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New Nitric Oxide or Hydrogen Sulfide Releasing Aspirins

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ABSTRACT. A new series of (((R-oxy)carbonyl)oxy)methyl esters of Aspirin (ASA), bearing nitric oxide (NO) or hydrogen sulfide (H_2S) releasing groups were synthesized and evaluated as new ASA co-drugs. All the products were quite stable in buffered solution at pH 1 and 7.4. By contrast, all of them were fast metabolized with production of ASA and of the related NO/H_2S releasing moieties used for their preparation. As consequence of ASA release, the compounds were capable of inhibiting collagen induced platelet aggregation of human platelet rich plasma (PRP). The simple NO/H_2S donor substructures were able to relax contracted rat aorta strips with a mechanism NO- and H_2S-dependent, respectively, but they did not trigger antiaggregatory activity or displayed antiplatelet potencies definitively lower than that of the related co-drug. These new products could represent an alternative to the use of ASA in a variety of clinical applications.

KEYWORDS: nitric-oxide, hydrogen sulfide, platelet aggregation, co-drugs.

Introduction.

Gastrointestinal adverse effects of Aspirin (ASA, acetyl salicylic acid (**1**), Chart 1), which is the most widely used nonsteroidal anti-inflammatory drug (NSAID), in spite of its introduction into the market over 100 years ago, are the most prominent drawbacks of this medicine.[1-3] A number of strategies have been proposed to overcome this problem, including the "gaseous solution".[2,4,5] This approach consists in the conjugation of a nitric oxide (NO) or a hydrogen sulfide (H_2S) releasing moiety with ASA by an ester link. NO is an important gaseous messenger which mediates a variety of physiological actions, including gastroprotection.[6] Indeed, NO is able to defense gastric mucosa against adverse effects consequent to NSAID-induced COX-1 inhibition, such as decrease of mucosal blood flow, reduction of mucus and bicarbonate secretion, promotion of neutrophil adherence and activation, and over-expression of inflammatory mediators.[7,8] In recent years also H_2S has been recognized as an important gaseous signaling molecule. Similarly to NO, it presents dual personality, namely at endogenous concentrations it displays a variety of beneficial effects, but detrimental at superphysiological concentrations. It shares many biological activities with NO, including reduction of neutrophil adhesion, attenuation of inflammatory mediator expression, protection against gastric injury through mechanisms similar to those of NO.[4,9-12] The NicOx NO-donor aspirins **2**, **3**, **4**, **5** and the H_2S-donor CTG Pharma aspirin **6** (Chart 1), which are the prototypes of this class of drugs, were designed following the aforementioned approach.[13,14] The problem with these products is that they are rapidly metabolized in human plasma into salicylates and then into salicylic acid and NO/H_2S releasing moieties, without any formation of ASA. This is due to the loss of the negative charge on the ASA moiety, which induces high enzymatic liability of the acetyloxy group.[15] A number of similar products have been reported in literature but, in our knowledge, only few well documented examples of "true" ASA-NO donor co-drugs are known.[16,17] None true ASA H_2S-donor co-drug has been so far described. This paper deals with the synthesis of a new class of aryloxy and alkyloxy carbonyloxymethyl esters of ASA, bearing either nitrooxy NO-donor moieties (der.s **9a-d**, Scheme 1) or the H_2S-donor residue (3-thioxo-3*H*-1,2-dithiol-5-yl) (der.s **16a**,**16b**, Scheme 2), and shows that all these products are stable in acid and

physiological pH solutions, but are able to release aspirin when incubated in human serum. The antiaggregatory properties of the products, and the vasodilator effects of the simple NO-/H_2S-donor moieties used for their preparation are discussed as well.

