Journal of Medicinal Chemistry

ARTICLE

pubs.acs.org/jmc

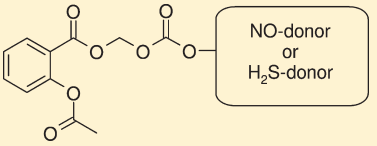
New Nitric Oxide or Hydrogen Sulfide Releasing Aspirins

Loretta Lazzarato, Konstantin Chegaev, Elisabetta Marini, Barbara Rolando, Emily Borretto, Stefano Guglielmo, Sony Joseph, Antonella Di Stilo, Roberta Fruttero, and Alberto Gasco*

Dipartimento di Scienza e Tecnologia del Farmaco, Università degli Studi di Torino, Via Pietro Giuria 9, 10125 Torino, Italy

Supporting Information

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, was synthesized, and the compounds were evaluated as new ASA co-drugs. All the products were quite stable in buffered solution at pH 1 and 7.4. Conversely, they were all rapidly metabolized, producing ASA and the NO/H_2S releasing moiety used for their preparation. Consequent on 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 NO- and H_2S-dependent mechanism, respectively, but they either did not trigger antiaggregatory activity or displayed antiplatelet potency markedly below that of the related co-drug. The new products might provide a safer and improved alternative to the use of ASA principally in its anti-inflammatory and antithrombotic applications.



INTRODUCTION

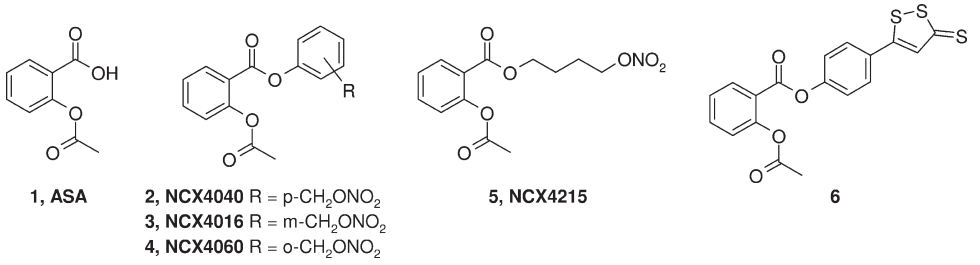
Although it was introduced onto the market over 100 years ago, the adverse gastrointestinal effects of aspirin (ASA, acetylsalicylic acid (**1**), Chart 1), which is the most widely used nonsteroidal anti-inflammatory drug (NSAID), are still a significant drawback to its use.[1−3] A number of strategies have been proposed to overcome this problem, including the "gaseous solution".[2,4,5] This approach consists of conjugating a nitric oxide (NO) or a hydrogen sulfide (H_2S) releasing moiety with ASA via an ester link. NO is an important gaseous messenger that mediates a variety of physiological actions, including gastroprotection.[6] NO defends the gastric mucosa against adverse effects consequent on NSAID-induced COX-1 inhibition, including decreased mucosal blood flow, reduced mucus and bicarbonate secretion, promotion of neutrophil adherence and activation, and modulation of inflammatory mediators.[7−9] In addition it is a key determinant of vascular health exerting antiplatelet, antithrombotic, and vasodilating effects.[10] In recent years, H_2S has also been recognized as an important gaseous signaling molecule. Like NO, it possesses a "dual personality", since at endogenous concentrations it displays a variety of beneficial effects but is detrimental at superphysiological concentrations. It shares many biological activities with NO, including reduction of neutrophil adhesion, modulation of inflammatory mediator release, protection against gastric injury, and beneficial effects on cardiovascular system including vasodilator activity.[4,11−14] 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.[15,16] The drawback of 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 negative charge on the ASA moiety, which induces high enzymatic lability of the acetyloxy group.[17] A number of similar products have been developed, but to our knowledge, few well-documented examples of "true" ASA NO-donor co-drugs are known,[18,19] and no true ASA H_2S-donor co-drug has yet been described. This paper reports the synthesis of a new class of aryloxy and alkyloxy carbonyloxymethyl esters of ASA, bearing either nitrooxy NO-donor moieties (**9a**−**d**, Scheme 1) or the H_2S-donor residue (3-thioxo-3*H*-1,2-dithiol-5-yl) (**16a**,**b**, Scheme 2). All these products are shown to be 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 also discussed.

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**,**b** (Scheme 2) required the use of the known H_2S-donor 5-(4-hydroxyphenyl)-3*H*-1,2-dithiole-3-thione (**10**)[14] 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 azodicarboxylate (DIAD)). The THP group was removed under the

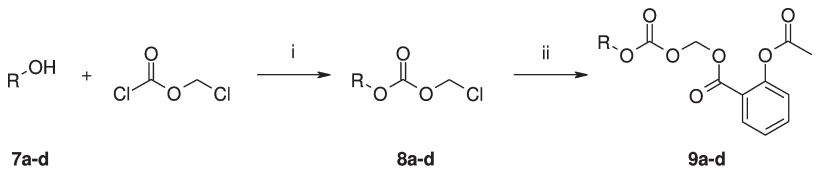
Received: April 26, 2011
Published: June 21, 2011

Chart 1. Examples of NO-Donors and H_2S-Donor Aspirin



1, ASA | **2, NCX4040** R = p-CH_2ONO_2; **3, NCX4016** R = m-CH_2ONO_2; **4, NCX4060** R = o-CH_2ONO_2 | **5, NCX4215** | **6**

Scheme 1[a]

