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ARTICLE *in* JOURNAL OF MEDICINAL CHEMISTRY · MAY 2006

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New NO-Releasing Pharmacodynamic Hybrids of Losartan and Its Active Metabolite: Design, Synthesis, and Biopharmacological Properties

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Received January 9, 2006

In a preliminary work, we reported two NO-sartans, possessing the characteristics of an AT₁ antagonist and a “slow NO donor”, obtained by adding NO-donor side chains to losartan **1**. The NO release from an NO-sartan should be modulated in order to strengthen the antihypertensive activity of the native drug and to ensure additional effects, such as the antiplatelet and anti-ischemic ones. To obtain a collection of prototypical NO-sartans, showing different rates of NO release, new NO-donor moieties have been linked to **1** or its active metabolite **2** (EXP 3174). Almost all the synthesized compounds exhibited both AT₁-antagonist and NO-mediated vasorelaxing properties, with a wide range of NO-releasing rates. Further pharmacological investigation on compound **4a** showed that it possessed antihypertensive and cardiac antihypertrophic effects similar to those of the reference AT₁-blocking or ACE-inhibiting drugs. Furthermore, the additional anti-ischemic cardio-protective properties and antiplatelet effects of **4a** have been preliminarily investigated.

Introduction

An effective approach for treating hypertension is offered by the possibility of modulating the activity of the renin-angiotensin system (RAS). The effector hormone in the RAS is the octapeptide angiotensin II (AII), produced in vivo from angiotensin I by the nonspecific carboxydiptidase angiotensin-converting enzyme (ACE).¹ By interacting with its type 1 receptor (AT₁), AII determines a direct vasoconstrictor action and a rise in the release of aldosterone, a mineral corticoid hormone which causes renal retention of Na⁺ ions and water, thus exerting a hypertensive effect.

The RAS offers several pharmacologically distinct approaches for antihypertensive therapy, and among these, the inhibition of ACE is of particular interest. ACE inhibitors, such as captopril and enalapril, are widely used in the treatment of hypertension. However, ACE is not a selective enzyme for the conversion of angiotensin I into AII: actually, it degrades other biologically active peptides such as bradykinin, substance P, and enkephalins.² In particular, bradykinin stimulates the endothelium to release nitric oxide (NO), a substance generated from its precursor L-arginine by nitric oxide synthase (NOS), whose principal biological action includes vascular smooth muscle relaxation through activation of guanylate cyclase and the production of c-GMP as the second messenger. Thus, by inhibiting the catabolism of bradykinin, ACE inhibitors exert some of their beneficial pharmacological effects by increasing NO production.^{3,4}

However, while on one hand, bradykinin conservation can be considered responsible for additional cardiovascular activities of ACE inhibitors, on the other hand, it is the cause of some of their adverse effects such as the cough. Besides, increases in bradykinin and substance P levels are also thought to be contributing factors to angioedema.⁵ In addition, ACE inhibitors do not lead to a complete blockade of RAS; indeed, AII can be

produced via alternative pathways by enzymes such as chymase.⁶

The inability of ACE inhibitors to induce a complete block of the production of AII at the receptor site, and their adverse effects (such as cough and angioedema), have led to the development of “sartans” as a new class of drugs for the treatment of hypertension. These drugs selectively block AT₁ receptors and, compared with ACE inhibitors, have fewer side effects, because they do not inhibit the catabolism of bradykinin carried out by ACE enzyme, and therefore they do not induce cough and angioedema. However, this biopharmacological peculiarity is also responsible for the weaker effectiveness of drugs of this class, compared with that of ACE inhibitors, because of their inability to increase the bradykinin level and therefore to enhance the NO-induced vasorelaxing activity.

The physiological levels of endogenous NO mediate multiple fundamental processes in the cardiovascular system. NO donors are pharmacologically active substances that release NO spontaneously or through enzymatic pathways. Organic nitrate and nitrite esters represent a class of NO-donor agents used in cardiovascular diseases since the 19th century. Treatment with these conventional esters is limited by their therapeutic half-life, their systemic absorption with potentially adverse hemodynamic effects, and problems of drug tolerance.⁷ To overcome these limitations, novel NO donors have been developed that offer selectivity, a prolonged half-life, and a reduced incidence of problems of drug tolerance.

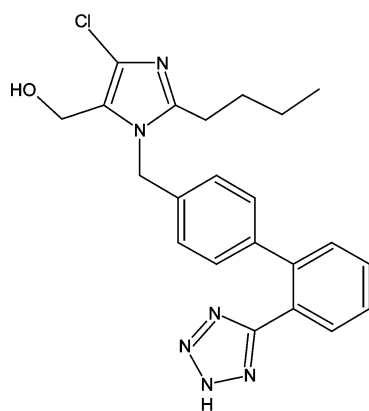
In the past few years, we have witnessed the flourishing of studies on several hybrid drugs, in which a well-known molecule with a particular pharmacological pattern has been linked with an NO-donor group, with the aim of improving the pharmacological profile or reducing the adverse effects. In this field, many NO-releasing antiinflammatory drugs have emerged as an interesting new class of effective antiinflammatory compounds with reduced side effects (gastric or intestinal ulceration), with respect to those of the classic nonsteroidal antiinflammatory drugs (NSAIDs) used so far,^{8,9} designed on the basis of a knowledge of the biological properties of NO, which protects the gastric mucosa. Thus, a variety of nitric esters of aspirin¹⁰

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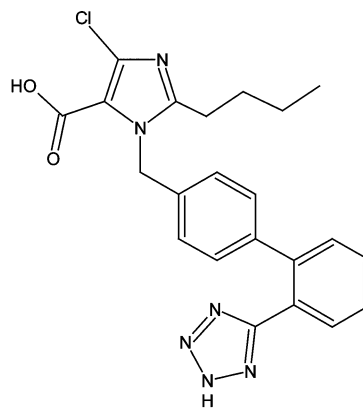
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Chart 1



1: losartan



2: EXP3174

have been synthesized, and recently, nitrosothiol esters of diclofenac¹¹ and NO-releasing derivatives of prednisolone¹² have also been described. As regards cardiovascular drugs, NO-releasing ACE inhibitors, NO-releasing calcium antagonists, and NO-releasing β -blocking agents have been synthesized,^{13–18} to improve the antihypertensive effects of the “native” drugs.

As regards sartans, we decided to add an NO-donor group to a sartan molecule, in an attempt to increase the antihypertensive activity. This chemical manipulation generated an original new class of drugs (NO-sartans)¹⁹ consisting of pharmacodynamic hybrids with AT₁-antagonist properties and additional NO-mediated, but bradykinin-independent, cardiovascular effects.

Initially, on the basis of the consideration that losartan (**1**) and its active metabolite **2** (EXP 3174) (Chart 1) possess both the high activity and the molecular features (i.e., the presence of an easily esterifiable group) useful for our purposes, we synthesized two lead compounds (**3** and **4a**),¹⁹ in which an aliphatic (as in **3**) or an aromatic (as in **4a**) moiety is inserted between the AT₁ antagonist (**1**) and a nitric ester function.

The results of in vitro and in vivo preliminary studies¹⁹ indicate that these NO-sartans (**3**, **4a**) are really pharmacodynamic hybrids possessing the desired dual activity and that the nature of the linker may affect their biopharmacological properties. On this basis, as a further step, a series of new dual molecules (**4b–d**, **5**, **6**), in which losartan itself is linked to different molecular portions bearing a nitric ester moiety, were synthesized. Furthermore, the hybrids (**7**, **8a,b**) in which an aromatic nitric ester is linked to the active metabolite of losartan (**2**) and one (**9**) in which the nitric ester function is directly linked to losartan itself were synthesized (Chart 2). The new “NO-donor linkers” used in this work (**10**, **11a–d**, **12–15a,b**) (Chart 3) present a pyridine system (**13**) or methyl group(s) directly linked to the aromatic systems (**12**) or to the carbon adjacent to the nitric ester function (**11c,d**). These linkers were selected on the basis of the hypothesis that steric and/or electronic differences, due to their different molecular structures, may modulate the rate of NO release and therefore affect the biopharmacological responses.

Chemistry

The synthesis of compounds **3–6** is represented in Scheme 1. 5-Chloropentanoic acid (**16**) was converted into 5-nitroxypentanoic acid (**10**) by treatment with silver nitrate in acetonitrile at room temperature and in the dark. Condensation of the nitro ester (**10**) with losartan (**1**) in tetrahydrofuran (THF), in the presence of *N,N'*-dicyclohexylcarbodiimide (DCC) and a cata-

lytic amount of *N,N*-dimethylaminopyridine (DMAP), afforded compound **3**.

Derivatives **4a** and **4b** were prepared from **11a** and **11b**, obtained by the reaction of the corresponding commercially available chloro derivatives **17** and **18** with silver nitrate in acetonitrile. Compounds **11a,b** were condensed with losartan (**1**), using DCC and DMAP in THF, to give the corresponding hybrids **4a** and **4b**.

The reduction of the appropriate acetylbenzoic acid (**19**, **20**) by LiAlH₄ in THF furnished the corresponding alcohols (**21**, **22**). The subsequent reaction with nitric acid and acetic anhydride at -10°C afforded the nitro esters **11c** and **11d**, which were condensed with losartan (**1**), to give compounds **4c** and **4d**.

The hybrid **5** was obtained from the condensation of losartan with the benzoic acid **12** following the same procedure described above. 2,6-Dimethylbenzoic acid (**23**) was treated with paraformaldehyde, concentrated HCl, and glacial acetic acid at 80°C to give compound **24**. This chloro derivative was converted into the nitro ester **12** by treatment with silver nitrate in acetonitrile.

