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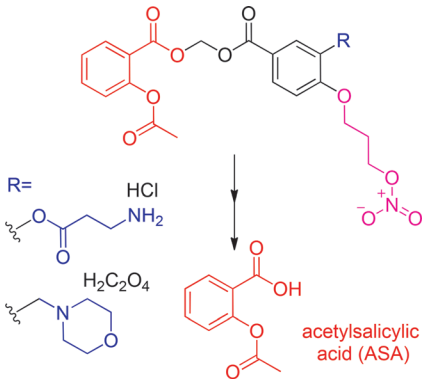
A new look for aspirin: Acetylsalicylic acid (ASA) derivatives bearing NO-releasing nitrooxy group and a solubilizing moiety at the benzoyl ring were designed as ASA prodrugs. When evaluated, all the products displayed good antiaggregatory properties, and two derivatives (shown) also exhibited improved in vivo anti-inflammatory properties and decreased gastrotoxicity when compared with ASA.



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

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

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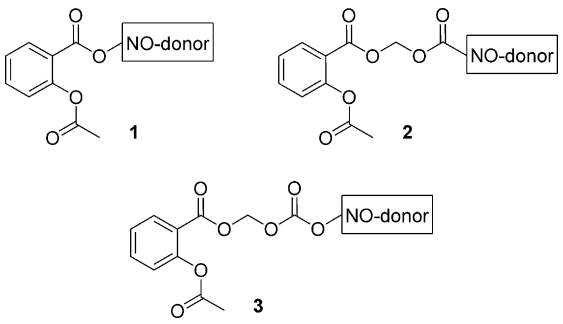
A series of water-soluble (benzoyloxy)methyl esters of acetylsalicylic acid (ASA), commonly known as aspirin, are described. The new derivatives each have alkyl chains containing a nitric oxide (NO)-releasing nitrooxy group and a solubilizing moiety bonded to the benzoyl ring. The compounds were synthesized and evaluated as ASA prodrugs. After conversion to the appropriate salt, most of the derivatives are solid at room temperature and all possess good water solubility. They are quite stable in acid solution (pH 1) and less stable at physiological pH. In human serum, these compounds are immediately metabolized by esterases, producing a mixture of ASA, salicylic acid (SA), and of the related NO-donor benzoic acids, along with other minor products. Due to ASA release, the prodrugs are capable of inhibiting collagen-induced platelet aggregation of human platelet-rich plasma. Simple NO-donor benzoic acids 3-hydroxy-4-(3-nitrooxypropoxy)benzoic acid (**28**) and 3-(morpholin-4-ylmethyl)-4-[3-(nitrooxy)propoxy]benzoic acid (**48**) were also studied as representative models of the whole class of benzoic acids formed following metabolism of the prodrugs in serum. These simplified derivatives did not trigger antiaggregatory activity when tested at 300 μM. Only **28** displays quite potent NO-dependent vasodilatatory action. Further in vivo evaluation of two selected prodrugs, {[2-(acetyloxy)benzoyl]oxy}methyl-3-[(3-[aminopropanoyl)oxy]-4-[3-(nitrooxy)propoxy]benzoate·HCl (**38**) and {[2-(acetyloxy)benzoyl]oxy}methyl 3-(morpholin-4-ylmethyl)-4-[3-(nitrooxy)propoxy]benzoate oxalate (**49**), revealed that their anti-inflammatory activities are similar to that of ASA when tested in the carrageenan-induced paw edema assay in rats. The gastrotoxicity of the two prodrugs was also determined to be lower than that of ASA in a lesion model in rats. Taken together, these results indicated that these NO-donor ASA prodrugs warrant further investigation for clinical application.

Introduction

Nitric oxide (NO)-donor acetylsalicylic acid (ASA) derivatives are a class of products that has received particular attention in recent years.[1,2] The pharmacological rationale for developing these agents is that NO displays gastrosparing properties and consequently could be expected to decrease the well-known gastrotoxicity of ASA, without negatively effecting its antithrombotic and anti-inflammatory properties.[3–5] NO-donor ASA (NO-ASA) derivatives have the general structure **1**, in which the carboxylic group of ASA is linked through an enzymatically labile ester bridge to a substructure containing a NO-donor moiety, typically belonging to the nitrate, furoxan, or NONOate class. Examples of such hybrid agents have been reported in the literature.[6–8] The pharmacological action of these substances is complex, and there is no clear evidence to date that they are true prodrugs of ASA, namely that they are capable of releasing ASA in 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.[9] These products should thus be considered prodrugs of salicylic acid (SA) rather than of ASA.



We recently showed that (nitrooxyacyloxy)methyl esters of ASA and related carbonates are true ASA prodrugs (**2** and **3**).[10,11] 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

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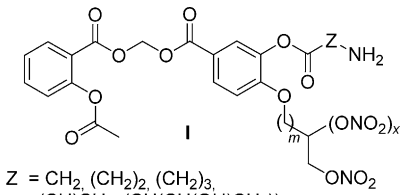
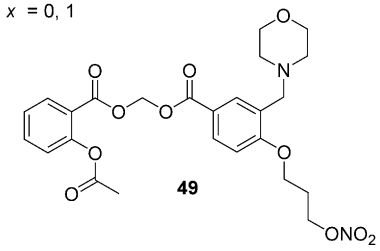
esters is their poor water solubility; many are hydrophobic in nature. This study describes a new series of (nitrooxyacyloxy)-methyl esters of ASA, which are true ASA prodrugs and that possess good water solubility and are solid at room temperature. In these products, the NO-donor nitrooxyacyloxy moiety comprises the benzoyloxy scaffold, bearing a nitrooxyalkyloxy chain at the *para* position, and a solubilizing substructure at the *meta* position. In most of the derivatives (**38**–**44**), the solubilizing substructure is a metabolically labile aminoacyloxy group (**I**); however, in the case of compound **49**, the morpholinomethyl solubilizing substructure is directly joined to the aromatic ring. The synthesis, physical properties (solubility, stability in different media, release of ASA), and the in vitro platelet antiaggregatory profile of these new prodrugs are also reported for two selected compounds (**38** and **49**).