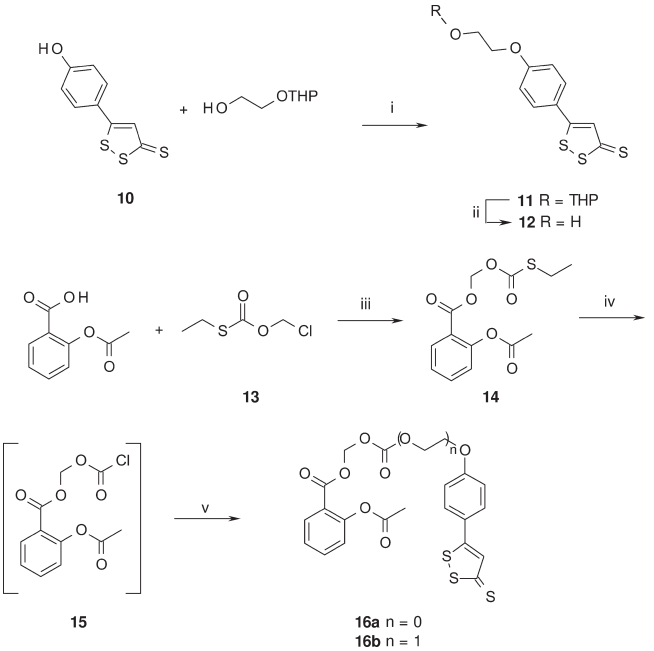


7a-d **8a-d** **9a-d**

a R = $(CH_2)_3ONO_2$;
b R = $CH_2CH(ONO_2)CH_2ONO_2$;
c R = 4-$C_6H_4(CH_2)_3ONO_2$;
d R = 4-$C_6H_4CH_2CH(ONO_2)CH_2ONO_2$;

[a] Conditions: (i) Py, CH_2Cl_2, −15 °C; (ii) ASA, Cs_2CO_3, DMF, room temp.

Scheme 2[a]

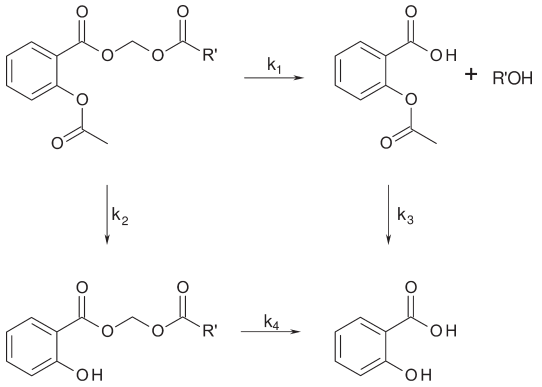


10 **11** R = THP, **12** R = H **13** **14** **15** **16a** n = 0, **16b** n = 1

[a] Conditions: (i) PPh_3, DIAD, THF dry, −15 °C to room temp; (ii) PPTS, MeOH, 55 °C; (iii) Cs_2CO_3, DMF, room temp; (iv) SO_2Cl_2; (v) **10** or **12**, 4-methylmorpholine, CH_2Cl_2, −15 °C.

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

Scheme 3. Possible Hydrolytic Routes of Compounds



(**13**) in the presence of Cs_2CO_3 in DMF. The resulting product **14** was treated with neat SO_2Cl_2 to give **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** and **16b**.

Hydrolysis Studies. The possible hydrolytic routes of the new carbonates are reported in Scheme 3. In order to be true ASA NO/H_2S donor co-drugs, the products must have a 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 remained more than 96% unchanged after 3 h of incubation in acid solution. The same occurred at physiological pH, with the exception of **9b** and **16a**, which were transformed more extensively (85% and 80% unchanged, respectively). This was not the case in human serum, in which it is known that a variety of esters are hydrolyzed by

dx.doi.org/10.1021/jm2004514 |*J. Med. Chem.* 2011, 54, 5478–5484

Table 1. Stability of the Compounds 9a−d, 16a, 16b in Buffered Solutions (Percentage of Unchanged Compound after 3 h) and in Human Serum (Half-Life, Percent of Maximal Amounts of Aspirin Released, and AUC Values of Aspirin Released over the First 10 min of Incubation Time) and Antiaggregatory Activities of Compounds 9a−d, 16a, 16b

	stability in buffered solutions, % of unchanged at 3 h[a]		stability in human serum			
compd	pH 1.0	pH 7.4	$t_{1/2}$ (min)[b]	% max of ASA released[c]	ASA $AUC_{0-10min}$ (μM min)	platelet aggregation IC_{50} (95% CL) (μM)
ASA	90	90	63			54 (49−60)
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%.

carboxylesterases:[20] both the nitrogen and the sulfur containing compounds were very rapidly hydrolyzed, 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 to one-phase exponential decay equation (GraphPad Prism software, version 5).

As shown in Scheme 3, ASA, *R*-oxycarbonyloxymethyl esters of salicylic acid (salicylates), related hydroxy derivatives, and salicylic acid were detected during hydrolysis in human serum. For all the compounds, the final metabolites were salicylic acid and hydroxyl derivatives. The time-course of the degradation products, monitored over 10 min and 4 h of incubation time, in the case of compound **16a**, is reported in Figure 1 as an example. The peak amounts of ASA detected for each compound, expressed as % of the initial carbonate concentration, are given in Table 1, together with the areas under the ASA concentration−time curves, measured for each compound over the first 10 min of incubation (AUC_{0-10}). Among the nitrooxy-containing compounds, the aromatic carbonates **9c** and **9d** released ASA more extensively than the aliphatic compounds (**9a** and **9b**). Both the sulfur-containing esters **16a** and **16b** were quite good ASA producers. In conclusion, all the products act 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), taking aspirin as reference standard. The inhibitory activity was assessed by adding 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, under the same conditions used to test the corresponding co-drugs. Alcohols **7c** and **12** acted slightly differently, showing 163 and 184 μM IC_{50} values, respectively, potencies markedly below those of the related co-drugs **9c** and **16b**. Consequently, the antiaggregatory activities of all the products may reasonably be principally attributed to their capacity to release ASA.