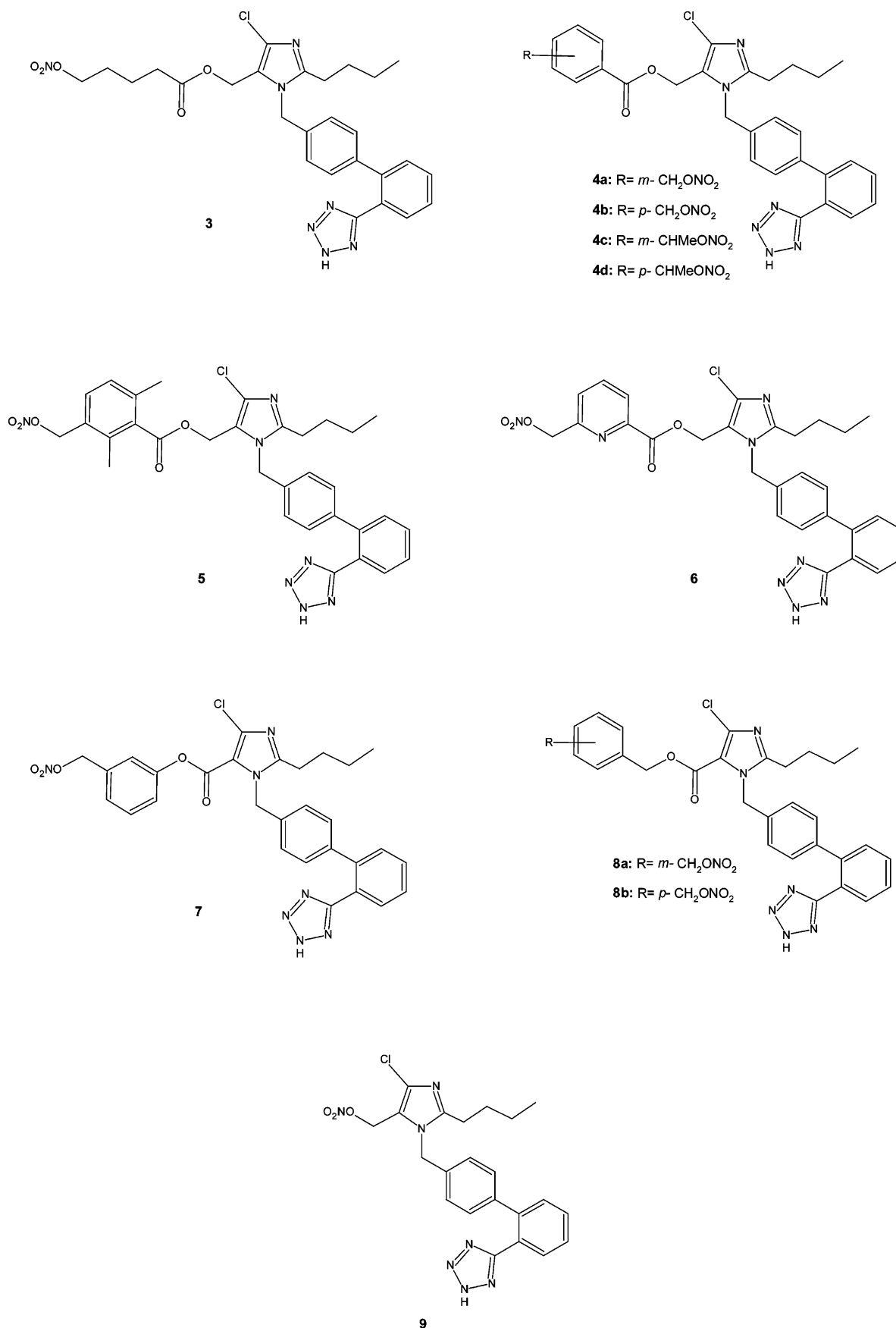
The 2,6-pyridindicarboxylate (**25**) was reduced with NaBH₄ in a 2:1 ratio to give monoalcohol **26**.²⁰ The subsequent hydrolysis of monoester **26** with NaOH in methanol under microwave irradiation afforded the acid **27**, which was nitrated with nitric acid and acetic anhydride at -10°C to give compound **13**. Finally, the ester derivative **6** was obtained by the coupling of **13** with the losartan (**1**), using DCC and DMAP.

Derivatives **7** and **8a,b** were prepared following the procedures illustrated in Scheme 2. The 3-hydroxybenzyl alcohol (**28**) was transformed into the corresponding chloro derivative **29** by chlorination with thionyl chloride in chloroform. The subsequent reaction with silver nitrate in acetonitrile gave the nitric ester **14**. This compound was condensed with **2** to give compound **7**. Compound **2** was prepared from losartan (**1**), which, initially, was oxidized with manganese dioxide in water under microwave irradiation to the corresponding aldehyde²¹ (**30**) and subsequently was oxidized with NaClO₂ and NaH₂PO₄ in water and *t*-BuOH to the corresponding acid **2**.

The appropriate dihydroxybenzyl alcohol (**31a,b**) was transformed into the corresponding monochloro derivative **32a,b** by treatment with concentrated HCl in toluene at 25°C . The subsequent reaction with silver nitrate afforded the nitric esters **15a** and **15b** which were condensed with **2**, to give compounds **8a** and **8b**.

Compound **9** was obtained by direct nitration of losartan (**1**) with nitric acid and acetic anhydride at -10°C .

Chart 2

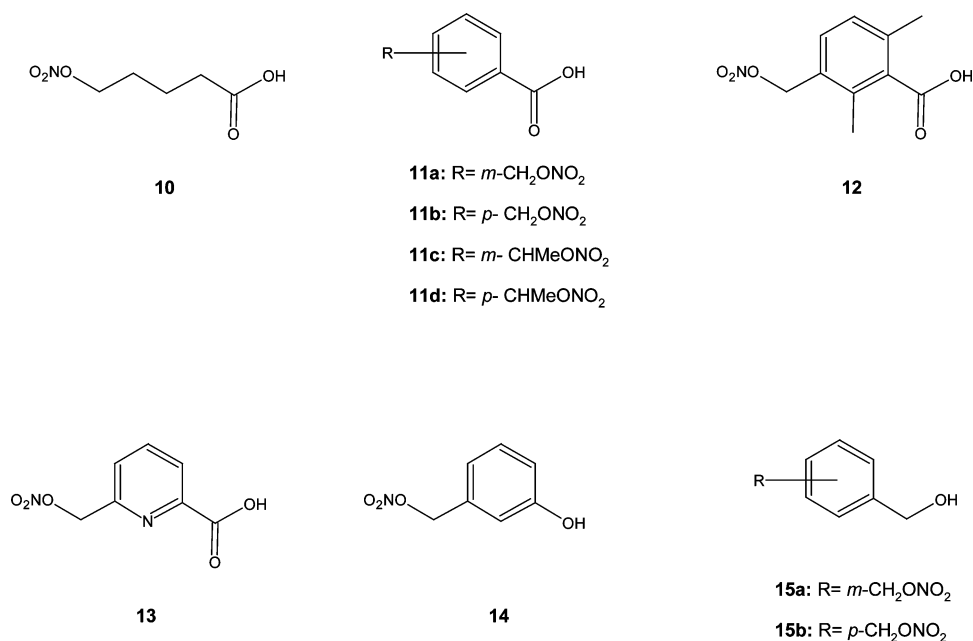


Pharmacological Results

NO-Mediated Vasorelaxing Activity. All the tested compounds (**3–9**), with the only exception of **4c** which exhibited

only a partial efficacy, evoked concentration-dependent vasorelaxing responses, with full efficacy, on rat aortic rings precontracted by 30 mM KCl (Table 1). All these responses were

Chart 3



significantly antagonized by the inhibition of guanylate cyclase, achieved by the administration of 1 μ M 1-*H*-[1,2,4]-oxadiazole-[4,3-*a*]-quinoxalin-1-one (ODQ), thus indicating that the vasorelaxing effect was due to the release of NO from the hybrid drugs and therefore to the triggering of the NO–cGMP pathway.

In particular, compounds **8a,b** showed a vasorelaxing activity with potency parameters almost comparable with those exhibited by **3** and **4a**. Compound **4b** showed the strongest vasorelaxing activity, with a potency parameter (pIC₅₀) higher than those exhibited by the previously described pioneer compounds **3** and **4a** (Figures 1 and 2). In comparison with **3** and **4a**, compounds **4d** and **7** showed a moderate decrease in the pIC₅₀ values (about 1 order of magnitude), while **5**, **6**, and **9** exhibited a greatly reduced potency index (about 2 orders of magnitude). Finally, compound **4c** showed only partial vasorelaxing efficacy (<50%, at the highest concentration administered), which did not allow the calculation of the potency index.

The experimental results emerging from the time-course protocols are fully consistent with the pIC₅₀ values recorded with the cumulative concentration–response curves, thus indicating that the differences in the potency are linked to different rates of NO release. As expected, the concentration of 1 μ M of compounds **4c,d**, **5–7**, and **9** evoked modest vasorelaxing responses (<25%) in this experimental protocol, because of their low pIC₅₀. Compound **4a** (1 μ M) determined a reduction to approximately one-half of the contractile tone of the vessels (Figure 2). The most potent compound **4b** (1 μ M) caused an almost complete vasorelaxing effect (Figure 2). Although compounds **8a,b** showed vasorelaxing potencies lower than that of **4b**, their time-course vasorelaxing profiles were similar to that of **4b** (Figure 2). Moreover, a comparison of the time-course vasorelaxing effects of the synthesized compounds with those of sodium nitroprusside (SNP, a rapid NO donor selected as a reference drug) showed that the release of NO by NO–sartans is a slow process (Figure 2).

AT₁-Antagonist Activity. After 20 min of incubation, compounds **4b** and **6** exhibited AT₁-antagonist properties, with potency parameters (*K_b*) in the nanomolar range, similar to those shown by the pioneer drugs **3** and **4a** and to that of the reference antagonist losartan **1** (Table 2).

