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Losartan-Antioxidant Hybrids: Novel Molecules for the Prevention of Hypertension-Induced Cardiovascular Damage

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We report the first examples of a new series of antioxidant-sartan hybrids (AO-sartans), which were made by adding an antioxidant fragment to the hydroxymethyl side chain of losartan. Experiments performed in cultured cells demonstrate that these new hybrids retain the ability to block the angiotensin II effect with increased antioxidant ability. In hypertensive rats, these compounds show properties that suggest they may be more useful than losartan for controlling hypertension and preventing hypertension-induced cardiovascular damage.

Introduction

Although it is believed that lowering blood pressure is the main protective mechanism of antihypertensive treatments, other possibilities to explain the beneficial effects of these drugs have been proposed. It is a well-known fact that local changes that take place at the vascular level, such as the increased oxidative stress, may also be a relevant mechanism of cardiovascular disease progression in hypertensive patients. Thus, treatments that interfere with this oxidative stress could be useful tools for management of these individuals.

Among antihypertensive agents, angiotensin converting enzyme (ACE[a]) inhibitors have been widely used in the treatment of hypertension. However, in the past two decades, research focused on drugs that can replace ACE inhibitors in hypertension therapy, without their side effects (mainly coughing), has led to the discovery of sartans (Chart 1).[1] Drugs of this class are antagonists of angiotensin II at the AT1 receptor and block the action of angiotensin II in a potentially more complete and specific way than ACE inhibitors.

Lowering blood pressure is not the only beneficial effect of angiotensin II blockade. Angiotensin II plays a significant role in the progression of tissue damage in cardiovascular diseases,[2] and the beneficial effects of these drugs in the prevention of cardiovascular morbidity and mortality cannot be solely explained by their antihypertensive action. In this context, some chemical interventions have been performed on sartan molecules in order to modify, where possible, their antihypertensive potency, but mainly to increase their ability to prevent tissue damage in the cardiovascular system.[3]

Oxidative stress is a well-known mechanism that is responsible for the development of vascular damage.[4] Different pathogenic stimuli involved in cardiovascular diseases, such as activated macrophages, hyperglycaemia, oxidized low density lipoprotein (LDL), and even angiotensin II, exert their harmful effects, at least partially, through an increased local synthesis of reactive oxygen species.[5−8] These active metabolites induce a significant endothelial dysfunction[9] and are able to modify the normal balance among proliferation, apoptosis, and extracellular matrix synthesis in heart and arterial walls.[10−12] Some beneficial effects of antihypertensive molecules have been attributed, at least partially, to their antioxidant ability.[13−15]

The above-mentioned considerations prompted us to develop a new class of sartan derivatives by adding an antioxidant fragment to these drugs. We hypothesized that the administration of these hybrid compounds would be a more specific or efficient means to target antihypertensive or antioxidant molecules to cardiovascular cells. Herein, we report our strategy of incorporating a variety of antioxidant moieties onto the primary alcohol group of losartan through an ester linkage to generate new losartan-antioxidant hybrids, which retain the ability to block the angiotensin II effect, with increased antioxidant ability.

Chemistry

Among the sartans shown in Chart 1, losartan[16] was chosen as the appropriate candidate for chemical manipulation on the basis of its high activity as an angiotensin II receptor blocker, along with the presence in its structure of a hydroxymethyl moiety, which made it suitable for attaching the antioxidant fragment in a simple synthetic chemical process. Among the antioxidant candidates to be incorporated in the losartan structure, a series of phenols were selected as the best choices because of their well-known redox properties.

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[a] Abbreviations: ABTS, 2,2′-azino-di-(3-ethyl benzothiazoline-6-sulfonic acid); ACE, angiotensin converting enzyme; AO, antioxidant; Bn, benzyl; EDTA, ethylenediamine tetraacetic acid; DMEM, Dulbecco's Modified Eagle Medium; HNE, 4-hydroxy-2-nonenal; HPLC-MS/MS, high-performance liquid chromatography-tandem mass spectrometry; LDH, lactate dehydrogenase; LDL, low density lipoprotein; L-NAME, *Nω*-Nitro-L-arginine methyl ester hydrochloride; PCSA, planar cell surface area; QqQ, triple-quadrupole; TBAF, tetra *n*-butyl ammonium fluoride; TBDMS, *tert*-butyldimethylsilane; THF, tetrahydrofuran; VSMC, vascular smooth muscle cells.

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Chart 1. Sartans, a Family of Angiotensin II Receptor Antagonists

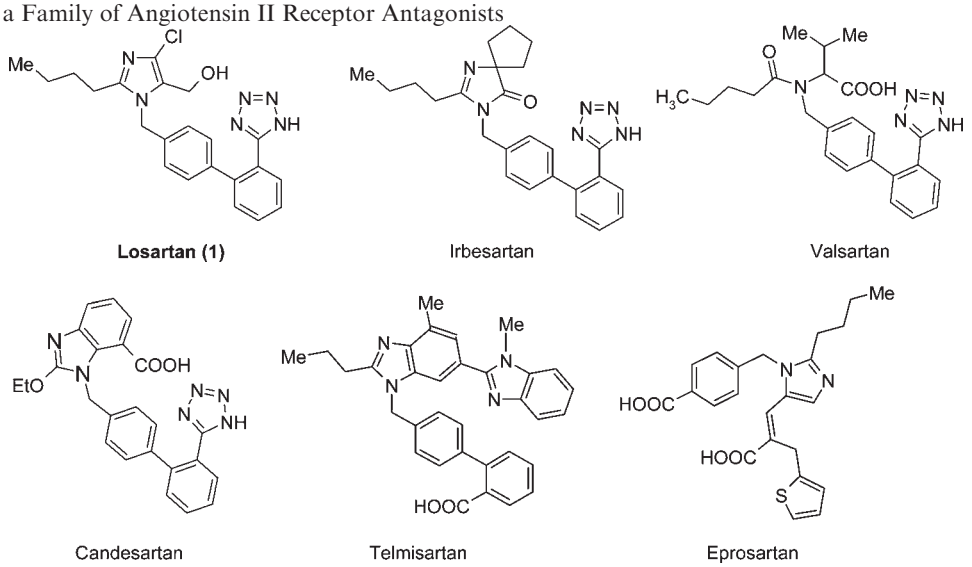
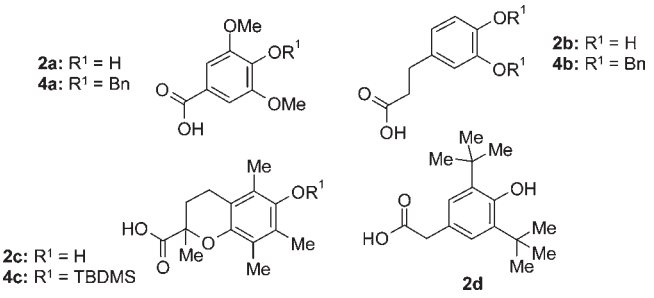