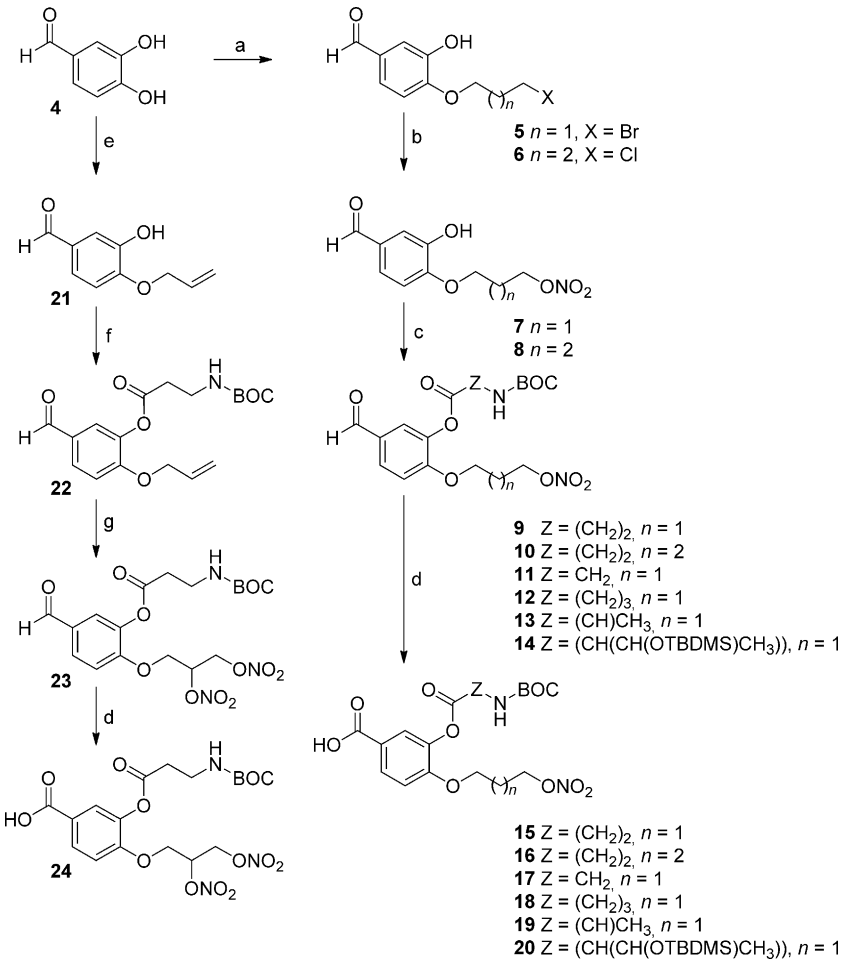




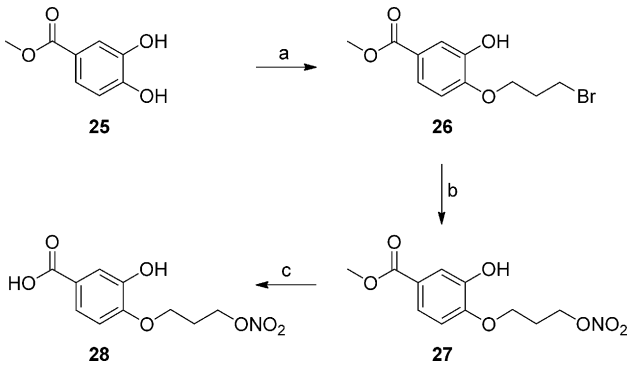
Results and Discussion

Chemistry

The products bearing aminoacyloxy groups were obtained as described in Schemes 1–3. Commercially available 3,4-dihydroxybenzaldehyde (**4**) was bromoalkylated with 1,3-dibromopropane in acetonitrile to give **5**, which in turn was transformed by action of silver nitrate into the related nitrooxy analogue (**7**). This intermediate was reacted under inert atmosphere with the appropriate *tert*-butoxycarbonyl (BOC)-protected amino acid in dichloromethane in the presence of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC·HCl) and 4-(dimethylamino)pyridine (DMAP) to afford aminoacyloxy-substituted aldehydes **9**, **11**–**14**. Corresponding acids **15**, **17**–**20** were obtained by oxidation with potassium permanganate in acetone. Acid **16** and its intermediates **10** and **8** were obtained in the same manner as its short-chain derivative **15**, the only difference being that the starting material was chloro-substituted compound **6** (Scheme 1). Coupling of acid derivatives **15**–**20** with ASA chloromethyl ester **29**, in the presence of cesium carbonate, gave oxymethyloxy esters **30**–**35**. Subsequent removal of the BOC protection under acidic conditions (HCl in dioxane) yielded desired compounds **38**–**43** (Scheme 3). For the preparation of dinitrooxy-substituted prodrug **44**, 3,4-dihydroxybenzaldehyde (**4**) was alkylated with allylbromide to give un-

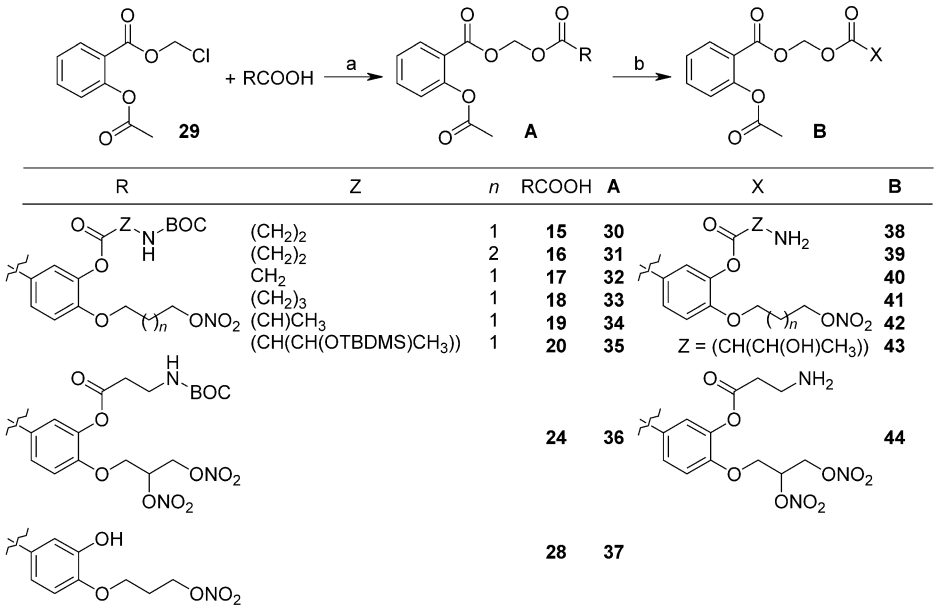


Scheme 1. *Reagents and conditions*: a) $Br(CH_2)_3Br$ or $Br(CH_2)_4Cl$, $KHCO_3$, CH_3CN, 70 °C, 4–14 h; b) $AgNO_3$, CH_3CN, 70 °C, 14 h; c) EDC·HCl, DMAP, *N*-BOC-amino acid, CH_2Cl_2, RT, 3 h; d) $KMnO_4$, acetone, 0 °C→RT, 1 h; e) $BrCH_2CH{=}CH_2$, $KHCO_3$, CH_3CN, 70 °C, 4 h; f) EDC·HCl, DMAP, $HOOC(CH_2)_2NHBOC$, CH_2Cl_2, RT, 3 h; g) $AgNO_3$, I_2, CH_3CN, 70 °C, 16 h.



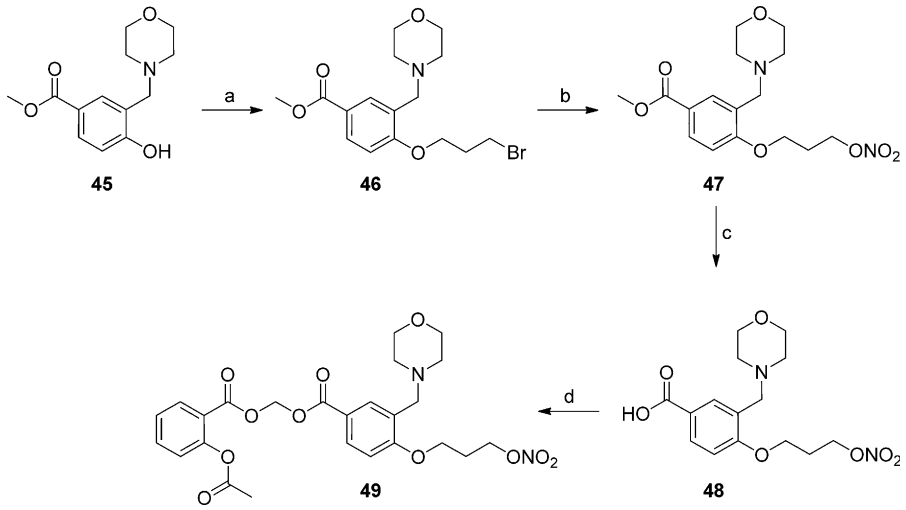
Scheme 2. *Reagents and conditions*: a) $Br(CH_2)_3Br$, $KHCO_3$, CH_3CN, 70 °C, 5 h; b) $AgNO_3$, CH_3CN, 70 °C, 14 h; c) NaOH, THF/H_2O (1:1), 40 °C, 4 h.

saturated aldehyde **21**. The 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 silver nitrate into dinitrooxyester **23**, which in turn was oxidized with potassium permanganate to the corresponding acid (**24**). Final compound **44** was obtained from **24** through intermediate **36** using the procedures described above for the preparation of the aminoacyloxy-substituted prodrugs from the related acids. Simple NO-donor benzoic acid derivative **28** was prepared as shown in Scheme 2. In particular, ester derivative **27** was obtained from the methyl ester of 3,4-dihydroxybenzoic acid **25**, in the same manner as its aldehyde analogue **7**. Intermediate **27** was then hydrolyzed to the corresponding carboxylic acid (**28**) by using a solution of sodium hydroxide in tetrahydrofuran/water with heating to 40 °C.



R	Z	*n*	RCOOH	A	X	B
BOC-NH-CH(Z)-CO-O-	$(CH_2)_2$	1	**15**	**30**	NH_2	**38**
	$(CH_2)_2$	2	**16**	**31**		**39**
	CH_2	1	**17**	**32**		**40**
	$(CH_2)_3$	1	**18**	**33**		**41**
	$(CH)CH_3$	1	**19**	**34**		**42**
	$(CH(CH(OTBDMS)CH_3))$	1	**20**	**35**	$Z = (CH(CH(OH)CH_3))$	**43**
BOC-NH-CH(CH$_2$CH$_2$CO-O-)			**24**	**36**	NH_2	**44**
HO-			**28**	**37**		

Scheme 3. *Reagents and conditions*: a) Cs_2CO_3, DMF, RT, 24 h; b) 1,4-dioxane HCl, CH_2Cl_2, RT, 3 h.



Scheme 4. *Reagents and conditions*: a) $Br(CH_2)_3Br$, CH_3CN, K_2CO_3, 70 °C, 6 h; b) $AgNO_3$, CH_3CN, 70 °C, 14 h; c) 1 N NaOH, MeOH, RT, 4 h; d) **29**, Cs_2CO_3, DMF, RT, 24 h.

Target compound **49** containing a morpholino moiety as a solubilizing group was synthesized as shown in Scheme 4. Ester **45** was treated with 1,3-dibromopropane in acetonitrile to give bromo compound **46**. Treatment of this product with a solution of silver nitrate in acetonitrile transformed it into the nitrooxy analogue (**47**), which was subsequently hydrolyzed to corresponding acid **48**. The acid was finally coupled with **29** to give the desired product **49**.