Results and Discussion

Chemistry. The synthetic routes used to prepare the ASA NO-donor co-drugs are summarized in Scheme 1. Chloromethyl chloroformate was treated, in CH_2Cl_2 solution, in the presence of pyridine (Py), with the appropriate NO-donor alcohols **7a,b** and phenols **7c,d**. The resulting chloromethylcarbonates **8a-d** were used for the next reaction with ASA, in the presence of Cs_2CO_3 in DMF, to give the final carbonates **9a-d**. The preparation of ASA-H_2S donor co-drugs **16a**,**16b** (Scheme 2) required the use of the known H_2S-donor 5-(4-hydroxyphenyl)-3H-1,2-dithiole-3-thione (**10**)[12] and of the new intermediates **12** and **14**. The former product was synthesized treating **10** with O-tetrahydropyranyl (THP) protected ethylene glycol under Mitsunobu conditions (triphenylphosphine (PPh$_3$), diisopropyl azidodicarboxylate (DIAD)). The THP group was removed under the action of pyridinium p-toluensulfonate (PPTS) in methanol to give the expected **12**. The latter intermediate was obtained by reaction of ASA with O-(chloromethyl)-S-ethyl thiocarbonate (**13**) in the presence of Cs_2CO_3 in DMF. Resulting product **14** was treated with neat SO_2Cl_2 to afford **15**. Finally, reaction of **15** with phenol **10** or alcohol **12** in the presence of 4-methylmorpholine in CH_2Cl_2 gave the desired carbonates **16a**,**16b**.

Hydrolysis Studies. The possible hydrolytic routes of the new carbonates we developed are reported in Scheme 3. To be true ASA NO/H_2S donors co-drugs it is necessary that the products have the rate constant of deacetylation k_2 slower than the hydrolytic constant k_1. The stability of all the compounds was assessed by high-performance liquid chromatography (HPLC) in buffered solution at pH 1.0 and 7.4, as well as in human serum. The results are reported in Table 1. All the products resulted unchanged for more than 96% after 3 h of incubation in acid solution. A similar behavior was observed at physiological pH, with the exception of **9b** and **16a**, that were transformed slightly more extensively

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(unchanged 85% and 80% respectively). A different situation occurred in human serum, in which it is known that a variety of esters are hydrolyzed by carboxylesterases.[18] Both the nitrogen and sulfur containing compounds were hydrolyzed very fast, following pseudo-first-order kinetics. The observed pseudo-first-order rate constants (k_{obs}) and the half-lives ($t_{1/2}$, Table 1) were determined by fitting the data with one phase exponential decay equation (Graph Pad, Prism software vers. 5).

According to Scheme 3, ASA, R-oxycarbonyloxymethyl esters of salicylic acid, related hydroxy derivatives, and salicylic acid were detected during hydrolysis in human serum. For all the compounds salicylic acid and hydroxyl derivatives were the final metabolites. The time-course of the degradation products detected over 10 min and 2h in the case of products **16a**, is reported in Figure 2 as example. The maximal amount of ASA detected for each compound, expressed as % of the initial carbonate concentration, are collected in Table 1 as well as the areas under the ASA release curves (AUC), measured for each compound after ten minutes. Among the nitrooxy containing compounds, the aromatic carbonates **9c** and **9d** were better ASA releasing than the aliphatic ones (**9a** and **9b**). Both the sulfur containing esters **16a** and **16b** are quite good ASA producers. In conclusions all the products behave as true ASA co-drugs.

Platelet Antiaggregatory Activity. Antiaggregatory effects of the new ASA co-drugs were studied on collagen induced platelet aggregation of human rich plasma (PRP), taken aspirin as reference. The inhibitory activity was assessed by addition of each product to PRP 10 min before addition of the stimulus. The calculated antiaggregatory potencies (IC_{50}) are reported in Table 1. The NO-donor structures **7a,b,d**, and the H_2S-donor structure **10**, used to hybridize ASA, did not trigger any antiaggregatory action, when tested at 300 μM concentration, under the same conditions used to test the corresponding co-drugs. Alcohols **7c** and **12** behave in a slightly different manner showing 174 and 184 μM IC_{50} value respectively, potencies definitively lower than those of the related co-drugs **9c** and **16b**. Consequently, the antiaggregatory activities of all the products are reasonably attributable principally to their capacity to release ASA.

In a previous work, we showed that the areas under the ASA release curves (AUC) measured at ten minutes for a series of (nitrooxyacyloxy)methyl esters of ASA linearly correlate with the corresponding antiaggregatory potencies.[16] The antiaggregatory IC_{50} values versus AUC values for the carbonates object of the present work, fit quite well with this line; the only exception is represented by **16b** (Figure 2). Synergism between ASA and alcohol **12** could be responsible for this behavior. Indeed, when the antiaggregatory potency was evaluated using 1:1 (10μM, mol/mol) mixtures of the products, at a concentration in which the two compounds separately were inactive, a significant inhibition of platelet aggregation was observed (data not shown)