Chart 2. Antioxidant Phenolic Derivatives



A selection of the most potent antioxidant phenols are compounds **2a**−**d**[17] (Chart 2).

Although there are different possibilities for the linkage between losartan and the antioxidant, the simplest approach involves an esterification reaction, and this was the initial choice from a trityl-protected losartan prepared according to the literature procedure.[16] Attempts to carry out the esterification reaction using some unprotected phenols failed or gave very low yields of the corresponding ester. As a result, the hydroxy groups in phenols **2a**,**b** were protected with the benzyl group (Bn) and **2c** was protected with *tert*-butyldimethylsilane (TBDMS). The hydroxy group in phenol **2d** was difficult to protect, probably due to the steric hindrance of the two vicinal *tert*-butyl groups, and this compound was used without protection.

The esterification reaction between the protected losartan **3** and **2d** and protected phenols **4a**−**c** was attempted using different conditions and reagents for the activation of the carboxylic acid and the primary alcohol. The best results were obtained using Mitsunobu conditions[18] with the corresponding esters **5a**−**d** being obtained in moderate yields (Scheme 1). Deprotection of losartan derivatives **5a**−**d** to give the desired esters was carried out under different conditions depending on the protecting group. In all cases, deprotection of the tetrazole ring was required, so compound **5d** was used as a model to establish the best conditions for removing the trityl group. Hydrogenolysis in the presence of Pt/C (5%) afforded the best yield for the ester **6d**. Under these conditions, compound **5b** was transformed into the corresponding derivative **6b** by removal of both the benzyl and trityl protecting groups, whereas the formation of **6c** required the removal of the silyl protecting group with tetrabutyl ammonium fluoride (TBAF) in tetrahydrofuran (THF) followed by hydrogenolysis. The attempted deprotection of **5a** under these conditions needed higher amounts of the catalyst in order to complete its conversion, thus giving **7a** as the main product, and only traces of **6a** were obtained.

In the attempts to remove the trityl group from **5d** using palladium on carbon, we detected the formation of the derivative **7d** as the main product, which results from the deprotection and dehalogenation of **5d**. This unexpected result was convenient to establish the role of the halogen in the target compounds. Derivatives **7a**−**c** were also prepared under the same conditions used for **7d**. The series of losartan-antioxidant hybrids obtained are shown in Chart 3.

Results and Discussion

After the syntheses of **6** and **7**, the antioxidant properties were assessed.[17] Losartan displayed minimal antioxidant ability, which increased 4−8-fold in the synthesized compounds, with **6b**, **7a**, and **7b** showing the best antioxidant ability (Table 1). At concentrations of 1 mmol/L, these products display the same antioxidant ability as 1 mmol/L of compound **2b** (0.36 ± 0.03 mmol/L).

To test the ability of the synthesized compounds to block angiotensin II *in vitro*, two kinds of experiments were performed in cultured mesangial cells. These human cells express angiotensin II receptors and show a contractile response in the presence of this peptide.[19] First, the binding of radiolabeled angiotensin II was tested in the presence of the different compounds.[20] All of them inhibited the binding of the labeled peptide. The maximum inhibition was observed with losartan (47%), followed by **6b** (41%) and **7b** (40%), although the differences observed between the different compounds were not statistically significant (Table 1).

Second, the ability of angiotensin II to reduce the planar cell surface area (PCSA) in cells pretreated with the same compounds was analyzed. Changes in PCSA are considered to be a consequence of cell contraction and are due to the interaction of angiotensin II with its receptor.[20] The different compounds tested inhibited the angiotensin II-induced PCSA reduction. This inhibition ranged between 62% with losartan and 77%