All of the final products were successfully transformed into the hydrochloride salts, with the sole exception of morpholino derivative **49**, which was characterized as an oxalate. Most of these salts are solid at room temperature with well-defined melting points (Table 1). Two products were oils at room temperature: compounds **39** and **41**, both of which are long-chain derivatives of **38**, the former at the aminoacyloxy chain, and the latter at the nitrooxyalkyloxy moiety. Compound **44**, derived from **38** by the introduction of a second nitrooxy group at the α-position, is a foam at room temperature.

Table 1. Physicochemical characterization, stability in buffer solutions, and stability in human serum of test compounds.

Compd	mp[a] [°C]	Solubility[b] [g L^{-1}]	Stability in buffer pH 1.0 % unchanged[c]	Stability in buffer pH 7.4 $t_{1/2}$[d] [min]	Stability and ASA released in human serum $t_{1/2}$ [min]	[%] max[g]	$AUC_{0-10\,min}$[h]	Platelet aggregation IC_{50}[i] [μM]
ASA[10]	–	3.0	90	90	63	–	954	54 (49–60)
38	94.5–95.5	56	88	57	<1	55	528.0	23 (20–28)
39	oil	52	98	86	<1	50	336.2	47 (41–54)
40	60.5–62.5	110	70	12	<1	57	381.6	40 (34–48)
41	oil	32	90	10	<1	50	217.3	139 (126–154)
42	65.5–69.0	86	70	10	<1	55	232.0	107 (97–119)
43	87.5–88.5	70	90	15	<1	56	397.5	35 (31–40)
44	foam	63	80	20	<1	50	251.8	93 (71–123)
49	135–139 dec.	2.0	98	98[e]	2.2[f]	30	189.2	77 (70–84)
28	–	–	–	–	–	–	–	0.87 ± 0.44[j]
48	–	–	–	–	–	–	–	7.4 ± 5.0[j]

[a] Melting point (mp). [b] Water solubility; data are the mean ($n=3$); standard error of the mean (SEM) <1. [c] Percent unchanged after 3 h; data are the mean ($n=3$); SEM <1%. [d] $n=3$; SEM <0.5. [e] Value is the percent unchanged after 3 h at pH 7.4. [f] $n=3$; SEM = 0.2. [g] Maximal amount (%) of ASA released—data are the percent of the initial concentration of the compound; $n=3$; SEM <1.5%. [h] Area under the curve (AUC) values at 10 min. [i] IC_{50} values derived from 3–5 experiments; 95% confidence levels are given in parentheses. [j] Due to the low activity of the compound, the IC_{50} value could not be calculated; value given is inhibition (%) ± SEM at 300 μM.

Physicochemical evaluation

Water solubility

The water solubility 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^{-1}, The Merck Index 14th Edition). For the solid hydrochloride salts, a general trend was observed—higher solubility was associated with a lower melting point.

Hydrolysis studies

Most of the prodrugs described here (**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 be hydrolyzed are shown in Scheme 5. Hydrolysis was assessed by high-performance liquid chromatography (HPLC) in pH 1 and pH 7.4 buffer solutions, as well as in human serum.

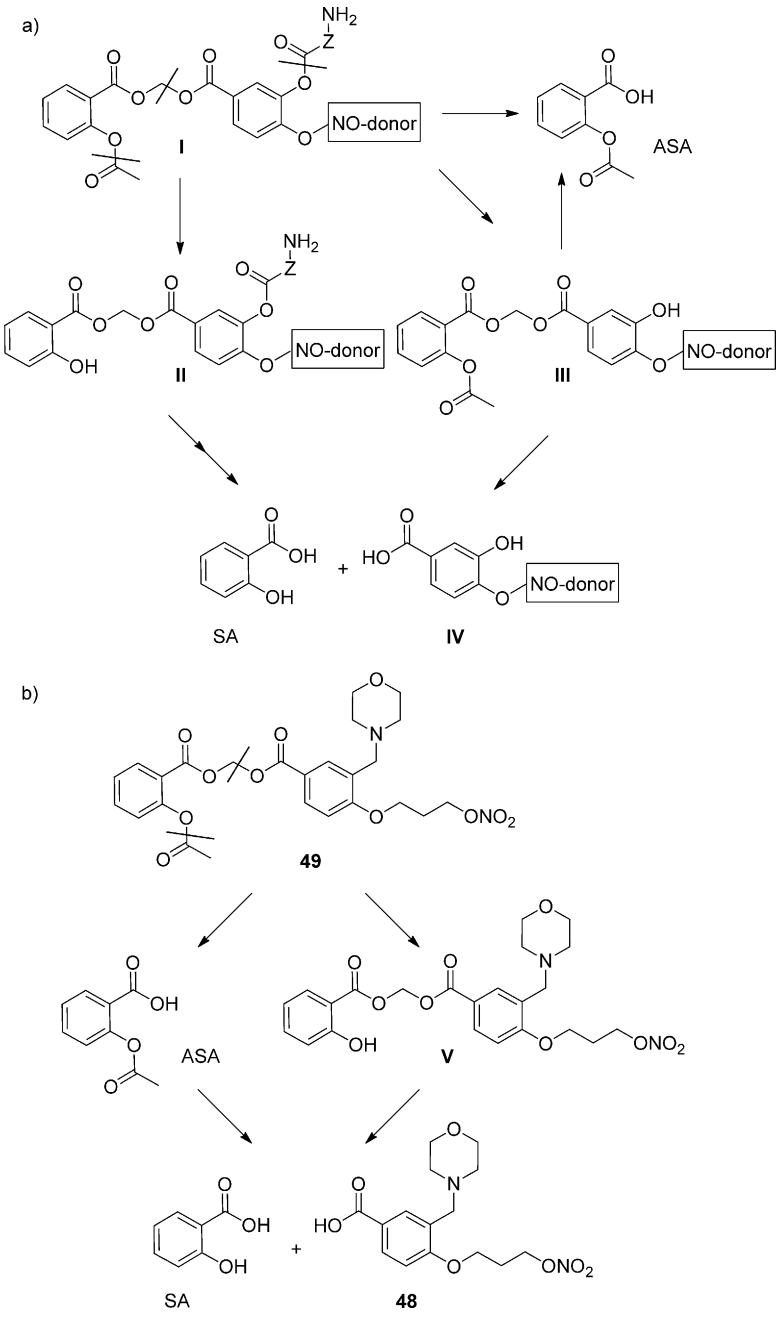
In human serum, hydrolysis is catalyzed by carboxylesterases—ubiquitous enzymes that display broad substrate specificity; the same ester can often be hydrolyzed by more than one enzyme.[12] In human serum, the fate of all compounds was very similar; the observed half-lives were less than one minute (Table 1). The metabolites were monitored for six hours. After ten minutes, only ASA, SA, **IV**, and traces of **II** and **III** were present. This indicates that the aminoacyloxy and carbonyloxymethyloxycarbonyl moieties hydrolyze simultaneously and much more rapidly than the related acetyloxy functionalities. After six hours, only SA and **IV**, accompanied by trace amounts 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. The relationship shown is an interesting result and shows that these products are true ASA prodrugs. Maximal ASA production is reached for all compounds after around two to five minutes (Table 1). Values for all products are similar and fall within the range 50–57%. Table 1 also gives The area under the curve (AUC) values for ASA release measured after a ten minute incubation period do not entirely parallel the corresponding maximal ASA release values (Table 1), 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 at physiological pH (phosphate buffer) than in serum; after an incubation period of three hours, the sole metabolite detected was **III**. The hydrolysis strictly followed first-order kinetics, and the observed pseudo-first-order constants (k_{obs}) were calculated from the slopes of linear plots of the logarithm of the remaining ester against time. The corresponding half-lives (Table 1) were obtained from Equation (1).

$$t_{1/2} = 0.693/k_{obs} \quad (1)$$

The most stable prodrugs were found to be compounds **39** (86 min), **38** (57 min), and **44** (20 min), bearing a β-aminopropionyloxy chain at the 3-position of the benzoyl ring. The other compounds exhibited $t_{1/2}$ values in the range 10–15 minutes. In pH 1 buffer solution, all products remained 70–90% unchanged after three hours, and only hydrolysis product **III** had formed at this point (Table 1).

Unlike the products discussed above, prodrug **49** bears a water-solubilizing morpholinomethyl group directly linked to the benzoyl scaffold, rather than through a vulnerable ester bridge. Consequently, only the acetyloxy and carbonyloxymethyloxycarbonyl moieties undergo hydrolysis, with a consequent decrease in the number of possible metabolites (Scheme 5). Compound **49** is more stable in serum than the other prodrugs discussed above, due to the lack of the very vulnerable aminoacyloxy function. Like the other prodrugs, its deacetylation rate constant is lower than the hydrolytic rate constant of the carbonyloxymethyloxycarbonyl moiety, which



Scheme 5. Possible hydrolytic routes of prodrugs bearing a) aminoacyloxy and b) morpholino solubilizing substructures.

enables ASA to be present among the metabolites. The hydrolysis strictly follows first-order kinetics. Its half-life, calculated from the observed pseudo-first-order rate constant, is 2.2 minutes. Figure 2 shows the concentrations of the metabolites over time for this product. The maximum concentration of ASA is reached after six minutes, and the amount of ASA released (% max and AUC values) is lower than the values measured for all other prodrugs (Table 1). After six hours, only **48**, SA, and traces of ASA were present. The compound is very stable both at pH 1 and at physiological pH.