In a previous study, we showed that the areas under the ASA release-time curves (AUC_{0-10}) in human serum, measured for a series of (nitrooxyacyloxy)methyl esters of ASA, correlate linearly with the corresponding antiaggregatory potencies.[18]

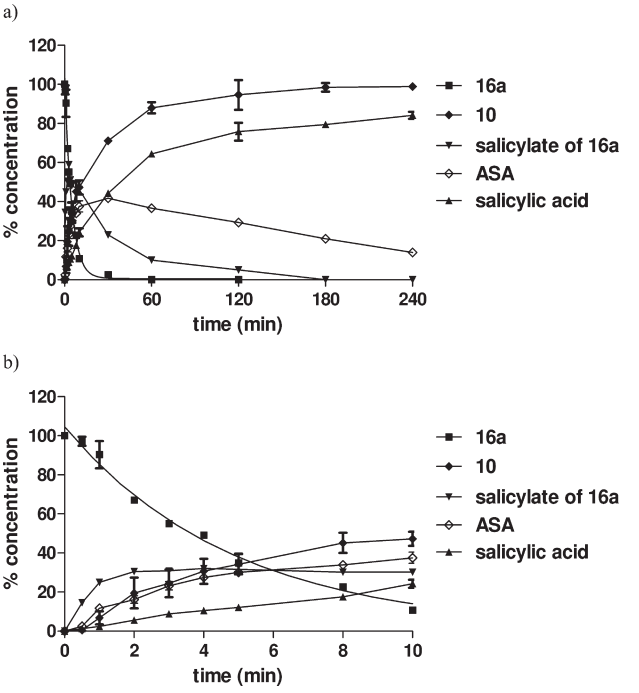


Figure 1. Time-course of the degradation products of compound **16a** at 4 h (a) and at 10 min (b) incubation time in human serum. Values are the mean ± SEM (SEM ≤ 3; number of determinations is 3).

The antiaggregatory IC_{50} values versus AUC values for the carbonates object of the present research fit this line quite well; the only exception is **16b** (Figure 2). Synergism between ASA and alcohol **12** could be responsible for this difference: when the antiaggregatory potency was evaluated using 1:1 mixtures of the products, at a concentration at which both compounds separately are inactive (10 μM, mol/mol), a significant inhibition of platelet aggregation (~66%) was observed.

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 caused relaxation of the contracted tissue in a concentration-dependent manner. Their potencies, expressed as EC_{50}, are in Table 2. As expected, both among the

dx.doi.org/10.1021/jm2004514 |*J. Med. Chem.* 2011, 54, 5478–5484

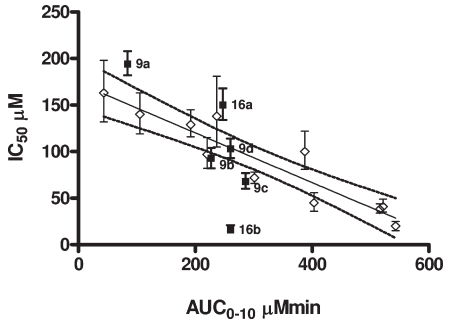


Figure 2. Antiaggregatory IC_{50} values versus AUC_{0-10} values of aspirin released in human serum from compounds **9a**−**d**, **16a**, **16b** on the linear correlation between antiaggregatory IC_{50} values and AUC_{0-10} values of aspirin released in human serum obtained in a previous study (ref 16) from a series of (nitrooxyacyloxy)methyl esters of ASA.

Table 2. Vasodilator Activities of Compounds 7a−d, 10, and 12

	vasodilator activity (EC_{50} ± SEM) (μM)	
compd		with 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.

aliphatic and among the aromatic carbonates, the dinitrooxy-substituted products are more potent than their 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's being the mechanism underlying 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 those 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 shifted rightward. This is consistent 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 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 activity or display 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 role exerted by NO and H_2S in the gastrointestinal tract and in the cardiovascular system, as well as in the regulation of the inflammatory processes, this new class of ASA co-drugs might represent a safer and improved alternative to ASA in the antithrombotic and anti-inflammatory therapies.

EXPERIMENTAL SECTION

Chemistry. ^1H and ^{13}C NMR spectra were recorded on a Bruker Avance 300 at 300 and 75 MHz, respectively, using $SiMe_4$ as internal standard. Low resolution mass spectra were recorded with a Finnigan-Mat TSQ-700. Melting points were determined with a capillary apparatus (Büchi 540). Flash column chromatography was performed on silica gel (Merck Kieselgel 60, 230−400 mesh ASTM); PE stands for 40−60 petroleum ether. The progress of the reactions was followed by thin layer chromatography (TLC) on 5 cm × 20 cm plates with a layer thickness of 0.2 mm. Anhydrous magnesium sulfate was used as drying agent for the organic phases. Organic solvents were removed under vacuum at 30 °C. Elemental analyses (C, H, N) of the target compounds were performed by Section de Pharmacie, Service de Microanalyse (Geneva), and the results are within 0.4% of the theoretical values. Target compounds were prepared, as assessed using the aforementioned standard spectroscopic techniques and elemental analyses, in ≥95% purity. Compounds **7a**,[21] **7b**,[22] **7c**,[23] **7d**,[24] **10**,[25] and **13**[26] were obtained as described elsewhere.

