous buffered solutions.** A solution of each compound (10 mM) in acetonitrile/water (50/50, v/v) was added to HCl 0.1 M or to phosphate buffer pH 7.4 (50 mM) preheated to 37 °C; the final concentration of the compound was 100 µM. The resulting solution was maintained at 37 ± 0.5 °C and, at appropriate time intervals, a 20 µL aliquot of reaction solution was analyzed by RP-HPLC, as described below.

**Evaluation of stability in human serum.** A solution of each compound (10 mM) in acetonitrile/water (50/50, v/v) was added to human serum (from human male AB plasma, Sigma) preheated to 37 °C; the final concentration of the compound was 200 µM. The resulting solution was incubated at 37 ± 0.5 °C and, at appropriate time intervals, 300 µL of reaction mixture were withdrawn and added to 300 µL of acetonitrile containing 0.1% trifluoroacetic acid, in order to deproteinize the serum. The sample was sonicated, vortexed and then centrifuged for 10 min at 2150 g; the clear supernatant was filtered through 0.45 µm PTFE filters (Alltech) and analyzed by RP-HPLC. The reverse-phase HPLC procedure separated and quantitated the remaining compound and the products of hydrolysis (ASA, SA, nirooxy-substituted carboxylic 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), and a diode-array detector (DAD) (model G1315B) integrated into the HP1100 system. Data were analyzed by the 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 samples were analyzed using a gradient method, employing a mobile phase consisting of acetonitrile/water with 0.1% trifluoroacetic acid 40/60 (v/v) over the first 2 min, grading to 60/40 at 11 min, keeping 60/40 until 16 min, and then returning to 40/60 at 20 min. 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 all prodrugs and the majority of metabolites) and at 240 nm (for salicylic acid) referenced against a 600 nm wavelength. Quantitation was done using calibration curves of compounds and the relative metabolites, chromatographed under the same conditions; the linearity of the calibration curves was determined in a concentration range of 1-200µM (r² > 0.99).

**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 the 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 placed in 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 a submaximal concentration (0.8-1.5 µg/mL) was used as platelet activator in PRP. The compounds under study were preincubated with PRP for 10 min before addition of the stimulus (collagen). Drug vehicle alone (0.5% DMSO) added to PRP did not affect platelet function in control samples.

The antiaggregatory activity of the tested compounds is expressed as % inhibition of platelet aggregation compared to control samples. For most of the active compounds, IC₅₀ 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 of the studies and the protocols used have been approved by the Ministry of Health, Rome, Italy. The endothelium was removed and the vessels were helically cut: four to six strips were obtained from each aorta. The tissue was 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, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.0, NaHCO₃ 12.0, glucose 11.1, maintained at 37 °C and gassed with 95% O₂-5% CO₂ (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 EC₅₀ ± SE (µM). The effects of 1 µM ODQ on relaxation were evaluated in separate series of experiments, in which the compound was added to the organ bath 5 minutes before contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. Addition of drug vehicle (DMSO) had no appreciable effect on contraction.
Anti-inflammatory 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. Acute edema was induced in conscious rats by intraplantar injection into the right hind paw of 0.1 ml of 1% carrageenan, suspended in 1% carboxymethylcellulose (CMC). Immediately after carrageenan injection, compound or vehicle (1% CMC) was administered intragastrically to different groups of rats in a volume of 10 mL kg⁻¹. ASA was administered as reference drug at the dose of 120 mg kg⁻¹ and the two selected NO-donor ASA pro-drugs, 38 and 49, were administered at a dose equimolar to 120 mg kg⁻¹ ASA. Groups of 6 animals were used. Paw volume was measured with a water plethysmometer (Basile, Comerio, Italy) immediately before carrageenan injection and 3 hours afterwards. The edema reduction in treated animals was expressed as percentage inhibition of the edema observed in vehicle-treated animals, taken as 100. The results are presented as means ± SEM. Statistical analysis was performed with ANOVA, followed by the 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. By the intragastric route, groups of rats (n = 6) were given ASA 120 mg kg⁻¹ or equimolar doses of the two selected NO-donor ASA pro-drugs, 38 and 49 (1% CMC as vehicle). Rats were killed 3 h after administration of the compound. Immediately after death, the stomach was removed, opened along the lesser curvature and examined, to assess mucosal lesions. The stomach was 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 are presented as means ± SEM. Statistical analysis was performed with ANOVA, followed by the Newman-Keuls test.

Acknowledgements
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Keywords: anti-inflammatory agents · acetylsalicylic acid · nitric oxide · prodrugs · water solubility.
References


Chart 1. General structure of NO-donor ASA prodrugs.
Chart 2. General structure of water soluble ASA prodrugs.

\[
\begin{align*}
    &\text{Chart 2. General structure of water soluble ASA prodrugs.} \\
    \text{I: } z = \text{CH}_2, (\text{CH}_2)_2, (\text{CH}_2)_3, (\text{CH})\text{CH}_3, \\
    &\quad (\text{CH(CH(OH))CH}_3) \\
    &m = 1, 2 \\
    &x = 0, 1
\end{align*}
\]
Scheme 1.