Pharmacological studies

Platelet antiaggregatory activity

The antiaggregatory activity of the new NO-ASA derivatives was assessed through a collagen-induced platelet aggregation of human platelet-rich plasma (PRP) assay, using ASA as a reference agent. The inhibitory effect of a compound was tested by adding it to PRP ten minutes before addition of the stimulus. All products displayed a concentration-dependent inhibitory effect. Their antiaggregatory potencies, expressed as IC_{50} values, are given in Table 1. As previously mentioned, compounds **IV** and **48** are the metabolites formed rapidly under the action of serum esterases from the prodrugs under study. The antiaggregatory action of **28** and **48**, chosen as prototypes of these acid metabolites, was also evaluated. No antiaggregatory activity was observed when the products were tested at a concentration of 300 μM, meaning that NO does not play a significant role in the antiaggregatory effect of these prodrugs and that this activity is largely due to their capacity to produce ASA. The relationship between $AUC_{0-10\,min}$ and IC_{50} values for the entire series of prodrugs studied is depicted in Figure 3.

Vasodilator activity

All the prodrugs 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.

Compound **28**, which contains the *meta*-hydroxybenzoic acid moiety that is common to all the aminoacyloxy-substituted prodrugs, was found to relax the contracted tissue in a concentration-dependent manner ($EC_{50} = 11 \pm 1$ μM). When the experiments were repeated in the presence of 1 μM 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ), a known inhibitor of soluble guanylate cyclase (sGC), the potency of **28** de-

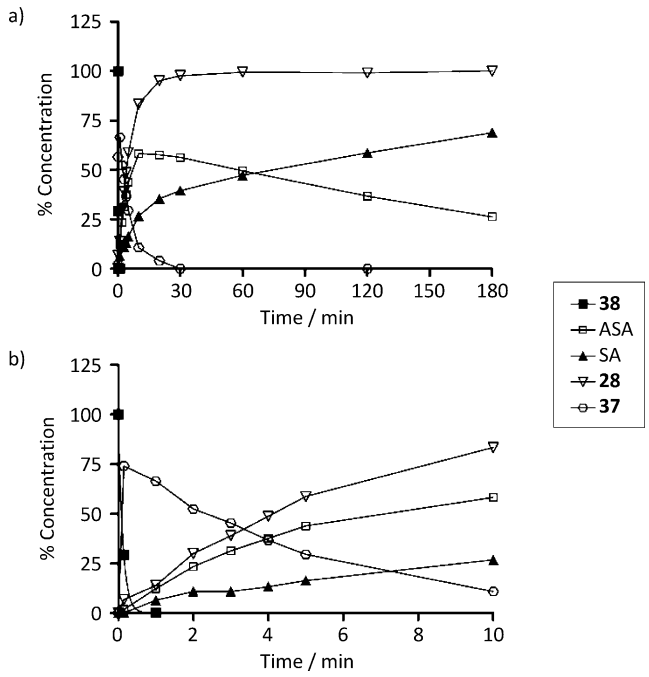


Figure 1. a) Concentration over time of prodrug **38** and its metabolites (**28** and **37**) in human serum during an incubation period of 3 h. b) A detail look at the concentration of compounds during the first 10 min of incubation. Values are mean of $n=3$ experiments; SEM <1.

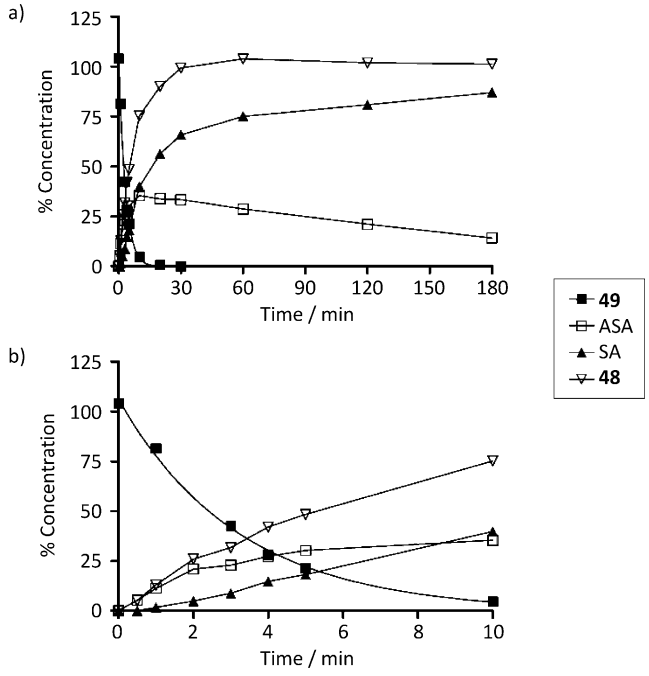


Figure 2. a) Concentration over time of prodrug **49** and its metabolites in human serum during an incubation period of 3 h. b) A detail look at the concentration of compounds during the first 10 min of incubation. Values are mean of $n=3$ experiments; SEM <1.

creased ($EC_{50}>100$ μM). This observation is in keeping with the proposed mechanism of vasodilation through NO-induced activation of sGC.

Conversely, upon evaluation in the same assay, compound **48** was found to be inactive as a vasodilator, even at concentrations of up to 100 μM. While compound **48** contains the same *para*-nitrooxy alkyl chain as **28**, the water solubilizing group is covalently attached to the benzoic acid scaffold. The lipophilic–hydrophilic balance of the two compounds differs (**28**, $\log D^{7.4}=-0.80$; **48**, $\log D^{7.4}=-1.30$) as do their steric and electronic properties. At physiological pH, compound **28** exists principally as an anionic species (anion form $>99\%$; $pK_{a(COOH)}=4.41$) while **48** exists as a mixture of the anion and zwitterion species (anion form $=54\%$, zwitterion form $=46\%$; $pK_{a(COOH)}=3.90$, $pK_{a(morpholine)}=7.33$).

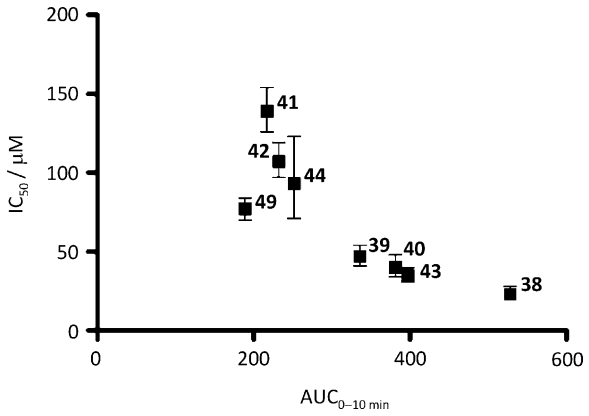


Figure 3. Correlation between ASA released during the first 10 min of incubation in human serum (AUC_{0-10}) and antiaggregatory activity (IC_{50}).

As was observed in our assay, these diverse physicochemical profiles give the two compounds different capacities both to reach and to interact with the putative target enzyme involved in NO production (i.e., sGC), thus affording different vasodilator behavior. However, despite these observations, compound **48** could still 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 (NOx), the oxidized metabolites of NO derived from biotransformation of organic nitrates (data not shown).

Anti-inflammatory activity

NO-donor ASA prodrugs **38** and **49**, and ASA as a reference drug, were tested for their anti-inflammatory activity using a carrageenan-induced paw edema test in conscious rats. The intraplantar injection of carrageenan produced an immediate increase in paw volume, with the inflammatory response peaking four to five hours after carrageenan injection. The intragastric administration of ASA (120 mg kg^{-1}) just prior to carrageenan injection led to a significant decrease in edema (57.4 ± 6.8%; $p<0.01$ versus vehicle-treated controls) assessed three hours after ASA administration. Both prodrugs **38** and **49** administered at doses equimolar to that used for ASA (i.e., 120 mg kg^{-1}) caused a significant decrease in paw edema (54.9 ± 6.6% and 51.5 ± 4.3%, respectively; $p>0.01$ versus vehicle-treated controls). The extent of decrease achieved following the administration of the two prodrugs was comparable to that observed with ASA (Figure 4a).