Vasodilator Activities. The vasodilator activity of the NO-donor moieties **7a-d**, used to hybridize ASA, was evaluated on endothelium denuded rat aorta strips precontracted with phenylephrine. All the products were capable of relaxing the contracted tissue, in a concentration dependent manner. Their potencies, expressed as EC_{50}, are collected in Table 2. As expected, both in the aliphatic and aromatic carbonates, the dinitrooxy substituted products are more potent than the mononitrooxy analogues. The vasodilator effect was abolished by the presence of 1 μM ODQ (1*H*-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one), a well known inhibitor of the soluble guanylate cyclase (sGC), in keeping with NO-induced activation of this enzyme as the mechanism which underlies the effect. The vasodilator actions of the H_2S releasing products **10** and **12** were evaluated on endothelium denuded rat aorta strips precontracted with KCl. The two alcohols showed similar vasodilator potencies, but lower than the ones of the related nitrooxy carbonates (Table 2). In experiments performed in the presence of glibenclamide, a well known potent blocker of ATP-modulated K^+ channels, the dose-response curves obtained for the two compounds were significantly rightward shifted. This result is in keeping with the involvement of H_2S in the vasodilator action of the compounds.

Conclusions. We were able to develop a new class of NO-donor and, for the first time, of true H_2S donor ASA co-drugs. All the products are capable of fast ASA release, when incubated in human serum, following pseudo-first order kinetics. By contrast they are rather stable in acid and physiological pH.

They are able to inhibit collagen induced platelet aggregation of human platelet rich plasma. The simple NO-donor and H_2S-donor moieties, used to prepare the final products do not trigger antiaggregatory properties antiaggregatory activity or displayed antiplatelet potencies definitively lower than that of the related co-drug. By contrast, they display NO-dependent and H_2S-dependent vasodilator activities, respectively. In view of the gastroprotective effect exerted both by NO and H_2S, these products could represent an improved alternative of ASA in a variety of clinical applications.