Scheme 1. Synthesis of Losartan-Antioxidant Hybrids

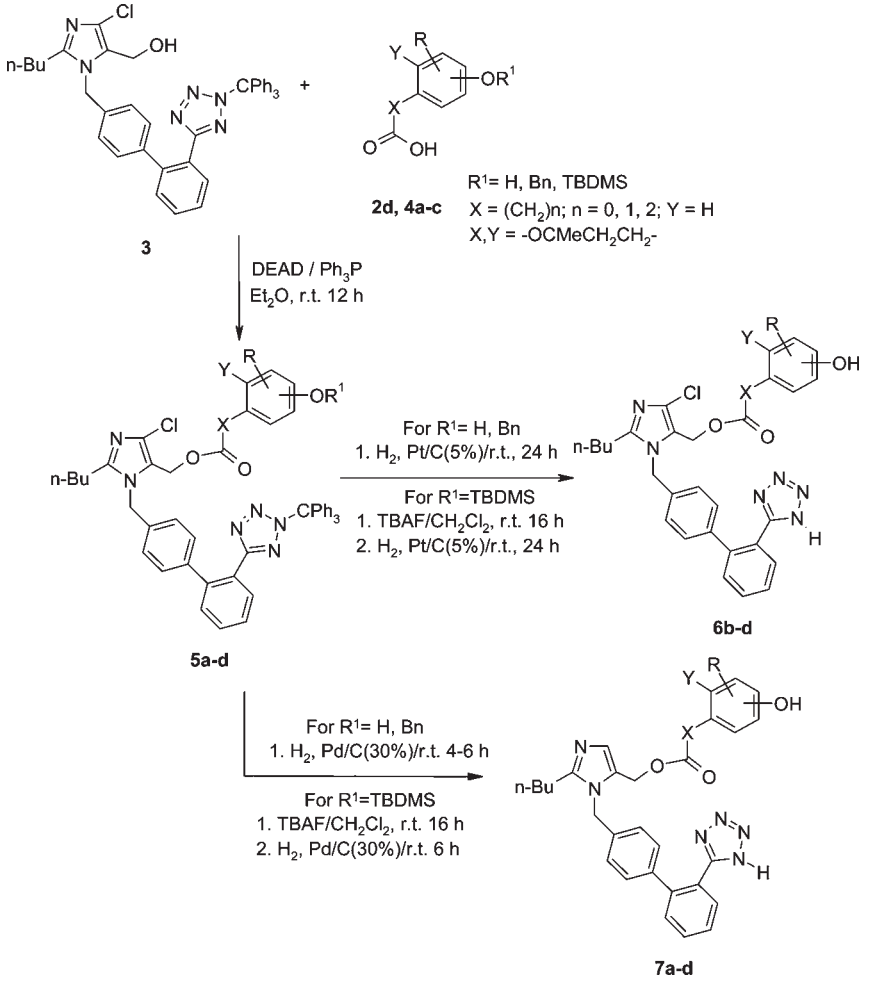
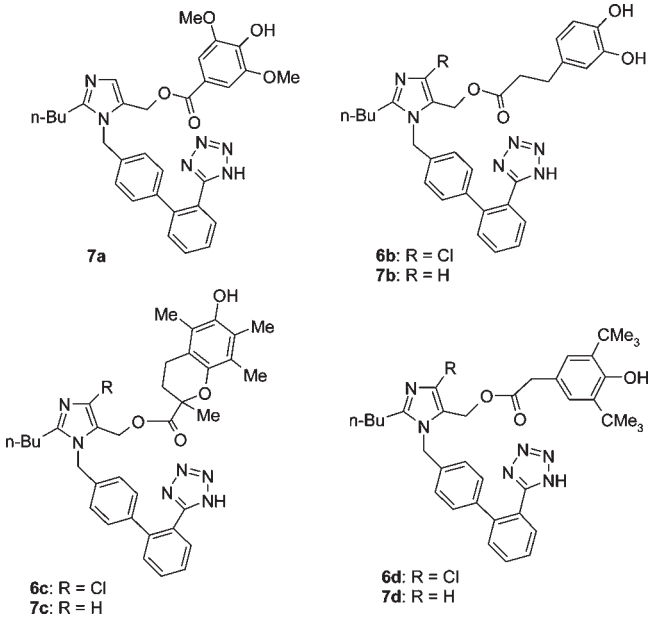


Chart 3. Losartan-Antioxidant Hybrids Prepared



with **7b**, but no statistically significant differences were observed in the inhibition of the contraction showed by the different compounds.

Table 1. Antioxidant Ability and Angiotensin II Binding of Losartan Derivatives **6** and **7**[a]

compound	antioxidant ability	Ang II binding
Losartan	0.04 ± 0.01	$53 \pm 8\%$
6b	0.31 ± 0.03^b	$59 \pm 7\%$
6c	0.25 ± 0.03^b	$68 \pm 7\%$
6d	0.16 ± 0.02^b	$65 \pm 10\%$
7a	0.31 ± 0.04^b	$66 \pm 9\%$
7b	0.31 ± 0.04^b	$60 \pm 6\%$
7c	0.27 ± 0.03^b	$65 \pm 11\%$
7d	0.24 ± 0.02^b	$77 \pm 11\%$

[a] Antioxidant ability is expressed as antioxidant equivalent concentration, in mmol/L, per 1 mmol/L of product. Ang II binding is expressed as percentage of the binding of labeled angiotensin II (100%) in the presence of the same concentration of the products (1 μmol/L). Results are the mean ± SEM of 8 independent experiments. [b] $p < 0.05$ vs losartan.

These results show that the attachment of an antioxidant moiety to losartan is able to increase its antioxidant ability without modifying its basic properties as an angiotensin II receptor blocker. However, these *in vitro* results should be confirmed by an *in vivo* approach. For this purpose, **6b** or/and **7b** were selected as representative hybrids. Wistar rats were treated for 8 weeks with an inhibitor of the synthesis of nitric oxide, *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), to induce hypertension, and received losartan, **6b** or **7b**, for the last 4 weeks. Two additional groups of

Table 2. Summary of the Main Biochemical Parameters for the Different Groups of Animals[a]

	C	L-NAME	LOS	AO (**2b**)	LOS+AO	**6b**	**7b**
glucose (mg/dL)	144 ± 5	165 ± 10[b]	149 ± 8	170 ± 14[b]	168 ± 14[b]	154 ± 10	149 ± 11
cholest (mg/dL)	49 ± 3	57 ± 6	48 ± 1	58 ± 6	54 ± 3	52 ± 4	55 ± 2
triglyc (mg/dL)	52 ± 14	84 ± 15[b]	65 ± 13	70 ± 7	69 ± 11	52 ± 12	60 ± 17
Ca (mg/dL)	8.5 ± 0.3	8.5 ± 0.3	8.3 ± 0.4	8.8 ± 0.2	8.5 ± 0.3	8.6 ± 0.4	8.6 ± 0.3
protein (mg/dL)	4.5 ± 0.3	4.5 ± 0.5	5 ± 0.3	5.2 ± 0.4	4 ± 0.4	4.5 ± 0.5	4.5 ± 0.47
LDH (UI/L)	588 ± 20	643 ± 4[b]	622 ± 26	591 ± 33	610 ± 7	561 ± 49	600 ± 12
AST (UI/L)	85 ± 9	93 ± 12	91 ± 4	77 ± 12	93 ± 10	83 ± 4	85 ± 7
ALT (UI/L)	28 ± 3	32 ± 5	24 ± 1	24 ± 1	22 ± 4	24 ± 2	25 ± 2
creatinine (mg/dL)	0.42 ± 0.02	0.44 ± 0.01	0.42 ± 0.02	0.47 ± 0.02	0.44 ± 0.01	0.42 ± 0.01	0.43 ± 0.01
urea (mg/dL)	25 ± 3	29 ± 4	24 ± 3	27 ± 1	24 ± 3	24 ± 5	24 ± 4
Na^+ (mmol/L)	146 ± 3	151 ± 4	151 ± 3	148 ± 7	148 ± 4	151 ± 4	151 ± 3
K^+ (mmol/L)	4.4 ± 0.1	4.5 ± 0.2	4.7 ± 1	4.1 ± 1.3	4.2 ± 0.2	4.6 ± 0.2	4.4 ± 0.2