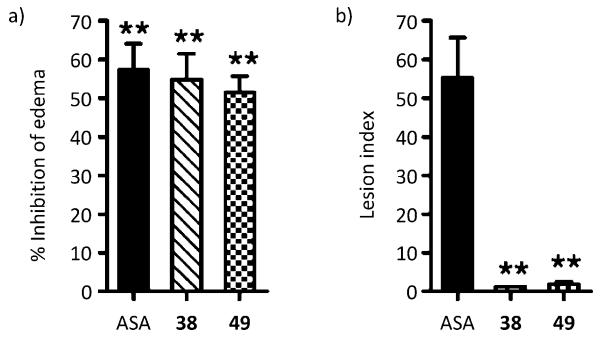


Figure 4. a) Anti-inflammatory effects of ASA and NO-donor ASA prodrugs **38** and **49** on carrageenan-induced paw edema in conscious rats. Results are expressed as the percent inhibition of edema versus the vehicle-treated group (set to 100). ** $p < 0.01$ versus vehicle (ANOVA, followed by Dunnett's test). Values are the mean ± SEM ($n = 6$ rats per group). b) Gastric ulcerogenic effects of ASA and NO-donor ASA prodrugs **38** and **49** in conscious rats. Gastric lesions were measured along the greatest length, and the cumulative length (mm) was designated as the lesion index for each stomach. All compounds tested produced significantly less gastric damage than ASA. ** $p < 0.01$ (ANOVA and the Newman–Keuls test). Values are means ± SEM ($n = 6$ rats per group).

Acute gastric mucosal damage

NO-donor ASA prodrugs **38** and **49**, and ASA as a reference drug, were administered intragastrically to conscious rats, and after three hours, the lesion index of each rat stomach was assessed. At a dose of 120 mg kg^{-1}, ASA treatment resulted in the development of macroscopically detectable lesions located in the gastric mucosa, characterized by necrosis and hemorrhage (LI = 55.3 ± 10.4 mm). In contrast, when administered at a dose equimolar to 120 mg kg^{-1} ASA, neither prodrug caused macroscopically detectable damaging effects on the gastric mucosa (Figure 4 b).

Conclusions

We have developed a new class of (benzoyloxy)methyl esters of acetylsalicylic acid (ASA), commonly known as aspirin. These new derivatives have both alkyl chains containing NO-releasing nitrooxy groups and aminoacyloxy solubilizing moieties appended to the benzoyl ring. When converted to the corresponding salts, most of the derivatives were solid, with well-defined melting points, and the entire class display good water solubility and acid stability.

The compounds are rapidly metabolized under physiological conditions. In human serum, under the action of esterases, the aminoacyloxy and the carbonyloxymethyloxycarbonyl moieties simultaneously hydrolyze, much more rapidly than the related acetyloxy functionalities. The resulting mixture of metabolites includes significant amounts of ASA, and consequently these compounds can be considered true NO-donor ASA prodrugs.

All of the compounds display good antiaggregatory properties as evaluated in a collagen-induced platelet aggregation assay using human platelet-rich plasma. This activity is roughly related to the amount of ASA released in the first ten minutes of incubation. The benzoic acid derivative containing a *meta*-hydroxybenzoic acid moiety (**28**), which was studied as a prototype of the acid metabolites derived from the aminoacyloxy-substituted prodrugs, is endowed with NO-dependent vasodilator activity, unlike compound **48**, in which the solubilizing group remains covalently attached to the benzoic acid scaffold. The related prodrugs **38** and **49** display in vivo anti-inflammatory properties and decrease gastrotoxicity in rats.

Based on their pharmacological and physicochemical profiles, the compounds described here represent an improved class of NO-donor ASA prodrugs more suitable for clinical application than related less soluble prodrugs previously described.

Experimental Section

Synthesis

General: Flash column chromatography was performed on silica gel (Merck kiesel gel 60, 230–400 mesh ASTM). PE refers to petroleum ether 40–60. The progress of the reactions was monitored by thin layer chromatography (TLC) on 5 cm × 20 cm plates, with a layer thickness of 0.25 mm. Anhydrous Na_2SO_4 was used as the drying agent of organic phases during work-up. Organic solvents were removed in vacuo using a water bath at 30 °C.

^{1}H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 at 300 and at 75 MHz, respectively, using $SiMe_4$ as an 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 using a Finnigan-Mat TSQ-700 mass spectrometer using chemical ionization (CI) with isobutane; ESI mass spectra were recorded using a Waters Micromass Quattro micro API. Melting points (mp) were determined using a capillary apparatus (Buchi 540) and are uncorrected. Elemental analyses (C, H, N) were performed by Redox Analyticals (Monza, Italy), and results for all compounds tested were within 0.4% of the theoretical values. Compounds **29**[13] and **45**[14] were synthesized as described in the literature.

4-(3-Bromopropoxy)-3-hydroxybenzaldehyde (5): A solution of **4** (5.00 g, 36.22 mmol), $Br(CH_2)_3Br$ (7.35 mL, 72.44 mmol) and $KHCO_3$ (4.35 g, 43.46 mmol) in CH_3CN (100 mL) was heated at reflux for 4 h. The mixture was then poured into H_2O (50 mL) and extracted with EtOAc (50 mL); the organic layer was washed with saturated aq $NaHCO_3$ (2 × 50 mL) and extracted with 2 N NaOH (2 × 50 mL). The aqueous extracts were acidified with 6 M HCl and then extracted with EtOAc (3 × 50 mL). The combined organic layers were dried, filtered, and concentrated in vacuo to give **5** as a pale yellow semisolid (4.4 g, 47%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.43$ (qi, 2H, OCH_2CH_2), 3.60 (t, 2H, CH_2Br), 4.32 (t, 2H, OCH_2), 5.88 (s, 1H, O*H*), 7.01 (d, 1H, C_6H_3), 7.42 (m, 1H, C_6H_3), 7.44–7.46 (m, 1H, C_6H_3), 9.85 ppm (s, 1H, C*H*O); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 29.3$, 31.8, 66.9, 111.2, 114.4, 124.6, 130.8, 146.2, 150.9, 191.1 ppm; MS (CI): *m/z* 259/261 $[M+H]^+$.

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 $KHCO_3$ (870 mg, 8.69 mmol) in CH_3CN (20 mL) was heated at reflux for 14 h. The mixture was then poured into H_2O (30 mL) and extracted with EtOAc (30 mL); the organic layer was washed with saturated aq $NaHCO_3$ (30 mL) and extracted with 2 N NaOH (3 × 30 mL). The aqueous extracts were acidified with 6 M HCl and then extracted with EtOAc (3 × 30 mL). The combined organic layers were dried, filtered, and concentrated in vacuo to give **6** as a white solid (1.0 g, 60%): mp: 58–59 °C (PE/toluene, 1:1 *v/v*);

^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.95$–2.12 (m, 4H, $OCH_2CH_2CH_2$), 3.65 (t, 2H, CH_2Cl), 4.20 (t, 2H, OCH_2CH_2), 5.93 (s, 1H, OH), 6.97 (d, 1H, C_6H_3), 7.41–7.46 (m, 2H, C_6H_3), 9.84 ppm (s, 1H, CHO); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 26.9$, 29.6, 45.0, 68.9, 111.4, 114.8, 125.0, 131.2, 146.7, 151.6, 191.6 ppm; MS (CI): *m/z* 229/231 $[M+H]^+$.

4-(Allyloxy)-3-hydroxybenzaldehyde (21): A solution of **4** (2.00 g, 14.48 mmol), allyl bromide (1.20 mL, 14.48 mmol) and $KHCO_3$ (1.74 g, 17.37 mmol) in CH_3CN (20 mL) was heated at reflux for 4 h. The mixture was then poured into H_2O (50 mL) and extracted with EtOAc (50 mL); the organic layer was washed with saturated aq $NaHCO_3$ (2×50 mL) and extracted with 2 N NaOH (2×50 mL). The aqueous extracts were acidified with 6 M HCl and then extracted with EtOAc (3×50 mL). The combined organic layers were dried, filtered, and concentrated in vacuo to give **21** as a pale yellow solid (1.0 g, 40%): mp: 57–60 °C (*i*Pr_2O); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta =$ 4.69 (d, 2H, OCH_2), 5.34–5.46 (m, AMX-like system, 2H, $CH{=}CH_2$), 5.99–6.13 (m, AMX-like system, 2H, $CH{=}CH_2 + OH$), 6.96 (d, 1H, C_6H_3), 7.38–7.46 (m, 2H, C_6H_3), 9.83 ppm (s, 1H, CHO); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 69.9$, 111.5, 114.4, 119.2, 124.4, 130.6, 131.8, 146.3, 150.9, 191.2 ppm; MS (CI): *m/z* 179 $[M+H]^+$.