General Procedure for the Preparation of 8a−d. To a solution of the appropriate alcohol or phenol (2.5 mmol) and chloromethyl chloroformate (0.25 mL, 2.7 mmol) in dry CH_2Cl_2 (15 mL), stirred at −15 °C, a solution of Py (0.22 mL, 2.7 mmol) in dry CH_2Cl_2 (10 mL) was added dropwise. At the end of the addition, the ice−salt bath was removed and the reaction mixture allowed to reach room temperature. After 15 min, the solvent was removed and the resulting oil was purified by flash chromatography. Chromatographic eluents and yields of the products were as follows.

Chloromethyl-3-nitrooxypropyl Carbonate (8a). Eluent (PE/CH_2Cl_2 7/3 v/v); colorless oil; yield 83%. ^1H NMR ($CDCl_3$) δ 2.15 (qi, 2H, $-CH_2CH_2ONO_2$), 4.35 (t, 2H, $-OCH_2CH_2-$), 4.58 (t, 2H, $-CH_2ONO_2$), 5.74 (s, 2H, $-CH_2Cl$). ^{13}C NMR ($CDCl_3$) δ 26.3, 64.9, 69.1, 72.3, 153.2.

Chloromethyl-2,3-bis(nitrooxy)propyl Carbonate (8b). Eluent (PE/CH_2Cl_2 1/1 v/v); colorless oil; yield 80%. ^1H NMR ($CDCl_3$) δ 4.44 (dd, 1H, $-CHHO-$), 4.56−4.70 (m, 2H, $-CHHONO_2$ + $-CHHO-$), 4.81 (dd, 1H, $-CHHONO_2$), 5.48−5.54 (m, 1H, $-CHONO_2$), 5.72−5.77 (m, 2H, $-CH_2Cl$). ^{13}C NMR ($CDCl_3$) δ 64.6, 68.2, 72.6, 75.6, 152.9.

Chloromethyl-4-(3-nitrooxypropyl)phenyl Carbonate (8c). Eluent (PE/CH_2Cl_2 8/2 v/v); colorless oil, which solidified on standing in the freezer; yield 75%. ^1H NMR ($CDCl_3$) δ 2.05 (m, 2H, $-CH_2CH_2ONO_2$), 2.75 (t, 2H, $-CH_2CH_2CH_2ONO_2$), 4.45 (t, 2H, $-CH_2ONO_2$), 5.82 (s, 2H, $-CH_2Cl$), 7.13−7.26 (m, 4H, C_6H_4). ^{13}C NMR ($CDCl_3$) δ 28.3, 31.1, 72.1, 72.5, 120.9, 129.5, 138.6, 149.2, 152.1.

4-(2,3-Bis(nitrooxy)propyl)phenylchloromethyl Carbonate (8d). Eluent (PE/CH_2Cl_2 6/4 v/v); yellowish oil; yield 50%. ^1H NMR ($CDCl_3$) δ 2.99−3.14 (m, 2H, $-CH_2CH-$), 4.44 (dd, 1H, $-CHHONO_2$), 4.73 (dd, 1H, $-CHHONO_2$), 5.40−5.47 (m, 1H, $-CHONO_2$), 5.82 (s, 2H, $-CH_2Cl$), 7.20−7.30 (m, 4H, C_6H_4). ^{13}C NMR ($CDCl_3$) δ 34.9, 70.0, 72.6, 79.2, 121.5, 130.5, 132.6, 150.2, 151.9.

5-(4-(2-Hydroxyethoxy)phenyl)-3*H*-1,2-dithiole-3-thione (12). To a solution of Ph_3P (0.28 g, 1.1 mmol) in dry THF (10 mL), stirred under positive nitrogen pressure at −15 °C, DIAD (0.22 mL, 1.1 mmol) was added. The reaction mixture was stirred for 15 min until a white precipitate formed, and **10** (0.20 g, 0.90 mmol) was added,

dx.doi.org/10.1021/jm2004514 |*J. Med. Chem.* 2011, 54, 5478–5484

followed by 2-(tetrahydropyran-2-yloxy)ethanol (0.13 g, 0.90 mmol). The resulting mixture was stirred for 24 h at room temperature, then poured into H_2O (10 mL) and extracted with Et_2O (3 × 10 mL). The combined organic layers were washed with brine, dried, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/acetone 9/1 v/v) to give 5-(4-(2-(tetrahydropyran-2-yloxy)ethoxy)phenyl)-3*H*-1,2-dithiole-3-thione (**11**) as a reddish-brown oil; yield 70%.^{1}H NMR ($CDCl_3$) δ 1.52−1.89 (m, 6H, 3CH_2 pyran), 3.51−3.58 (m, 1H), 3.81−4.25 (m, 5H), (CH_2Opyran, −OCH_2CH_2O−), 4.70−7.72 (m, 1H, −OC*H*O−), 7.02 (d, 2H, C_6H_4), 7.40 (s, 1H, C_3S_3H), 7.62 (d, 2H, C_6H_4). ^{13}C NMR ($CDCl_3$) δ 19.4, 25.4, 30.5, 62.3, 65.6, 67.8, 99.1, 115.5, 124.2, 128.6, 134.6, 162.3, 173.1, 215.1. MS (CI) m/z 355 $(M + 1)^+$.