Figure 1. The time-course of the degradation products of compound **16a** at 2 hours and at 10 min incubation time in human serum: values are mean ± SEM (SEM ≤ 3; number of determinations = 3).
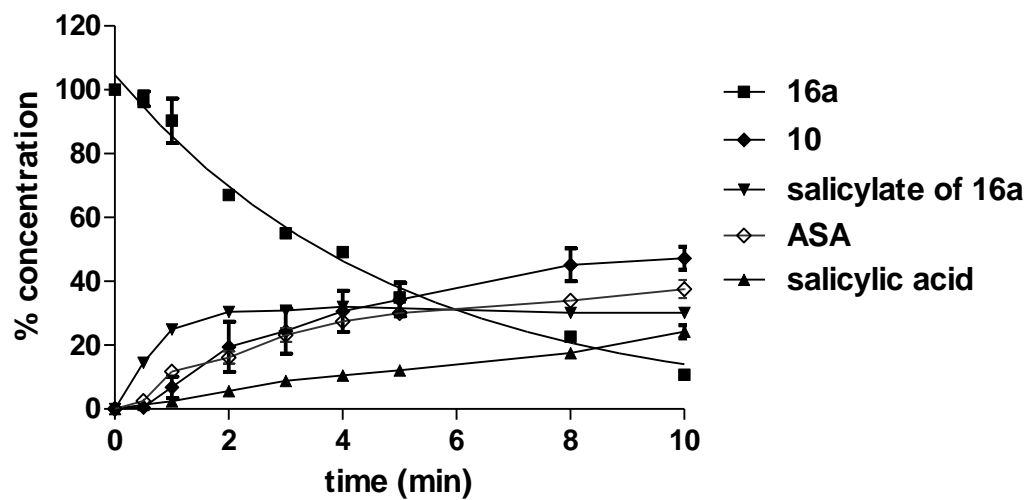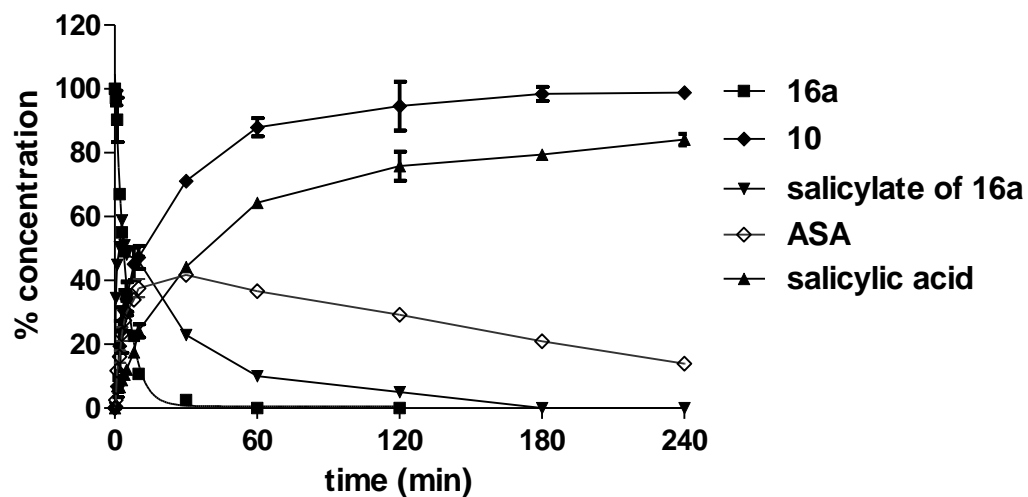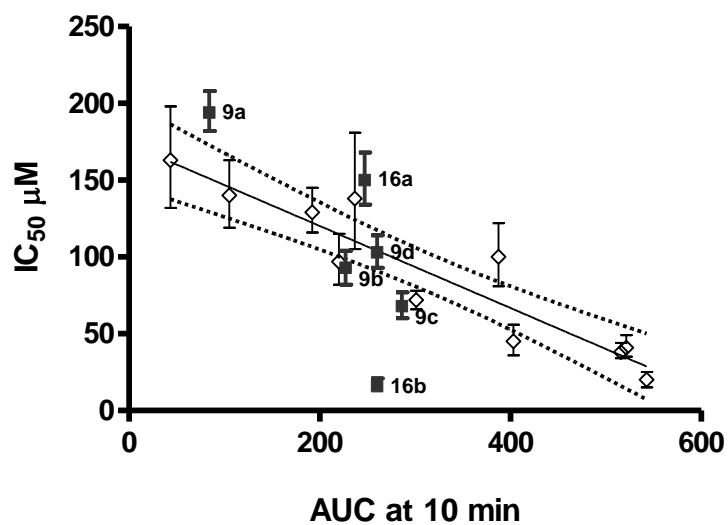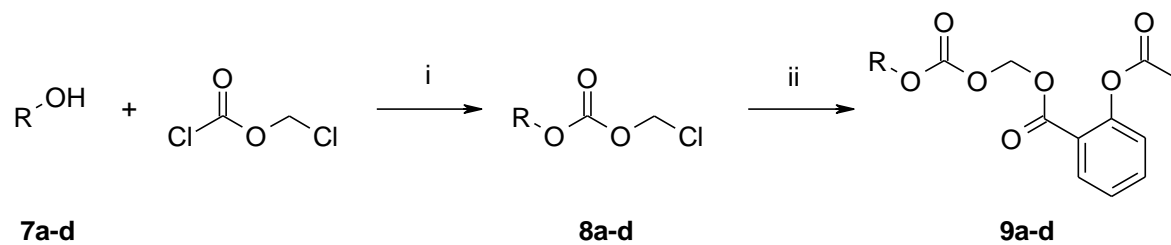