1) Br(CH$_2$)$_3$Br or Br(CH$_2$)$_4$Cl, KHCO$_3$, CH$_3$CN, 70 °C; ii) AgNO$_3$, CH$_3$CN, 70 °C; iii) EDC-HCl, DMAP, N-BOC-amino acid, CH$_2$Cl$_2$; iv) KMnO$_4$, acetone; v) BrCH$_2$CH=$\text{CH}_2$, KHCO$_3$, CH$_3$CN, 70 °C; vi) EDC-HCl, DMAP, HOOC(CH$_2$)$_2$NHBOC, CH$_2$Cl$_2$; vii) AgNO$_3$, I$_2$, CH$_3$CN, 70 °C
Scheme 2.

\[ \begin{align*}
\text{i)} & \text{ Br(CH}_2\text{)}_3\text{Br, KHCO}_3, \text{ CH}_3\text{CN, 70 °C}; \\
\text{ii)} & \text{ AgNO}_3, \text{ CH}_3\text{CN, 70 °C}; \\
\text{iii)} & \text{ NaOH, THF/H}_2\text{O, 40 °C}. 
\end{align*} \]
Scheme 3.

\[
\text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{R} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{Cl} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{O} \quad \text{X} \quad \text{B} \quad \text{RCOOH}
\]

\(i\) Cs\(_2\)CO\(_3\), DMF; \(ii\) 1,4-dioxane HCl or TFA, CH\(_2\)Cl\(_2\)

<table>
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<th>R</th>
<th>z</th>
<th>n</th>
<th>RCOOH number</th>
<th>A number</th>
<th>X</th>
<th>B number</th>
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</table>
Scheme 4.

Br(CH₂)₂Br, CH₃CN, K₂CO₃, 70 °C; ii) AgNO₃, CH₃CN, 70 °C; iii) 1N NaOH, MeOH, r.t.; iv) Cs₂CO₃, DMF.
Scheme 5. Possible hydrolytic routes of prodrugs bearing aminoacyloxy (A) and morpholino (B) solubilizing substructure.

A

B
**Table 1.** Physicochemical characterization (melting point and water solubility), stability in buffered solutions, stability in human serum (half-life, maximal amounts of aspirin released (%) and AUC values at 10 min) of compounds under study.

<table>
<thead>
<tr>
<th>compd</th>
<th>M.P. (°C)</th>
<th>Solubility (g L⁻¹)</th>
<th>Stability in buffered solutions</th>
<th>Stability and ASA released in human serum</th>
<th>Platelet aggregation</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>pH 1 % unchanged after 3 h[^b]</td>
<td>pH 7.4 t[½] (min)[^c]</td>
<td>% max of ASA released[^f]</td>
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<tr>
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<td>90</td>
<td>63</td>
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<tr>
<td>38</td>
<td>94.5−95.5</td>
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<td>oil</td>
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<td>86</td>
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<tr>
<td>40</td>
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<td>90</td>
<td>10</td>
<td>&lt; 1</td>
</tr>
<tr>
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<td>86</td>
<td>70</td>
<td>10</td>
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<tr>
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<td>70</td>
<td>90</td>
<td>15</td>
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<tr>
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<td>80</td>
<td>20</td>
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<td>98[^g]</td>
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<td>-</td>
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<tr>
<td>48</td>
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Figure 1. A. Concentration over time of the prodrug 38 and its metabolites in human serum during 3 hours’ incubation. B. Detail of concentration of compounds during the first 10 min of incubation. Values are means ± SEM (SEM < 1; n=3).
Figure 2. A. Concentration over time of the prodrug 49 and its metabolites in human serum during 3 hours’ incubation. B. Detail of concentration of compounds over the first 10 min of incubation. Values are means ± SEM (SEM < 1; n=3).
**Figure 3.** Correlation between ASA released during the first 10 minutes of incubation in human serum (area under curve, AUC$_{0-10}$) and antiaggregatory activity (IC$_{50}$).
Figure 4. A. Anti-inflammatory effects of aspirin and NO-donor ASA prodrugs, 38 and 49, on carrageenan-induced paw edema in conscious rats. The aspirin-like compounds were administered by the intragastric route at doses equimolar to aspirin, 120 mg/kg, at the same time as carrageenan, and their effects were evaluated 3 h later. Results are expressed as the percentage inhibition of edema versus the vehicle-treated group, taken arbitrarily as 100. (** *) \( p < 0.01 \) versus vehicle (ANOVA, followed by the Dunnett test). Values are means ± SEM (n= 6 rats per group). B. Gastric ulcerogenic effects of aspirin, and NO-donor ASA prodrugs, 38 and 49, in conscious rats. The aspirin-like compounds were administered by the intragastric route at doses equimolar to aspirin, 120 mg/kg, and the stomach 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 the Newman-Keuls test). Values are means ± SEM (n= 6 rats per group).