11 (0.44 g, 1.20 mmol) was dissolved in MeOH (15 mL), and a catalytic amount of PPTS was added. The resulting mixture was heated at 55 °C for 2 h, then concentrated under reduced pressure. The crude product was purified by flash chromatography (PE/acetone 6/4 v/v) to give a reddish solid, which was recrystallized from EtOH to give the title compound as a yellowish-orange solid. Yield 40%; mp 117.5 °C (from EtOH). ^{1}H NMR ($CDCl_3$) δ 2.12 (sbr, 1H, O*H*), 4.02 (t, 2H), 4.16 (t, 2H)(−OCH_2CH_2OH), 7.00 (d, 2H, C_6H_4), 7.39 (s, 1H, C_3S_3H), 7.61 (d, 2H, C_6H_4). ^{13}C NMR ($CDCl_3$) δ 61.2, 69.6, 115.5, 124.5, 128.6, 134.7, 162.0, 172.9, 215.1. MS (CI) m/z 271 $(M + 1)^+$.

General Procedure for the Preparation of 9a−d and 14. To a solution of acetylsalicylic acid (0.22 g, 1.2 mmol) in DMF (5 mL) was added Cs_2CO_3 (0.20 g, 0.60 mmol), and the resulting mixture was vigorously stirred for 15 min. The appropriate amount of chloromethyl carbonate (1.0 mmol) was then added, and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with Et_2O (25 mL) and washed with H_2O, saturated solution of $NaHCO_3$, and brine. The organic layer was dried, filtered, and concentrated under reduced pressure. The crude product thus obtained was purified by flash chromatography. Chromatographic eluents and yields of the products are listed below.

((3-Nitrooxypropyl)carbonyl)oxymethyl 2-(Acetyloxy)-benzoate (9a). Eluent (PE/EtOAc 9/1 v/v); colorless oil; yield 75%. ^{1}H NMR ($CDCl_3$) δ: 2.12 (qi, 2H, −$CH_2CH_2ONO_2$), 2.36 (s, 3H, −CH_3), 4.31 (t, 2H, −OCH_2CH_2−), 4.55 (t, 2H, −CH_2ONO_2), 5.95 (s, 2H, −OCH_2O−), 7.12 (d, 1H), 7.34 (t, 1H), 7.61 (t, 1H), 8.08 (d, 1H) (C_6H_4). ^{13}C NMR ($CDCl_3$) δ: 21.0, 26.3, 64.4, 69.2, 82.2, 121.7, 124.1, 126.2, 132.5, 134.9, 151.2, 153.8, 162.7, 168.2. MS (CI) m/z 358 $(M + 1)^+$. Anal. ($C_{14}H_{15}NO_{10}$) C, H, N.

((2,3-Bis(nitrooxy)propyl)carbonyl)oxymethyl 2-(Acetyloxy)-benzoate (9b). Eluent (PE/EtOAc 8/2 v/v); colorless oil; yield 29%. ^{1}H NMR ($CDCl_3$) δ: 2.36 (s, 3H, −CH_3), 4.39 (dd, 1H, −CH*H*ONO_2), 4.54 (dd, 1H, −CH*H*ONO_2), 4.64 (dd, 1H, −CH*H*O−), 4.80 (dd, 1H, −CH*H*O−), 5.45−5.51 (m, 1H, −*CH*ONO_2), 5.97 (s, 2H, −OCH_2O), 7.13 (d, 1H), 7.35 (t, 1H), 7.62 (t, 1H), 8.08 (d, 1H) (C_6H_4). ^{13}C NMR ($CDCl_3$) δ: 20.9, 64.3, 68.2, 75.7, 82.5, 121.6, 124.1, 126.3, 132.3, 135.0, 151.2, 153.5, 162.6, 169.7. MS (CI) m/z 419 $(M + 1)^+$. Anal. ($C_{14}H_{14}N_2O_{13}$) C, H, N.

(4-(3-Nitrooxypropyl)phenoxycarbonyl)oxymethyl 2-(Acetyloxy)benzoate (9c). Eluent (PE/EtOAc 8/2 v/v); white solid; mp 52.5−53 °C (*i*-Pr_2O); yield 64%. ^{1}H NMR ($CDCl_3$) δ: 2.04 (qi, 2H, −$CH_2CH_2ONO_2$), 2.37 (s, 3H, −CH_3), 2.74 (t, 2H, −$CH_2CH_2CH_2ONO_2$), 4.45 (t, 2H, −CH_2ONO_2), 6.05 (s, 2H, −OCH_2O−), 7.13−7.22 (m, 5H), 7.37 (t, 1H), 7.65 (t, 1H), 8.11 (d, 1H) ($2C_6H_4$). ^{13}C NMR ($CDCl_3$) δ: 21.0, 28.3, 31.1, 72.1, 82.5, 121.0, 121.7, 124.1, 126.2, 129.5, 132.3, 135.0, 138.4, 149.3, 151.2, 152.7, 162.7, 169.7. MS (CI) m/z 434 $(M + 1)^+$. Anal. ($C_{20}H_{19}NO_{10}$) C, H, N.