Figure 2. Antiaggregatory IC_{50} values versus AUC values of aspirin released in human serum from the compounds **9a-d**, **16a-b** on the linear correlation between antiaggregatory IC_{50} values and AUC values of aspirin released in human serum obtained in a previous work (ref. 16) from a series of (nitrooxyacyloxy)methyl esters of ASA.
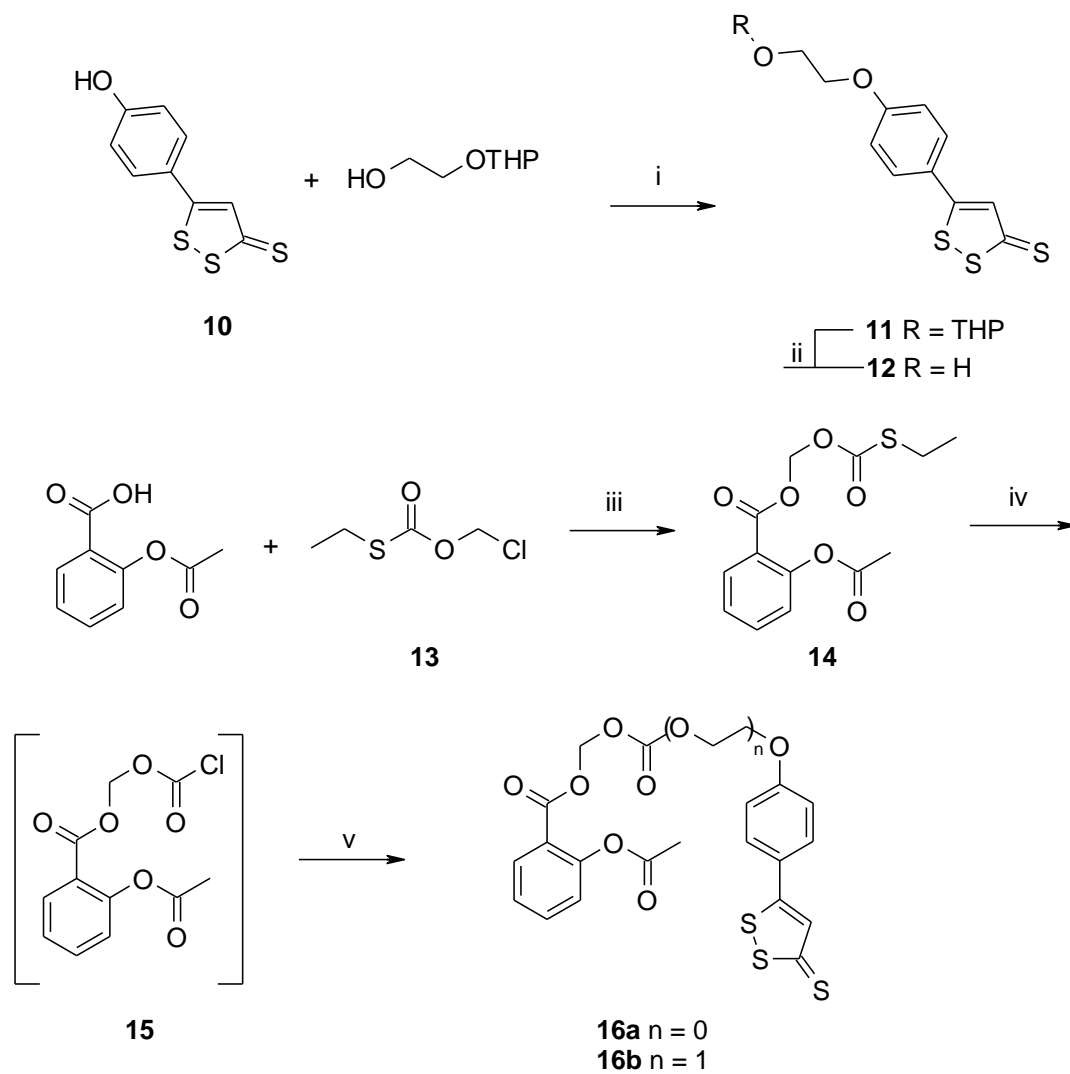
Scheme 1.[a]