[a] C, Control; L-NAME, *Nω*-Nitro-L-arginine methyl ester hydrochloride; LOS, losartan; AO, Antioxidant (**2b**); LOS+AO, losartan+antioxidant; Ca, calcium; LDH, lactic dehydrogenase; AST, aspartate transaminase; ALT, alanine transaminase. Results are the mean ± SEM of 10 rats. [b] $p < 0.05$ vs control group.

animals subjected to an 8 week treatment with L-NAME were also included, some rats that received just the antioxidant compound **2b**, and another group of rats that were cotreated with losartan and **2b**, simultaneously but separately. In these two groups, the treatments were also administered for the last 4 weeks. All compounds were administered in equimolar concentrations. The main biochemical parameters of the different groups of animals are given in Table 2. Although the nature of the molecules under investigation (i.e., losartan and **2b**) did not suggest the potential appearance of adverse effects, this analysis was performed to evaluate the possible toxic effect of drug administration. In short, metabolic, hepatic, and renal parameters were studied. An 8 week treatment with L-NAME induced a slight but statistically significant increase in serum glucose, triglycerides, and LDH concentrations. Similar serum glucose changes were observed in rats treated with L-NAME plus **2b**, with or without losartan, whereas the biochemical parameters of the other animals, those treated with L-NAME plus losartan, **6b** and **7b**, were comparable to those of control animals. These results enabled us to rule out a toxic effect of the newly synthesized hybrids, at least on the basis of the parameters studied.

Losartan decreased systolic blood pressure in rats treated with L-NAME, but the values observed after 4 weeks of treatment did not reach the control values. Similar results were observed when the antioxidant molecule was administered at the same time as losartan. Treatment with **6b** and **7b** not only diminished systolic blood pressure but normalized it (Figure 1A). Two macroscopic characteristics were selected to evaluate the consequences of these changes in blood pressure, heart weight, and aorta thickness. In the case of heart weight (Figure 1B), losartan and **6b** and **7b** were able to normalize the increased heart weight induced by L-NAME, whereas in terms of aorta thickness (Figure 1C), the three compounds prevented the effect of L-NAME, albeit only partially. The administration of the antioxidant molecule did not modify either blood pressure or aorta thickness, and only minimally decreased the L-NAME-induced cardiac hypertrophy (Figure 1).

The analysis of the changes in blood pressure, as well as the evaluation of heart weight and aorta thickness, suggest that **6b** and **7b** are at least as potent as losartan in the control of hypertension and in the prevention of cardiovascular damage. Moreover, a more careful analysis of the data suggests that the two newly synthesized compounds may have an increased antihypertensive effect and, in the case of **6b**, a more marked protective effect in heart damage than losartan. However, differences are subtle and, in the case of heart damage, not statistically significant.

For this reason, a more sophisticated analysis of vascular damage was performed: the vascular content of two extracellular matrix proteins, fibronectin and collagen type I, as well as the local protein oxidative damage, measured as the accumulation of 4-hydroxy-2-nonenal-lysine (HNE-lysine), were evaluated in the different groups of animals. L-NAME administration induced an increased accumulation of fibronectin (Figure 2A) and collagen I (Figure 2B) as well as increased oxidative damage (Figure 2C). Losartan slightly but significantly decreased the collagen I content in vessel walls, and when administered together with **2b**, it also decreased the fibronectin content and the HNE-lysine accumulation without reaching the values observed in the control animals. The compounds **6b** and **7b** completely normalized the changes induced by L-NAME in vascular walls (Figure 2). These results, together with the changes in blood pressure, suggest that long-term treatment with these new compounds may prevent more efficiently the cardiovascular damage induced by hypertension than with the use of standard angiotensin II blockers. In addition, the ability of the new compounds to prevent the accumulation of extracellular matrix proteins suggests that, on a long-term basis, it is possible that these drugs minimize significantly the structural damage induced by hypertension in vascular walls.

In order to analyze the mechanisms responsible for the differences observed between the different groups of animals, we measured the blood levels of losartan, its main metabolite EXP-3174, **2b** and **6b**, after chronic exposure to losartan, **2b**, losartan + **2b**, and **6b**. We wanted to gain insight into the stability of the hybrid to esterases and ensure that the observed pharmacological effects are associated with the presence of circulating levels of the hybrid rather than hydrolysis products or even the carboxy-metabolite of losartan EXP-3174 (more potent than losartan at the AT1 receptor level). We also wanted to compare the steady-state circulating levels of losartan, **2b**, and the hybrid after chronic administration, to analyze the possibility that significant differences in circulating levels could explain, at least partially, the observed effects. Before performing the analysis of plasma, we carried out a stability study of the **6b**-containing drinking water solution. After 2 days, **6b** remains mainly nonhydrolyzed (95.9%) in the drinking water, and only a low amount of losartan was detected (4.1%).