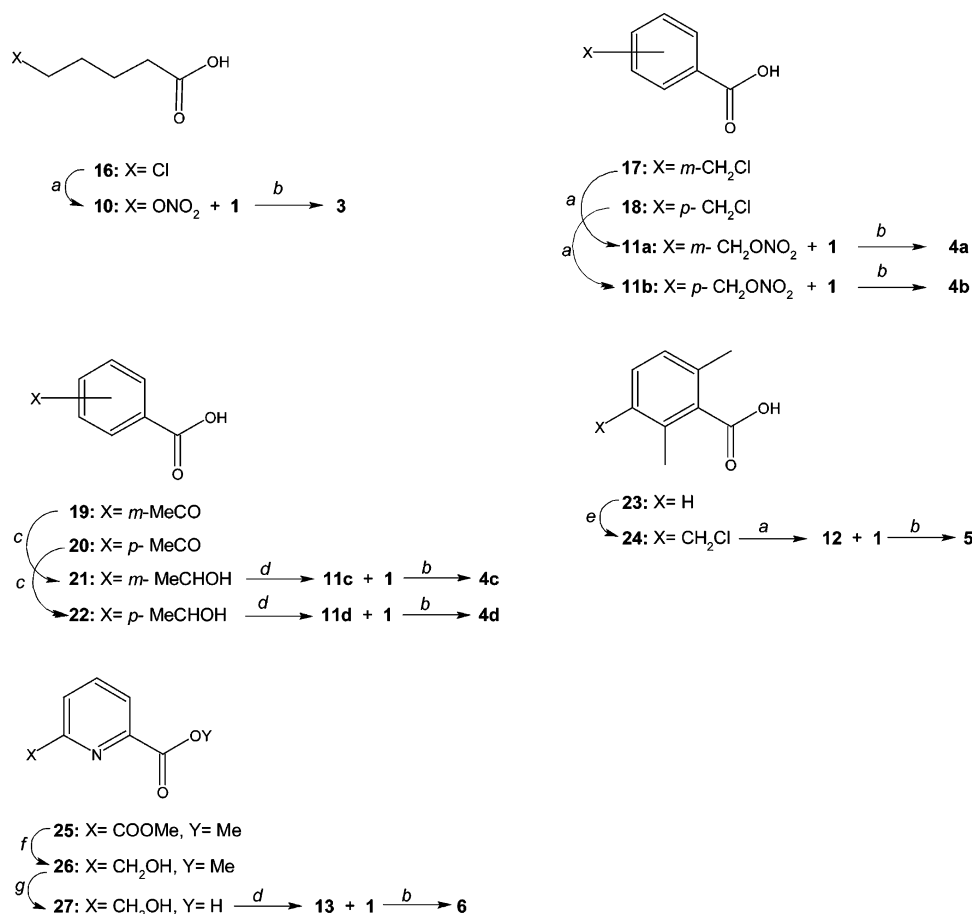
In these experimental conditions, compounds **4c,d**, **5**, and **9** failed to exert any significant antagonism, while a prolonged incubation time (60 min) allowed compounds **4c,d** and **9** to exhibit a significant AT₁ antagonism, with *K_b* which again reached the nanomolar range (Table 2). As regards compounds **7** and **8a,b**, hybrid derivatives of **2**, after a “brief” incubation time (20 min) only compound **7** (0.1 μ M) determined an almost complete abolition of the vasocontractile effects of angiotensin II, exhibiting the profile of insurmountable antagonism typical of **2** (Table 2). Compounds **8a,b** were ineffective after a “brief” incubation time and required a prolonged one (60 min) to release the “native” drug **2** and thus to exert an insurmountable antagonism (Table 2).

Although the reference AT₁ antagonists **1** and **2**, as well as the hybrid derivative **4a**, showed the antagonist properties after 20 min of incubation time (“brief” period), they were also incubated for 60 min (prolonged incubation). Their antagonist profiles recorded in these latter experimental conditions were almost equivalent to those observed after the “brief” incubation time (Table 2).

Antihypertensive Effects. As previously reported,¹⁹ following both the oral and the subcutaneous treatment of spontaneously hypertensive rats (SHRs), the ACE inhibitor captopril, the AT₁ antagonist losartan (**1**), and compound **4a** caused a significant lowering of the values of systolic blood pressure, which, after 4 weeks of treatment, were almost the same as those recorded in normotensive animals. In particular, the antihypertensive effects of **4a** were not lower (and, perhaps, they were even slightly higher) than those exhibited by the “native” drug losartan **1**.

Cardiac Antihypertrophic Effects. As widely reported in international literature,²² the SHRs used in this study showed a significant cardiac hypertrophy, almost exclusively due to an increased mass of the left ventricle (Figure 3).

As expected, the administration of the AT₁ antagonist losartan (**1**) or the ACE inhibitor captopril led to a reduction in the left ventricle mass and, consequently, in the overall cardiac mass, which went down to the levels recorded in the normotensive animals. In the same type of experiment, compound **4a** exhibited antihypertrophic effects, which proved to be very similar to those shown by losartan **1** and captopril (Figure 3).

Scheme 1^a

^a Key: (a) AgNO_3 , CH_3CN , 25°C ; (b) DCC, DMAP, THF, 25°C ; (c) LiAlH_4 1 M, THF, 0°C ; (d) HNO_3 , Ac_2O , -10°C ; (e) HCHO , HCl , AcOH , 80°C ; (f) NaBH_4 , MeOH , -10°C ; (g) NaOH , MeOH , mw, 2.5 min, 160°C .

Anti-Ischemic Effects. Losartan (**1**) and compound **4a** were tested on an experimental model of cardiac ischemia–reperfusion. The ischemic injury, evaluated with the functional parameters of postischemic inotropism (evaluated as rate pressure product, RPP) and coronary flow (CF), was not reduced by **1**, while compound **4a** induced a marked improvement of the two parameters: the RPP parameter reached an almost complete recovery of the levels recorded in the preischemic time, and the CF recorded in the reperfusion time was almost 2-fold higher than that recorded in the preischemic period (Figure 4).

Antiplatelet Effects. A concentration of $5\ \mu\text{M}$ adenosine diphosphate (ADP) determined on rat platelet-rich plasma (PRP) a significant platelet aggregation ($67 \pm 1\%$; Figure 5). The effect of ADP was partially reduced by losartan (**1**) ($50 \pm 5\%$; Figure 5). Compound **4a** caused a more pronounced antiplatelet activity, determining an about half-reduction of the aggregating effects of ADP ($41 \pm 4\%$; Figure 5).

Discussion

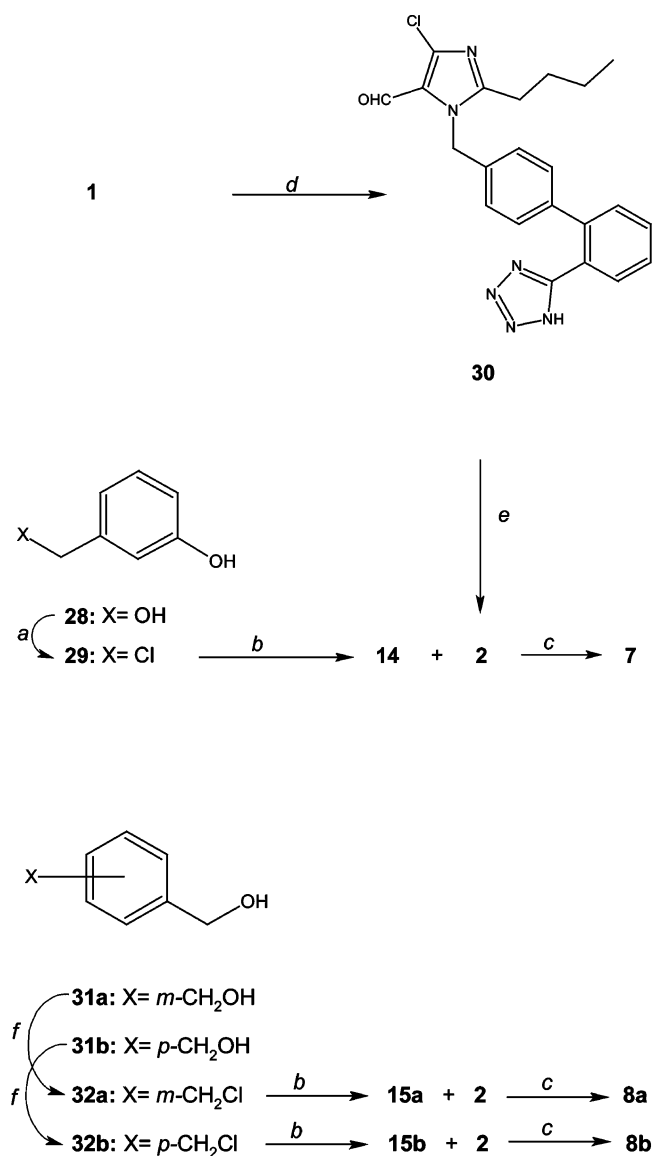
In a previous preliminary report, we introduced a new class of potential cardiovascular drugs, the NO–sartans, which exhibit a dual pharmacodynamic profile.¹⁹ Indeed, the two reported pioneer compounds (**3** and **4a**) showed both NO-releasing effects and AT_1 -antagonist properties. In particular, compounds **3** and **4a** produced vasorelaxing effects, showing pIC_{50} in the micromolar range. These vasorelaxing effects were shown to be due to the release of NO, because they were antagonized by the guanylate cyclase inhibitor ODQ ($1\ \mu\text{M}$).

Both these compounds also exhibited the desired AT_1 -antagonist effects. As the hybrid compound **4a** exerted AT_1 -antagonist effects in eserine-free conditions, while the presence of eserine (esterase inhibitor) dramatically reduced this antagonism, it was clear, as previously demonstrated, that the AT_1 -blocking activity was due to the hydrolytic cleavage of the side chain and the release of the “native” losartan. Consistently, the AT_1 -antagonist properties of **3** and **4a** ($K_b = 16$ and $6\ \text{nM}$, respectively) were slightly lower (**3**) than, or substantially equivalent (**4a**) to, those shown by losartan itself ($K_b = 4\ \text{nM}$).

This experimental evidence demonstrated the concrete possibility of developing such a class of pharmacodynamic hybrids.

As regards hybrid “dual” molecules, the presence of both the desired mechanisms of action represents the first necessary condition. Another fundamental aspect concerns the correct balancing of the two pharmacodynamic properties, to obtain drugs that can profitably be applied in therapy. In particular, the NO release from an NO–sartan should be correctly modulated in order to strengthen the antihypertensive activity of the native drug and, in particular, to ensure additional desired beneficial cardiovascular effects such as the antiplatelet and anti-ischemic ones, without exasperating the negative effects, such as an excessive hypotensive response. According to this view, the NO-modulated vasorelaxing effects should be moderate, because the additional contribution of a strong vasodilator activity seems to be inessential (or even deleterious) for drugs already possessing a good antihypertensive efficacy, such as sartans.

Therefore, to develop a representative collection of prototypical NO–sartans differing from each other in their variable NO–

Scheme 2^a

^a Key: (a) $SOCl_2$, $CHCl_3$, 25 °C; (b) $AgNO_3$, CH_3CN , 25 °C; (c) DCC, DMAP, THF, 25 °C; (d) MnO_2 , H_2O , mw, 2 min, 160 °C; (e) $NaClO_2$, NaH_2PO_4 , H_2O , *t*-BuOH, 25 °C; (f) HCl_{conc} , toluene, 25 °C.

releasing rates, compound **4a** underwent various structural modifications of the NO-donor moiety. The first step consisted of the direct insertion of the nitric ester on the hydroxyl group of losartan. The target compound **9** exhibited a low vasorelaxing potency and, consistently, the administration of **9** at a concentration of 1 μM did not produce any vasorelaxing effect in the time-course protocols. This can be explained by the hypothesis of a very slow release of NO and, consequently, of free losartan from the hybrid. This hypothesis seems to be confirmed by the observation of the AT_1 antagonism of this compound. Indeed, **9** did not antagonize the AT_1 receptor after a “brief” incubation (20 min), indicating that such a short period does not allow the hybrid to release losartan. On the contrary, a longer incubation time (60 min) allowed the compound to reach a losartan-like AT_1 antagonism. Moreover, the unsatisfactory NO-releasing properties of **9** suggested the need for the presence of a “linker” moiety between the native sartan and the nitrate ester, ensuring a significant, albeit modulated, release of NO. Therefore, a series of NO-releasing side chains, analogues of **11a**, were synthesized. Preliminary experiments demonstrated that all these side chains

Table 1. pIC_{50} and E_{max} ^a

compound	pIC_{50}	E_{max} %
3	6.07 ± 0.11	100
4a	6.56 ± 0.08	100
4b	7.18 ± 0.03	100
4c	not calculable	47 ± 1
4d	5.11 ± 0.03	100
5	4.49 ± 0.02	100
6	4.91 ± 0.06	100
7	5.22 ± 0.08	100
8a	6.18 ± 0.03	100
8b	6.56 ± 0.02	100
9	4.82 ± 0.07	100

^a The NO-mediated vasorelaxing efficacy of the synthesized compounds was evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 30 mM KCl. The parameter of potency was expressed as pIC_{50} , calculated as the negative logarithm of the molar concentration of the test compounds, evoking 50% reduction of the contractile tone induced by 30 mM KCl. The pIC_{50} could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as means \pm standard error, for 5–10 experiments.