(4-(2,3-Bis(nitrooxy)propyl)phenoxycarbonyl)oxymethyl 2-(Acetyloxy)benzoate (9d). Eluent (PE/EtOAc 8/2 v/v); colorless oil; yield 51%.^{1}H NMR ($CDCl_3$) δ 2.36 (s, 3H, −CH_3), 2.97−3.13 (m, 2H, −CH_2CH−), 4.43 (dd, 1H, −CH*H*ONO_2), 4.73 (dd, 1H, −CH*H*ONO_2), 5.38−5.46 (m, 1H, −*CH*ONO_2), 6.05 (s, 2H, −OCH_2O−), 7.13−7.38 (m, 6H), 7.61 (t, 1H), 8.11 (d, 1H) ($2C_6H_4$). ^{13}C NMR ($CDCl_3$) δ 21.0, 34.9, 70.0, 79.2, 82.5, 121.7, 124.1, 126.2, 130.5, 132.3, 135.0, 150.3, 151.3, 152.5, 162.7, 169.7. MS (CI) m/z 495 $(M + 1)^+$. Anal. ($C_{20}H_{18}N_2O_{13}$) C, H, N.

(Ethylthiocarbonyl)oxymethyl 2-(Acetyloxy)benzoate (14). Eluent (PE/EtOAc 8/2 v/v); colorless oil; yield 87%. ^{1}H NMR ($CDCl_3$) δ: 1.33 (t, 3H, −CH_2CH_3), 2.36 (s, 3H, −CH_3), 2.90 (q, 2H, −CH_2CH_3), 6.00 (s, 2H, −OCH_2O−), 7.13 (d, 1H), 7.35 (t, 1H), 7.60 (t, 1H), 8.07 (d, 1H) (C_6H_4). ^{13}C NMR ($CDCl_3$) δ: 14.8, 21.0, 25.5, 80.5, 121.9, 124.1, 126.1, 132.3, 134.8, 151.2, 162.7, 169.6, 170.8. MS (EI) m/z 298 $(M)^+$.

General Procedure for the Preparation of 16a and 16b. SO_2Cl_2 (0.88 mL, 10.9 mmol) was added dropwise to **14** (3.24 g, 10.9 mmol), and the mixture was stirred at 0 °C. The mixture was allowed to reach room temperature and stirred for 1 h. The reaction mixture was then concentrated under reduced pressure to give **15** as a colorless oil that was used in the next synthetic step without further purification.

To a solution of **15** (0.85 g, 3.1 mmol) in CH_2Cl_2 (30 mL), stirred at −15 °C, a solution of the appropriate hydroxy derivative (2.6 mmol) and *N*-methylmorpholine (0.31 mL, 2.6 mmol) in CH_2Cl_2 (30 mL) was added dropwise. The reaction mixture was stirred for 1 h at −15 °C, then for 24 h at room temperature, then poured into H_2O (20 mL) and extracted with CH_2Cl_2 (3 × 20 mL). The combined organic layers were washed with brine, dried, filtered, and concentrated under reduced pressure. The crude product was purified by flash chromatography. Chromatographic eluents and yields of the products were as follows.

((4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)carbonyloxy)-methyl 2-(Acetyloxy)benzoate (16a). Eluent (PE/EtOAc 8/2 v/v); reddish-brown solid, which was recrystallized from EtOH to give the title compound as a yellowish-orange solid; yield 30%; mp 103.5−104 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.37 (s, 3H, −CH_3), 6.07 (s, 2H, −OCH_2O−), 7.15 (d, 1H, C_6H_4), 7.33 − 7.40 (m, 4H, $2C_6H_4$ + C_3HS_3), 7.61−7.71 (m, 3H), 8.13 (d, 1H) ($2C_6H_4$). ^{13}C NMR ($CDCl_3$) δ 21.0, 82.6, 121.5, 122.2, 124.1, 126.2, 128.4, 129.8, 132.3, 135.0, 136.2, 151.3, 151.8, 153.3, 162.6, 169.8, 171.4, 215.5. MS (CI) m/z 463 $(M + 1)^+$. Anal. ($C_{20}H_{14}O_7S_3$) C, H, N.

((2-(4-(3-Thioxo-3*H*-1,2-dithiol-5-yl)phenoxy)ethoxy)carbonyloxy)methyl 2-(Acetyloxy)benzoate (16b). Eluent (PE/EtOAc 75/25 v/v); reddish-brown solid, which was recrystallized from EtOH to give the title compound as a yellowish-orange solid; yield 20%; mp 105 °C (EtOH). ^{1}H NMR ($CDCl_3$) δ 2.36 (s, 3H, −CH_3), 4.28 (t, 2H), 4.58 (t, 2H) (−OCH_2CH_2O−), 5.98 (s, 2H, −OCH_2O−), 6.96 (d, 2H), 7.13 (d, 1H), 7.33 (t, 1H) ($2C_6H_4$), 7.37 (s, 1H, C_3HS_3), 7.56−7.65 (m, 3H), 8.8 (d, 1H) ($2C_6H_4$). ^{13}C NMR ($CDCl_3$) δ 21.0, 65.7, 66.4, 82.3, 115.6, 121.7, 124.1, 124.7, 126.2, 128.6, 132.2, 134.8, 134.9, 151.2, 153.9, 161.5, 162.6, 169.7, 172.7, 215.2. MS (CI) m/z 507 $(M + 1)^+$. Anal. ($C_{22}H_{18}O_8S_3$) C, H, N.

Evaluation of Stability in Buffered Solutions and in Human Serum. *Hydrolysis in Acidic Medium (pH 1.0) and in Phosphate Buffer (pH 7.4).* A 2 mL aliquot of 0.5 mM solution of each compound in DMSO was diluted to 10 mL using 0.1 M HCl to obtain pH 1.0 or 50 mM phosphate buffer to obtain pH 7.4. The resulting solution was maintained at 37 ± 0.5 °C, and at appropriate time intervals, an amount of 20 μL of the solution was analyzed by RP-HPLC. All experiments were performed in triplicate.