Wistar rats were treated with losartan, **2b**, losartan plus **2b**, and **6b** for 4 weeks, plasma was obtained at sacrifice, and the

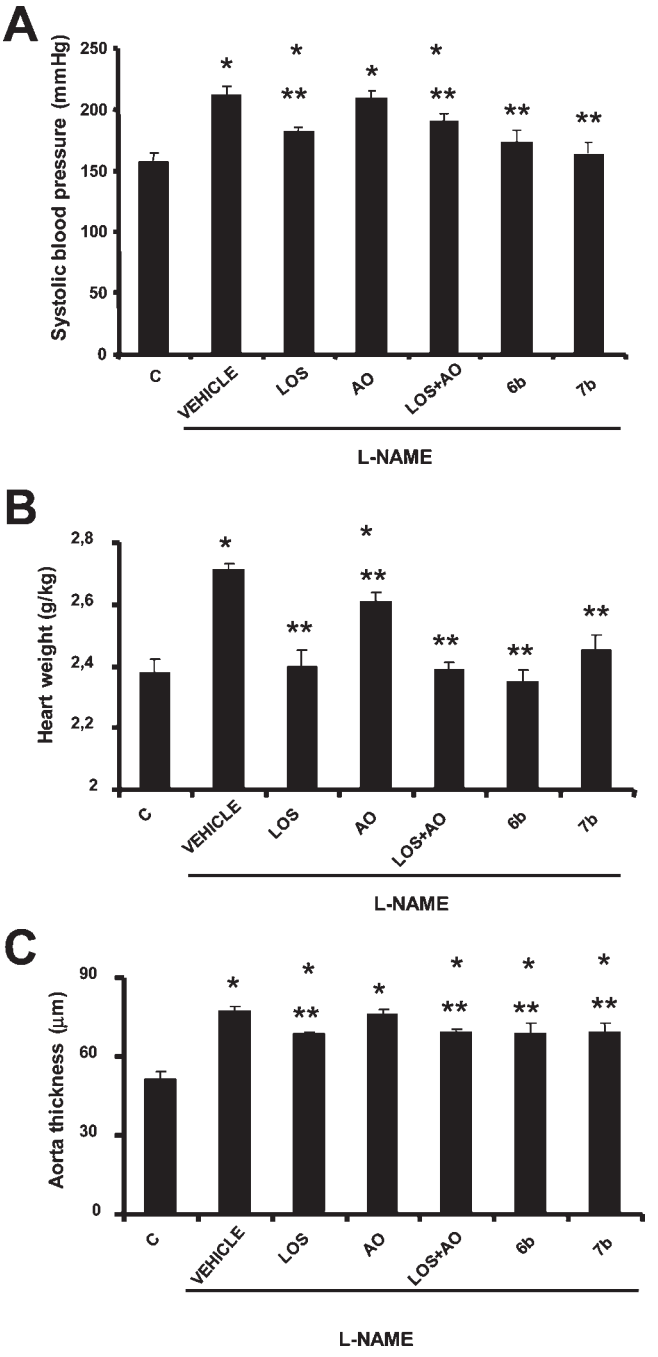


Figure 1. Changes in systolic blood pressure (A); heart weight (B); aorta thickness (C). Wistar rats were treated with *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) for 8 weeks and Losartan (LOS), the antioxidant compound **2b** (AO), LOS+AO, **6b**, and **7b** were administered for the last 4 weeks. In the cases of heart weight, values were corrected to the animal weight. Results are mean ± SEM of 10 rats. * $p < 0.05$ vs control. ** $p < 0.05$ vs vehicle.

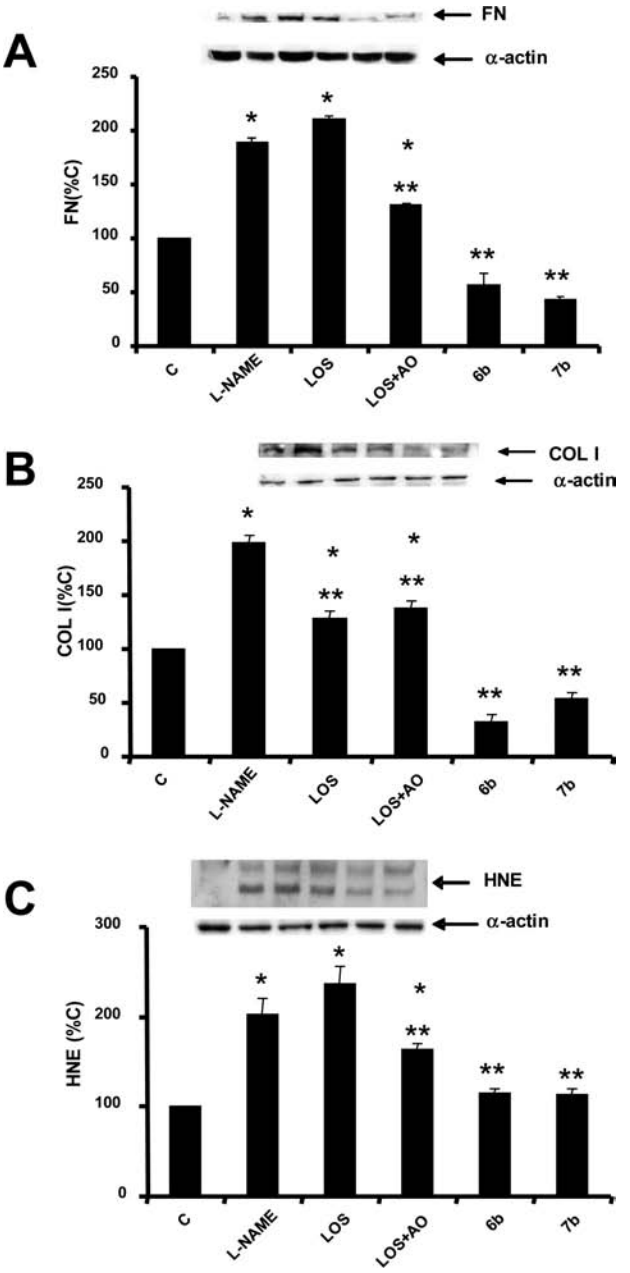


Figure 2. Analysis of the vascular content of two extracellular matrix proteins: fibronectin (FN) (A) and Col I (collagen type I) (B) and the local protein oxidative damage, measured as HNE (accumulation of HNE-lysine) (C). Wistar rats were treated with *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) for 8 weeks and losartan (LOS), LOS+AO, **6b**, and **7b** were administered for the last 4 weeks. Representative Western blots and densitometric analysis from 10 separate experiments are shown. Results are expressed as percent of the basal value, and they are the mean SEM of the densiometric values. * $p < 0.05$ vs control. ** $p < 0.05$ vs L-NAME.

levels of losartan, EXP-3174, **2b**, and **6b** were measured. Two different plasma samples from each animal were analyzed with basically identical results. A significant amount of **6b** (90.3−93.5%) was detected in plasma, with only minimal amounts of losartan (3.1−3.3%) and EXP-3174 (3.3−6.6%) (see Supporting Information), thus supporting the high metabolic stability of the ester linkage in **6b** and the low levels of circulating losartan and EXP-3174 after the oral exposure of Wistar rats to **6b**. Moreover, total plasma concentrations of antihypertensive molecules, including losartan, EXP-3174, and **6b**, were higher in the animals receiving the **6b** compound than in the other groups (Table 3). No significant amounts of **2b** were detected in plasma samples.