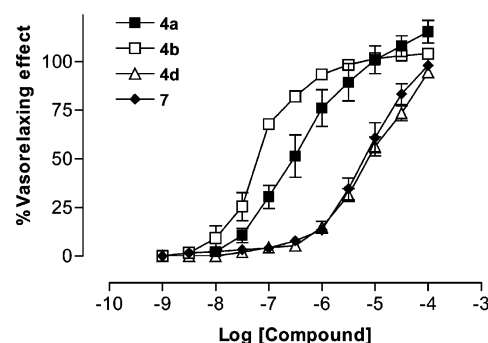


Figure 1. Representative concentration–vasorelaxing effect curves of selected test compounds. Compounds showing a pIC_{50} value lower than 5 are not shown. X axis: logarithm of the molar concentration of the test compounds. Y axis: vasorelaxing effect, expressed as a percentage (%) of the contractile tone induced by 30 mM KCl. Bars: standard errors.

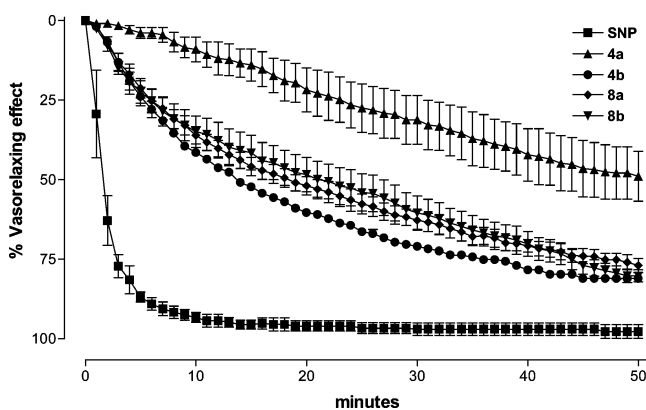


Figure 2. Representative time-course profile of the vasorelaxing effect evoked by a 1 μM concentration of selected test compounds. Compounds presenting a final vasorelaxing effect lower than 25% are not shown. X axis: time expressed in minutes. Y axis: vasorelaxing effect, expressed as a percentage (%) of the contractile tone induced by 30 mM KCl. Bars: standard errors.

showed ODQ-sensitive vasorelaxing effects due to the release of NO, with IC_{50} ranging around the micromolar order of magnitude.

The pyridine compound **6**, with the heterocycle ring replacing the benzene one of **4a**, showed a significant decrease in the vasorelaxing potency and a consistent lack of vasoactive effects in the time-course protocol. Compound **6** exhibited a losartan-

Table 2. K_b Values^a

compounds	K_b (20 min)	K_b (60 min)
1	4 nM	9 nM
2	insurmountable*	insurmountable*
3	16 nM	N.T.
4a	6 nM	10 nM
4b	19 nM	N.T.
4c	N.A.	12 nM
4d	N.A.	8 nM
5	N.A.	N.T.
6	8 nM	N.T.
7	insurmountable*	N.T.
8a	N.A.	insurmountable*
8b	N.A.	insurmountable*
9	N.A.	3 nM

^a Antagonist potency values of the test compounds (expressed as K_b) after a "brief" (20 min) and a "longer" (60 min) period of incubation. N.A. (no activity) means that the compound has no AT₁-antagonist properties after a "brief" period of incubation. N.T. (not tested) means that the compound which showed AT₁-antagonist properties after 20 min was not tested in the 60 min incubation protocol. The asterisk (*) indicates that the compound exhibited an insurmountable antagonist feature, typical of nonreversible receptor antagonists, such as 2.

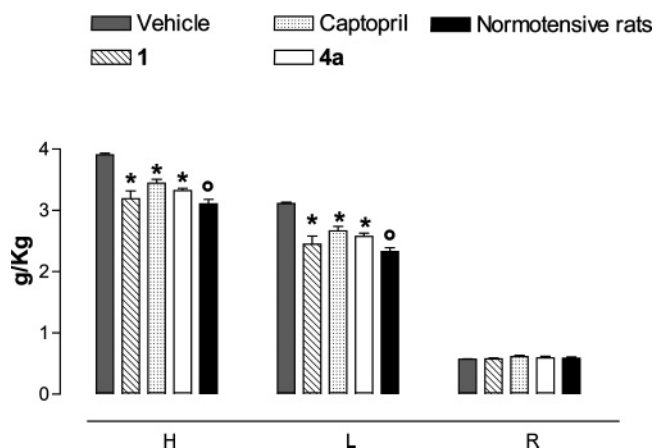


Figure 3. Histograms representing the ratios expressed in g/kg. H indicates the ratio value between the whole heart weight and the rat body weight. L indicates the ratio value between the left ventricle weight and the rat body weight. R indicates the ratio value between the right ventricle weight and the rat body weight. Vertical bars indicate the standard error. The asterisk (*) indicates significant statistical differences between a treated group and the corresponding vehicle group. The circle (O) indicates a significant statistical difference between the normotensive group and the vehicle group.

like AT₁-antagonist potency after the "brief" incubation time (20 min), indicating a rapid hydrolytic cleavage of the side chain and a rapidly exhaustive release of free losartan.

The introduction of (a) methyl group(s) on the nitrooxymethyl chain carbon or on the benzene ring of **4a**, giving compounds **4c** and **5**, respectively, determined a negative impact on the NO-releasing properties. Indeed, the vasorelaxing potencies fell dramatically both in **4c** (pIC_{50} not calculable because of the vasodilator efficacy <50%) and in **5**. Of course, these two compounds were devoid of any vasorelaxing effects in the time-course protocols. Although the lack of a significant NO-releasing property makes these two compounds uninteresting for further development, their AT₁-antagonist profile was also evaluated. Neither of the compounds exhibited any significant AT₁ antagonism after the "brief" incubation time (20 min). Compound **4c** was arbitrarily selected (as representative of both of them) for the evaluation of the AT₁ antagonism after a longer incubation period (60 min), and in these experimental conditions it showed an AT₁-blocking potency similar to that shown by losartan. This evidence seems to indicate that the hydrolytic

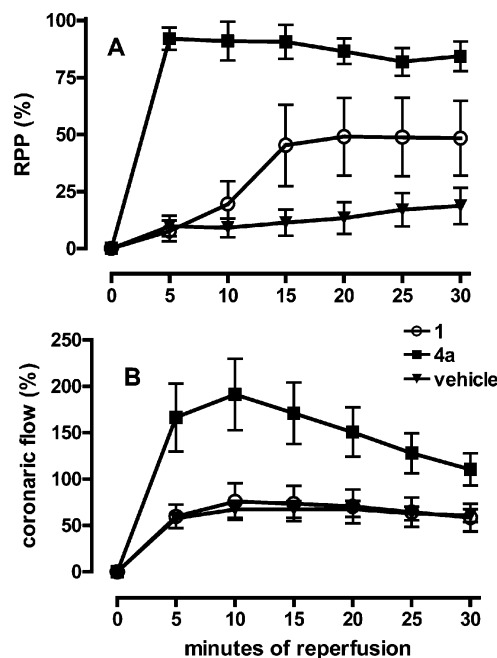


Figure 4. Parameters of RPP and CF, recorded during the 30 min postischemic period and expressed as a percent of the respective values observed in the preischemic period, on Langendorff perfused hearts from rats pretreated with vehicle, compound **1**, or compound **4a**. The vertical bars indicate the standard error.

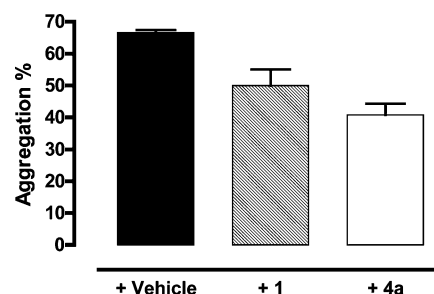


Figure 5. Histograms representing the aggregating effects induced by 5 μ M ADP on rat PRP, in the presence of the vehicle, **1**, or compound **4a**. The vertical bars indicate the standard error.

cleavage of the side chain, allowing the release of free losartan, could be slowed by the presence of (a) methyl substituent(s).

The shift of the nitrooxymethyl chain from the *meta* position of **4a** to the *para* position of **4b** led to a substantial increase of vasorelaxing potency. This vasorelaxing activity was ODC-sensitive, indicating the involvement of the release of NO. Consistently, **4b** also exhibited the highest vasorelaxing effect in the time-course protocol, leading to an almost complete recovery of the basal tone of the vascular smooth muscle precontracted by KCl. However, it is noteworthy that a comparison of the time-course profiles of the vasorelaxing effects induced by **4b** and by SNP demonstrated that **4b** can be considered as a slow NO donor. After the "brief" incubation time (20 min), **4b** demonstrated a clear AT₁ antagonism, although it showed a potency index slightly lower than that of losartan, probably because of a significant, but not yet complete, release of the free "native" drug.

As clearly emerged from the comparison between the two analogues **4a** and **4c**, also the introduction of a methyl substituent on the nitrooxymethyl chain of **4b**, giving the analogue **4d**, caused a slowing down of the NO-releasing rate, represented by the lowering of the potency index. The time-course protocol revealed a weak vasorelaxing effect induced

by 1 μ M **4d**. As observed in the couple of analogues **4a** and **4c**, also for the couple **4b** and **4d** the presence of the methyl group seems to cause a delayed hydrolytic cleavage of the side chain and a delayed release of free losartan, because the AT₁ antagonism was not recorded after the "brief" incubation (20 min), but it appeared after 60 min of incubation, with losartan-like levels of potency.