Methyl 4-(3-bromopropoxy)-3-hydroxybenzoate (26): A solution of methyl 3,4-dihydroxybenzoate (2.10 g, 12.5 mmol), $Br(CH_2)_3Br$ (3.80 mL, 37.5 mmol) and $KHCO_3$ (1.50 g, 14.53 mmol) in CH_3CN (20 mL) was heated at reflux for 5 h. The mixture was then poured into H_2O (50 mL) and extracted with EtOAc (2×30 mL); the combined organic layers were washed with brine (10 mL), dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (PE/EtOAc, 9:1→7:3 *v/v*) to give **31** as a white solid (0.91 g, 25%): mp: 106.5–107.5 °C (*i*Pr_2O); ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.39$ (qi, 2H, OCH_2CH_2), 3.58 (t, 2H, CH_2Br), 3.88 (s, 3H, OCH_3), 4.26 (t, 2H, OCH_2CH_2-), 5.75 (br s, 1H, OH), 6.90 (d, 1H, C_6H_3), 7.57–7.61 ppm (m, 2H, C_6H_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 29.4$, 31.8, 50.0, 66.7, 110.8, 115.9, 122.8, 132.1, 145.3, 149.4, 166.8 ppm; MS (CI): *m/z* 289/291 $[M+H]^+$.

Methyl 4-(3-bromopropoxy)-3-(morpholin-4-ylmethyl)benzoate (46): A solution of **27** (2.00 g, 7.90 mmol) in CH_3CN (12 mL) was treated with K_2CO_3 (1.66 g, 12.0 mmol) and 1,3-$Br(CH_2)_3Br$ (3.96 mL, 39.0 mmol), and the mixture was heated at reflux for 6 h. The reaction mixture was then cooled, poured into H_2O (50 mL), and extracted with EtOAc (3×50 mL). The combined organic layers were washed with H_2O (25 mL) and brine (25 mL), then dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (PE/EtOAc, 60:40 *v/v*) to give **46** as a colorless oil (1.12 g, 38%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.36$ (qi, 2H, OCH_2CH_2), 2.48 (t, 4H, CH_2NCH_2), 3.53 (*s*, 2H, $ArCH_2N$), 3.64–3.73 (m, 6H, CH_2OCH_2, CH_2Br), 3.89 (s, 3H, OCH_3), 4.19 (t, 2H, OCH_2), 6.90 (d, 1H, C_6H_3), 7.94 (d, 1H, C_6H_3), 8.01 ppm (s, 1H, C_6H_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 29.8$, 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 ppm; MS (CI): *m/z* 372/374 $[M+H]^+$.

General procedure for the preparation of nitrooxy derivatives 7, 8, 27, 47: A solution of the appropriate bromo/chloro derivative (1.93 mmol) and $AgNO_3$ (0.82 g, 4.82 mmol) in CH_3CN (15 mL) was stirred at 70 °C for 14 h. Brine was then added to precipitate excess $AgNO_3$, and the mixture was filtered through Celite. The filtrate was concentrated in vacuo, and the residue was partitioned between EtOAc (50 mL) and H_2O (50 mL). After separation, the aqueous layer was extracted with EtOAc (2×10 mL). The combined organic layers were dried, filtered, and concentrated in vacuo. The crude product was purified, if necessary, by flash chromatography. Chromatography conditions and characterization data for these products are given in the Supporting Information.

General procedure for the preparation of N-BOC-protected derivatives 9–14, 22: A stirred solution of BOC-protected amino acid (9.38 mmol) in dry CH_2Cl_2 (60 mL) under an inert atmosphere was treated at RT with EDC·HCl (3.96 g, 20.66 mmol) and DMAP (0.12 g, 0.92 mmol). After 30 min, the appropriate phenol derivative (1.70 g, 7.05 mmol) was added. The reaction reached completion after 3 h. The reaction mixture was washed with H_2O (50 mL), dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography. Chromatography conditions and characterization data for these products are given in the Supporting Information.

2-[2,3-Bis(nitrooxy)propyl]-5-formylphenyl 3-[(tert-butoxycarbonyl)amino]propanoate (23): I_2 (0.70 g, 2.78 mmol) was added portionwise to a stirred solution of **22** (0.97 g, 2.78 mmol) and $AgNO_3$ (0.47 g, 2.78 mmol) in CH_3CN (10 mL) at −15 °C. At the end of the addition, stirring was continued for 1 h. Additional $AgNO_3$ (1.18 g, 6.95 mmol) was then added, and the mixture was heated to 70 °C for 16 h. After cooling, the mixture was filtered through Celite, and the filtrate was concentrated in vacuo. The residue was dissolved in H_2O (40 mL) and extracted with EtOAc (3×20 mL). The combined organic layers were washed with brine (20 mL), dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography (PE/EtOAc, 80:20 *v/v*) to give **23** as a yellow oil (0.41 g, 31%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.46$ (s, 9H, *t*Bu), 2.84 (t, 2H, CH_2CH_2NH), 3.51–3.56 (m, 2H, CH_2CH_2NH), 4.38 (d, 2H, OCH_2CH), 4.69–4.90 (m, AMX-like system, 2H, $CHCH_2ONO_2$), 5.03 (svbr, 1H, NH), 5.62–5.66 (m, AMX-like system, 1H, $CHCH_2ONO_2$), 7.08 (d, 1H, C_6H_3), 7.64–7.65 (m, 1H, C_6H_3), 7.76–7.81 (m, 1H, C_6H_3), 9.90 ppm (s, 1H, CHO); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 14.2$, 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 ppm; MS (CI): *m/z* 473 $[M+H]^+$.

General procedure for the preparation of carboxylic acids 15–20, 24: $KMnO_4$ (0.52 g, 3.27 mmol) was added to a stirred solution of the appropriate aldehyde (2.18 mmol) in acetone (50 mL) at 0 °C. The reaction was allowed to reach RT; it was complete after 1 h (TLC, CH_2Cl_2/MeOH, 95:5 *v/v*). Oxalic acid was added, and the mixture was filtered. The filtrate was diluted with CH_2Cl_2 (100 mL), and the organic layer was washed with H_2O (2×50 mL), then dried, filtered, and concentrated in vacuo to give the desired product. Characterization data for these products are given in the Supporting Information.

3-Hydroxy-4-(3-nitrooxypropoxy)benzoic acid (28): A solution of **27** (0.56 g, 2.06 mmol) in THF (10 mL) and H_2O (10 mL) was treated with NaOH pellets (165 mg, 4.13 mmol). The mixture was heated to 40 °C for 4 h. The residue was treated with EtOAc (10 mL) and H_2O (10 mL). After separation, the aqueous layer was acidified with 6 N HCl and extracted with EtOAc (2×20 mL). The combined organic layers were dried, filtered, and concentrated in vacuo to give **28** as a yellow solid (0.31 g, 59%): mp: 166.5–169.5 °C (toluene); ^{1}H NMR (300 MHz, [D_6]DMSO): $\delta = 2.14$ (qi, 2H, OCH_2CH_2), 4.12 (t, 2H, OCH_2CH_2), 4.74 (t, 2H, CH_2ONO_2), 7.00 (d, 1H, C_6H_3), 7.35–7.42 (m, 2H, C_6H_3), 9.27 (br s, 1H, OH), 12.58 ppm (br s, 1H, COOH); ^{13}C NMR (75 MHz, [D_6]DMSO): $\delta = 26.0$, 64.4, 70.8, 112.4, 116.1, 121.4, 123.4, 146.2, 150.4, 167.0 ppm; MS (CI): *m/z* 258 $[M+H]^+$.

3-(Morpholin-4-ylmethyl)-4-[3-(nitrooxy)propoxy]benzoic acid (48): A solution of **29** (0.56 mmol) in MeOH (5 mL) and 1 N NaOH (2 mL) was stirred at RT for 4 h. The solvent was then removed in vacuo, the residue was redissolved in H_2O (10 mL), the pH was ad-

justed to 7, and the product extracted in continuous cycle for 18 h with EtOAc (200 mL). The organic layer was dried, filtered, and concentrated in vacuo to give **48** as colorless oil (0.15 g, 67%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.28$ (qi, 2H, OCH_2CH_2), 2.79 (m, 4H, CH_2NCH_2), 3.77 (m, 4H, CH_2OCH_2), 3.85 (s, 2H, NCH_2Ar), 4.21 (t, 2H, OCH_2), 4.74 (m, 2H, CH_2ONO_2), 7.07 (d, 1H, C_6H_3), 7.98 (d, 1H, C_6H_3), 8.04 ppm (s, 1H, C_6H_3); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 28.0$, 54.2, 57.1, 66.1, 66.9, 71.9, 112.2, 123.7, 126.4, 133.0, 134.7, 161.8, 171.0 ppm; MS (CI): *m/z* 341 $[M+H]^+$.

General procedure for the preparation of ASA esters 30–37, 49: A solution of **29** (0.58 g, 2.52 mmol) in DMF (8 mL) was treated with the appropriate carboxylic acid derivative (2.52 mmol) and Cs_2CO_3 (0.41 g, 1.26 mmol). The mixture was stirred at RT for 24 h, then poured into H_2O (30 mL) and extracted with EtOAc (3×5 mL). The combined organic layers were dried, filtered, and concentrated in vacuo. The crude product was purified by flash chromatography. Chromatography conditions and characterization data for these products are given in the Supporting Information.