The high concentration of angiotensin II blocking molecules detected after **6b** administration was probably responsible for the complete normalization of blood pressure in these animals. Even in these conditions, no significantly hypotensive effect was demonstrated. Moreover, these high levels of antihypertensive molecules, together with the presence of **6b** in

Table 3. Concentration (nM) of Losartan, EXP-3174 and **6b** in Rat Plasma Samples from the Different Groups of Rats[a]

	groups		
	losartan (n = 4)	losartan + **2b** (n = 4)	**6b** (n = 6)
losartan	24.4 ± 2.6	11.1 ± 1.4	8.0 ± 1.1
EXP-3174	39.0 ± 5.2	19.4 ± 2.3	13.0 ± 1.3
6b	---;	---;	171.0 ± 16.4
total	63.4 ± 5.3	30.5 ± 4.2	192.0 ± 20.1

[a] Each data represents the mean ± SEM.

the circulation, a substance with antioxidant properties, could explain the decreased vascular content in extracelular matrix proteins and HNE-lysine observed in the **6b**-treated animals. It would be expected that the inhibition in the HNE-lysine content, the selected index of oxidative tissue damage, would be similar when comparing administration of losartan + **2b** with **6b** or **7b** treatments, but this was not the case. As previously mentioned, the circulating levels of angiotensin II blocking molecules were higher in the **6b** treated animals than in the rats receiving losartan + **2b**, and angiotensin II blockade may diminish oxidative stress.[21] Moreover, the circulating levels of **2b** were undetectable after oral administration, probably as a consequence of its rapid removal from the circulation,[22] and this fact may also contribute to a lower antioxidant effect. Finally, the observed differences in the antioxidant effect could be also explained by the fact that HNE lysine was only analyzed in vascular walls, a tissue with a higher Ang II receptor content, to which the newly synthesized molecules could gain access more specifically.

In summary, the results provide *in vitro* evidence that these losartan/antioxidant hybrids retain the ability to block the angiotensin II effect, with increased antioxidant ability. In hypertensive rats, the compounds show properties that suggest they may be more useful than losartan to control hypertension and to prevent the hypertension-induced cardiovascular damage.

Experimental Section

General Chemical Methods. Melting points were determined with a Gallenkamp apparatus and are uncorrected. ^{1}H NMR and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, on a Varian Gemini 200. Chemical shifts are given in ppm relative to solvent. Signals are quoted as singlet (s), broad singlet (bs), doublet (d), triplet (t), quartet (q), quintet (quin), hexaplet (hex), and multiplet (m). Coupling constants (J) are given in hertz (Hz). MS were recorded on a Hewlett-Packard 5988A or a Hewlett-Packard 1100MSD mass spectrometers. Elemental analyses were performed on a Heraeus CHN rapid analyzer. FTIR spectra were recorded on a Perkin-Elmer FTIR 1725X spectrophotometer. Reagents and solvents were obtained from commercial sources and used without further purification. TLC was carried out on Alugram Sil G/UV$_{254}$ silica gel plates. Preparative gravity column chromatography was performed on Merck silica gel. Reported yields are not optimized. Purities of the hybrids and new compounds were determined by elemental analysis, which indicated >95% purity of each product (see Supporting Information).

Chemistry. General Procedure for the Synthesis of 4a−b. Benzyl bromide (1.5 equiv/hydroxy or carboxy group) and K_2CO_3 (2.0 equiv/hydroxy or carboxy group) were added over a solution of **2a−c** (0.55 M) in DMF under an argon atmosphere and heated at 60 °C for 20 h. The reaction mixture was cooled at room temperature and filtered, and the solvent was removed under reduced pressure. Residue was dissolved in EtOAc and washed with 1 M HCl and brine and dried over $MgSO_4$. After filtering, solvent was removed under reduced pressure, the residue was dissolved in MeOH (0.35 M in relation to starting material) and treated with 2.3 M NaOH (1.2 equiv), and the mixture was heated at reflux for 1 h. Solvents were evaporated under reduced pressure, and the residue was dissolved in water and extracted with Et_2O. Aqueous phase was made acidic (pH 1−2) with 1 M HCl, extracted with EtOAc, and the organic extracts dried over $MgSO_4$. After filtering and removing solvent, **4a−c** were obtained as white solids.

General Procedure for the Synthesis of 5a−d. A solution of **3** (0.025 M) and PPh_3 (0.025 M) in Et_2O was added dropwise over a solution of **4a−d** (0.025 M) in Et_2O under an argon atmosphere. The mixture was stirred at room temperature for 12 h, $OPPh_3$ was filtered off and the solvent was evaporated under reduced pressure. Residue was chromatographed on silica gel using hexane/EtOAc 1:1 to give **5a−d** as yellowish oils.

General Procedure for the Catalytic Hydrogenation of 5a,b,d and 8c. Catalyst was added over a solution of **5a**,**b**,**d** or **8c** in the corresponding solvent, and the reactor was flushed with argon for 10 min. Then, reaction mixture was kept under a hydrogen atmosphere at 1 atm and stirred at room temperature for the time indicated in each case. Mixture was filtered through Celite, and solvent was evaporated under reduced pressure. Residue was chromatographed on silica gel using the more convenient eluent in each case to give **6b−d** or **7a−d**.