7a-d   **8a-d**   **9a-d**

    a $R = (CH_2)_3ONO_2$;

    b $R = CH_2CH(ONO_2)CH_2ONO_2$;

    c $R = 4\text{-}C_6H_4(CH_2)_3ONO_2$;

    d $R = 4\text{-}C_6H_4CH_2CH(ONO_2)CH_2ONO_2$;

[a] Conditions: i) C_5H_5N, CH_2Cl_2, 0 °C; ii) ASA, Cs_2CO_3, DMF, rt.

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Scheme 2.[a]

10

11 R = THP
12 R = H

13

14

15

16a n = 0
16b n = 1

[a] Conditions: i) PPh₃, DIAD, THF dry, 0 °C to rt; ii) PPTS, MeOH, 55 °C; iii) Cs₂CO₃, DMF, rt; iv) SO₂Cl₂; v) **10** or **12**, 4-methylmorpholine, CH₂Cl₂, -15 °C.
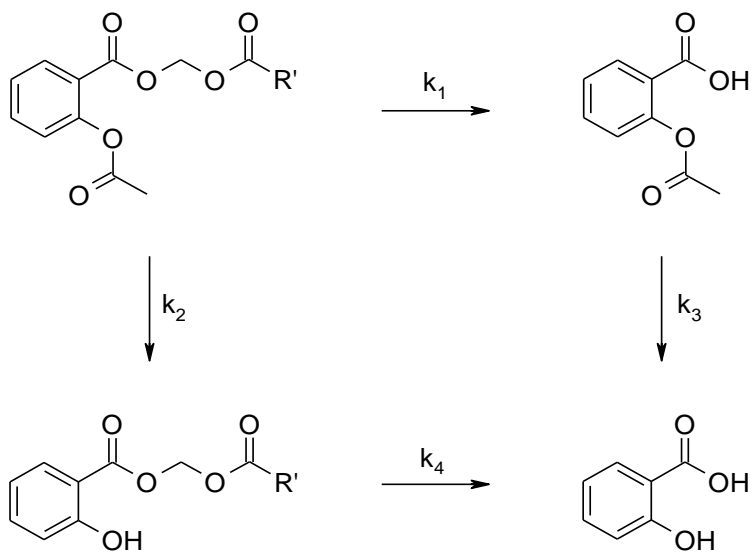
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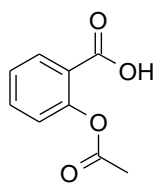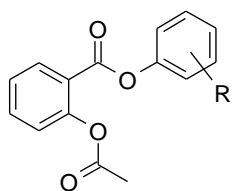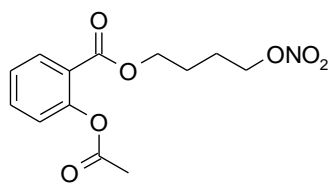**Scheme 3.** Possible hydrolytic routes of compounds.

Chart 1. Examples of NO-donors and H$_2$S-donor aspirin.

1, ASA

2, NCX4040 R = p-CH$_2$ONO$_2$
3, NCX4016 R = m-CH$_2$ONO$_2$
4, NCX4060 R = o-CH$_2$ONO$_2$

5, NCX4050
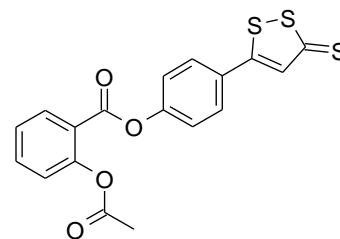
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Table 1. Stability of the compounds **9a-d**, **16a**, **16b** in buffered solutions (percentage of unchanged compound after 3 hours) and in human serum (half-life, percent of maximal amounts of aspirin released and AUC values at 10 min); antiaggregatory activities of the compounds **9a-d**, **16a**, **16b**.

Compound	Stability in buffered solutions % of unchanged at 3 h [a]		Stability in human serum			Platelet Aggregation IC$_{50}$ (µM) (CL 95%)
	pH 1.0	pH 7.4	$t_{1/2}$ (min) [b]	% max of aspirin released [b]	AUC at 10min	
9a	98	98	<1	9	84	194 (182-208)
9b	98	85	<1	25	227	93 (82-104)
9c	98	98	1.6	40	286	68 (60-77)
9d	98	95	2.2	34	260	103 (93-114)
16a	96	80	2.1	36	247	150 (134-168)
16b	98	96	3.0	42	260	16 (13-21)

[a] SEM ≤ 1%. [b] SEM ≤ 0.1. [c] SEM ≤ 1.5%.

Table 2. Vasodilator activities of the compounds **7a-d**, **10** and **12**.

Compound	Vasodilator activity (EC$_{50}$ µM ± SEM)	
		+ inhibitor
7a	4.0 ± 0.6 [a]	> 100 µM [a,b]
7b	1.7 ± 0.3 [a]	> 100 µM [a,b]
7c	1.0 ± 0.2 [a]	> 100 µM [a,b]
7d	0.13 ± 0.03 [a]	> 100 µM [a,b]
10	5.9 ± 0.6 [c]	17 ± 1 [c,d]
12	8.0 ± 0.5 [c]	16 ± 2 [c,d]

[a] Aorta strips precontracted with 1 µM phenylephrine. [b] Inhibitor: 1 µM ODQ.

[c] Aorta strips precontracted with 25 mM KCl. [d] Inhibitor: 10 µM glibenclamide.

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NEW NITRIC OXIDE OR HYDROGEN SULFIDE RELEASING ASPIRINS (((R-OXY)CARBONYL)OXY)METHYL ESTERS OF ASPIRIN BEARING NITRIC OXIDE- OR HYDROGEN SULPHIDE RELEASING GROUPS

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