{[2-(Acetyloxy)benzoyl]oxy}methyl 3-hydroxy-4-(3-nitrooxypropoxy)benzoate (37): Eluent: PE/EtOAc (90:10→70:30 *v/v*); white solid (0.42 g, 37 %); mp: 84.5–85.5 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.26$ (qi, 2H, -OCH_2CH_2-), 2.35 (s, 3H, CH_3CO-), 4.22 (t, 2H, OCH_2CH_2-), 4.65 (t, 2H, -CH_2ONO_2), 5.62 (s, 1H, *OH*), 6.16 (s, 2H, -OCH_2O-), 6.85 (d, 1H, C_6H_3), 7.12 (d, 1H, C_6H_4), 7.32 (t, 1H, C_6H_4), 7.56–7.67 (m, 3H, $C_6H_3+C_6H_4$), 8.07–8.10 ppm (m, 1H, C_6H_4); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 21.0$, 26.9, 65.1, 69.6, 79.8, 110.8, 116.5, 122.1, 122.5, 123.5, 124.0, 126.1, 132.3, 134.6, 145.4, 149.8, 151.0, 163.1, 164.7, 169.8 ppm; MS (CI): *m/z* 450 $[M+H]^+$; Anal. calcd for $C_{20}H_{19}NO_{11}$: C 53.46, H 4.26, N 3.12, found: C 53.08, H 4.42, N 3.10.

{[2-(Acetyloxy)benzoyl]oxy}methyl 3-(morpholin-4-ylmethyl)-4-[3-(nitrooxy)propoxy]benzoate·$H_2C_2O_4$ (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 colorless 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 $H_2C_2O_4$ (0.06 g, 1 equiv) in EtOAc (2 mL), and the title compound (0.25 g, 20%) was obtained by filtration as a white solid; mp: 135–139 °C (dec.); ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 2.17$–2.25 (m, 5H, -OCH_2CH_2-+-$COCH_3$), 2.71 (m, 4H, —CH_2NCH_2-), 3.64 (m, 4H, -CH_2OCH_2-), 3.83 (s, 2H, -NCH_2Ar), 4.20 (t, 2H, -OCH_2-), 4.74 (t, 2H, -CH_2ONO_2), 6.14 (s, 2H, -OCH_2O-), 7.19 (d, 1H, C_6H_4), 7.27 (d, 1H, C_6H_3), 7.42 (t, 1H, C_6H_4), 7.73 (t, 1H, C_6H_3), 7.96–8.05 (m, 3H, $C_6H_3+C_6H_4$), 8.06 ppm (s, 1H, C_6H_4); ^{13}C NMR (75 MHz, [D$_6$]DMSO): $\delta =$ 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 ppm; MS (ESI$^+$): *m/z* 533 $[M+H]^+$; Anal. calcd for $C_{25}H_{17}N_2O_{11}\cdot H_2C_2O_4$: C 52.09, H 4.86, N 4.50, found: C 52.04, H 4.82, N 4.48.

General procedure for the preparation of amino acid 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 in vacuo. Purification details and characterization data for these products are given below.

{[2-(Acetyloxy)benzoyl]oxy}methyl-3-[(3-[aminopropanoyl)oxy]-4-[3-(nitrooxy)propoxy]benzoate·HCl (38): The crude product was triturated with dry Et_2O to give **38** as a white solid (0.99 g, 89%): mp: 94.5–95.5 °C; ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 2.16$ (qi, 2H, OCH_2CH_2), 2.25 (s, 3H, CH_3CO), 3.01–3.06 (m, 2H, $CH_2CH_2NH_2$), 3.11–3.13 (m, 2H, $CH_2CH_2NH_2$), 4.21 (t, 2H, OCH_2CH_2), 4,63 (t, 2H, CH_2ONO_2), 6.14 (s, 2H, OCH_2O), 7.26–7.34 (m, 2H, $C_6H_3+C_6H_4$), 7.44 (t, 1H, C_6H_4), 7.71–7.77 (m, 1H, C_6H_4), 7.82–7.83 (m, 1H, C_6H_3), 7.93–8.00 (m, 2H, $C_6H_3+C_6H_4$), 8.22 ppm (br s, 2H, NH_2); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 21.0$, 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 ppm; MS (ESI$^+$): *m/z* 521 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}N_2O_{12}\cdot$HCl: C 49.60, H 4.52, N 5.03, found: C 49.24, H 4.50, N 5.04.

{[2-(Acetyloxy)benzoyl]oxy}methyl-3-[(3-[aminopropanoyl)oxy]-4-[3-(nitrooxy)butoxy]benzoate·HCl (39): The crude product was purified by flash chromatography (H_2O/CH_3CN/HCl, 60:40:0.1 *v/v/v*) to give **39** as a colorless oil (0.57 g, 50%): ^{1}H NMR (300 MHz, [D$_6$]DMSO + $CDCl_3$): $\delta = 1.78$–1.94 (m, 4H, $OCH_2CH_2CH_2$), 2.28 (s, 3H, CH_3CO), 3.09 (t, 2H, CH_2CH_2NH), 3.21 (t, 2H, CH_2CH_2NH), 4.13 (t, 2H, OCH_2CH_2), 4.58 (t, 2H, CH_2ONO_2), 6.14 (s, 2H, OCH_2O), 7.17–7.21 (m, 2H, $C_6H_3+C_6H_4$), 7.42 (t, 1H, C_6H_4), 7.81 (t, 1H, C_6H_4), 7.79–7.80 (m, 1H, C_6H_3), 7.94–8.03 (m, 2H, $C_6H_3+C_6H_4$), 8.34 ppm (br s, 2H, NH_2); ^{13}C NMR (75 MHz, [D$_6$]DMSO + $CDCl_3$): $\delta = 21.4$, 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 ppm; MS (ESI$^+$): *m/z* 535 $[M+H]^+$; Anal. calcd for $C_{24}H_{26}N_2O_{12}\cdot$HCl·2.5$H_2O$: C 46.80, H 5.24, N 4.55, found: C 46.74, H 4.71, N 4.38.

{[2-(Acetyloxy)benzoyl]oxy}methyl-3-[(2-amino)acetoxy]-4-[3-(nitrooxy)propoxy]benzoate·HCl (40): The crude product was triturated with dry Et_2O to give **40** as a white solid (0.43 g, 40%): mp: 60.5–62.5 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.16$ (m, 2H, OCH_2CH_2), 2.31 (s, 3H, CH_3CO), 3.99 (m, 2H, $CH_2NH_2+OCH_2CH_2$), 4.20 (t, 2H, $CH_2NH_2+OCH_2CH_2$), 4.53 (t, 2H, CH_2ONO_2), 6.16 (s, 2H, OCH_2O), 6.83 (d, 1H, C_6H_3), 7.08 (d, 1H, C_6H_4), 7.27 (t, 1H, C_6H_4), 7.55 (t, 1H, C_6H_4), 7.71 (s, 1H, C_6H_3), 7.86 (d, 1H, C_6H_3), 8.03 (d, 1H, C_6H_4), 8.69 ppm (br s, 3H, NH_3^+); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 20.9$, 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 ppm; MS (ESI$^+$): *m/z* 507 $[M+H]^+$; Anal. calcd for $C_{22}H_{20}N_2O_{12}\cdot$HCl·0.5$H_2O$: C 47.88, H 4.38, N 5.08, found: C 47.82, H 4.35, N 5.37.

{[2-(Acetyloxy)benzoyl]oxy}methyl-3-[(4-amino)butanoyl]oxy-4-[3-(nitrooxy)propoxy]benzoate·HCl (41): The crude product was purified by flash chromatography (H_2O/CH_3CN/HCl, 70:30:0.1 *v/v/v*) to give **41** as a colorless oil (0.48 g, 42%): ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 2.14$–2.18 (m, 4H, $OCH_2CH_2+CH_2CH_2NH_2$), 2.32 (s, 3H, CH_3CO), 2.74 (t, 2H, $COCH_2CH_2$), 3.10–3.20 (m, 2H, $CH_2CH_2NH_2$), 4.07 (t, 2H, OCH_2CH_2), 4.56 (t, 2H, CH_2ONO_2), 6.12 (s, 2H, OCH_2O), 6.90 (d, 1H, C_6H_3), 7.09 (d, 1H, C_6H_4), 7.29 (t, 1H, C_6H_4), 7.56 (t, 1H, C_6H_4), 7.75–7.76 (m, 1H, C_6H_3), 7.90–7.94 (m, 1H, C_6H_3), 8.02–8.06 (m, 1H, C_6H_4), 8.30 ppm (br s, 3H, NH_3^+); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 21.0$, 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 ppm; MS (ESI$^+$): *m/z* 535 $[M+H]^+$; Anal. calcd for $C_{24}H_{26}N_2O_{12}\cdot$HCl: C 44.58, H 3.81, N 4.00, found: C 44.24, H 3.86, N 3.81.