3-(3,4-Dihydroxy-phenyl)propionic Acid 2-butyl-5-chloro-3-[2′-(2*H*-tetrazol-5-yl)-biphenyl-4-ylmethyl]-3*H*-imidazol-4-ylmethyl ester (6b). Pt/C (5%) (1.1 equiv/protecting group), **5b** (1.3×10^{-2} M; MeOH/EtOAc 4:3), 48 min, eluent EtOAc/MeOH/AcOH (60:20:5), Rf $_{\text{EtOAc/MeOH/AcOH60:20:5}}$ 0.63, oil, yield 10%. IR (KBr) [cm^{-1}] 2957, 2361, 1732, 1604, 1528, 1460, 1352, 1259, 1146, 1115, 956, 816, 759, 667, 634. ^{1}H NMR (200 MHz, CD_3OD) δ 7.72−7.36 (m, 4H), 7.11 (d, J = 8.2 Hz, 2H), 6.87 (d, J = 8.5 Hz, 2H), 6.71−6.53 (m, 3H), 5.08 (s, 2H), 5.00 (s, 2H), 2.78 (t, J = 7.2 Hz, 2H), 2.47 (t, J = 7.8 Hz, 2H), 2.34 (t, J = 7.7 Hz, 2H), 1.58 (quin, 2H, J = 7.7 Hz), 1.39 (hex, 2H, J = 7.2 Hz), 0.90 (t, J = 7.2 Hz, 3H). ^{13}C NMR (50 MHz, CD_3OD) δ 175.1, 157.0, 150.7, 147.1, 147.0, 142.6, 140.5, 136.5, 132.2, 131.6, 131.5, 130.7, 129.9, 128.9, 126.9, 124.8, 124.4, 123.1, 122.1, 121.9, 118.0, 78.4, 73.4, 62.2, 55.7, 32.0, 30.9, 27.3, 26.0, 23.3, 22.0, 14.0, 12.9, 12.1, 11.9. ES-MS m/z: 587 (M+1). Anal. Calcd. for $C_{55}H_{53}N_6O_4Cl$: C (63.42), H (5.32), N (10.90); found C (63.53), H (5.21), N (11.04).

3-(3,4-Dihydroxy-phenyl)-propionic Acid 2-Butyl-3-[2′-(2*H*-tetrazol-5-yl)-biphenyl-4-ylmethyl]-3*H*-imidazol-4-ylmethyl Ester (7b). Pd/C (30%) (0.76 equiv/protecting group), **5b** (3.3×10^{-2} M; MeOH/$CHCl_3$ 2:1), 4 h, eluent MeOH/EtOAc 1:5, Rf $_{\text{EtOAc/MeOH5:1}}$ 0.3, oil, yield 46%. IR (KBr) [cm^{-1}] 3441, 3137, 3058, 2958, 2931, 2871, 2505, 1941, 1736, 1603, 1519, 1458, 1354, 1283, 1143, 1114, 1005, 966, 819, 784, 759, 635. ^{1}H NMR (200 MHz, CD_3OD) δ 7.58−7.31 (m, 4H), 7.10 (d, J = 7.1 Hz, 3H), 6.80 (d, J = 8.2 Hz, 3H), 6.71−6.59 (m, 2H), 6.40 (dd, J = 2.0 Hz, J = 8.2 Hz, 1H), 5.08 (s, 2H), 5.00 (s, 2H), 2.66 (t, J = 7.4 Hz, 2H), 2.64 (t, J = 7.4 Hz, 2H), 2.34 (t, 2H, J = 7.3 Hz), 1.58 (quin, 2H, J = 7.5 Hz), 1.31 (hex, 2H, J = 7.2 Hz), 0.89 (t, 3H, J = 7.1 Hz). ^{13}C NMR (50 MHz, CD_3OD) δ 173.9, 162.0, 151.8, 146.0, 144.4, 142.4, 141.8, 135.9, 133.0, 131.6, 131.1, 130.6, 130.2, 130.1, 128.4, 128.1, 127.8, 126.5, 120.3, 116.3, 116.2, 56.1, 36.7, 31.2, 30.7, 27.2, 23.3, 14.0 . ES-MS m/z: 553 (M+1). Anal. Calcd. for $C_{31}H_{32}N_6O_4$: C (67.37), H (5.83), N (15.20); found C (67.42), H (5.61), N (15.06).

Pharmacology. Experimental *in Vitro* and *in Vivo* Methods. Measurement of the Total Antioxidant Ability. The antioxidant ability of the different compounds was evaluated with a commercial kit (Total Antioxidant Status Assay Kit, Calbiochem, La Jolla, CA).[17] The assay relies on the ability of antioxidants to inhibit the oxidation of a chromogen (ABTS) by metmyoglobin and hydrogen peroxide. Oxidation is monitored by reading the absorbance at 600 nm. The antioxidant ability is proportional to the reduction of the absorbance, and it is expressed as mM.

Experimental Studies in Cells. Vascular smooth muscle cells (VSMC) were obtained from thoracic aortas of Wistar rats by methods described previously.[20] Briefly, thoracic aortas from Wistar rats were removed, cleaned, dissected into small strips, and incubated in DMEM/Ham′s F-12 medium (Biowhittaker, Walkersville, MD) with collagenase type IV at 37 °C for 45 min. The digested strips were seeded onto dishes and maintained in 10 mL of DMEM/Ham′s F-12 medium with 10% fetal calf serum (Biowhittaker), at 37 °C, in a humidified atmosphere of 5% CO_2. Culture media were changed every 3 days. The primary cultures on days 20 to 22 were passaged by trypsinization (trypsin-EDTA). The cells were used between the third and fifth passages.