Finally, the investigation of the impact due to the "inversion" of the ester bond linking the side chain to the sartan was performed by studying **7** and **8a,b**. These compounds exhibited a significant vasorelaxing potency. In particular, the vasorelaxing potency of compound **7**, a phenolic ester, was lower than that of **4a**. The insertion of a benzylic linker (**8a,b**) led to a significant increase of the potency parameters, which resulted in a value almost similar to that exhibited by **4a**. Consistently with what observed with the couples of analogues discussed above (**4a** vs **4b** and **4c** vs **4d**), the shift of the nitrooxymethyl chain from the *meta* position to the *para* position (**8a** vs **8b**) caused, again, an increase of the vasorelaxing potency. As expected, the vasorelaxing effect of **7**, at a concentration of 1 μ M, was negligible in the time-course protocol, while compounds **8a,b** showed time-course vasorelaxing profiles similar to that of **4b**.

As regards the AT₁ antagonism, **7** showed a high antagonist effectiveness after the "brief" incubation time (20 min), indicating a relatively rapid cleavage of the "inverse" phenolic ester and a complete release of active **2**. The presence of the "inverse" benzylic ester of **8a,b** caused a slowing down of the hydrolytic cleavage; indeed, compounds **8a,b** showed significant AT₁ antagonism (due to the release of **2**) only after 60 min of incubation time.

As the rational strategy leading to the development of a generic pharmacodynamic hybrid aims to confer additional positive properties, without decreasing the fundamental profile of effectiveness of the native drug, it is evident that such a hybrid must fit in with the "golden paradigm": it must not show any reduction of the basic pharmacological properties of the native drug itself. As far as sartans are concerned, this pharmacological class is considered as fully satisfactory for the treatment of important cardiovascular diseases, such as hypertension and/or cardiac hypertrophy. Consequently, the first inevitable step in the development of NO-sartans is a demonstration that, in such hybrids, the presence of the NO-donor property does not compromise (by possible pharmacodynamic interactions and/or chemical influences) the effectiveness of the native drugs, as regards their main therapeutic indications. In other words, the possible addition of new properties (for example, antiplatelet and anti-ischemic effects, expected as a result of the release of NO) is subordinate to the maintenance of intact (or improved) antihypertensive and antihypertrophic effects, since their reduction can undermine the rational basis for the development of this novel class of cardiovascular drugs.

Consequently, in the light of the impossibility of identifying the NO-sartan exhibiting the optimal balance between the two pharmacodynamic properties, compound **4a** was selected as a representative NO-sartan for further preliminary investigation, since it presented intermediate NO-releasing rate levels, among the various NO-sartans synthesized in this work. As previously reported,¹⁹ the chronic oral or subcutaneous administration of **4a** demonstrated significant antihypertensive effects on SHR. These effects were almost the same as those exerted by the ACE inhibitor captopril and equivalent to (or perhaps slightly better than) those shown by the equimolar doses of the reference AT₁ antagonist losartan. Furthermore, in this work it has been

demonstrated that **4a** also showed significant pharmacological effects in the reversion of the left ventricle hypertrophy, typical of SHRs. Again, these antihypertrophic effects were very similar to those exhibited by the reference compounds captopril and losartan.

Finally, important, albeit preliminary, experimental data demonstrated that **4a** seems to possess additional pharmacological features, not shown (or poorly shown) by the respective "native" drug losartan. In particular, **4a** exerted a significant cardio-protective activity against the myocardial injury induced by an ischemia-reperfusion cycle. Furthermore, compound **4a** was more effective than losartan in the reduction of the aggregating effect induced by ADP on rat platelets. These additional properties will be studied more in depth in future pharmacological investigations.

Another essential future experimental approach will aim to identify, among the various NO-sartans synthesized to date, the one(s) exhibiting an optimal balance of the two pharmacodynamic mechanisms, i.e., the one(s) possessing the most convenient NO-releasing rate.

Experimental Section

Chemistry. Melting points were determined on a Kofler hot-stage apparatus and are uncorrected. NMR spectra were obtained with a Varian Gemini 200 MHz spectrometer. Chemical shifts (δ) are reported in parts per million downfield from tetramethylsilane and referenced from solvent references. Mass spectra were obtained on a Hewlett-Packard 5988 A spectrometer using a direct injection probe and an electron beam energy of 70 eV. The elemental compositions of the compounds agreed to within $\pm 0.4\%$ of the calculated value. Chromatographic separation was performed on silica gel columns by flash (Kieselgel 40, 0.040–0.063 mm; Merck) or gravity column (Kieselgel 60, 0.063–0.200 mm; Merck) chromatography. Reactions were followed by thin-layer chromatography (TLC) on Merck aluminum silica gel (60 F₂₅₄) sheets that were visualized under a UV lamp. Evaporation was performed in vacuo (rotating evaporator). Sodium sulfate was always used as the drying agent. The microwave-assisted procedures were carried out with a CEM Discover LabMate microwave. Commercially available chemicals were purchased from Sigma-Aldrich.

5-(Nitrooxy)pentanoate of Losartan (3). Preparation of **3** and characterization data have already been reported.¹⁹

3-[(Nitrooxy)methyl]benzoate of Losartan (4a). Preparation of **4a** and characterization data have already been reported.¹⁹ ¹³C NMR (CDCl₃) δ : 165.50, 149.48, 140.54, 139.74, 135.15, 133.76, 133.11, 131.16, 131.03, 130.61, 130.12, 130.30, 129.89, 129.25, 128.43, 125.88, 123.48, 120.64, 66.13, 55.16, 47.63, 30.06, 26.91, 22.59, 13.87.

4-[(Nitrooxy)methyl]benzoate of Losartan (4b). A solution of DCC (350 mg, 0.7 mmol) in dry THF (11 mL) was added dropwise to a stirred solution of losartan **1** (600 mg, 1.4 mmol), **11b** (276 mg, 1.4 mmol), and DMAP (12 mg) in dry THF (14 mL). The suspension was stirred at room temperature for 12 h, then the precipitate was removed by filtration, and the filtrate was concentrated. The solid was washed with acetone, and the solvent was evaporated. The crude product was purified by column chromatography eluting with AcOEt/*n*-hexane (8:2) to give **4b** (464 mg, 0.8 mmol, 55% yield) as a white solid: mp 84–86 °C. MS (*m/z*) 538 ($M^+ - \text{ONO}_2$, 2%); Anal. C₃₀H₂₈ClN₇O₅.

3-[2-(Nitrooxy)ethyl]benzoate of Losartan (4c). Compound **4c** was synthesized from **11c** (275 mg, 1.30 mmol) following the same procedure described above for the preparation of **4b**. This compound was purified by precipitation with *n*-hexane from AcOEt, and the solid was collected to give **4c** (396 mg, 0.6 mmol, 46% yield) as a yellow solid: mp 88–90 °C. MS (*m/z*) 552 ($M^+ - \text{ONO}_2$, 8%); Anal. C₃₁H₃₀ClN₇O₅.

4-[2-(Nitrooxy)ethyl]benzoate of Losartan (4d). Compound **4d** was synthesized from **11d** (200 mg, 0.94 mmol) following the same

procedure described above for the preparation of **4b**. The crude product was purified by column chromatography eluting with AcOEt/*n*-hexane (7:3) to give **4d** (148 mg, 0.24 mmol, 25% yield) as a white solid: mp 70–72 °C. MS (*m/z*) 552 ($M^+ - \text{ONO}_2$, 6%); Anal. $\text{C}_{31}\text{H}_{30}\text{ClN}_7\text{O}_5$.

2,6-Dimethyl-3-[(nitrooxy)methyl]benzoate of Losartan (5). Compound **5** was synthesized from **12** (200 mg, 0.47 mmol) following the same procedure described above for the preparation of **4b**. This compound was purified by column chromatography eluting with AcOEt/*n*-hexane (3:7) to give **5** (101 mg, 0.16 mmol, 34% yield) as a white solid: mp 105–107 °C. MS (*m/z*) 566 ($M^+ - \text{ONO}_2$, 20%); Anal. $\text{C}_{32}\text{H}_{32}\text{ClN}_7\text{O}_5$.

6-[(Nitrooxy)methyl]pyridine-2-carboxylate of Losartan (6). Compound **6** was synthesized from **13** (270 mg, 1.36 mmol) following the same procedure described above for the preparation of **4b**. This compound was purified by column chromatography eluting with $\text{CHCl}_3/\text{MeOH}$ (9:1) to give **6** (343 mg, 0.57 mmol, 42% yield) as a solid: mp 102–104 °C. MS (*m/z*) 166 ($M^+ - \text{O}_2\text{NOCH}_2\text{Py}$, 100%); Anal. $\text{C}_{29}\text{H}_{27}\text{ClN}_8\text{O}_5$.

3-[(Nitrooxy)methyl]phenyl Ester of EXP 3174 (7). A solution of DCC (161 mg, 0.8 mmol) in dry THF (5 mL) was added dropwise to a stirred solution of **2** (283 mg, 0.6 mmol), **14** (110 mg, 0.6 mmol), and DMAP (6 mg) in dry THF (6.6 mL). The suspension was stirred at room temperature for 12 h, then the precipitate was removed by filtration, and the filtrate was concentrated. The solid was triturated with acetone, and the solvent was evaporated. The crude product was purified by column chromatography eluting with hexane/AcOEt (1:1) to give **7** (46 mg, 0.08 mmol, 12% yield) as a yellow solid. MS (*m/z*): 588 (M^+ , 2%); Anal. $\text{C}_{29}\text{H}_{26}\text{ClN}_7\text{O}_5$.

3-[(Nitrooxy)methyl]benzoate of EXP 3174 (8a). Compound **8a** was synthesized from **15a** (105 mg, 0.57 mmol) following the same procedure described above for the preparation of **7**. This compound was purified by column chromatography eluting with AcOEt and then by precipitation with *n*-hexane from AcOEt. The solid was collected to give **8a** (79 mg, 0.131 mmol, 24% yield) as a white solid: mp 56–58 °C. MS (*m/z*): 602 (M^+ , 0.2%); Anal. $\text{C}_{30}\text{H}_{28}\text{ClN}_7\text{O}_5$.

4-[(Nitrooxy)methyl]benzoate of EXP 3174 (8b). Compound **8b** was synthesized from **15b** (66 mg, 0.36 mmol) following the same procedure described above for the preparation of **8a**. This compound was purified by column chromatography eluting with AcOEt and then by precipitation with *n*-hexane from AcOEt. The solid was collected to give **8b** (58 mg, 0.097 mmol, 27% yield) as a white solid: mp 72–74 °C. MS (*m/z*): 602 (M^+ , 0.6%); Anal. $\text{C}_{30}\text{H}_{28}\text{ClN}_7\text{O}_5$.