Hydrolysis in Human Serum. A solution of each compound (10 mM) in DMSO was added to human serum (sterile-filtered from human male AB plasma, Sigma-Aldrich) preheated at 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, an amount of 300 μL of the reaction mixture was 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 by 0.45 μm PTFE filters (Alltech) and analyzed by RP-HPLC. All experiments were performed at least in triplicate.

The reverse-phase HPLC procedure afforded separation and quantitation of the remaining compounds and of the products of hydrolysis (aspirin, salicylic acid, salicylate, and hydroxy derivatives bearing NO-donor or H_2S-donor moieties). HPLC analyses were performed with an HP 1100 chromatographic system (Agilent Technologies, Palo Alto, CA, U.S.) 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 analysis was done using an HP ChemStation system (Agilent Technologies). The injection volume was 20 μL (Rheodyne, Cotati, CA). The analytical column was a Nucleosil 100-5C18 Nautilus (250 mm × 4.6 mm, 5 μm particle size) (Macherey-Nagel) column, eluting with a flow rate of 1.2 mL/min. The samples were analyzed using a gradient method employing a mobile phase consisting of acetonitrile/water with 0.1% trifluoroacetic acid 55/45 over the first 4 min, grading to 70/30 at 6 min, maintaining 70/30 until 15 min, then returning to 55/45 at 20 min. The column effluent was monitored at 226 nm (for compounds, aspirin and NO-donor hydroxy derivatives), at 360 nm (for H_2S-donor hydroxy derivatives), and at 240 nm (for salicylic acid and salicylates) referenced against a 600 nm wavelength. Quantitation was done using calibration curves of compounds and relative metabolites chromatographed under the same conditions. The linearity of the calibration curves was determined in a concentration range of 1−200 μM ($r^2 > 0.99$).

Inhibition of Platelet Aggregation in Vitro. Venous blood samples were obtained from healthy volunteers who had not taken any drug for at least 2 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 210*g* for 20 min. Aliquots (500 μL) of PRP were added into aggregometer (Chrono-log 4902D) cuvettes, and aggregation was recorded as increased light transmission under continuous stirring (1000 rpm) at 37 °C for 10 min after addition of the stimulus. Collagen at submaximal concentration (0.8−1.5 μg/mL) was used as a platelet activator in PRP. Compounds under study were preincubated with PRP 10 min before addition of the stimulus (collagen). Vehicle alone (0.5% DMSO) added to PRP did not affect platelet function in control samples. At least five experiments for each compound were performed.

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

Vasodilator Activities. Thoracic aortas were isolated from male Wistar rats weighing 180−200 g. As few animals as possible were used. The goals and protocols of the studies were approved by the Ministry for Health, Rome, Italy. The endothelium was removed. The vessels were cut helically and four to six strips were obtained from each aorta. The tissues were mounted under 1.0 g of tension in organ baths containing 30 mL of Krebs bicarbonate buffer with the following composition (mM): NaCl 111.2, KCl 5.0, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.0, $NaHCO_3$ 12.0, glucose 11.1. The samples were maintained at 37 °C and gassed with 95% O_2−5% CO_2 (pH 7.4). The aortic strips were allowed to equilibrate for 1.5 h and then contracted with 1 μM L-phenylephrine or 25 mM KCl. When the response to the agonist reached a plateau, cumulative concentrations of the vasodilating agent were added. Results are expressed as $EC_{50} \pm$ SEM (μM). The effects of 1 μM ODQ or 10 μM glibenclamide on relaxation were evaluated in a separate series of experiments, in which the inhibitors were added 5 min before the contraction. In this protocol, the inhibitor is preincubated for at least 30 min before addition of the vasodilator compound. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. Addition of the drug vehicle, DMSO, had no appreciable effect on contraction degree. At least five experiments for each compound were performed.

■ ASSOCIATED CONTENT

Supporting Information. Elemental analysis data for the target compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +39 011 6707670. Fax: +39 011 6707286. E-mail: alberto.gasco@unito.it.

■ ABBREVIATIONS USED

NSAIDs, nonsteroidal anti-inflammatory drugs; PRP, platelet rich plasma; NO, nitric oxide; sGC, soluble guanylate cyclase; COX, cyclooxigenase; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one; ASA, acetylsalicylic acid; THP, tetrahydropyranyl; DIAD, diisopropyl azodicarboxylate; PPTS, pyridinium *p*-toluensulfonate

■ REFERENCES

(1) Vane, J. R.; Flower, R. J.; Botting, R. M. History of aspirin and its mechanism of action. *Stroke* **1990**, *21*, 12–23.

(2) Wolfe, M. M.; Lichtenstein, D. R.; Singh, G. Gastrointestinal toxicity of nonsteroidal antiinflammatory drugs. *N. Engl. J. Med.* **1999**, *340*, 1888–1899.

(3) Campbell, C. L.; Smyth, S.; Montalescot, G.; Steinhubl, S. R. Aspirin dose for the prevention of cardiovascular disease: a systematic review. *JAMA, J. Am. Med. Assoc.* **2007**, *297*, 2018–2024.

(4) Lim, Y. J.; Lee, J. S.; Ku, Y. S.; Hahn, K. B. Rescue strategies against non-steroidal anti-inflammatory drug-induced gastroduodenal damage. *J. Gastroenterol. Hepatol.* **2009**, *24*, 1169–1178.