Angiotensin II binding experiments were performed to assess the ability of the different compounds to block the angiotensin II receptor. For this purpose, VSMC were gently removed from the dishes and resuspended in assay buffer (20 mM Tris−HCl, 5 mM glucose, 130 mM NaCl, 5 mM KCl, 10 mM sodium acetate, pH 7.4), at a protein concentration of 0.5 μg/μL. Binding experiments were performed by incubating 0.15 nM ^{125}I-Angiotensin II (Amersham International, Buckinghamshire, UK) with 125 μg of cell protein, in the presence of losartan or the different synthesized compounds, for 45 min. The free radioactivity was separated from the bound radioactivity by centrifugation at 11 000 *g* for 2 min, and the resultant pellet was washed three times with ice-cold 0.15 M NaCl. The radioactivity was counted in a Kontron gamma counter (Kontron Instruments AG, Zurich, Switzerland). Nonspecific binding was controlled by incubating some samples with an excess of angiotensin II (10^{-4} M).[20]

The ability of the different compounds to block angiotensin II-induced cell contraction was also tested. This analysis was performed by studying the changes in planar cell surface area (PCSA) under different experimental conditions.[20] In every experiment, cells were washed and placed in Tris-glucose buffer (20 mM Tris, 130 mM NaCl, 5 mM KCl, 10 mM sodium acetate, and 5 mM glucose, pH 7.45) containing 2.5 mM $CaCl_2$. Cells were incubated with 1 μM angiotensin II, in the presence of losartan or the different synthesized compounds, for 30 min. Photographs (TMS-F Photomicroscope, Nikon, Tokyo, Japan, magnification 150×) of the same cells were taken at times 0 and 30 min. PCSA was determined by computer-aided planimetric techniques.

Animal Studies. Two-month-old male Wistar rats were treated with *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME) in the drinking water (20 mg/kg/day). Four weeks after starting L-NAME, some rats were treated with losartan (20 mg/kg/day), **2b** (10 mg/kg/day), losartan plus **2b** (20 mg/kg/day plus 10 mg/kg/day), **6b** (28 mg/kg/day), and **7b** (30 mg/kg/day). These treatments were administered daily, for a controlled period of time (12.00−18.00 h), also in drinking water. Ten animals were included in each experimental group. Arterial pressure was monitored in conscious animals with a tail cuff sphygmomanometer (LE 5001 Pressure Meter, Letica Scientific Instruments, Hospitalet, Spain).[23] After 8 weeks, animals were sacrificed under halothane anesthesia, between 9.00 and 10.00 h. Blood samples were taken, and heart and aorta removed. Blood was centrifuged and serum stored until analysis. Heart was weighed. A portion of descending thoracic aorta was transferred to ice-cold Hank′s, cleaned and flushed with 4% formaldehyde solution. Then, it was cut into 2−3 mm rings, fixed on slides, and photographed. Media thickness was measured by computer-aided planimetric techniques.

Serum biochemical parameters were measured with an automatic analyzer (Hitachi 717, Boehringer Mannheim, Mannheim, Germany). Vascular samples were lysed in protein lysis buffer, and total extract proteins (μg/lane) were size-fractionated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After blocking these membranes with 5% nonfat milk for 1 h, they were incubated with rabbit anticollagen type I or antifibronectin monoclonal antibodies (Chemicon, La Jolla, CA) or rabbit anti HNE-lysine polyclonal antibody (Chemicon). Membranes were washed, incubated with the secondary antibody conjugated with horseradish peroxide (goat antirabbit IgG; Bio-Rad Laboratories, Richmond, CA) for 30 min, and washed extensively. Blots were developed by chemiluminescence using the ECL Western blotting system (Pharmacia-Biotech, Uppsala, Sweden). Densitometric analysis was performed with appropriate software (NIH Image 1.55; Bethesda). Blots were stripped and reblotted with an antibody against β-tubulin, as a control of charge.[24]

Blood Level Determination of Losartan, Losartan Metabolites, 2b, and 6b after Chronic Exposure to Drugs. Additional groups of animals were treated for 4 weeks with losartan (n = 4), **2b** (n = 4), losartan plus **2b** (n = 4), and **6b** (n = 6), sacrificed, and blood was obtained. The administration of the treatments and the sacrifice were performed as previously reported. Plasma circulating levels of the drugs were analyzed using HPLC-MS/MS (QqQ). For the blood level determination of losartan, EXP-3174 and **6b**, SPE-C18 cartridges were conditioned with MeOH and equilibrated with 2% formic acid. A plasma aliquot (100 μL) was diluted up to 1 mL with 2% formic acid and applied on the cartridge, washed with MeOH/water 1:1 (1 mL) and eluted with MeOH (1 mL). Aliquots (2 μL) from the eluting fraction were taken and injected in a HPLC-MS-QqQ instrument (HPLC model 1200 coupled through an orthogonal electrospray interface model G1607A to a triple quadrupole model 6410 all of them from Agilent Technologies) using an Ascentis Express column (25 mm × 2.1 mm i.d.; 2.7 μm from Supelco) and an isocratic mobile phase (25% MeOH with 0.1% v/v formic acid plus 45% water with 0.1% v/v formic acid; 0.5 mL/min) at 40 °C. For the blood level determination of **2b** and its metabolites, a plasma aliquot (100 μL) was diluted with 0.1 mM sodium acetate buffer (pH = 5, 100 μL) and 200 mM HCl/MeOH (200 μL). The mixture was vortexed and ultrasonicated (5 min). Then, the homogenized sample was extracted with EtOAc (6 × 600 μL). For each extraction, the sample was shaken for 7 min, centrifuged (6200*g*) for 5 min, and phases were separated. Organic extracts were pooled and dried under N_2. Dried extracts were diluted with 15% MeCN/H_2O (100 μL). Aliquots (5 μL) were taken and injected in a HPLC-MS-QqQ instrument (see above) using a C18 Tripple End-capping LUNA column (250 mm × 4.6 mm i.d.; 5 μm Phenomenex) and a gradient (A, water with 0.1% v/v formic acid; B, MeOH with 0.1% v/v formic acid; starting mobile phase, 75% A plus 25% B; 0.5 mL/min) at 25 °C.

Statistical Analysis. In every case, data shown are the mean ± SEM of a variable number of experiments (see figure legends), and in some cases, they are expressed as percentages of the control values. Because the number of data points in each distribution was never over 10, nonparametric statistics was selected to compare the different groups of results. p values < 0.05 were considered statistically significant.

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Supporting Information Available: Chemical experimental procedures for the synthesis and fully characterization of compounds **4**−**7**. HPLC chromatograms from **6b**-containing dinking water and plasma samples of rats treated with **6b**, losartan and losartan + **2b**. This material is available free of charge via the Internet at http://pubs.acs.org.

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