Nitrooxy Ester of Losartan (9). Compound **9** was synthesized from losartan **1** (200 mg, 0.5 mmol) following the same procedure described above for the preparation of **11c**. The crude product was purified by precipitation with *n*-hexane from AcOEt to give **9** (140 mg, 0.3 mmol, yield 55%) as a yellow solid: mp 92–95 °C. MS (*m/z*): 467 (M^+ , 3%); Anal. $\text{C}_{22}\text{H}_{22}\text{ClN}_6\text{O}_2$.

5-(Nitrooxy)pentanoic Acid (10). Preparation of **10** and characterization data have already been reported.¹⁹

3-[(Nitrooxy)methyl]benzoic Acid (11a). Preparation of **11a** and characterization data have already been reported.¹⁹

4-[(Nitrooxy)methyl]benzoic Acid (11b). A solution of 4-(chloromethyl)benzoic acid (**18**) (1.00 g, 5.3 mmol) in a small amount of CH_3CN (5 mL) was added to a stirred solution of AgNO_3 (3.59 g, 21.2 mmol) in CH_3CN (19 mL). Stirring was continued over 2 h at room temperature in the dark, and then the precipitate (silver chloride) was filtered off, and the solvent was evaporated. The crude product was triturated with CHCl_3 (20 mL) and filtered off to remove the unreacted silver nitrate and AgCl. The solvent was evaporated to give **11b** (563 mg, 2.9 mmol, yield 54%). MS (*m/z*): 197 (M^+ , 3%); 135 ($M^+ - \text{ONO}_2$, 46%); Anal. $\text{C}_8\text{H}_7\text{NO}_5$.

3-[(2-Nitrooxyethyl)benzoic Acid (11c). A solution of **21** (200 mg, 1.2 mmol) in Ac_2O (2 mL) was added to a solution of HNO_3 (0.34 mL) and Ac_2O (0.70 mL) cooled at –10 °C. The mixture was stirred at –10 °C for 2 h. Then water was added, and the resulting aqueous phase was extracted with AcOEt. The organic

phase was dried, and the solvent was evaporated to give **11c** (206 mg, 1.0 mmol, yield 81%) as a yellow solid: mp 90–92 °C. MS (*m/z*): 211 (M^+ , 2%); 149 ($M^+ - \text{ONO}_2$, 100%); Anal. $\text{C}_9\text{H}_9\text{NO}_5$.

4-[(2-Nitrooxyethyl)benzoic Acid (11d). Compound **11d** was synthesized from **22** (268 mg, 1.6 mmol) following the same procedure described above for the preparation of **11c**. The crude product was purified by precipitation with *n*-hexane from AcOEt to give **11d** (100 mg, 0.5 mmol, yield 30%) as a white solid. MS (*m/z*): 149 ($M^+ - \text{ONO}_2$, 100%); Anal. $\text{C}_9\text{H}_9\text{NO}_5$.

2,6-Dimethyl-3-[(nitrooxy)methyl]benzoic Acid (12). Compound **12** was synthesized from **24** (730 mg, 3.7 mmol) following the same procedure described above for the preparation of **11b**. The crude product was triturated with CHCl_3 (20 mL) and filtered to remove the unreacted silver nitrate and AgCl. The solvent was evaporated to give **12** (740 mg, 3.3 mmol, yield 90%) as a white solid: mp 123–125 °C. MS (*m/z*): 225 (M^+ , 6%); 163 ($M^+ - \text{ONO}_2$, 85%); Anal. $\text{C}_{10}\text{H}_{11}\text{NO}_5$.

6-[(Nitrooxy)methyl]pyridine-2-carboxylic Acid (13). A solution of **27** (300 mg, 2.0 mmol) in Ac_2O (2 mL) was added to a solution of HNO_3 (0.55 mL) and Ac_2O (1.15 mL) cooled at –10 °C. The mixture was stirred at –10 °C for 2 h. Then the solvent was evaporated to give **13** (277 mg, 1.4 mmol, yield 69%) as a white solid: mp 58–63 °C. MS (*m/z*): 107 ($M^+ - \text{NO}_2$, – CO_2 , 100%); 123 ($M^+ - \text{CH}_2\text{ONO}_2$, 69%); Anal. $\text{C}_7\text{H}_6\text{N}_2\text{O}_5$.

3-Hydroxybenzyl Nitrate (14). A solution of 3-hydroxy-benzyl chloride **29** (608 mg, 4.3 mmol) in a small amount of CH_3CN was added to a stirred solution of AgNO_3 (2.90 g, 17.1 mmol) in CH_3CN (14 mL). Stirring was continued over 1 h at room temperature in the dark, and then the precipitate (silver salt) was filtered off, and the solvent was evaporated. The crude product was purified by column chromatography eluting with hexane/AcOEt (3:1) to give **14** (232 g, 1.4 mmol, 32% yield) as a yellow oil. MS (*m/z*): 170 (M^+ , 11%); 107 (100%, – ONO_2); Anal. $\text{C}_7\text{H}_7\text{NO}_4$.

3-(Hydroxymethyl)benzyl Nitrate (15a). Compound **15a** was synthesized from **32a** (638 mg, 4.08 mmol) following the same procedure described above for the preparation of **11b**. The crude product was triturated with CHCl_3 (20 mL) and filtered to remove the unreacted silver nitrate and AgCl. The solvent was evaporated to give **15a** (681 mg, 3.72 mmol, yield 91%) as a yellow oil. MS (*m/z*): 183 (M^+ , 15%); 121 ($M^+ - \text{ONO}_2$, 100%); Anal. $\text{C}_8\text{H}_9\text{NO}_4$.

4-(Hydroxymethyl)benzyl Nitrate (15b). Compound **15b** was synthesized from **32b** (454 mg, 2.90 mmol) following the same procedure described above for the preparation of **11b**. The crude product was triturated with CHCl_3 (10 mL) and filtered to remove the unreacted silver nitrate and AgCl. The solvent was evaporated to give **15b** (390 mg, 2.13 mmol, yield 74%) as a yellow oil. MS (*m/z*): 183 (M^+ , 10%); 121 ($M^+ - \text{ONO}_2$, 100%); Anal. $\text{C}_8\text{H}_9\text{NO}_4$.

3-(1-Hydroxyethyl)benzoic Acid (21). A solution of 3-acetylbenzoic acid (**19**) (400 mg, 2.4 mmol) in THF (2 mL) was added to a solution of 1 M LiAlH_4 in THF (2.44 mL, 2.4 mmol) cooled at 0 °C. The mixture was stirred at 0 °C for 1 h, then the solvent was evaporated, and water was added. The aqueous phase was acidified with 1 M HCl and extracted with AcOEt. The organic phase was dried, and the solvent was evaporated to give **21** (341 mg, 2.1 mmol, 84% yield) as a yellow solid: mp 90–92 °C. Anal. $\text{C}_9\text{H}_{10}\text{O}_3$.

4-(1-Hydroxyethyl)benzoic Acid (22). Compound **22** was synthesized from **20** (500 mg, 3.0 mmol) following the same procedure described above for the preparation of **21**. The crude product was purified by crystallization from CHCl_3/n -hexane to yield **22** (251 mg, 1.5 mmol, yield 50%) as a solid. MS (*m/z*): 167 (M^+ , 2%), 123 ($M^+ - \text{CO}_2$, 31%); Anal. $\text{C}_9\text{H}_{10}\text{O}_3$.

2,6-Dimethyl-3-(chloromethyl)benzoic Acid (24). Paraformaldehyde (7.9 g) and HCl_{conc} (15.9 mL) were added to a solution of 2,6-dimethylbenzoic acid (**23**) (1.60 g, 10.0 mmol) in AcOH glacial (15.9 mL). The resulting suspension was heated at 80 °C for 5 h. Then water was added, and the solid precipitate was collected to give **24** (1.47 g, 7.4 mmol, yield 74%); mp 55–57 °C. Anal. $\text{C}_{10}\text{H}_{11}\text{ClO}_2$.

Methyl-6-(hydroxymethyl)pyridine-2-carboxylate (26). A solution of NaBH₄ (0.30 g, 7.9 mmol) in H₂O (8.8 mL) was added dropwise to a solution of dimethyl 2,6-pyridinedicarboxylate **25** (1.0 g, 5.26 mmol) in MeOH (24.8 mL) cooled at -10 °C. The mixture was stirred at room temperature for 4 h, then the solvent was evaporated, and the aqueous phase was extracted with AcOEt. The organic phase was dried, and the solvent was evaporated to give **26** (514 mg, 3.1 mmol, yield 39%) as a white solid: mp 68–70 °C. MS (*m/z*): 152 (M⁺, 100%); Anal. C₈H₉NO₃.

6-(Hydroxymethyl)pyridine-2-carboxylic Acid (27). A solution of compound **26** (220 mg, 1.31 mmol) in MeOH (4.9 mL) and NaOH 50% (0.2 mL), was heated for 10 min by microwave irradiation at 100 °C and with a power of 150 W. After irradiation, the solution was acidified with 1 N HCl, and the aqueous layer was extracted with CHCl₃. The organic phase was dried over sodium sulfate, and the solvent was evaporated to give **27** (184 mg, 1.2 mmol, yield 90%) as a white solid. MS (*m/z*): 152 (M⁺, 100%); 107 (M⁺ - COOH, 58%); Anal. C₇H₇NO₃.

3-Hydroxybenzyl Chloride (29). Thionyl chloride (1.2 mL, 16.3 mmol) was added dropwise to a cold solution (0 °C) of 3-hydroxybenzyl alcohol **28** (675 mg, 5.4 mmol) in CHCl₃ (14 mL). The resulting mixture was stirred for 1 h at room temperature. The CHCl₃ layer was washed with water and dried over sodium sulfate, and the solvent was removed to afford **29** (608 mg, 4.3 mmol, 78% yield) as a yellow oil. Anal. C₇H₇ClO.

Imidazole-5-carboxaldehyde Derivative of Losartan (30). A mixture of losartan (**1**) (400 mg, 0.9 mmol) and activated MnO₂ (786 mg, 8.9 mmol) in H₂O (6 mL) was heated for 10 min by microwave irradiation (power = 150 W, *T* = 160 °C, ramp = 2 min, pressure = 200 psi). After irradiation, the excess of MnO₂ was filtered off and the solvent was evaporated to afford **30** (174 mg, 0.4 mmol, 42% yield) as a white solid: mp 154–156 °C. MS (*m/z*): 419 (M⁺, 2%); Anal. C₂₂H₂₁ClN₆O.