(5) Wallace, J. L. Building a better aspirin: gaseous solutions to a century-old problem. *Br. J. Pharmacol.* **2007**, *152*, 421–428.

(6) Kerwin, J. F.; Lancaster, J. R.; Feldman, P. L. Nitric oxide: a new paradigm for second-messengers. *J. Med. Chem.* **1995**, *38*, 4343–4362.

(7) Wallace, J. L.; Granger, D. N. The cellular and molecular basis of gastric mucosal defense. *FASEB J.* **1996**, *10*, 731–740.

(8) Lanas, A. Role of nitric oxide in the gastrointestinal tract. *Arthritis Res. Ther.* **2008**, *10* (2), S4.

(9) Wallace, J. L. Nitric oxide as a regulator of inflammatory processes. *Mem. Inst. Oswaldo Cruz* **2005**, *100* (Suppl. 1), 5–9.

(10) Jin, R. C.; Loscalzo, J. Vascular nitric oxide: formation and function. *J. Blood Med.* **2010**, *1*, 147–162.

(11) Szabó, C. Hydrogen sulphide and its therapeutic potential. *Nat. Rev. Drug Discovery* **2007**, *6*, 917–935.

(12) Wallace, J. L. Hydrogen sulfide-releasing anti-inflammatory drugs. *Trends Pharmacol. Sci.* **2007**, *28*, 501–505.

(13) Lowicka, E.; Beltowski, J. Hydrogen sulfide (H_2S): the third gas for interest for pharmacologists. *Pharmacol. Rep.* **2007**, *59*, 4–24.

(14) Caliendo, G.; Cirino, G.; Santagata, V.; Wallace, J. L. Synthesis and biological effects of hydrogen sulfide (H_2S): development of H_2S-releasing drugs as pharmaceuticals. *J. Med. Chem.* **2010**, *53*, 6275–6286.

(15) Del Soldato, P.; Sorrentino, R.; Pinto, A. NO-aspirins: a class of new antiinflammatory and antithrombotic agents. *Trends Pharmacol. Sci.* **1999**, *20*, 319–323.

(16) Sparatore, A.; Perrino, E.; Tazzari, V.; Giustarini, D.; Rossi, R.; Rossoni, G.; Erdman, K.; Schroder, H.; Del Soldato, P. Pharmacological profile of a novel H_2S-releasing aspirin. *Free Radical Biol. Med.* **2009**, *46*, 586–592.

(17) Nielsen, N. M.; Bundgaard, H. Evaluation of glycolamide esters and various other esters of aspirin as true aspirin prodrugs. *J. Med. Chem.* **1989**, *32*, 727–734.

(18) Lazzarato, L.; Donnola, M.; Rolando, B.; Chegaev, K.; Marini, E.; Cena, C.; Di Stilo, A.; Fruttero, R.; Biondi, S.; Ongini, E.; Gasco, A. (Nitrooxyacyloxy)methyl esters of aspirin as novel nitric oxide releasing aspirins. *J. Med. Chem.* **2009**, *52*, 5058–5068.

(19) Jones, M.; Inkielewicz, I.; Medina, C.; Santos-Martinez, M. J.; Radomski, A.; Radomski, M. W.; Lally, M. N.; Moriarty, L. M.; Gaynor, J.; Carolan, C. G.; Khan, D.; O'Byrne, P.; Harmon, S.; Holland, V.; Clancy, J. M.; Gilmer, J. F. Isosorbide-based aspirin prodrugs: integration of nitric oxide releasing groups. *J. Med. Chem.* **2009**, *52*, 6588–6598.

(20) Buchwald, P. Structure−metabolism relationships: steric effects and the enzymatic hydrolysis of carboxylic esters. *Mini-Rev. Med. Chem.* **2001**, *1*, 101–111.

(21) Kawashima, Y.; Ikemoto, T.; Horiguchi, A.; Hayashi, M.; Matsumoto, K.; Kawarasaki, K.; Yamazaki, R.; Okuyama, S.; Hatayama, K. Synthesis and pharmacological evaluation of (nitrooxy)alkyl apovincaminate. *J. Med. Chem.* **1993**, *36*, 815–819.

(22) Dunstan, I.; Griffiths, J. V.; Harvey, S. A. Nitric esters. Part I. Characterisation of the isomeric glycerol dinitrates. *J. Chem. Soc.* **1965**, 1319–1324.

(23) Boschi, D.; Tron, G. C.; Lazzarato, L.; Chegaev, K.; Cena, C.; Di Stilo, A.; Giorgis, M.; Bertinaria, M.; Fruttero, R.; Gasco, A. NO-donor phenols: a new class of products endowed with antioxidant and vasodilator properties. *J. Med. Chem.* **2006**, *49*, 2886–2897.

(24) Li, L.; Rossoni, G.; Sparatore, A.; Lee, L. C.; Del Soldato, P.; Moore, P. K. Antiinflammatory and gastrointestinal effects of a novel diclofenac derivative. *Free Radical Biol. Med.* **2007**, *42*, 706–719.

(25) Wallace, J. L; Cirino, G.; Caliendo, G.; Sparatore, A.; Santagada, V.; Fiorucci, S. Derivatives of 4- or 5-Aminosalicylic Acid. International Patent WO2006125293, November 30, 2006.

(26) Folkmann, M.; Lund, F. J. Acyloxymethyl carbonochloridates. New intermediates in prodrug synthesis. *Synthesis* **1990**, *12*, 1159–1166.