{[2-(Acetyloxy)benzoyl]oxy}methyl-3-[(2-amino)propanoyl]oxy-4-[3-(nitrooxy)propoxy]benzoate·HCl (42): The crude product was purified by flash chromatography (H_2O/CH_3CN/HCl, 80:20:0.1→60:40:0.1 *v/v/v*) to give **42** as a white solid (0.5 g, 44%): mp: 65.5–69.0 °C; ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.78$ (d, 3H, $CHCH_3$), 2.09–2.11 (m, 2H, OCH_2CH_2), 2.32 (s, 3H, CH_3CO), 4.03 (m, 2H, OCH_2CH_2), 4.40 (m, 1H, $CHCH_3$), 4.53 (t, 2H, CH_2ONO_2), 6.12 (s, 2H, OCH_2O), 6.84 (d, 1H, C_6H_3), 7.10 (d, 1H, C_6H_4), 7.29 (t, 1H, C_6H_4), 7.65 (t, 1H, C_6H_4), 7.75 (s, 1H, C_6H_3), 7.86 (d, 1H, C_6H_3), 8.02 (d, 1H, C_6H_4),

8.84 ppm (br s, 3H, NH_3^+); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 16.1$, 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 ppm; MS (ESI^+): *m/z* 521 $[M+H]^+$; Anal. calcd for $C_{23}H_{24}N_2O_{12}\cdot HCl\cdot 0.5H_2O$: C 48.82, H 4.63, N 4.95, found: C 48.47, H 4.44, N 5.00.

{[2-(Acetyloxy)benzoyl]oxy}methyl3-[2-amino-3-hydroxybutoxy]-4-(3-nitrooxypropoxy)benzoate·HCl (43): The crude product was triturated with dry Et_2O to give **43** as a white solid (0.82 g, 70%): mp: 87.5–88.5 °C; ^1H NMR (300 MHz, $CDCl_3$): $\delta = 1.40$ (d, 3H, $CHCH_3$), 2.08 (m, 2H, OCH_2CH_2), 2.24 (s, 3H, CH_3CO), 3.95 (t, 2H, OCH_2CH_2), 4.26 (m, 1H, $CHCH_3$), 4.35 (m, 1H, OH), 4.44 (t, 2H, CH_2ONO_2), 6.01 (s, 2H, OCH_2O), 6.90 (d, 1H, C_6H_3), 7.00 (d, 1H, C_6H_4), 7.22 (t, 1H, C_6H_4), 7.47 (t, 1H, C_6H_4), 7.73 (s, 1H, C_6H_3), 7.82 (d, 1H, C_6H_3), 7.95 (d, 1H, C_6H_4), 8.83 ppm (br s, 3H, NH_3^+); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 13.2$, 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 ppm; MS (ESI^+): *m/z* 551 $[M+H]^+$; Anal. calcd for $C_{24}H_{26}N_2O_{13}\cdot HCl\cdot H_2O$: C 48.94, H 4.96, N 4.75, found: C 48.96, H 4.70, N 4.79.

{[2-(Acetyloxy)benzoyl]oxy}methyl-3-[(3-[aminopropanoyl)oxy]-4-[2,3-bis(nitrooxy)propoxy]benzoate·HCl (44): The crude product was triturated with dry Et_2O to give **44** as a colorless foam (0.97 g, 79%): ^1H NMR (300 MHz, [D_6]DMSO): $\delta = 2.26$ (s, 3H, CH_3CO), 3.02–3.13 (m, 4H, $CH_2CH_2NH_2$), 4.46–4.62 (m, AMX-like system, 2H, OCH_2CH), 4.85–5.06 (m, AMX-like system, 2H, $CHCH_2ONO_2$), 5.82–5.87 (m, 1H, $CHCH_2ONO_2$), 6.15 (s, 2H, OCH_2O), 7.28 (d, 1H, C_6H_3), 7.36–7.47 (m, 2H, $C_6H_3+C_6H_4$), 7.71–7.74 (m, 1H, C_6H_4), 7.76–7.77 (m, 1H, C_6H_3), 7.95–8.00 (m, 2H, $C_6H_3+C_6H_4$), 8.33 ppm (br s, 2H, NH_2); ^{13}C NMR (75 MHz, [D_6]DMSO): $\delta = 20.6$, 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 ppm; MS (ESI^+): *m/z* 582 $[M+H]^+$. Due to the high hygroscopicity and instability of the product at RT, elemental analysis was not possible. The purity was determined by HPLC analysis to be >99%.

Evaluation of the physicochemical properties

Solubility: The solubility of test compounds in water was determined at 25±1 °C. Excess amounts of test compound was added to water in test tubes, and the mixtures were sonicated for 10 min and then stirred for a further 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 analyzed by HPLC for quantitation of remaining intact compound and any degradation products.

Stability: For evaluation in buffers, a solution of test compound (10 mM) in CH_3CN/H_2O (50:50, *v/v*) was added to 0.1 M HCl (pH 1), 50 mM phosphate buffer (pH 7.4) preheated to 37 °C. The final compound concentration 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. For evaluation in human serum, a solution of test compound (10 mM) in CH_3CN/H_2O (50:50, *v/v*) was added to human serum (male AB plasma, Sigma) preheated to 37 °C. The final compound concentration 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 CH_3CN containing 0.1% trifluoroacetic acid (TFA), 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, as described below. The reverse-phase HPLC procedure separated and quantitated the remaining test compound and the hydrolysis products (ASA, SA, nitrooxy-substituted carboxylic acid).

HPLC analyses were performed with a HP1100 chromatograph system (Agilent Technologies, Palo Alto, 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 using the HP ChemStation system (Agilent Technologies). The analytical column was a Nucleosil 100-5 C18 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 CH_3CN/H_2O with 0.1% TFA: 40:60 (*v/v*) over the first 2 min, increasing to 60:40 at 11 min, remaining at 60:40 until 16 min, and then returning to 40:60 at 20 min. The flow-rate was 1.2 mL min^{-1}. 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 for compounds and the metabolites, chromatographed under the same conditions; the linearity of the calibration curves was determined in a concentration range of 1–200 μM ($r^2 > 0.99$).

Biological evaluation

Ethical statement: Both the concept and protocols for studies involving animals were approved by the Italian Ministry of Health, and as few animals as possible were used in each experiment. Where human tissue was used, volunteers, who were treated according to the Helsinki protocol for biomedical experimentation, gave their informed consent for the use of samples for research purposes.

Animals: Male Wistar rats were obtained from Harlan Laboratories, San Pietro al Natisone, Italy and 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.

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. Platelet-rich plasma (PRP) was prepared by centrifugation of citrated blood at 200 *g* for 20 min. 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 min after addition of the stimulus. Collagen at a submaximal concentration (0.8–1.5 μg mL^{-1}) 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 percent inhibition of platelet aggregation compared with vehicle control samples. For most of the active compounds, IC_{50} values could be calculated by nonlinear regression analysis; when this was not possible, percent inhibition at maximal concentration tested (300 μM) is reported. The number of experiments was 3–5 for IC_{50} values, and 3 for determination of percent inhibition.

Vasodilator activity: Thoracic aortas were isolated from male Wistar rats weighing 180–200 g. The endothelium was removed, and the vessels were helically cut: 4–6 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: NaCl (111.2 mM), KCl (5.0 mM), $CaCl_2$ (2.5 mM), $MgSO_4$ (1.2 mM), KH_2PO_4 (1.0 mM), $NaHCO_3$ (12.0 mM), glucose (11.1 mM), 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 L-phenylephrine (1 μM). When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. Results are expressed as the EC_{50} value; data are the mean ± SEM of three experiments. The effects of ODQ (1 μM) on relaxation were evaluated in a separate series of experiments, in which the compound was added to the organ bath 5 min before contraction. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. Addition of drug vehicle (1% DMSO) had no appreciable effect on contraction.

Anti-inflammatory activity: Acute edema was induced in conscious male Wistar rats weighing 180–200 g by intraplantar injection of 1% carrageenan (0.1 mL suspended in 1% carboxymethylcellulose (CMC)) into the right hind paw. 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 a reference drug at the dose of 120 mg kg^{-1}, and test compounds were administered at a dose equimolar to 120 mg kg^{-1} ASA. Groups of six animals were used. Paw volume was measured with a water plethysmometer (Basile, Comerio, Italy) immediately before carrageenan injection and 3 h afterwards. The edema volume decrease in treated animals was expressed as percent inhibition of the edema observed in vehicle-treated animals, taken as 100. Data are the mean ± SEM. Statistical analysis was performed with ANOVA, followed by Dunnett's test.

Gastrotoxicity: Male Wistar rats weighing 180–200 g 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 test compound, using 1% CMC as the 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. Data are the mean ± SEM. Statistical analysis was performed with ANOVA, followed by the Newman–Keuls test.

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