EXP 3174 (2). A solution of NaClO₂ (546 mg, 6.0 mmol) and NaH₂PO₄ (552 mg, 4.6 mmol) in H₂O (5.1 mL) was added dropwise to a solution of compound **30** (280 g, 0.7 mmol) in *t*-BuOH (5.1 mL). The mixture was stirred for 5 h at room temperature. The organic phase was separated and washed with H₂O and NaCl. The AcOEt layer was dried, and the solvent was evaporated. The crude product was purified by crystallization from AcOEt/hexane to yield **2** (131 mg, 0.30 mmol, 45% yield) as a white solid: mp 176–178 °C. MS (*m/z*): 435 (M⁺); Anal. C₂₂H₂₁ClN₆O₂.

3-(Chloromethyl)benzyl Alcohol (32a). HCl_{conc} (1.85 mL) was added at room temperature to a stirred suspension of 1,3-benzenedimethanol (**31a**) (500 mg, 3.62 mmol) in toluene (18 mL). The resulting solution was stirred for 12 h at room temperature. Then, the solution was washed with aqueous NaHCO₃ and water, the organic phase was separated, and the aqueous layer extracted with CH₂Cl₂. The organic phase was dried, and the solvent was evaporated to afford **32a** as a colorless oil (470 mg, 3.00 mmol, 83% yield).

4-(Chloromethyl)benzyl Alcohol (32b). Compound **32b** was synthesized from **31b** (500 mg, 3.62 mmol) following the same procedure described above for the preparation of **32a**, to give **32b** (450 mg, 2.90 mmol, 80% yield) as a white solid.

Pharmacology. All the experimental procedures were carried out following the guidelines of the European Community Council Directive 86-609.

A. In Vitro Vascular Protocols. The effects of the compounds were tested on isolated thoracic aortic rings of male normotensive Wistar rats (250–350 g).

After light ether anesthesia, the rats were sacrificed by cervical dislocation and bleeding.

The aortas were immediately excised and freed from extraneous tissues, and the endothelial layer was removed by gently rubbing the intimal surface of the vessels with a hypodermic needle. Five millimeter wide aortic rings were suspended, under a preload of 2 g, in 20 mL organ baths, containing Tyrode solution (composition of saline in mM: NaCl, 136.8; KCl, 2.95; CaCl₂, 1.80; MgSO₄·7H₂O, 1.05; NaH₂PO₄, 0.41; NaHCO₃, 11.9; glucose, 5.5), thermostated at 37 °C, and continuously gassed with a mixture of O₂

(95%) and CO₂ (5%). Changes in tension were recorded by means of an isometric transducer (Grass FTO3), connected with a unirecord microdynamometer (Buxco Electronics).

After an equilibration period of 60 min, endothelium removal was confirmed by the administration of acetylcholine (ACh) (10 μM) to KCl (30 mM)-precontracted rings. A relaxation <10% of the KCl-induced contraction was considered to be indicative of an acceptable lack of the endothelial layer, while the organs showing a relaxation ≥10% (i.e., significant presence of the endothelium) were discarded.

B. NO-Mediated Vasorelaxing Effect. From 30 to 40 min after the confirmation of endothelium removal, the aortic preparations were contracted by a single concentration of KCl (30 mM), and when the contraction reached a stable plateau, 3-fold increasing concentrations of the test substances (from 1 nM to 100 μM) were added.

Preliminary experiments showed that the KCl (30 mM)-induced contractions remained in a stable tonic state for at least 40 min.

The same experiments were carried out in the presence of a well-known guanylate cyclase inhibitor, ODQ (1 μM), which was incubated in aortic preparations after confirmation of endothelium removal.

C. AT₁-Antagonist Activity. From 30 to 40 min after confirmation of endothelium removal, the test compounds were incubated at a concentration of 0.1 μM. As the concentration 0.1 μM of compounds **4a** and **4b** already induced a significant vasorelaxing effect, **4a** and **4b** were incubated together with 1 μM ODQ, to avoid any influence of the NO-mediated effects on the contractile response evoked by AII.

Then, after an incubation period of 20 or 60 min, aortic preparations were treated with AII, using 3-fold increasing concentrations from 0.1 nM to 1 μM.

In parallel sets of experiments, the control concentration–contractile response curves for AII were obtained after the preincubation of only the vehicle (or vehicle and 1 μM ODQ for **4a** and **4b**).

D. Time-Course of NO-Mediated Vasorelaxing Effect. From 30 to 40 min after confirmation of endothelium removal, aortic preparations were contracted with a single concentration of 30 mM KCl, and after a stable plateau had been reached, a single concentration (1 μM) of the compounds was added. The vasorelaxing effect of the added compounds was monitored for 50 min.

E. Data Analysis. The vasorelaxing efficacy was evaluated as the maximal vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 30 mM KCl. When the limit concentration of 100 μM (the highest concentration that could be administered) of the tested compounds did not reach the maximal effect, the parameter of efficacy represented the vasorelaxing response, expressed as a percentage (%) of the contractile tone induced by 30 mM KCl, evoked by this limit concentration. The parameter of potency was expressed as pIC₅₀, calculated as the negative logarithm of the molar concentration of the test compounds, evoking a 50% reduction of the contractile tone induced by 30 mM KCl. The pIC₅₀ could not be calculated for those compounds showing an efficacy parameter lower than 50%. The parameters of efficacy and potency were expressed as means ± standard error, for 5–10 experiments. Student *t* test was selected for statistical analysis, and *P* < 0.05 was considered to be indicative of a significant statistical difference. Experimental data were analyzed by a computer fitting procedure (software: GraphPad Prism 3.0).

As regards the AT₁ antagonism, the AII-contracting effects, expressed as efficacy and potency, were evaluated as a percentage (%) of the previous KCl (30 mM)-induced contraction.

The parameter of efficacy, corresponding to the maximal contractile effect, was calculated as *E*_{max} (mean ± standard error, from 6 to 12 experiments).

The parameter of potency of AII was calculated as EC₅₀, corresponding to the molar concentration of AII necessary to evoke 1/2*E*_{max}.

The antagonist potency was calculated by Gaddum's equation: $K_b = B/(DR - 1)$, in which K_b represents the antagonist/receptor dissociation constant and B is the antagonist concentration, while $DR = EC_{50}$ (in the presence of B)/ EC_{50} (in control conditions).

F. In Vitro Cardiac Protocols. Adult male Wistar rats (260–350 g) were randomly assigned to one of three groups. In the first group ($n = 5$), animals were treated with an intraperitoneal (ip) injection (about 0.3 mL) of compound **1** (15.0 mg/kg), the second group ($n = 5$) was treated with the equimolar dose of compound **4a** (21.3 mg/kg ip), and the third group ($n = 5$) received the vehicle (DMSO).

After 2 h, all the animals were anesthetized with sodium pentobarbital (100 mg/kg ip) and heparinized (100 UI ip) to prevent blood clotting. After the opening of the chest, the hearts were quickly excised and placed in a 4 °C Krebs solution (composition mM: NaHCO_3 , 25.0; NaCl , 118.1; KCl , 4.8; MgSO_4 , 1.2; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 1.6; KH_2PO_4 , 1.2; glucose, 11.5) equilibrated with 95% O_2 5% CO_2 , to stop the contraction and to reduce oxygen consumption. Rapidly, the ascending aorta was cannulated and hearts mounted on a Langendorff apparatus, then the perfusion with Krebs solution (thermostated at 37 °C and continuously bubbled with a gas mixture of 95% O_2 and 5% CO_2) was started at constant pressure (70–80 mm of Hg). The above procedure was executed within 2 min.

A water-filled latex balloon connected to a pressure transducer (Bentley Trantec, mod 800) was introduced into the left ventricle via the mitral valve, and the volume was adjusted to achieve a stable left ventricular end-diastolic pressure of 5–10 mm of Hg during initial equilibration. The heart rate (HR) and left ventricular developed pressure (LVDP) were monitored by a Biopac system (California, U.S.A.), and the parameter of RPP was calculated as $\text{RPP} = \text{HR} \times \text{LVDP}$.

The CF was measured volumetrically and was expressed as milliliters of the perfusate collected in 1 min.

After a 30 min equilibration preischemic period, the hearts were subjected to 30 min of global ischemia (no flow), then they were reperfused for 30 min (reperfusion period).

The functional parameters of RPP and CF were measured before the global ischemia and at 5 min intervals during the reperfusion time. The values of postischemic RPP and of postischemic CF, recorded in the reperfusion time, were reported as a percentage of the respective preischemic values. Hearts showing severe arrhythmia were excluded from the experiments.

G. In Vitro Antiplatelet Activity. Male Wistar rats (250–300 g) were anesthetized with sodium pentobarbital (100 mg/kg, ip), the chest was opened, and the blood (7–10 mL) was obtained through intracardiac puncture.

Blood was collected into 5 mL plastic syringes containing 3.8% sodium citrate (1:9 v/v) and then centrifuged.

Platelet-rich plasma (PRP) was obtained by centrifugation at 80g for 40 min at room temperature. Then the top layer, PRP, was removed, while the residual blood sample was centrifuged at 1550g for 25 min in order to obtain platelet-poor plasma (PPP). The PRP platelet number was adjusted at about 6×10^5 platelets/ μL , through dilution with normal saline (NaCl 0.9%).

Platelets aggregation was determined, in diluted PRP, by an optical method, using a turbidimetric aggregometer (Elvi 840, Milan). In the aggregometer, contents of cuvettes (500 μL) were maintained at 37 °C and stirred constantly at 1000 rpm. Changes in light transmission through the diluted PRP and PPP were used for calibration and represent minimum (0%, no aggregation) and maximum (100%, full aggregation) light transmission, respectively.

In preliminary experiments concentration–response curves to the aggregating agent (ADP) were obtained, and the 5 μM ADP concentration was selected. Aggregation % was recorded as increased light transmission after the addition of the aggregating stimulus and has been expressed as mean \pm standard error, from groups of 10–12 samples (the samples of each group were collected from at least three different animals). The inhibitory effects of compounds **1** (100 μM) and **4a** (100 μM) on the ADP (5 μM)-induced aggregation were evaluated. The tested compounds were

added 2 min before the administration of 5 μM ADP. Control responses were obtained in the presence of drug vehicle only (DMSO). The final concentration of DMSO was 0.5%.

H. In Vivo Protocols. The effects of the compounds were also tested on male 10-week-old SHR, (250 g).

In this protocol, the test substances were administered orally, in drinking water, or subcutaneously, to two sets of four groups, each composed of three rats. The order of magnitude of the oral doses of captopril and losartan was selected on the basis of similar experimental protocols, described in the literature.²³ **4a** was administered at a dose equimolar to that of losartan. For subcutaneous administration, the doses were reduced by one-half.

I. Oral Administration. We estimated that the daily water intake for a single rat was about 50 mL. (1) The first group (control group) received the vehicle (DMSO 1%, in drinking water). (2) The captopril group received 50 mg/kg/die captopril dissolved in the vehicle. (3) The losartan group received 10 mg/kg/die losartan dissolved in the vehicle. (4) The **4a** group received 14.2 mg/kg/die **4a** (equimolar to 10 mg/kg/die losartan).

J. Subcutaneous Administration. (1) The first group (control group) received the vehicle (DMSO 1%, in a dorsal subcutaneous injection, about 0.25–0.30 mL). (2) The captopril group received 25 mg/kg/die captopril dissolved in the vehicle. (3) The losartan group received 5 mg/kg/die losartan dissolved in the vehicle. (4) The **4a** group received 7.1 mg/kg/die **4a** (equimolar to 5 mg/kg/die losartan).

K. Common Procedures. The experimental protocol was divided: after an initial period of 2 weeks, during which the rats were daily conditioned to enter and to remain in a containment box, the animals' tails were exposed to 40 min of irradiation with an IR lamp to determine a vasodilation of tail vessels. Systolic blood pressure values were recorded with the "tail-cuff" method by a BP recorder (Ugo Basile 58500).

All the four groups were subjected to 4 weeks of treatment and to three measurements a week (on alternate days).

Finally, we also examined a group of three male normotensive Wistar rats (250 g) which received only drinking water; in these animals, after 1 week of conditioning, systolic blood pressure was recorded three times in only 1 week.

At the end of all the *in vivo* procedures, we evaluated cardiac and ventricular hypertrophy in SHR: we recorded the body weight of each animal and then, after light ether anesthesia, rats were sacrificed by cervical dislocation and bleeding.

The whole hearts were immediately excised, freed from extraneous tissues, and then subdivided into left and right ventricles and atria. After rapid but careful washing and drying, we recorded the weights of left ventricle, right ventricle, and atria, and we calculated the following ratios, as described in the literature:²² heart weight/body weight (g/kg); left ventricle weight/body weight (g/kg); right ventricle weight/body weight (g/kg).

The same ratios were also calculated in the sample ($n = 3$) of normotensive animals, not submitted to any pharmacological treatment.

L. Materials. The substances used in the pharmacological experimental protocols were KCl (Carlo Erba) dissolved (2 M) in Tyrode solution; acetylcholine chloride (Sigma) dissolved (0.1 M) in EtOH 95% and further diluted in twice-distilled water. Angiotensin II (Sigma) was dissolved (1 mM) in twice-distilled water and then diluted; ODQ (Sigma) was dissolved (1 mM) in EtOH 95% and further diluted in Tyrode solution; losartan was dissolved (10 mM) in DMSO, whereas the following dilutions were dissolved in Tyrode solution.

The reference NO-releasing drug SNP (Sigma) was dissolved (10 mM) in DMSO and then diluted in Tyrode solution. Sodium pentobarbital (Sessa) was dissolved in twice-distilled water. Heparin Vister was purchased by Pfizer as an injectable preparation. ADP (Sigma) was dissolved in twice-distilled water.

All the synthesized compounds were dissolved (10 mM) in DMSO and further diluted in Tyrode solution.

All the solutions were freshly prepared immediately before the pharmacological experimental procedures. Previous experiments showed a complete ineffectiveness of the administration of the vehicles.

Acknowledgment. We thank the MIUR (Italian Ministry of Schooling, Higher Education and Research) for partial support of this project through a grant.

Supporting Information Available: ^1H NMR, MS, and elemental analysis results of all compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) De Gasparo, M.; Catt, K. J.; Inagami, T.; Wright, J. W.; Unger, T. International union of pharmacology XXIII. The angiotensin II receptors. *Pharmacol. Rev.* **2000**, *52*, 415–472.
- (2) Mombouli, J. V.; Vanhoutte, P. M. Heterogeneity of endothelium-dependent vasodilator effects of angiotensin-converting enzyme inhibitors: role of bradykinin generation during ACE inhibition. *J. Cardiovasc. Pharmacol.* **1992**, *20*, S74–S82.
- (3) Mombouli, J. V.; Vanhoutte, P. M. Endothelial dysfunction: from physiology to therapy. *J. Mol. Cell. Cardiol.* **1999**, *31*, 61–74.
- (4) Linz, W.; Wiemer, G.; Gohlke, P.; Unger, T.; Schölkens, B. A. Contribution of kinins to the cardiovascular actions of angiotensin-converting enzyme inhibitors. *Pharmacol. Rev.* **1995**, *47*, 25–49.
- (5) McConnaughey, M. M.; McConnaughey, J. S.; Ingenito, A. J. Practical considerations of the pharmacology of angiotensin-receptor blockers. *J. Clin. Pharmacol.* **1999**, *39*, 547–559.
- (6) Okunishi, H.; Oka, Y.; Shiora, N.; Kawamoto, T.; Song, K.; Miyazaki, M. Marked species-difference in the vascular angiotensin II-forming pathways: human versus rodents. *Jpn. J. Pharmacol.* **1993**, *62*, 207–210.
- (7) Munzel, T.; Kurz, S.; Heitzer, T.; Harrison, D. G. New insights into mechanisms underlying nitrate tolerance. *Am. J. Cardiol.* **1996**, *77*, 24C–30C.
- (8) Del Soldato, P.; Sorrentino, R.; Pinto, A. NO-aspirins: a class of antiinflammatory and anti-thrombotic agents. *Trends Pharmacol. Sci.* **1999**, *20*, 319–323.
- (9) Keeble, J. E.; Moore, P. K. Pharmacology and potential therapeutic applications of nitric oxide-releasing nonsteroidal antiinflammatory and related nitric oxide-donating drugs. *Br. J. Pharmacol.* **2002**, *137*, 295–310.
- (10) Minuz, P.; Lechi, C.; Zuliani, V.; Tommasoli, R.; Lechi, A. NO-aspirins: anti-thrombotic activity of derivatives of acetylsalicylic acid releasing nitric oxide. *Cardiovasc. Drug Rev.* **1998**, *16*, 31–47.
- (11) Bandarage, U. K.; Chen, L.; Fang, X.; Garvey, D. S.; Glavin, A.; Janero, D. R.; Letts, L. G.; Mercer, G. J.; Saha, J. K.; Schroeder, J. D.; Shumway, M. J.; Tam, S. W. Nitrosothiol esters of diclofenac: synthesis and pharmacological characterisation as gastrointestinal-sparing prodrugs. *J. Med. Chem.* **2000**, *43*, 4005–4016.
- (12) Baraldi, P. G.; Romagnoli, R.; Del Carmen Nunez, M.; Perretti, M.; Paul-Clark, M. J.; Ferrario, M.; Govoni, M.; Benedini, F.; Ongini, E. Synthesis of nitro esters of prednisolone, new compounds combining pharmacological properties of both glucocorticoids and nitric oxide. *J. Med. Chem.* **2004**, *47*, 711–719.
- (13) Shaffer, J. E.; Lee, F.; Thomson, S.; Han, B. J.; Cooke, J. P.; Loscalzo, J. The hemodynamic effects of S-nitrosocaptopril in anaesthetised dogs. *J. Pharmacol. Exp. Ther.* **1991**, *256*, 704–709.
- (14) Nakae, I.; Takahashi, M.; Kinoshita, T.; Matsumoto, T.; Kinoshita, M. The effects of S-nitrosocaptopril on canine coronary circulation. *J. Pharmacol. Exp. Ther.* **1995**, *274*, 40–46.
- (15) Korzycka, L.; Owczarek, J.; Czarnecka, E. A study on organic nitrates, Part VI. Synthesis and pharmacological activity of 1-isopropylamino-3-(1-naphthylloxy)-2-propyl nitrate. *Acta Pol. Pharm.* **2002**, *59*, 365–369.
- (16) Villarroja, M.; Herrero, C. J.; Ruiz-Nuno, A.; De Pascual, R.; Del Valle, M.; Michelena, P.; Grau, M.; Carrasco, E.; Lopez, M. G.; Garcia, A. G. PF9404C, a new slow donor with β -receptor blocking properties. *Br. J. Pharmacol.* **1999**, *128*, 1713–1722.
- (17) Boschi, D.; Di Stilo, A.; Cena, C.; Lolli, M.; Fruttero, R.; Gasco, A. Studies on agents with mixed NO-dependent vasodilating and β -blocking activities. *Pharmacol. Res.* **1997**, *14*, 1750–1758.
- (18) Di Stilo, A.; Visentin, S.; Cena, C.; Gasco, A. M.; Ermondi, G.; Gasco, A. New 1,4-dihydropyridines conjugated to furoxanyl moieties, endowed with both nitric oxide-like and calcium channel antagonist vasodilator activities. *J. Med. Chem.* **1998**, *41*, 5393–5401.
- (19) Breschi, M. C.; Calderone, V.; Digiacomo, M.; Martelli, A.; Martinotti, E.; Minutolo, F.; Rapposelli, S.; Balsamo, A. NO-sartans: a new class of pharmacodynamic hybrids as cardiovascular drugs. *J. Med. Chem.* **2004**, *47*, 5597–5600.
- (20) Jew, S.; Park, B.; Lim, D.; Kim, M. G.; Chung, I. K.; Kim, J. H.; Hong, J. I.; Kim, J.-K.; Park, H.-J.; Lee, J.-H.; Park, H. Synthesis of 6-formyl-pyridine-2-carboxylate derivatives and their telomerase inhibitory activities. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 609–612.
- (21) Santagada, V.; Fiorino, F.; Perissutti, E.; Severino, B.; Terracciano, S.; Teixeira, C. E.; Caliendo, G. A convenient synthesis by microwave irradiation of an active metabolite (EXP 3174) of losartan. *Tetrahedron Lett.* **2003**, *44*, 1149–1152.
- (22) Lassila, M.; Davis, B. J.; Allen, T. J.; Burrell, L. M.; Cooper, M. E.; Cao, Z. Cardiovascular hypertrophy in diabetic spontaneously hypertensive rats: optimizing blockade of the renin–angiotensin system. *Clin. Sci.* **2003**, *104*, 341–347.
- (23) Rodrigo, E.; Maeso, R.; Munoz-Garcia, R.; Navarro-Cid, J.; Ruilope, L. M.; Cachafeiro, V.; Lacera, V. Endothelial dysfunction in spontaneously hypertensive rats: consequences of chronic treatment with losartan or captopril. *J. Hypertens.* **1997**, *15*, 613–8.

JM0600186